P-Glycoprotein Expression on Normal and Abnormally Expanded Natural Killer Cells and Inhibition of P-Glycoprotein Function by Cyclosporin A and Its Analogue, PSC833

By Motoki Egashira, Norihiko Kawamata, Koichi Sugimoto, Takako Kaneko, and Kazuo Oshimi

P-glycoprotein (P-gp), a transmembrane efflux pump encoded by the MDR1 gene, has been found to be expressed in many normal bone marrow and peripheral blood cells. Among normal leukocytes, CD3+ CD16+ or CD3+ CD56+ lymphocytes, ie, natural killer (NK) cells, express relatively high levels of P-gp, but little is known about P-gp in abnormally expanded NK cells. In this study, we examined the expression and activity of P-gp on NK cells derived from three normal donors, six patients with indolent NK cell-lineage granular lymphocyte-proliferative disorder (NK-GLPD), three patients with aggressive NK cell tumors (one NK cell leukemia and two nasal NK cell lymphoma), and two NK cell lines. By flow cytometric analysis using the monoclonal antibody (MoAb) MRK16 and rhodamine 123 dye (Rh123), P-gp expression and the efflux of Rh123 were found in all NK samples except one NK cell line. The Rh123 efflux of NK cells was inhibited by cyclosporin A (CsA) and its analogue PSC 833, but the aggressive NK tumor cells were less inhibited than were the other NK cells. The percent inhibition of efflux in the normal NK cells, indolent NK-GLPD cells and aggressive NK cell tumors was 81.8% ± 0.9%, 93.4% ± 3.1% and 36.9% ± 11.7%, respectively, by 1 µmol/L CsA, and 80.2% ± 3.6%, 91.7% ± 2.6% and 32.7% ± 10.1%, respectively, by 1 µmol/L PSC833. In reverse transcription-polymerase chain reaction (RT-PCR) analysis, the low inhibitory effect of P-gp modulators in aggressive NK cell tumors did not correlate to the expression level of MDR1 gene, multidrug resistance-associated protein gene, or human canalicular multispecific organic anion transporter gene. This phenomenon could be related to the presence of other transporters or to unknown cellular or membrane changes. Some patients with NK cell tumors have been reported to show a highly aggressive clinical course and to be refractory to chemotherapy, and this could be related to the expression of P-gp on NK cells. Our results suggest that, although the inhibitors for P-gp have been used in combination with chemotherapy in some hematologic tumors, these inhibitors may be less effective against aggressive NK cell tumors.

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Flow cytometric analysis of P-gp and NK-related antigens. The expression of P-gp was measured by immunofluorescence using a P-gp–reactive monoclonal antibody (MoAb), MRK16 (Kyorin Medex, Tokyo, Japan). NK cells were stained with a three-color immunofluorescence method as follows. First, 5 × 10⁶ cells were reacted with 5 µg of MRK16 or IgG2a control antibody for 30 minutes at 4°C. After two washes with phosphate-buffered saline (PBS), the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated F(ab)₂ goat anti-mouse immunoglobulin (Ig). The cells were washed twice and blocked with mouse IgG, then stained with phycoerythrin-cyanine-5 (PC5)-conjugated anti-CD16 MoAb (Immunotech, Marseilles, France) and phycoerythrin (PE)-conjugated anti-CD56 MoAb (Leu-19; Becton Dickinson, San Jose, CA). The P-gp expression on NK cells was analyzed by flow cytometry (CYTORON ABOLUTE; Ortho Diagnostic System, Raritan, NJ), gating on CD16⁺ or CD56⁺ populations.

Measurement of Rh123 efflux. The P-gp–mediated efflux in the NK cells was determined indirectly by measuring the retention of a fluorescent P-gp substrate, rhodamine 123 dye (Rh123; Sigma, St Louis, MO), as previously described.²⁶ Naive, 5 × 10⁶ cells were stained with 150 ng/mL Rh123 for 15 minutes at 37°C and, after two washes, they were incubated in 10 mL dye-free RPMI 1640 containing 10% FCS for 3 hours at 37°C with or without P-gp inhibitors, 1 µmol/L CsA (Sandoz Pharmaceuticals, Basel, Switzerland) or 1 µmol/L PSC833. PSC833 was a kind gift from Sandoz. After the 3-hour efflux period, the cells were labeled with PC5-conjugated anti-CD16 MoAb and PE-conjugated anti-CD56 MoAb, then washed, and immediately analyzed for fluorescence using the flow cytometer gating on CD16⁺ or CD56⁺ populations. The mean channel fluorescence was measured for each sample at the beginning (T0 hour) and end (T3 hour) of the assay. The percent inhibition of efflux was calculated by incorporating the Rh123 fluorescence values into the following formula:

\[
100 \times \frac{\text{mean channel fluorescence (sample in medium T3 hr) - mean channel fluorescence (sample in inhibitor T3 hr)}}{\text{mean channel fluorescence (sample in medium T3 hr)}}
\]

Analysis of mRNA expression and quantification of polymerase chain reaction (PCR) products. The expression of MDR1 gene, multidrug resistance-associated protein (MRP), human canalicular multispecific organic anion transporter (cMOAT), and β2-microglobulin (β2m) were detected by the reverse transcription (RT)-PCR method as previously described.²⁴ Twenty total cellular RNA was extracted from the purified NK cells by using the guanidium thiocyanate method with Trizol (GIBCO BRL) according to the manufacturer’s protocol. cDNA was synthesized with 5 µg of total cellular RNA using Ready To Go Prime First Strand Beads (Amersham Pharmacia Biotech, Buckinghamshire, UK). cDNA derived from 50 ng of RNA was next subjected to PCR for 35 cycles in a final volume of 100 µL using 2.5 U Taq polymerase (Takara, Tokyo, Japan). The contents of reaction mixtures have been described previously.²⁶ The PCR conditions are as follows: after an initial denaturation for 2 minutes at 94°C, each cycle consisted of 30 seconds at 94°C, 30 seconds at 55°C, and 60 seconds at 72°C. The sequences of the primers were: MDR1 forward 5’-CCCCATCAGGAATGCTAGGAG-3’ and reverse 5’-GGTCAAACCTTCTGTCCTGTA-3’; MRP forward 5’-TGGACGTTGAACTGCGC-3’ and reverse 5’-GAGGAATGCCCCAGCTCCTG-3’; cMOAT forward 5’-CCAATGCATTGCATGCTTAG-3’ and reverse 5’-ATGCTGTCCTCCTGCTGG-3’; and β2m forward 5’-ACCCCTCTCTCAGAAAAGATGA-3’ and reverse 5’-CTCTCCACCAAACTCTAGTAG-3’. The PCR products were 167, 293, 275, and 120 bp long, corresponding to MDR1, MRP, cMOAT and β2m, respectively. PCR products were separated on a 2% agarose gel. The bands were visualized by ethidium bromide and photographed. For quantification of MDR1 gene, the density of the bands was analyzed using BIO-CAPT V97 (Vilber Lourmat, Marne La Vallee, France) and ZERO-Dscan (Scanalytics, Billerica, MA). Intensity of MDR1 expression was depicted as a ratio of MDR1 and β2m. The cell lines, K562/ADM and HepG2, were used for positive controls, K562/ADM for MDR1 and β2m, and HepG2 for MRP and cMOAT. These cell lines were kindly provided by Dr Toshiko Motoji (Department of Hematology, Tokyo Women’s Medical College, Tokyo, Japan).

RESULTS

Characterization of the patients studied. The clinical findings of nine patients in our study are summarized in Table 1. Patients 1 through 6 were diagnosed to have indolent NK cell-lineage granular lymphocyte-proliferative disorder (NK-GLPD), patient 7 had aggressive NK cell leukemia, and patients 8 and 9 had nasal NK cell lymphoma. Patients 1 through 6 and 8 are described elsewhere.²⁷²⁸ Patient 9 showed leukemic change. The classification into indolent and aggressive disorders was principally based on their clinical course,²⁷ but the phenotype of NK cells, age, the presence of fever, and hepatosplenomegaly or lymphadenopathy were somewhat helpful for the classification.²⁷²⁹ NK cells were isolated from PBMC in patients 1 through 7 and 9 and from a metastatic lymph node in patient 8. None of the patients had received any treatment at the time when their PBMC or lymph node cells were isolated. Their surface phenotypes of PBMC or lymph node cells were CD16⁺ CD56⁻ in patients 1 through 4 and 8, CD16⁺ CD56⁺ in patients 5 and 6, and CD16⁻ CD56⁺ in patients 7 and 9. All four patients tested with an indolent clinical course, and only one of the three patients with aggressive clinical course had high NK cell-mediated cytotoxicity against K562 target cells. Patients 1 through 6 have been followed for at least 2 years and have exhibited a stable clinical course without any treatment. Patients 7 through 9 had a progressive clinical course and died within 6 months despite the administration of combination chemotherapy.

Expression and function of P-gp. P-gp was expressed on all of the specimens tested except for one NK-cell line, NKL (Fig 1). Two populations, P-gp–positive and -negative, were present in NK cells in patient 6 (Fig 1i). In normal blood NK cells, it is said that three subsets are present, CD16⁺CD56dim, CD16⁺CD56bright, and CD16⁻CD56bright. Because the subset of patients 1 to 6 had indolent NK cell-lineage granular lymphocyte-proliferative disorder (NK-GLPD), patient 7 had aggressive NK cell leukemia, and patients 8 and 9 had nasal NK cell lymphoma. Abbreviations: ND, not done; G, germ-line configuration; 1, indolent; A, aggressive.

*Surface phenotypes of PBMC (patients 1 through 7 and 9) or lymph node (patient 8) are shown in percentages.

†Cytotoxicity was assayed against K562 target cells at an effector-to-target cell ratio of 20:1 with 4 hours of incubation.

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Table 1. Patient Characteristics and Clinical Data

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age and Sex</th>
<th>Percentage of Positive Cells*</th>
<th>NK Cell-Mediated Cytotoxicity</th>
<th>Clinical Course</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CD2</td>
<td>CD3</td>
<td>CD4</td>
</tr>
<tr>
<td>1</td>
<td>62F</td>
<td>98</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>51F</td>
<td>92</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>34M</td>
<td>ND</td>
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<td>17</td>
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<tr>
<td>4</td>
<td>10M</td>
<td>89</td>
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<tr>
<td>5</td>
<td>51F</td>
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<td>6</td>
<td>66M</td>
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<tr>
<td>7</td>
<td>28M</td>
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<tr>
<td>8</td>
<td>57M</td>
<td>98</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>67F</td>
<td>87</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

Patients 1 to 6 had indolent NK cell-lineage granular lymphocyte-proliferative disorder (NK-GLPD), patient 7 had aggressive NK cell leukemia, and patients 8 and 9 had nasal NK cell lymphoma. Abbreviations: ND, not done; G, germ-line configuration; I, indolent; A, aggressive.
CD16<sup>d</sup>CD56<sup>b</sup> was a small population, P-gp was measured in the following two subsets, CD16<sup>+</sup>CD56 and CD16<sup>−</sup>CD56<sup>+</sup>. In all three normal donors, no difference was found in P-gp expression between these two subsets (Fig 1a through c).

P-gp function was measured by Rh123 efflux (Fig 2). After the 3-hour efflux period, P-gp–positive NK cells of all normal donors, indolent NK-GLPD, aggressive NK cell tumors, and those of the NK cell line extruded Rh123, but the P-gp–negative cell line, NK92, did not. Patient 6 had two populations of NK cells, with the presence or absence of efflux function (Fig 2i). In the three normal donors, no difference was found in Rh123 efflux between the subsets of CD16<sup>+</sup>CD56<sup>+</sup> and CD16<sup>−</sup>CD56<sup>+</sup> (data not shown).

Inhibition of P-gp function by CsA and PSC833. Rh123 efflux was examined in the presence of the P-gp inhibitors, CsA and PSC833 (Fig 2 and Table 2). When 1 µmol/L CsA or PSC833 was added, the percent inhibition of efflux in the normal donors, indolent NK-GLPD, aggressive NK cell tumors, and the cell line, NK92, were 81.8% ± 0.9%, 93.4% ± 3.1%, 36.9% ± 11.7% and 93.7%, respectively, by CsA and 80.2% ± 3.6%, 91.7% ± 2.6%, 32.7% ± 10.1% and 84.7%, respectively, by PSC833, indicating that the P-gp of aggressive NK cell tumors was less inhibited by CsA and PSC833 than were other NK cells.

Quantification of MDR1 gene. Next, quantitative RT-PCR for MDR1 gene was performed to examine whether the different effect of CsA and PSC833 on aggressive NK cell tumors was due to a higher MDR1 gene expression. MDR1 mRNA was detected on all the specimens tested except for one NK-cell line, NK92 (Fig 3), but the aggressive NK cell tumors did not show higher MDR1 expression than other NK cells (Fig 4).

MRP and cMOAT expression. To determine the presence of other non-P–gp transport mechanisms, MRP and cMOAT were analyzed by RT-PCR (Fig 3). MRP was detected in NK cells in two of the three normal donors, two of the six indolent NK-GLPD, one of the three aggressive NK cell tumors, and both NK cell lines. cMOAT was not detected in all of the NK cell samples tested. Thus, the low inhibitory effect of P-gp modulators did not correlate with the expression of MRP or cMOAT.

DISCUSSION

In this study, we examined the 14 NK cell samples (three normal, nine abnormal, two cell lines), and all except one (the
cell line, NKL) expressed P-gp. In normal blood cells, as we and other investigators have pointed out, CD56^hi cells highly express P-gp, but P-gp expression on NK cell subsets or their precursors has not been reported.

Using human colon carcinoma and neuroblastoma cell lines, Mickley et al\textsuperscript{31} and Bates et al\textsuperscript{32} found that the expression of MDR1 gene was upregulated with differentiation. NK cells are generated from CD34^hi hematopoietic stem cells under appropriate conditions in vitro and in vivo. Chaudhary et al\textsuperscript{5} have reported that CD34^+ cells express P-gp, but MDR1 expression in normal NK developmental pathway is still an enigma. In normal adult NK cells, it is said that three subsets are present in the peripheral blood, i.e., CD16^bright/CD56^dim, CD16^dim/CD56^bright, and CD16^neg/CD56^bright, and NK cells seem to differentiate inversely from the last to the first.\textsuperscript{30} We investigated the differences of P-gp expression and Rh123 efflux among the two...
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subsets CD16+CD56+ and CD16−CD56+. In all three normal donors tested, no difference was found between these two subsets (Fig 1 and data not shown). In this view, it thus seemed that there was no relationship between the differentiation and the P-gp expression in the late stage of NK cell development.

Many patients with NK-GLPD have a stable clinical course, while some have an aggressive clinical course. Among six patients with indolent NK-GLPD, the phenotype of the NK cells in patients 1 through 4 was CD16−CD56+. These NK cells were considered as mature NK cells and exhibited the same characteristics with normal mature NK cells in terms of P-gp expression and function. The phenotype of the NK cells in patients 5 and 6 was CD16+CD56−, and this phenotype is unusual in NK cells. Two populations, P-gp–positive and P-gp–negative, existed in NK cells in patient 6. P-gp–negative NK cells may exist in some particular population of NK cells.

According to their clinical course, three patients were classified as having aggressive NK cell tumors. As described before,28 we considered the NK cells of case 8 as activated mature NK cells rather than immature NK cells: these NK cells expressed activation markers such as HLA-DR, CD71, CD45RO, CD30 and IL-2Rα, and strong NK activity. On the other hand, NK cells of patients 7 and 9 did not express CD16 antigen, nor did NK activity, suggesting that they were derived from immature NK cells. The MDR1 gene expression level of them was not particularly higher or lower than that of other NK cells. However, because the sample size was too small, it will be difficult to discuss the relationship between NK cell differentiation and P-gp expression based on the present data.

Although the NK cell samples showed various degrees of P-gp expression, all P-gp–positive NK cells functionally extruded Rh123. The discordance between P-gp expression and functional Rh123 efflux, with the failure of Rh123 efflux despite P-gp expression, has been reported in some AML cases,34 but the P-gp–positive NK cells observed in our study and others seemed to be always functional. Several investigators have described the relationship between P-gp expression and NK cell-mediated cytolyis.35-38 Klimecki et al38 suggested that P-gp in NK cells functions as a protective mechanism to remove perforin monomers from the NK cell plasma membrane, or that P-gp is a transporter of cytotoxic factors, which is involved in the killing process. In fact, evidence exists that P-gp participates in the transport of cytokines (IL-2, IL-4, and interferon-γ) in normal peripheral T lymphocytes.39 Our finding of P-gp expression and absent cytotoxicity in patients 7 and 9, however, does not support previous findings of a strong association between P-gp and NK cell function.

In preliminary experiments, the concentrations of CsA and PSC833 in the culture were changed, and 1 µmol/L of CsA and PSC833 was found to efficiently inhibit P-gp function in the cell line, NK92, and in NK cells from one of the indolent NK-GLPD patients (data not shown). We therefore used 1 µmol/L CsA and PSC833 in the present study. The P-gp function in normal NK cells, indolent NK-GLPD cells, and the cell line, NK92, was

Table 2. Rh123 Efflux and Its Inhibition in NK Cells

<table>
<thead>
<tr>
<th></th>
<th>Mean Channel Fluorescence of Rh123</th>
<th>% Inhibition of Rh123 Efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0</td>
<td>T3</td>
</tr>
<tr>
<td>Normal NK cells (n = 3)</td>
<td>123.2 ± 14.6</td>
<td>19.1 ± 3.1</td>
</tr>
<tr>
<td>Indolent NK-GLPD (n = 6)</td>
<td>151.3 ± 11.9</td>
<td>34.6 ± 3.2</td>
</tr>
<tr>
<td>Aggressive NK cell tumors (n = 3)</td>
<td>131.8 ± 9.4</td>
<td>39.6 ± 18.9</td>
</tr>
<tr>
<td>Cell line (n = 1)</td>
<td>203.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

The values of mean channel fluorescence are shown after staining with Rh123 (T0), and after 3 hour efflux without inhibitors (T3), with inhibitors of 1 µmol/L CsA (T3/CsA), and of 1 µmol/L PSC 833 (T3/PSC833). Percent inhibition of efflux was calculated by incorporating Rh123 fluorescence values into the following formula: 100 (T3/CsA or T3/PSC833 – T0)/(T3 – T0).

Fig 3. Analysis of mRNA expression of MDR1, MRP, cMOAT and β2m genes using RT-PCR. Lane (N) is negative control, without cDNA, and lane (P) is positive controls, K562/ADM for MDR1 and β2m, HepG2 for MRP, and cMOAT. Lanes 1 through 3, 4 through 9, 10 through 12 and 13 through 14 are NK cells from the normal donors, indolent NK-GLPD, aggressive NK cell tumors, and NK cell lines, respectively. Lanes 4 through 12 correspond to patients 1 through 9 of Table 1, respectively. Lane 13 is the cell line NK92, and lane 14 is the cell line NKL.
CsA and PSC833. We therefore investigated the sequence of

4). Second, a mutant P-gp must be considered. Chen et al 40

did not modulated by CsA or PSC833. This cell line had a mutant

described a P-gp–positive human sarcoma cell line, which was

did not show higher
did not show higher

did not show higher
did not show higher

did not show higher

did not show higher

did not show higher

did not show higher

did not show higher

did not show higher

P-gp function of original NK cells from this patient.

P-gp function of this cell line was, in contrast, efficiently inhibited by

1 µmol/L CsA and PSC833, but interestingly, P-gp function in the aggressive NK cell tumors could not be inhibited as in the other NK cells (Fig 2 and Table 2). After increasing the concentration of CsA and PSC833, we examined the Rh123 efflux in two of the three cases of aggressive NK cell tumors, patients 8 and 9. When 10 µmol/L CsA or PSC833 was added, the percent inhibition of efflux was increased from 33% to 50% by CsA and from 22% to 65% by PSC833 in patient 8 and was increased from 50% to 65% by CsA and from 42% to 67% by PSC833 in patient 9 (data not shown). Thus, the aggressive NK cell tumors could not be inhibited sufficiently by even 10 µmol/L CsA and PSC833 and seem to be resistant to CsA and PSC833. The cell line, NK92, was established from a patient with an aggressive NK cell lymphoma, and the P-gp function of this cell line was, in contrast, efficiently inhibited by 1 µmol/L CsA and PSC833. It will be intriguing to examine the P-gp function of original NK cells from this patient.

Several explanatory possibilities will be pointed out regarding the question of why the P-gp function in the aggressive NK-cell tumors was not sufficiently inhibited by CsA or PSC833. First, high MDR1 gene expression in aggressive NK cell tumors was expected. However, aggressive NK cell tumors did not show higher MDR1 expression than other NK cells (Fig 4). Second, a mutant P-gp must be considered. Chen et al 40
described a P-gp–positive human sarcoma cell line, which was not modulated by CsA or PSC833. This cell line had a mutant

details in the results of the amino acid phenylalanine at position 335 of P-gp (Phe335), and they suggested that Phe335 was an important binding site on P-gp for CsA and PSC833. We therefore investigated the sequence of DNA from base pairs 1404-1501 containing codon 335 by PCR in patients 8 and 9, but a deletion of Phe335 was not found (data not shown). Last, MDR mechanisms other than P-gp, such as MRP41 and cMOAT,42 may participate in Rh123 efflux. MRP and cMOAT also belong to the ATP binding cassette of drug transporter proteins.41,42 MRP was detected in one of the three aggressive NK cell tumors and also in several other NK cells. cMOAT was not detected in all of the NK cell samples tested. Our Rh123 assay results therefore seem to be unrelated to MRP and cMOAT, but the involvement of other transporter proteins, such as MRP homologues (MRP3, MRP4, and MRP5)43 and lung resistance-related protein,44 cannot be ruled out.

In summary, almost all NK cells expressed P-gp, and their P-gp was functional. Some patients with NK cell tumors have been reported to show a highly aggressive clinical course and to be refractory to chemotherapy, and this could be related to the expression of P-gp in NK cells. When chemotherapy is required for NK cell tumors, it may be necessary to consider using anticancer drugs that are not a substrate for P-gp. For the purpose of P-gp modulation, CsA and PSC833 have been used in combination with chemotherapy in acute leukemia, multiple myeloma, and malignant lymphoma,18-21 and this is an alternative treatment for NK cell tumors, but our results suggest that these inhibitors may be less effective in aggressive NK cell tumors due to the presence of other transporters, or to unknown cellular or membrane changes. Further studies are required to elucidate the relationship between P-gp and NK cells.

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