Expression of P-Glycoprotein in Adult T-Cell Leukemia Cells

By Yasuo Kuwazuru, Shuichi Hanada, Tatsuhiko Furukawa, Akihiko Yoshimura, Tomoyuki Sumizawa, Atae Utsunomiya, Kazuki Ishibashi, Takeshi Saito, Kimiharu Uozumi, Masao Maruyama, Minoru Ishizawa, Terukatsu Arima, and Shin-ichi Akiyama

We have examined the expression of P-glycoprotein (P-gp) in adult T-cell leukemia (ATL) samples from 25 patients. Based on immunoblotting with a monoclonal antibody against P-gp, C219, 8 of 20 ATL patients were P-gp positive at the initial presentation. All 6 patients at the relapsed stage were P-gp positive, and refractory to chemotherapy. The expression of MDR1 mRNA in P-gp-positive ATL cells was increased at the relapsed stage of one patient. P-gp of this patient was photolabeled with [3H]azidopine and the labeling was inhibited with nimodipine, vinblastine and progesterone. These results suggest that P-gp expressed in ATL cells from patients at relapsed stage has the same binding site(s) for the drugs as that in multidrug resistant cells, and is correlated with the refractory nature of the cells to chemotherapy.

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Leukemia cells from patients. Peripheral blood was collected from 21 ATL patients at the initial presentation and/or at relapse (indicated by the presence of 17% or more of leukemia cells). Lymph node samples were obtained from 4 ATL patients. We examined the correlation between drug-resistance and P-gp expression in these samples.

Preparation of crude membrane fractions. Crude membrane fractions were prepared from leukemia- and KB-cells as follows. Approximately 10³ cells were washed with phosphate buffered saline (PBS), and the pellets were suspended and homogenized with a Dounce glass homogenizer to a concentration of 10⁷ cells per ml. The homogenates were centrifuged at 100,000g for 15 minutes to remove nuclei and cellular debris. The crude membranes were then suspended in 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L NaCl, 1.5 mmol/L MgCl₂, 2 mmol/L NaF, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 3 trypsin inhibitory units/ml aprotinin. The crude membranes were then suspended at 5 mg protein/ml in 10 mmol/L Tris-HCl (pH 7.4) containing 0.25 mol/L sucrose and 1 mmol/L PMSF, and stored at -80°C. The protein concentration was determined by the method of Bradford. Immuno blotting. Monoclonal antibody against hamster P-glycoprotein (C219) originally isolated by Kallner et al was obtained from Centocor (Malvern, PA). Crude membrane fractions (100 to 200 μg protein) were suspended in 50 μL of electrophoresis buffer containing 0.5% (w/v) sodium dodecyl sulfate (SDS) and 10% (v/v) glycerol. Electrophoresis was performed with 7.5% polyacrylamide gel was carried out according to Laemmlli, without heating the sample. Resolution and

MATERIALS AND METHODS

Cultured cells. We used a KB epidermal carcinoma cell line and its multidrug-resistant clone, KB-8-5 and KB-C2 cells as controls. These cells were cultured in a minimal essential medium (MEM) containing 10% newborn calf serum (NCS). KB-8-5 and KB-C2 cells are 4 and 123 times resistant to Adriamycin (ADM) and 18 and 939 times as resistant to VCR, respectively, as the parental KB cells. Based on RNA blot analysis, the MDR1 mRNA levels were found to be 15 and 70 times higher in KB-8-5 and KB-C2 cells, respectively, than those in KB cells. The human ADM-resistant myelogenous leukemia K562 cells (K562/ADM) was a gift from Dr T. Tsuruo (Cancer Chemotherapy Center, Japan). K562/ADM cells that were found to be 134 times more resistant to ADM than the parental K562 cells were shown to overexpress P-gp. This patient was photolabeled with [3H]azidopine and the labeling was inhibited with nimodipine, vinblastine and progesterone. These results suggest that P-gp expressed in ATL cells from patients at relapsed stage has the same binding site(s) for the drugs as that in multidrug resistant cells, and is correlated with the refractory nature of the cells to chemotherapy.

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reproducibility in the Laemmli system was higher than that in the Fairbanks system, as reported by Greenberger et al. and Yoshimura et al. Transfer to nitrocellulose paper was performed as previously described. For immunoblotting, the nitrocellulose paper was blocked with 3.0% skim-milk in buffer A (0.4 mol/L NaCl, Tris-HCl, pH 8, 0.05% Tween-20) for 1 hour at room temperature, and then incubated for 4 hours with IgG containing 3% skim-milk. The paper was washed four times with buffer A, and then incubated for 0.5 pCi/mL of 18S-labeled @-actin probe was performed as an internal standard to compare amounts of RNA loaded.

Slot blot analysis. Total cellular RNA was extracted with homogenization in guanidinium isothiocyanate followed by acid-phenol extraction. Nitrocellulose filter membrane was wetted in 10 x SSC (1 x SSC = 0.15 mol/L NaCl/15 mmol/L sodium citrate, pH 7) before being placed on the slot blotter (Hybri-Slot Manifold, BRL, Bethesda, MD). Samples were applied under vacuum, and the filter was dried at room temperature before it was cross-linked by UV irradiation in a stratalinker (Stratagene, La Jolla, CA). The filters were then hybridized with 10 pg/mL of denatured salmon sperm DNA/mL with 100 pCi of 32P-labeled P-glycoprotein cDNA probe/mL. pMDR1 probe that was contained in a 800 bp PvuI fragment of pBR322 was obtained from Dr M.M. Gottesman (NCI).

After hybridization, the filters were washed four times with 250 mL x 1 SSC/0.1% sodium dodecyl sulfate (SDS) for 15 minutes at 23°C, then washed twice with 500 mL 0.2 x SSC/0.1% SDS for 10 minutes at 50°C. Autoradiographs were exposed for 4 days. Hybridization with 32P-labeled @-actin probe was performed as an internal standard to compare amounts of RNA loaded.

A polymerase chain reaction (PCR) assay for MDR1 gene expression. The purified cellular RNA was first transcribed with reverse transcriptase. Fifty microliter reverse transcription reactions contained 50 mmol/L Tris (pH 8.3), 3 mmol/L MgCl2, 10 mmol/L DTT, 5 µg BSA, 0.5 mmol/L dNTPs, 50 units (U) RNasin, 15 ng Random hexanucleotides primer (Pharmacia, LKB Biotechnology AB, Uppsala, Sweden), 1,000 U M-MLV reverse transcriptase (BRL), and 18-base oligonucleotides were synthesized for use as PCR primers: MDR1 set 1 (target site, 200 bp); 5'-GAAGAAGAGATCGCCGCG-3' (Tg-Actin gene set 2 (target site, 300 bp); 5'-ATGAGAGGAAGTCCAGCC-3'

The following 18-base oligonucleotides were synthesized for use as PCR primers: MDR1 set 1 (target site, 200 bp); 5'-GAAGAAGAGATCGCCGCG-3'

Table 1. Clinical Data

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Abbreviation: NT, Not tested.
minute at 94°C, 3 minutes at 65°C, and 2 minutes at 72°C. The last cycle was followed by an additional 9 minute incubation at 72°C.  

Photoaffinity labeling. Crude membranes were incubated with 0.75 μmol/L [3H]azidopine (53 Ci/mmol) for 15 minutes at room temperature in the presence or absence of various drugs. After continuous irradiation at 366 nm for 20 minutes at 25°C, samples were solubilized in a sodium dodecyl sulfate sample buffer as described previously.  

SDS-PAGE. SDS-PAGE was carried out according to Laemmli. Heat inactivated photolabeled samples were subjected to electrophoresis on 6% polyacrylamide gels. Myosin (200,000), α-galactosidase (116,000), phosphorylase b (97,000) and BSA (66,000), were used as molecular weight standards. Gels were fixed, stained with Coomassie blue and subjected to fluorography using ENLIGHTEN (Du Pont-NEN Research Products). The dried gels were exposed to Kodak XAR film at -80°C.  

Chemicals. [3H]Azidopine (53 Ci/mmol) was obtained from Amersham Corp (Arlington Heights, IL). Nimodipine, vinblastine and progesterone were purchased from Sigma (St Louis, MO).

RESULTS  

Detection of P-gp in cultured cells, leukemia cells and lymph nodes from patients. We measured P-gp in crude membrane fractions from cultured human myelogenous leukemia cells (K562 and K562/ADM) and human epidermoid carcinoma cells (KB and KB-8-5) to ascertain the sensitivity and specificity of our immunoblotting assay system with C219. As shown in Fig 1, K562/ADM cells overexpressed P-gp of two molecular sizes, 150,000 and 130,000 daltons. The two bands were broadened and fused. A much reduced level of P-gp was found in K562 cells. P-gp with a molecular weight of 140,000 to 150,000 daltons was detected in KB-8-5 cells, but not in KB cells. The expression of P-gp in K562 cells was considerably higher than that in KB cells.  

Immunoblotting for leukemia cells (PMN) and lymph nodes (LN) from ATL patients showed that 40% of patients (8/20) were P-gp positive at the initial presentation. The percentage of initial case with P-gp positive leukemia cells was 31.3% (5/16) and that with P-gp positive lymph nodes was 75% (3/4). All 6 patients at the relapsed and refractory stage were P-gp positive. In cases 15 and 16, P-gp was negative at their initial presentation, and became positive at the relapsed stage. The level of P-gp expression in case 15 at the relapsed stage was the highest among 25 ATL cases tested.

A slot blot and PCR assay for MDRI RNA in ATL cells from a patient. The level of MDRI mRNA in the sample from a patient (case 15) at the relapsed stage was measured by a slot blot assay as well as by the PCR assay. RNAs from KB-3-1, KB-8-5, KB-C2 cells were also analyzed as controls. Based on the slot assay, the level of MDRI RNA in the sample of case 15 was higher than that of KB-8-5 but lower than that of KB-C2 (Fig 2A).

Set 1 and set 2 primers were used to detect MDRI and γ-actin gene mRNA, respectively, using PCR. The set 1 primers would amplify 200 bp MDRI cDNA products corresponding to position 4 to 203 of the MDRI cDNA.

Table 2. Clinical Data II

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<th>LDH (W.U.)</th>
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Abbreviations: NT, not tested; I, initial presentation; R, relapsed stage; P, post chemotherapy.
sequence, and the set 2 primers would amplify 300 bp γ-actin gene cDNA products.

When set 1 and set 2 primers were used for the PCR assay, DNA products with the expected lengths were generated. The intensity of the amplified MDR1 cDNA bands for KB, KB-8-5, and KB-C2 correlated very well with the expression of MDR1 mRNA in each cell line as measured by the slot blot assay (Fig 2). There was no difference in the intensity of

Fig 1. Immunoblotting of leukemia cells from 25 ATL patients at indicated stages. Membrane fractions (100 μg protein/lane) from leukemia cells were prepared and subjected to 7.5% SDS-PAGE and immunoblotting. Membrane fractions from KB, KB-8-5 (8 - 5) K562 (K) and K562/ADR (K/A) cells were similarly analyzed.

Fig 2. Analysis of MDR1 expression. (A) Slot blot analysis of RNA extracted from leukemia cells of a ATL patient (case 15). Serial dilutions of 10, 3 and 1 μg of the total RNA were applied to each well. Filter was rehybridized with β-actin probe. MDR1 expression in KB-3-1 (drug-sensitive parental KB cell line), KB-8-5 (low MDR cell line) and KB-C2 (high MDR cell line) is shown as controls. (B) Analysis of MDR1 mRNA by PCR. The reaction products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. DNA fragments generated from single-stranded cDNA synthesized from RNA by reverse transcriptase, were amplified by 30 cycles of PCR with each primer pair. Fragments of 200 bp were generated from cDNA of multidrug-resistant cell lines, KB-8-5 and KB-C2, and leukemia cells, by using MDR1 set 1 primers. Equal amounts of fragments of 300 bp were generated from cDNA of KB, KB-8-5, KB-C2 and leukemia cells from case 15 by using γ-actin set 1 primers.
the band for the γ-actin gene cDNA among KB, KB-8-5, KB-C2, and the leukemia cells. The expression of MDR1 mRNA in the leukemia cells was again considerably higher than that of KB-8-5 based on the PCR assay (Fig 2B).

**Photolabeling with [3H]azidopine.** A photoactive radioactive dihydropyridine analog, [3H]azidopine, photolabels P-gp in the plasma membranes of multidrug-resistant human carcinoma KB cells. The labeling was inhibited by vinblastine and some MDR-reversing agents. The binding site of [3H]azidopine on P-gp appears to be identical to or partially overlaps with that of vinblastine and other MDR-reversing agents.

To determine whether the P-gp in the sample from the ATL patient (case 15) possesses similar drug binding sites, we examined specific labeling with [3H]azidopine of crude membranes prepared from the patient’s leukemia cells. As shown in Fig 3, P-gp in the leukemia cells was photolabeled with [3H]azidopine, and the labeling was specifically and almost completely inhibited by 100 μmol/L nimodipine, and moderately inhibited by vinblastine and progesterone at the same concentration. The photolabeled molecule was not seen in P-gp negative leukemia cells (data not shown).

**DISCUSSION**

The incidence of T-cell lymphoma has been found to be much higher in Japan than in Western and Asian countries. T-cell lymphoma accounts for approximately 75% of all non-Hodgkin’s lymphomas in the southern part of Japan, and most are ATL. T-cell lymphoma, especially ATL, had a poorer response- and survival-rate than does B-cell lymphoma.

One of the reasons for the poor response of T-cell lymphoma to chemotherapy is the emergence of drug-resistant tumor cells. Increased levels of MDR1 mRNA have been detected in leukemias, lymphomas, sarcomas and carcinomas. The high MDR1 RNA levels in renal cell carcinomas appear to be associated with intrinsic multidrug-resistance in the carcinoma cells. Our previous study indicated that the expression of P-gp was closely related to the drug-resistance clinically observed in leukemia patients. Nine of 17 patients with AML and four of 11 patients with ALL had P-gp-positive results at the initial presentation, and most P-gp-positive patients did not respond to chemotherapy. P-gp expression was detected in 6 out of 9 refractory CML patients in blast crisis and in 5 out of 8 cases of refractory AML.

In the present study, we have observed the expression of P-gp in leukemia cells from 40% of ATL patients at their initial presentation. The high rate of P-gp positivity may be attributed to the high sensitivity of the immunoblot assay used in this study. We could detect P-gp in drug-sensitive K562 cells but not in drug-sensitive KB cells. However, even if we define a sample possessing higher level of P-gp expression than K562 cells as P-gp positive, 20% of ATL patients were P-gp positive at their initial presentation. All six ATL patients at their relapsed stage were P-gp positive and refractory to chemotherapy; all six of these ATL patients were P-gp negative at the initial presentation, and responded to chemotherapy. The population of leukemia cells that expresses P-gp may have survived and proliferated during and after the initial chemotherapy. Alternatively, treatment with anti-cancer agents containing vincristine and ADM may have activated the expression of P-gp in the leukemia cells.

Measurements of MDR1 RNA and P-gp in leukemia cells from a patient (case 15) at his relapsed stage indicate that the level of MDR1 RNA was also increased, and the P-gp expressed in ATL cells had similar drug-binding site(s) as that in MDR cells.

P-gp is closely associated with drug-resistance in CML, AML and ALL. The ADM resistance in myeloma and
lymphoma is also predicted with P-glycoprotein staining. Our results indicate that the increased expression of P-gp in relapsed patients may correlate very well with their resistance to treatment.

However, further study of the correlation between expression of P-gp in ATL cells and the clinical response to therapy is needed to establish whether the expression of P-gp, especially at the initial presentation, is correlated with resistance to chemotherapy. If such a correlation can be demonstrated, it may be necessary to change the chemotherapy drugs to those that are not involved in the MDR mechanism, or to use agents that overcome MDR in combination with anti-cancer drugs. Dalton et al. reported that the administration of verapamil along with chemotherapeutic agents partially circumvents the drug-resistance in myeloma and lymphoma patients whose tumors over-express P-gp. Measurements of P-gp expression may be useful for planning chemotherapy for ATL patients.

REFERENCES


