Multidrug resistance protein expression of adult T-cell leukemia/lymphoma

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Abstract

In adult T-cell leukemia/lymphoma (ATL), it is difficult to achieve remission and the reason for the resistance to chemotherapeutic agents may be linked to the presence of multidrug resistance (MDR) proteins. Lung resistance-related protein (LRP), multidrug resistance-associated protein and P-glycoprotein are three MDR proteins which we examined in ATL cells using multiparametric flow cytometry and real-time RT-PCR. LRP was highly expressed and suppressing LRP function increased doxorubicin accumulation in nuclei. This indicates LRP may be contributing to drug resistance in ATL patients, and the suppression of LRP function could be a new strategy for ATL treatment.

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Keywords: Adult T-cell leukemia/lymphoma; Multidrug resistance protein (MDR); Lung resistance-related protein (LRP); Multidrug resistance-associated protein (MRP); P-glycoprotein (P-gp)

1. Introduction

Adult T-cell leukemia/lymphoma (ATL) is a malignant tumor of mature helper T-cells infected with human T-cell leukemia virus type I (HTLV-I) [1–3]. In ATL, it is difficult to induce remission, and the prognosis for ATL has been known to be poor [4]. One of the reasons for resistance to chemotherapeutic agents may be the presence of multidrug resistance (MDR) proteins. Lung resistance-related protein (LRP) is an MDR protein which was separated from P-glycoprotein (P-gp) negative small cell lung carcinoma cell lines by Schepet et al. [5]. LRP was shown to be the major vault protein. Vaults are localized in nuclear pore complexes and involved in the nucleocytoplasmic transport of drugs [6]. Multidrug resistance-associated protein (MRP), which was isolated from a P-gp negative lung cancer cell line [7], has been proven to confer drug resistance using human carcinoma cells [8]. The clinical significance of MDR proteins such as LRP or MRP in ATL has not been examined extensively. Recently, overexpression of LRP or MRP1 in ATL cells was shown using RT-PCR and Northern/Slot blot analysis [9–11]. The poor response to chemotherapy of ATL cases was partially attributed to the expression of P-gp or MDR1 RNA [12,13] in early reports, though, there was a report that P-gp was not overexpressed in most ATL cells [9] when examined by RT-PCR, thus no definite conclusion has yet been drawn.

Multiparametric flow cytometry has proven to be a reliable and sensitive approach to analyze MDR proteins [14]. Real-time RT-PCR is a recognized method to examine gene expression quantitatively [15]. There are no reports in which measuring the presence of MDR proteins or gene expression in ATL cells has been done using these techniques. In the present study, we used three color immunofluorescence analysis and real-time RT-PCR to clarify the expression of LRP, MRP and P-gp in ATL. In addition to this, we performed an LRP function assay to clarify whether LRP affects doxorubicin concentration in nuclei.

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2. Materials and methods

2.1. Cell line

KK-1, an interleukin-2 (IL-2) dependent cell line established from an ATL patient by Yamada et al. [16], was maintained with 0.25 U/ml of recombinant IL-2 (kindly provided by Takeda Pharmaceutical, Tokyo, Japan). The cell surface phenotype of KK-1 was CD3+ CD4+ CD8+ CD25+ as previously reported [16]. Adriamycin resistant sublines K562/ADM and HL60/AD of K562 and HL60, respectively, were maintained in RPMI1640 medium (Life Science, Grand Island, NY) containing 10% fetal calf serum (FCS; Inter-gen, Purchase, NY). HL60/AD was kindly provided by Dr. Takemura [17].

2.2. Cell separation from ATL patients

Peripheral blood or bone marrow samples were taken from patients with ATL under written informed consent. The diagnosis of ATL was made based on a positive test for the antibody for HTLV-I and on the pathology of bone marrow or peripheral blood, and all patients were diagnosed as acute type ATL [18]. Mononuclear cells of each sample were separated through Ficoll–Conray density gradient centrifugation (density 1.077). Cells were suspended in alpha-modified Eagle’s minimal essential medium (α-MEM; Flow Laboratories, McLean, VA), and then were either used immediately or cryopreserved in liquid nitrogen with 10% dimethylsulphoxide (DMSO) and 50% fetal calf serum (FCS) until use, or 1 × 10^7 cells were frozen after washing with phosphate-buffered saline (PBS) until RNA extraction.

2.3. Detection of LRP, MRP and P-gp by flow cytometry

Cells were stained with a three color immunofluorescence method as follows. For detection of the intracellular epitopes of LRP and MRP, 1 × 10^6 cells were incubated with 100 μl fixation medium (Caltag Laboratories, Burlingame, CA) for 15 min at room temperature, then washed with PBS, and incubated with 10% rabbit serum (R&D Systems, McKinley Place, MN) and permeabilization medium (Caltag Laboratories) for 5 min. Cells were then incubated for 1 h at 4 °C with anti-LRP monoclonal antibody (MoAb) LRP56, with anti-MRP MoAb MRPm6 (both from Kamiya Biotech, Thousand Oaks, CA) or with mouse isotype-matched control MoAb. For detection of P-gp, 1 × 10^6 cells were incubated with human γ-globulin for blocking, and then with MRK16, anti-P-gp MoAb (Kyowa Medix, Tokyo, Japan) or subclass-matched IgG2a MoAb (Beckman Coulter, Miami, FL) for 30 min at 4 °C as in our previous reports [19,20]. Cells were then washed and incubated with a secondary antibody, goat anti-mouse IgG conjugated with phycoerythrin (PE), for 30 min at 4 °C. After washing and blocking with mouse IgG (Beckman Coulter), cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 MoAb (Immunotech, Marseille, France) and phycoerythrin–cyanine 5 (PC5)-conjugated anti-CD25 MoAb (Immunotech) for another 30 min, then washed and analysed.

The LRP, MRP or P-gp expression on ATL cells was analysed by gating on CD4+ and CD25+ populations using an EPICS-XL system II flow cytometer (Beckmann Coulter). MDR positivity was determined with a CD4 and CD25 double positive population, thus we could identify MDR positivity of leukemic ATL cells without contamination of normal T cells. No difference was observed in LRP, MRP or P-gp positivity between fresh and cryopreserved samples.

2.4. Detection of LRP, MRP1 and MDR1 mRNA by real-time RT-PCR

2.4.1. RNA isolation and cDNA synthesis

Total RNA was isolated by a guanidinium thiocyanate–phenol–chloroform extraction method of Chomczynski and Sacchi [21], using ISOGEN reagent (Nippongene, Tokyo, Japan). After being checked by gel electrophoresis, the RNA of each sample was stored at −80 °C until use. One microgram of total RNA was converted to single-stranded cDNA using a random primer and reverse transcriptase under conditions as described by the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). The resulting cDNA was diluted in diethylprocarbonate water and stored at −80 °C.

2.4.2. Quantitative real-time RT-PCR

Measuring mRNA levels of LRP, MRP1 and MDR1 was based on the TaqMan probe method [22] using an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA). The mRNA levels of an internal control gene, human β-actin (TaqMan endogeneous control kit, Applied Biosystems) was also measured and used to normalize the mRNA levels of the drug resistance genes. PCR products were detected using a dual-fluorescent non-extendable probe labeled with the reporter dye FAM and the quencher dye TAMRA for all reactions, except for the human β-actin reaction in which FAM and TAMRA were replaced by VIC and MGB, respectively. All primer pairs and probes were made according to the reports as follows [23,24].

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**LRP**

Forward primer 5'-CAG CTG GCC ATC GAG ATC A-3'
Reverse primer 5'-TCC AGT CTC TGA GCC TCA TGC-3'
Probe 5'-CAA CTC CCA GGA AGC GGC GGC GGC-3'

**MRP1**

Forward primer 5'-CAA TGC TGT GAT GGC GAT G-3'
Reverse primer 5'-GAT CCG ATT GTT GCT GCT TTG CA-3'
Probe 5'-AGA CCA AGA CGT ATG TGG TGC CCC AC-3'

**MDR1**

Forward primer 5'-GCT ATA ATG CGA CAG GAG ATT GGC TGG-3'
Reverse primer 5'-CAT TAC TAT TTT GCT ACC AAT AAC TT-3'
Probe 5'-CAC GAT GGT GTG GAG GTC CTT AAC ACC CG-3'

β-Actin (Applied Biosystems)
Table 1
Clinical data of ATL patients and expression of MDR proteins

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/sex</th>
<th>WBC (×10^9/l)</th>
<th>ATL cells (%)</th>
<th>CD2 (%)</th>
<th>CD3 (%)</th>
<th>CD4 (%)</th>
<th>CD8 (%)</th>
<th>CD25 (%)</th>
<th>CD4+ 25+ LRP+ (%)</th>
<th>CD4+ 25+ MRP+ (%)</th>
<th>CD4+ 25+ P-gp+ (%)</th>
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<tr>
<td>1</td>
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<td>207.0</td>
<td>77</td>
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<td>96.4</td>
<td>97.2</td>
<td>3.8</td>
<td>96.9</td>
<td>81.9</td>
<td>1.4</td>
<td>2.3</td>
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<tr>
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<td>29.3</td>
<td>49</td>
<td>76.5</td>
<td>43.4</td>
<td>38.9</td>
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<td>57.2</td>
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<td>93.3</td>
<td>84.8</td>
<td>88.4</td>
<td>4.8</td>
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</table>

Peripheral blood was used except for case number 8 (pleural effusion) and case number 10 (bone marrow aspirate). ND, not done because of insufficient cell number.

The real-time amplification reaction was performed in the presence of 300 nM of each forward and reverse primer, 200 nM of each TaqMan probe, and 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems) in a total volume of 25 μl. In the reaction, 2.5 μl of diluted cDNA was used as a template in triplicate for all samples. Samples were heated for 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C. Under this assay, serially diluted cDNA prepared from KK-1, K562/ADM or HL60/AD was used as a positive control for each reaction. To compare the expression levels among different patient samples, the relative expression of the MDR genes and β-actin levels were calculated using the comparative standard curve method [25]. From each standard curve, which showed a linear detection range of 6–7 logs, the threshold cycle values were set for each target gene and β-actin. We then obtained the mean relative gene expression value for each sample. The difference between

Fig. 1. Expression of LRP, MRP and P-gp by using three color flow cytometric analysis. Flow cytometric analysis of case number 8. A1–A3, B1–B3 and C1–C3 represent CD4+CD25+ cell populations expressing LRP (89.8%), MRP (2.1%) and P-gp (0.6%), respectively.

the slopes of each target gene and β-actin was <0.1 in every experiment.

2.5. Measurement of [14C] doxorubicin in isolated nuclei

To investigate whether LRP affects doxorubicin accumulation in nuclei, the nuclei of ATL cells were isolated according to the report by Newmeyer and Wilson [26]. Nuclei were suspended in solution A [250 mM sucrose; 1 mM dithiothreitol; 80 mM KCl; 15 mM EDTA; 15 mM piperazine diethanesulfonic acid, NaOH (pH 7.4); 0.5 mM spermidine; 0.2 mM spermine; 1 mM phenylmethylsulfonyl fluoride] (Wako, Osaka, Japan). To inhibit the function of LRP, anti-LRP MoAb LRP56 or LRP mediated reversing agent PAK-104P (2-[4-(diphenylmethyl)-1-piperazinyl]ethyl-5-(trans-4,6-dimethyl-1, 3, 2-dioxaphosphorinan-2-yl)-2, 6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P oxide) (provided by Dr. Akiyama [27]), was added just before 1 μM of [14C] doxorubicin (Amersham Biosciences, Buckinghamshire, UK) to the nuclei suspension, which was then incubated for 10 min at 37°C. The nuclei were washed twice with ice cold solution A, suspended in Aqueous Counting Scintillant (Amersham Biosciences) and radioacitivity was determined with the liquid scintillation counter system (LSC-700 Aloka., Tokyo, Japan) after 10 min. In our preliminary experiment, the MoAb LRP56 caused a 1.4-fold increase in [14C] doxorubicin accumulation in KK-1 cell nuclei.

3. Results

3.1. Characterization of ATL patients

The hematological findings of 11 patients and cell surface phenotype of ATL cells are summarized in Table 1. As CD4 and CD25 were positive in all 11 ATL patients’ leukemic cells, we examined MDR expression of ATL cells in the CD4 and CD25 double positive cell population. In normal mononuclear cells from healthy individuals the CD4 and CD25 double positive cell population was less than 1%.

3.2. Expression of MDR proteins in ATL cells

As shown in Table 1, LRP was positive in 8 out of 11 samples and the positivity varied from 41.9 to 89.8%. For two of the remaining three, case numbers 3 and 10, positivity was 13.6 and 11.6% and CD25 expression was relatively low. The LRP positivity was 5.6% in case number 11 in spite of the 62% positivity of CD25 expression. On the other hand, there was no case with significant MRP or P-gp expression. Fig. 1 shows a representative result of three color immunofluorescence analysis of CD4 and CD25 with LRP, MRP or P-gp. KK-1 cells were positive for LRP in 97% of the cells, for MRP in 8.2%, and for P-gp in 12%.

Fig. 2. Relative mRNA expression levels of MDR genes. Relative mRNA expression levels were calculated as follows: MDR protein expression/β-actin expression. KK-1, HL60/AD and K562/ADM were used as positive controls of LRP, MRP1 and MDR1, respectively.

3.3. MDR gene expression in ATL cells

The relative mRNA expression levels of MDR by real-time PCR is shown in Fig. 2. LRP was expressed on all of the cases. The relative mRNA expression levels of LRP varied from 0.7 to 7.0 compared to the KK-1 cells. LRP expression was higher than the KK-1 cells in 7 of 11 patients (64%). MRP1 was weakly expressed in all cases with a lower magnitude of expression than that of HL60/AD. Expression of MDR1 was not observed in any of the ATL cells.

3.4. Accumulation of [14C] doxorubicin in isolated nuclei

Fig. 3 shows accumulation of [14C] doxorubicin in isolated nuclei from five patients in which enough cells were avail-
Fig. 3. Accumulation of [14C] doxorubicin in isolated nuclei. Anti-LRP antibody (A) LRP reversing agent PAK-104P (B) significantly increased accumulation of [14C] doxorubicin. Each value represents the mean of triplicate measurements (DPM).

able. LRP56 or PAK-104P resulted in a significant increase of [14C] doxorubicin accumulation compared with an absence of LRP inhibitor (paired t-test).

4. Discussion

Our present results indicate that LRP is highly expressed in leukemic cells in most ATL patients. We used three color flow cytometric analysis, which can eliminate the effect of residual normal T cells, and it revealed high expression of LRP in ATL cells. The results of real-time RT-PCR also demonstrated overexpression of LRP. For each case, however, these two test results do not always correlate. The reason for the discrepancy may be due to translation regulation, glycosylation [28], degradation, contamination by normal cells, etc. LRP is thought to be a drug transporter between the nucleus and cytoplasm. There are two reports [9,10] that show leukemic cells from chronic ATL patients overexpressed mRNA of the LRP gene. Sakaki et al. [29] reported that transfection of the Tax gene activated LRP expression in leukemic clones established from ATL patients. They demonstrated Tax transfected cells acquired drug resistance to doxorubicin and vincristine, suggesting that LRP expression caused the drug resistance in ATL cells. As well, we also showed that an anti-LRP MoAb or PAK-104P, an LRP inhibitor, increased doxorubicin accumulation in nuclei, suggesting that LRP confers drug resistance. Reversing LRP function may augment efficacy of anti-leukemic agents.

Our results show that MRP expression was low in the CD4+ CD25+ population of ATL cells and also MRP1 gene expression was low using real-time PCR. The MRP efflux function tests with calcein acetoxymethyl ester, which becomes fluorescent calcein by cleavage of the ester bond by intracellular esterases, was not affected in ATL cell lines by probenecid, a specific modulator of MRP [14,30] (data not shown). MRP appears not to have a role with drug resistance in ATL cells. Ohno et al. [11] reported that expression of MRP1 mRNA was higher with patients who have large numbers of peripheral blood abnormal lymphocytes, and with patients in chronic phase. Our results did not show any correlation between the MRP1 mRNA and leukemic cell counts. Ikeda et al. [9] showed a difference in MRP expression between the chronic type and control sample, and Ohno et al. [11] showed a difference between the chronic and lymphoma types, but neither found a significant difference between the acute type and others. Some of the acute type ATL cases may have high MRP expression, as in Ohno’s report (3 of 32 cases) [11]. Ikeda et al. [9] reported the correlation of MRP1 and LRP mRNA levels in chronic ATL cells. Although the LRP gene is localized close to the MRP1 gene, on the short arm of chromosome 16, there is a report that MRP1 and LRP genes are rarely coamplified and are not normally located within the same amplicon [31]. Our cases are all acute type, and there was no correlation between LRP and MRP1 mRNA levels.

For P-gp and MDR1 expression in ATL cells, there has been no definite conclusion from other studies [9,12,13]. Our results demonstrated no expression of P-gp or MDR1 in ATL cells.

In conclusion, our present results suggest that LRP overexpression may contribute to MDR in ATL patients. LRP mediated reversing agents such as PAK-104P or MoAb against LRP may be useful to improve the effect of chemotherapy for patients with LRP positive ATL cells. The suppression of LRP function would be a new strategy in ATL treatment.

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