Abstract. There is a controversy over the link between phenotypes of multidrug resistance (MDR) and clinical outcome in leukemia/lymphoma patients. This may be because the process behind the induction and loss of expression of genotypes and phenotypes by which MDR develops and the role of MDR in fresh cells of human leukemia/lymphoma are not clearly defined. P-glycoprotein (P-gp) increased and decreased along with mdr-1 expression in three cell lines out of five vincristine (VCR)-resistant cell lines. MRP appeared with increased mrp expression in the other two cell lines. After the drug was removed from the culture system, mdr-1/P-gp changed in parallel with the level of VCR resistance, although mrp and MRP did not. It was concluded that P-gp is directly derived from mdr-1 and that mdr-1/P-gp supports the VCR-resistance but mrp/MRP is not directly linked to the VCR-resistance. These results should contribute to a better understanding of MDR phenomenon in cancer.

Multidrug resistance (MDR), a major impediment to the treatment of cancer, can occur through altered drug transport. Permeability-related glycoprotein (P-gp) and multidrug resistance-associated protein (MRP) were initially found to cause MDR by altering drug transport (1). P-gp is encoded by the mdr-1 gene, up-regulation of which results in a decreased intracellular concentration of anti-cancer drugs such as anthracyclins, epipodophyllotoxins and vinca-alkaloids. At the cellular level, the function of P-gp has been extensively investigated in many types of cancer cells (2). In leukemia patients, cellular drug resistance profiles determined in vitro at the time of presentation showed a strong correlation with outcome (3, 4), especially in studies using mRNA (5, 6). However, other reports found no such correlation (7, 8).

MRP encoded by the mrp gene was subsequently identified in cell lines showing a typical MDR phenotype without elevated P-gp levels. MRPI is a subtype of MRP, which has been detected in all human tissues and in all cell types from peripheral blood. Like P-gp, MRPI is involved in altering drug distribution within intracellular components in the cytoplasm, leading to altered concentrations of cytoplasmic drugs at their target sites (9). However, MRPI expression at diagnosis was usually not correlated with outcome in the patients with leukemia (4, 7, 8), although mrp expression has been reported to be correlated with clinical response and survival in cases of leukemia (5, 6).

Thus, the role of MRP in determining prognosis in patients with leukemia remains to be understood. In addition, it is not known whether malignant cells that have attained the MDR phenotype can recover their drug sensitivity and how the MDR-related phenotypes behave during the intermission of therapy. These issues may be resolved by clarifying the process by which phenotypes of MDR develop and disappear.

Materials and Methods

Cultured cell lines derived from leukemia/lymphoma. Five cell lines derived from human leukemias and lymphomas were used: K-562 from chronic myelogenous leukemia (10), HL-60 from acute promyelocytic leukemia (11), BL-TH from non-endemic Burkitt’s lymphoma (12), A4 from B-type immunoblastic lymphoma (13) and SKW-3 from T-type chronic lymphocytic leukemia (14). To develop MDR cell lines, cells were made resistant by stepwise incubation using only vincristine (VCR). The cell lines were confirmed to be highly resistant at least to VCR and adriamycin. The cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 μg/mL penicillin-G and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

Chemosensitivity for VCR. To determine the 50% inhibitory concentration for VCR, 0.5-2×10^5 cells/mL in 60 μL of fresh medium containing various concentrations of drug were cultured in a 96-well round bottom microtest plate. On the fourth day of culture, 10 μL of 0.15 mg/mL propidium iodide (Carbiochem, CA, USA) dissolved in phosphate-buffered saline (PBS) containing 1.2% polyoxyethylene octylphenyl ether was added to each well.
After 20 min at room temperature, the fluorescence intensity was measured in a MPV-MT2 automated reading system (Leitz, Germany) (15).

Detection of P-gp and MRP. To detect P-gp, 5×10^5 cells were incubated for 30 min at 4°C with MRK-16 (Kyowa Medics, Tokyo) or mouse IgG (Cappel, PA, USA) as a negative control. Cells were washed and then incubated again with FITC-goat anti-mouse IgG (Ortho, NJ, USA) for 30 min on ice. Cells were analyzed using an Epics XL laser flow cytometer (Beckman-Coulter, FL, USA) (16). To detect MRP, 5×10^5 cells were treated with 2% formaldehyde in acetone for 10 seconds. After two washes with a buffer that consisted of PBS containing 2% FBS and 0.1% NaN3, the cells were subsequently incubated for 30 min at 4°C with a rat monoclonal antibody to human MRP1 (Alexis, CA, USA) or rat serum (Cappel, Ott, USA) as a negative control. After three washes with the buffer, cells were incubated with FITC-conjugated mouse monoclonal anti-rat IgG2a (ARP, MA, USA). They were washed and analyzed using the flow cytometer.

RNA extraction and RT-PCR. Total RNA from 1×10^7 cells was extracted by a one-step acid guanidine thiocyanate-phenol-chloroform method using an ISOGEN-LS total RNA purifying kit (Nippon Gene, Tokyo) (17). RT was carried out for 60 min at 37°C in a total volume of 20 μL containing 26 units of Ribonuclease inhibitor, 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase RNase H Minus with 100 pmol of random primer and 2 μg of total cellular RNA. The polymerase chain reaction (PCR) was performed with 50 ng of cDNA, 25 pmol of each primer, 1 mM dNTP and 1 unit AmpliTaq polymerase in 25 μL of PCR buffer using a GeneAmp PCR system (Perkin Elmer Cetus) (16). The sequences of the oligonucleotide primers for mdr-1 and mrp are described elsewhere (18, 19). The level of mRNA expression was analyzed using NIH image. The expression was shown relative to the intrinsic control, β2MG.

Results

Change of mdr-1 and P-gp expression in VCR-resistant cells transferred to cultures without drugs. Among wild-type cells, mdr-1 is weakly expressed in only K-562, in which 5.3% of P-gp-positive cells was detected. As shown in Figure 1a, the level of mdr-1 in K-562/VCR and HL-60/VCR transferred to cultures without drugs increased transiently in the fifth week and gradually decreased thereafter. However, the considerable expression of the mdr-1 gene lasted until the 25th week. BL-TH/VCR quickly lost mdr-1.
Figure 1b shows the change of P-gp expression in the VCR-resistant cell lines K-562/VCR, HL-60/VCR and BL-TH/VCR after VCR was removed. The percentage of P-gp-positive cells in HL-60/VCR and BL-TH/VCR peaked in the first week of the culture and it was much lower at 25 weeks. In contrast, K-562/VCR retained a high percentage of P-gp-positive cells for at least 25 weeks.

The correlation between P-gp and mdr-1 was analyzed using data shown in Figure 1a and 1b. As indicated in Figure 2, the correlation between mdr-1 and P-gp expression in HL-60/VCR and BL-TH/VCR was significant, although the level of significance was lower in K-562/VCR.
Change of mrp and MRP expression in VCR-resistant cells transferred to cultures without drugs. A positive mrp expression was detected in all five cell lines. However, no MRP-positive cells were found in any of the five cell lines. As shown in Figure 1c, the mrp gene expression in K-562/VCR, HL-60/VCR and BL-TH/VCR transferred to cultures without drugs decreased but was still positive in the 25th week. A4/VCR and SKW-3/VCR retained higher levels than their wild-type counterparts at 25 weeks.

Figure 1d shows the change in number of MRP-positive cells after the transfer to a drug-free culture. Among A4/VCR cells, 9.8% were positive in the first week, increasing to 43.6% in the 9th week and declining thereafter to be 0% in the 25th week. In contrast, 10.8% of SKW-3/VCR cells were positive in the first week, increasing continuously until the 25th week, where the percentage was 68.1%. The correlation between MRP and mrp in these two cell lines was analyzed using data from Figure 1c and 1d. A negative correlation was found as shown in Figure 3.

Change of chemo-sensitivity to VCR in VCR-resistant cells transferred into drug-free cultures. As shown in Figure 4, the relative increase in resistance that each cell line attained as compared to the parental cells ranged widely. The increase in three VCR-resistant cell lines, K-562/VCR, HL-60/VCR and SKW-3/VCR, was respectively 32-fold, 284-fold and 47.6-fold in the first week, increasing to 56.5-, 750- and 85-fold in the 5th week. In the 25th week of culture, the chemoresistance of these cell lines declined respectively to 20.2-fold, 176-fold and 45.5-fold that of the parental cells. In contrast, the resistance of BL-TH/VCR and A4/VCR whose initial increase was 36.1- and 57.6-fold gradually decreased. However, the resistance level of A4/VCR increased again up to 71.4-fold in the 25th week.

Correlation between gene expression and drug resistance. The correlation between gene expression and drug resistance was analyzed. As shown in Figure 5, resistance to VCR in the cells that expressed the mdr-1 gene was obviously correlated with mdr-1 levels, where significant R2 values were obtained in K-562/VCR, HL-60/VCR and BL-TH/VCR. The relation between the percentage of P-gp-positive cells and relative rate of drug resistance was significant in K-562/VCR and BL-TH/VCR. In the HL-60/VCR cells, the drug resistance level in the 1st week affected negatively the significance. However, a significant R2 value for the combination of mrp or MRP and the drug resistance rate was not obtained in any of the five cell lines (data not shown).

Discussion

This study demonstrates a significant correlation between mdr-1 or P-gp and the level of resistance to VCR, indicating that P-gp produced directly from the mdr-1 gene supports VCR resistance. In contrast, MRP expression in the wild-type cells was not evaluated as obviously positive in any of the five cell lines. Two cell lines, A4 and SKW-3, expressed MRP when they became resistant to VCR. However, the MRP level in these cell lines was not precisely associated with the level of drug resistance during the drug-free-culture. Work was performed to find genes that may explain this dissociation among lung resistance protein, lrp, found in the
cytoplasm of tumor cells showing the MDR phenotype (20), bcl-2 which belong to the apoptosis cascade and Wilm’s tumor suppressor gene, wt1, which has been linked to a poor prognosis in leukemia. However, none of the genes could satisfy this purpose (data not shown). Thus, there may be some factors that modulate the process from transcription through translation in the expression of MRP from mdr.

Increased expression of P-gp or mdr-1 has been associated with a poor prognosis for many hematologic malignancies (3-6, 21, 22). In contrast, MRPl expression at diagnosis is usually not correlated with clinical response and survival in patients with AML and ALL (4, 7, 8). The results of the present study may be consistent with the clinical experiences reported and should contribute to a better understanding of the MDR phenomenon in cancer therapy.

References


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