Prediction of clinical response to chemotherapy by \textit{in vitro} chemosensitivity assay in acute leukemia

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ABSTRACT

Multidrug resistance is a major cause of treatment failure in hematological malignancies. In this study, we investigated \textit{in vitro} chemosensitivity of blasts isolated from patients with acute myeloid leukemia (AML) (n=9) and acute lymphocytic leukemia (ALL) (n=5) using MTT assay. P-gp expression in blast cells was measured by flow cytometry using JSB-1 antibody. There was a very strong correlation between LD\textsubscript{50} values of Ara-C and doxorubicin HCl (r\textsubscript{s}=0.94, p<0.001). There was also correlation between mitoxantrone and doxorubicin HCl cytotoxicities (r\textsubscript{s}=0.70, p=0.12) and between LD\textsubscript{50} values of mitoxantrone and Ara-C (r\textsubscript{s}=0.85, p=0.067). There was not any false negative result in comparing \textit{in vitro} sensitivity with the clinical response. P-gp expression in blasts ranged between 0 and 83\%. Our results indicate that MTT assay is an essential tool for prediction of clinical response in patients with acute leukemia and \textit{in vitro} chemosensitivity testing may be recommended at diagnosis. [Turk J Cancer 2004;34(2):75-80]

KEY WORDS:

MTT assay, acute leukemia, P-glycoprotein

INTRODUCTION

Treatment failures and unsuccessful attempts to treat relapsed patients with acute leukemia have been a major challenge. Drug resistance proteins differing in structure and mechanism of action have been shown to be involved in chemotherapy resistance (1).

Multidrug resistance (MDR) is the innate or acquired resistance of tumor cells to a wide range of chemotherapeutic agents with different mechanisms of action, such as anthracyclins, vinca alkaloids, taxanes and etoposide. The best understood mechanism of MDR involves P-glycoprotein (P-gp), a 170 kDa membrane protein encoded by the MDR-1 gene, which transports chemotherapeutic agents out of the tumor cell. That is, P-gp acts as an efflux pump, excreting drugs from cells that results in drug resistance (2,3).

P-gp is positive around 40\% of adult acute myeloid leukemia (AML) cases at diagnosis while it may also be overexpressed after induction therapy. P-gp expression has been identified as a poor prognostic factor for remission induction and disease free survival in AML (4-8). However, studies with adult acute lymphocytic leukemia (ALL) show varying results on impact of P-gp in drug resistance (9,10). P-gp is overexpressed around 10-20\% of cases. In addition,
it was reported that high expression of MDR-1 was shown not to be in correlation with treatment failure (9).

Among several in vitro cytotoxicity assays, the nonclonogenic MTT assay has been widely used for evaluating in vitro chemosensitivity of leukemic blasts to chemotherapeutic agents. This assay is based on the reduction of the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to a blue formazan product by living cells. The formazan product can be quantitated using a microplate spectrophotometer (11).

This study aimed to compare in vitro chemosensitivity of blasts with in vivo response to chemotherapy. We further analyzed the correlations between LD<sub>50</sub> values (the concentration lethal to 50% of the cells) of drugs and the correlations of LD<sub>50</sub> values with P-glycoprotein expression. Our results indicate that MTT assay is a sensitive tool for predicting in vivo sensitivity to drugs for patients affected by acute leukemia.

**MATERIALS AND METHODS**

**Cells**

Heparinized bone marrow or peripheral blood samples were obtained from 14 patients with acute leukemia. These were 6 with initial AML, 2 with ALL, 3 with relapsed AML, and 3 with relapsed ALL.

Blast cells were isolated by ficoll hypaque density gradient centrifugation. After isolation the cells were washed twice and resuspended in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. We studied samples with ≥ 80% leukemic cells at the start of culture. The cell viability was determined by trypan blue dye exclusion.

**Drugs**

Doxorubicin HCl was dissolved in distilled water and diluted to stock concentration of 5 mM. Mitoxantrone, idarubicin HCl, cytosine arabinoside, and vincristine sulfate were obtained as drugs for injection and were diluted in 0.9 % NaCl to stock concentrations. Stock concentrations were prepared as 5 mM for mitoxantrone and idarubicin HCl, 50 mM for cytosine arabinoside (Ara-C) and 10 µM for vincristine sulfate. Concentrated stock solutions were stored at –20°C. Drugs were diluted daily in culture medium for MTT assay with the highest concentration being 10<sup>–3</sup> times the stock concentration.

**Chemosensitivity assay**

MTT assay was used with minor modifications to evaluate cell viability (12). Briefly, 2x10<sup>4</sup> cells /well in 50 µl were seeded in 96-well plates and 50 µl drug was added into each well. After 72 h incubation, 25 µl MTT solution (1 mg/mL final concentration) was added to each well and the plates were incubated for further 4 h. The produced formazan was solubilized by adding 80 µl lysing buffer of 23% sodium dodecyl sulfate (SDS) dissolved in a solution of 45% N,N-dimethylformamide (DMF) (pH=4.7). After an overnight incubation at 37°C, the optical densities (OD) at wavelength of 540 nm were measured using microplate reader (Organon Teknika Reader 530). Cells incubated in culture medium alone served as a control for cell viability (untreated wells). All assays were performed in quadruplicate and mean ± SD values were used to estimate cell viability. LD<sub>50</sub> value was calculated from the dose-response curve and used as measure for drug resistance.

**P-gp expression measured by flow cytometry**

P-gp expression in blasts was determined by indirect immunofluorescence using JSB-1 monoclonal antibody (MoAb) (Boehringer Mannheim) which has a specificity for an internal epitope (13). Briefly, cells were incubated for 5 minutes in 70% cold methanol at –20°C for fixation and permeabilization. The cells were washed twice and resuspended in phosphate buffered saline (PBS) + 0.05% NaN<sub>3</sub>. After then, 1x10<sup>6</sup> cells were incubated either with the antibody prepared at a final concentration of 10 µg/ml or isotype-matched mouse control IgG (Sigma) for 30 minutes at 4°C and then washed and incubated with fluorescein isothiocyanate-conjugated sheep antimouse IgG serum (Dako) for 30 minutes at 4°C. The cells were then washed and analyzed on a flow cytometer (EPICS 541 Coulter). P-gp positivity was expressed as percent of cells stained. P-gp expression of 10% was considered as cut-off value for MDR positivity (9).
Statistical analysis

The correlation among LD_{50} values of different drugs as well as among P-gp expression and LD_{50} values were estimated using Spearman rank correlation coefficient (r_{s}) analysis.

RESULTS

Relationship between in vitro chemosensitivity and clinical response to chemotherapy

The relationships between in vitro chemosensitivity and in vivo drug response in patients with newly diagnosed acute leukemia are given in table 1. In two patients (patient 1 and 2) complete remission was achieved in accordance with MTT assay. Two patients (patient 3 and 8) that were shown to be resistant to chemotherapeutic agents by in vitro assay died either before therapy or with no response to drugs. In two patients (patient 6 and 7) that were shown to be sensitive by MTT assay, clinical response could not be evaluated because of early death before therapy. Among eight patients with newly diagnosed acute leukemia, partial remission was achieved in two patients (patient 4 and 5) that were shown to be sensitive by MTT assay.

The relationships between in vitro chemosensitivity and in vivo drug response in patients with relapsing disease are given in table 2. All the patients in this group, who had already received chemotherapy before MTT assay, were clinically resistant to treatment. Among these patients, two (patient 12 and 14) were found to be sensitive to the drugs by in vitro assay that was discordant with clinical outcome.

Correlation between LD_{50} values of chemotherapeutic drugs

LD_{50} values of patients for the chemotherapeutic drugs tested are given in table 3. There was a very strong correlation between Ara-C and doxorubicin HCl cytotoxicities (r_{s}=0.94, p<0.001). There was also correlation between mitoxantrone and doxorubicin HCl cytotoxicities (r_{s}=0.70, p=0.12) and between mitoxantrone and Ara-C cytotoxicities (r_{s}=0.85, p=0.067).

Correlation between LD_{50} values of chemotherapeutic drugs and P-gp expression

P-gp expression in patients' samples are given in table 3. Protein expression in blasts ranged between 0 and 83%. Among patients evaluated for P-gp expression, 50% of cases (6/12) were considered to be MDR(+) with expression level > 10%. There was an inverse correlation between

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, sex</th>
<th>Diagnosis</th>
<th>Sample</th>
<th>In vitro sensitivity</th>
<th>In vivo sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43 years, male</td>
<td>AML</td>
<td>PB</td>
<td>S: Mtx, Ara-C, Ida</td>
<td>CR: Mtx, Ara-C</td>
</tr>
<tr>
<td>2</td>
<td>61 years, male</td>
<td>APL</td>
<td>BM</td>
<td>S: Ida, Ara-C</td>
<td>CR: Ida, Ara-C</td>
</tr>
<tr>
<td>3</td>
<td>25 years, male</td>
<td>AML</td>
<td>BM</td>
<td>R: Dox, Ara-C, Ida</td>
<td>exitus before therapy</td>
</tr>
<tr>
<td>4</td>
<td>36 years, male</td>
<td>ALL</td>
<td>BM</td>
<td>S: Ara-C, Dox, Mtx; R: Vin</td>
<td>PR: Mtx, vin</td>
</tr>
<tr>
<td>5</td>
<td>67 years, female</td>
<td>AML</td>
<td>PB</td>
<td>S: Mtx, Ida, Ara-C</td>
<td>PR: Ida, Ara-C</td>
</tr>
<tr>
<td>6</td>
<td>50 years, female</td>
<td>AML</td>
<td>PB</td>
<td>S: Ara-C, Ida, Mtx</td>
<td>exitus before therapy</td>
</tr>
<tr>
<td>7</td>
<td>53 years, male</td>
<td>AML</td>
<td>PB</td>
<td>S: Ara-C, Dox</td>
<td>exitus before therapy</td>
</tr>
<tr>
<td>8</td>
<td>35 years, female</td>
<td>ALL</td>
<td>BM</td>
<td>R: Ara-C, Dox, Vin</td>
<td>NR, exitus</td>
</tr>
</tbody>
</table>

AML: acute myeloid leukemia; APL: acute promyelocytic leukemia; ALL: acute lymphocytic leukemia; PB: peripheral blood; BM: bone marrow; S: sensitive; R: resistant; Ara-C: cytosine arabinoside; Dox: doxorubicin HCl; Mtx: mitoxantrone; Ida: idarubicin HCl; Vin: vincristine sulfate; CR: complete remission; PR: partial remission; NR: no response.
Table 2
Comparison of in vitro and in vivo sensitivity to chemotherapy for relapsing acute leukemia patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, sex</th>
<th>Diagnosis</th>
<th>Sample</th>
<th>In vitro sensitivity</th>
<th>In vivo sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>24 years, female</td>
<td>ALL</td>
<td>BM</td>
<td>S: Mtx; R: Ara-C, Dox, Vin</td>
<td>CNS relaps, exitus</td>
</tr>
<tr>
<td>10</td>
<td>57 years, male</td>
<td>ALL</td>
<td>BM</td>
<td>S: Mtx, Ara-C, Dox; R: Vin</td>
<td>NR, exitus</td>
</tr>
<tr>
<td>11</td>
<td>32 years, female</td>
<td>ALL</td>
<td>BM</td>
<td>S: Mtx; R: Ara-C, Dox, Vin</td>
<td>NR, exitus</td>
</tr>
<tr>
<td>12</td>
<td>30 years, female</td>
<td>AML</td>
<td>PB</td>
<td>S: Mtx, Ida, Ara-C, Dox</td>
<td>NR, exitus</td>
</tr>
<tr>
<td>13</td>
<td>46 years, male</td>
<td>AML</td>
<td>PB</td>
<td>S: Mtx; R: Ara-C, Dox</td>
<td>NR, exitus</td>
</tr>
<tr>
<td>14</td>
<td>40 years, male</td>
<td>AML</td>
<td>BM</td>
<td>S: Ara-C, Mtx, Ida</td>
<td>NR, exitus</td>
</tr>
</tbody>
</table>

AML: acute myeloid leukemia; ALL: acute lymphocytic leukemia; PB: peripheral blood; BM: bone marrow; S: sensitive; R: resistant; Ara-C: cytosine arabinoside; Dox: doxorubicin HCl; Mtx: mitoxantrone; Ida: idarubicin HCl; Vin: vincristine sulfate; NR: no response; CNS: central nervous system

Table 3
LD50 values and P-gp expression in patients’ samples

<table>
<thead>
<tr>
<th>Patients</th>
<th>Ara-C*</th>
<th>Dox*</th>
<th>Mtx*</th>
<th>Ida*</th>
<th>P-gp (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.186</td>
<td>ND</td>
<td>0.530</td>
<td>0.0104</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>8.167</td>
<td>ND</td>
<td>0.395</td>
<td>0.150</td>
<td>74</td>
</tr>
<tr>
<td>3</td>
<td>25.566</td>
<td>ND</td>
<td>0.261</td>
<td>0.395</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>1.615</td>
<td>ND</td>
<td>2.216</td>
<td>0.206</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>1.206</td>
<td>ND</td>
<td>1.55</td>
<td>ND</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>50†</td>
<td>2.5†</td>
<td>ND</td>
<td>0.175</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>50†</td>
<td>2.5†</td>
<td>ND</td>
<td>1.118</td>
<td>26</td>
</tr>
<tr>
<td>8</td>
<td>0.001</td>
<td>ND</td>
<td>0.960</td>
<td>ND</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>50†</td>
<td>2.5†</td>
<td>ND</td>
<td>1.288</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>3.608</td>
<td>0.241</td>
<td>0.003</td>
<td>0.016</td>
<td>83</td>
</tr>
<tr>
<td>11</td>
<td>50†</td>
<td>2.5†</td>
<td>1.880</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>6.507</td>
<td>ND</td>
<td>1.890</td>
<td>0.151</td>
<td>ND</td>
</tr>
</tbody>
</table>

* LD50 value was given as µM of drug concentration determined by MTT assay
** P-gp expression was measured by flow cytometry using JSB-1 MoAb
Ara-C: cytosine arabinoside; Dox: doxorubicin HCl; Mtx: mitoxantrone; Ida: idarubicin HCl; ND: not determined

LD50 values of mitoxantrone and P-gp expression (r=-0.69, p= 0.058). Besides, there was not any correlation between P-gp expression and either LD50 values of Ara-C or doxorubicin HCl.

DISCUSSION

Drug resistance is an important factor that limits the successful treatment of acute leukemia. This study addresses the determination of in vitro chemosensitivity of blasts before chemotherapy. Here, we report the relationship between in vitro sensitivity and in vivo sensitivity of patients to the chemotherapeutic agents.

MTT assay provides a rapid assessment of drug sensitivity. Principally, it is a short-term culture assay that is nonradioactive (12,14). On the other hand, this assay is unable to evaluate cytostatic effect of chemotherapeutic drugs (15). Cellular damage due to cytotoxicity is quantitated by measuring the capacity of viable cells to reduce tetrazolium salt (MTT) into a formazan product. In our experiments with various drugs, 72 hours of incubation was chosen for precise evaluation of cytotoxicity and/or chemoresistance. Incubation duration was also reasonable for control cells’ survival.

MTT chemosensitivity assay has been reported to have high sensitivity (91%) and specificity (78%), besides there
was not any false negative result in our study (16). That is, there was not any case who achieved remission despite prediction of resistance in MTT assay. The main limitation for comparison with the clinical response was early death of some newly diagnosed cases before chemotherapy (patients 3, 6, 7). In relapsing patients, two cases with AML (patients 12 and 14) were resistant to therapy in spite of in vitro sensitivity to the drugs. This discordance may be due to in vivo factors and other poor prognostic factors such as cytogenetic abnormalities and prior treatment failure in AML as well. A significant inverse association exists between MDR1 expression and the achievement of complete remission in patients with acute leukemia (4,10). Accordingly, patient 12 had a remarkably high level of (%83) P-gp expression. Nevertheless, in vivo resistance cannot be attributed solely on P-gp expression. MDR proteins other than P-gp may contribute to multidrug resistance in tumor cells (17,18). In evaluating the relative contributions of MDR proteins to prognosis in clinical trials, functional tests have been widely used (19). In a recent consensus, functional tests were recommended to evaluate MDR gene activity rather than mRNA expression. Therefore, discordance between in vitro results and clinical response should be regarded with caution and further evaluation of MDR proteins’ activities along with proteins’ expression in patients may improve clarifying mechanisms underlying drug resistance.

MDR phenotype confers a cross-resistance status to tumor cells against chemotherapeutic drugs of different mechanisms of action. With this purpose, we evaluated the correlations between chemosensitivities of drugs by comparing LD50 values. These values were further analysed for correlation with P-gp expression.

Ara-C is a nucleoside analogue which is not a substrate for P-gp. There is a weak correlation between cytotoxicities of Ara-C and the other MDR drugs. In contrast, we found a significant correlation between LD50 values of Ara-C and doxorubicin HCl. There was also a correlation between LD50 values of Ara-C and mitoxantrone, the latter of which is also not a P-gp substrate. These observations together support the idea that, more than one mechanism of drug resistance may co-exist in blasts (20).

There was no correlation between P-gp expression and in vitro Ara-C chemosensitivity. This finding is in concordance with the mechanisms involved in Ara-C resistance which are unrelated to P-gp (21,22).

In our study, we did not observe a correlation between LD50 values of doxorubicin HCl and P-gp expression. Doxorubicin HCl is a well-known P-gp substrate that exerts cytotoxicity mainly by intercalating with DNA or by inhibition of topoisomerase II. In multidrug resistant cells, factors that enable the drug to reach nuclear targets may have much more impact on drug resistance than P-gp dependent drug efflux (23). Nevertheless, less number of samples in this study necessitates further examination of this correlation.

We found an inverse correlation between LD50 values of mitoxantrone and P-gp expression. Mitoxantrone-resistant HL-60 cells do not display P-gp overexpression (24). Then, this inverse correlation can be explained with the drug’s not being a P-gp substrate. Additionally, correlation between LD50 values of mitoxantrone and doxorubicin HCl points out multifactorial mechanism underlying drug resistance in leukemia.

In conclusion, MTT assay may be recommended as a sensitive tool for prediction of clinical response in patients with acute leukemia. Drug resistance mechanisms contributing to treatment failure in acute leukemia should be examined utilizing functional assays along with the determination of MDR proteins in blasts either at protein or transcriptional level. The further clarification of mechanisms will lead to selection of circumvention strategies by specific resistance modifiers.

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References


