THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

NEW METHODS IN THE DIAGNOSTIC AND PROGNOSTIC EVALUATION OF ACUTE LEUKEMIAS

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INTRODUCTION

The correct diagnosis and determination of the exact type of acute leukemia is necessary for the selection of appropriate treatment protocol and thus can contribute to the effectiveness of chemotherapy. A complex genetic and immunological characterization of leukemic cells has diagnostic significance but it also enables the detection of residual leukemic cells (minimal residual disease – MRD) and the early recognition of relapse. In order to achieve these important features a multiparametric analysis of the malignant cells - providing both structural and functional information - is required. In our studies technical conditions of sample preparation in the detection of intracellular markers were investigated in order to establish the optimal combination of permeabilizing reagent, antibody clones and fluorochromes. The possible diagnostic importance of a new marker CD162 (P-selectin glycoprotein ligand-1, PSGL-1) in the identification of AML subtypes was also evaluated.

Detection of different prognostic factors at the time of diagnosis can provide information about possible treatment outcome and can also influence the selection of appropriate chemotherapy. Drug resistance mechanisms - among which the presence and activity of membrane transporters is believed to be

the most frequent cause - as prognostic factors were also investigated. Membrane transporters extrude several chemically unrelated molecules from the cells by active pump mechanism. Among their substrates are numerous cytotoxic drugs that are used in the treatment of malignant hematological disorders. As a result of the activity of these transporters cells become resistant to several type of cytotoxic drugs – this phenomenon is called multidrug resistance (MDR).

Diagnostic importance of immunophenotyping

In the diagnostics of hematological malignancies and detection of MRD directly labeled monoclonal antibodies measured by flow cytometry is the method of choice. The detection of intracellular markers is the most reliable tool for identification of malignant lineage since the earliest and most specific markers for the different lymphoid and myeloid cell lineages are frequently absent from the cell surface but usually detectable at the intracellular level. The most commonly used markers to differentiate between myeloid, T and B cell lineage are MPO, cy CD3 and cyCD79a. Appropriate selection of the permeabilizing solution, monoclonal antibody clones and fluorochromes can provide a sensitive and specific procedure in lineage assessment.

Though hematological malignancies can usually be classified by the commonly used markers it is also important to find new markers that enable us to differentiate undoubtedly between leukemia subtypes. Besides multiple labeling of the cells (three and four color analysis) a new diagnostic approach is the application of markers that are present on the cell surface in different intensity depending on cellular maturation. Quantitative differences in the expression of several markers like CD10, CD34 etc. on normal and leukemic cells have been demonstrated. Molecules of particular interest are the ones that have a role in the functions of malignant cells like adhesion, aggregation, transendothelial migration (integrins, selectins and selectin ligands). One of these proteins is the P-selectin glycoprotein ligand-1 (PSGL-1, CD162) that is a counter receptor for all three selectins.

Multidrug resistance mechanisms

Immunological detection of membrane proteins does not always correlate with their function so it is biologically more relevant to detect the functional activity of these proteins. It is particularly valid for the detection of membrane transporters since their activity can confer drug resistance.

Membrane transporters work as an ATP dependent efflux pump and are able to remove hydrophobic molecules including cyctotoxic drugs and dyes from the cells. This activity maintains the level of a wide range of currently used

antineoplastic drugs below the cell-killing threshold. These transporters belong to the large family of ABC transporters. Their common feature is the ATP binding cassette region localized in the intracellular part of the protein. Unfavorable prognostic effect like therapy failure and decreased survival rates in AML have been proved by several studies. The most important proteins in this group are the P-glycoprotein (Pgp), members of the MRP family (multidrug resistance related proteins) and the recently described BCRP (breast cancer related protein).

There are at least three different approaches to detect MDR: (i) functional tests measuring drug or dye efflux/accumulation, (ii) detection of expression level by monoclonal antibodies with flow cytometry or immunocytochemistry (iii) detection of mRNA levels for transporter proteins. Functional tests measure the transport activity of the protein, that is the clinically relevant parameter.

AIM OF THE STUDIES

1. Diagnostic investigations (immunophenotyping):

a. To determine the optimal combination of permeabilization reagent, fluorophor and antibody clones that provides the most sensitive and specific detection of the lineage

b. To evaluate the possible diagnostic significance of a surface marker P-selectin glycoprotein ligand-1 (PSGL-1, CD162) that has not yet been used as a marker in leukemia diagnostics.

2. Investigation of prognostic markers (multidrug resistance)

a. To adapt and optimize the calcein assay as a routine diagnostic tool

b. To determine the MDR activity in different cell populations and to examine the correlation between MDR activity and antigen expression

c. To determine the prognostic role and predictive value of the calcein test on a large cohort of de novo acute leukemia patients

MATERIALS AND METHODS

Permeabilization and staining procedures

A total of 21 normal and acut leukemic samples were measured and the comparative analysis of 6 commercially available permeabilization/fixation kits was performed in two centers (University of Debrecen, Department of Clinical Biochemistry and Molecular Pathology and Cytometry Unit of the University of Salamanca). All acute leukemia patients were studied at the onset of the disease before chemotherapy. The permeabilization kits were used strictly according to the recommendation of the manufacturers. During the study Cytofix/Cytoperm (Pharmingen), Fix and Perm (Caltag Laboratories), Intraprep (Immunotech), Intrastain (DAKO), Permeacyte (Bio-E) and the Permeafix (Ortho) kits were compared.

The most important lineage specific intracellular markers, MPO, cyCD79a, cyCD3 and their FITC (fluorescein isothiocyonate) and PE (phycoerythrin) conjugates were detected in all samples using direct immunofluorescence technique. In leukemic samples the selective analysis of malignant cells was provided by surface CD45 staining and cells with dim CD45 labeling were selected. Measurements were carried out on FACScan and FACSCalibur flow cytometers. Statistical analyses were performed using Wilcoxon and Kruskal –Wallis tests.

Quantitative determination of PSGL-1 molecule

P-selectin glycoprotein ligand-1 (PSGL-1, CD162) mediates leucocyte rolling on endothelial cells covered by P- and E selectins and it is the major counter receptor for leucocyte L-selectin. PL-1 neutralizing monoclonal antibody to PSGL-1 and PE conjugated anti-CD162 KPL-1 (BD, Pharmingen, CD162) antibody was used for the detection of the molecule. The PL-1 was applied in quantitative flow cytometry tests, while the KPL-1 PE conjugate was used in 3-color stainings.

Altogether twenty-five normal and twenty leukemic samples were analyzed (peripheral blood and/or bone marrow). To measure the quantitative expression of PSGL-1, calibrated beads (QIFI kit DAKO) were used: PSGL-1 copy number was calculated by determining fluorescence intensity at the FL-1 channel. Results were expressed as antibody binding capacity (ABC) obtained from the calibration curve using beads coated with fix amounts of antibodies and subsequently stained with the same secondary reagent. Samples were measured with constant voltage settings on Becton Dickinson FACScan flow cytometer. Leukemic blasts for PSGL-1 expression were selected by backgating CD45 dim cells on to FS-SS dot plot, while in normal bone marrow samples CD33/CD34 double positive cells were

analyzed. Receptor numbers in patients and control subjects and in different leukocyte subsets were compared using Mann-Whitney U test.

Calcein assay

Calcein–acetoxymethylester, the hydrophobic derivative of calcein passively enters the cells and is actively extruded from resistant cells by both Pgp and MRP1. The consequent reduction in the cellular accumulation of the fluorescence free calcein relative to that seen in the presence of an inhibitor e.g verapamil provides a quantitative measure of the transport activity. If these transporters are not present or not work, fluorescent calcein is produced by cellular esterases. Results were expressed as multidrug resistance activity factor (MAF) calculated by the formula [FI(Vp+) - FI(Vp-)]/FI (Vp+) x 100 that measures the transport activity of both Pgp and MRP1, and the results are correlated with the level of extrusion of Pgp/MRP1 substrate cytotoxic drugs. Cells were loaded with calcein-AM following an incubation period with or without the inhibitor (verapamil). After a centrifuge step propidium-iodid labeling was applied to detect the viability of the cells. Samples were stored at 4°C and measured within 6 hours on a Becton Dickinson FACScalibur or FACScan flow cytometer.

Sample storage for calcein assay

In functional tests the viability of the cells has particular importance and ATP supply for the cells has to be provided. In order to test the duration of appropriate sample storage that has no effect on MFI and MAF values, whole bone marrow samples and isolated calcein loaded mononuclear cells were stored and analyzed at different time points. Both sample types were stored at room temperature and 4°C up to 24 hours.

The combination of calcein assay with cell surface staining

The pan leukocyte marker CD45 was used in our experiments, that shows low expression on immature cells. CD45dim cells were selectively analyzed that refers to the MAF value of blast cells.

Determination of Pgp copy number

For the quantitative determination of Pgp number, the calibration curve of the QIFI kit beads were used. The beads were run simultaneously with each measurement by using constant voltage settings. Results were expressed as antibody binding capacity (ABC). The MRK-16 antibody reacts with external loops of the PgP-MDR1 and thus has been shown to be a suitable clone for the quantitation of surface associated PgP. ABC values and MAF values were compared on MDR+ cell lines and clinical samples.

Patients and chemotherapy

The study was performed in two hematological centers, at the University of Debrecen and at the National Institute of Hematology and Immunology, Budapest. Samples of 93 untreated do novo acute leukemia patients were analyzed. Immunophenotypic analysis of surface and intracellular markers was performed using standard three-color methodology. Patients were uniformly treated with the 7+3 remission induction protocol, consisting of cytosine – arabinoside 200 mg/m^2 for 7 days and anthracycline for 3 days (daunoblastin 60 mg/$m^{2,}$ adriamycin 45 mg/m^2 or idarubicin 12 mg/m^2) supplemented with 100 mg/ m^2 etoposide in the case of AML M4 and M5. Patients were given 1 or 2 cycles of the above protocol depending on the response to therapy. To evaluate treatment efficiency, bone marrow and peripheral blood were examined morphologically at 4 weeks. Differences for significance between groups were analyzed by unpaired Student's t-test. Multiple logistic regression models were used for evaluating therapy response and Cox regression was used for analysis of survival data. Kaplan-Meier survival curves were evaluated statistically by log-rank test.

RESULTS

Comparative analysis of permeabilization techniques

1. Light scatter signals and autoflorescence

All fixation/permeabilization kits induced considerable change in forward (FSC) and side (SSC) scatter characteristics that is particularly striking on FSC-SSC dot plots in comparison to the plots of fixed but not permeabilized normal peripheral sample. Analyzing the numerical values of FSC in each cell type significant differences were obtained with all six permeabilizing agents and it was most remarkable in the population of peripheral and bone marrow lymphoid cells. Increments in autofluorescence levels as recorded through MFI values of the FL1 and FL2 channel were detected in case of all permeabilizing reagents.

Myeloperoxidase

Comparing the MFI values of three clones (MPO-7, CBL-MPO-1 and H-43-5) the sensitivity of MPO-7 clone was significantly higher in contrast to the two other clones. Pairwise comparison of FITC and PE conjugates of the same clone revealed that PE conjugates yielded significantly higher MFI values. False positive reaction was seen following the use of Cytofix/Cytoperm reagent where all three anti-MPO clones stained B-ALL and T-ALL blasts dimly.

3. cyCD79a

The Pharmingen CD79aPE monoclonal antibody yielded significantly lower MFI than the DAKO and Immunotech CD79aPE. Similar percentage of positive cells were detected in all samples and none of the anti-CD79a clones showed false positive reactions with T-ALL or AML blasts.

4. cyCD3

The S4.1 clone yielded significant false positive reactions therefore further analyses were restricted of two other anti-CD3 clones where UCTH-1PE clone showed significantly higher MFI values and percentage of positive cells were also more than in case of Hit3aPE clone.

5. Permeabilizing solutions

Sensitivity of the permeabilizing solutions were investigated comparing the MFI values of positive cells of each antibody pooled for the different fixation/permeabilization reagent, respectively. No significant differences were found in MFI values obtained with the different permeabilizing solutions.

Quantitative PSGL-1 determination on normal and malignant cells

1. PSGL-1 expression on normal hematopoietic cells

Based on the examination with normal peripheral blood cells B lymphocytes showed low CD162 labeling while PSGL-1 expression of T and NK cells

was significantly higher. Monocytes and neutrophils displayed intensive CD162 labeling. In normal bone marrow cells PSGL-1 expression was stronger in mature cells than in myeloid precursors and the expression of the molecule was increased during the maturational process. Monocytic cells showed significantly higher CD162 expression comparing to granulocytes.

2. Quantitative data of PSGL-1 expression

Numerical values of PSGL-1 copy number were analyzed in order to determine the exact differences between cell populations. The following results were revealed:

a. Leukemic myeloblasts showed significantly lower PSGL-1 expression than mature cells.

b. Monocytic precursors displayed CD162 labeling in a broad range that did not prove significantly different from that of mature monocytes.

c. Numerical values showed significantly higher CD162 expression on monoblasts contrary to myeloblasts and there was no overlap between the values of the two populations. Based on this observation we suggest that quantification of surface PSGL-1 may aid in differentiating these two subsets of leukemic cells: low CD162 labeling in blast cells refers to the presence of myeloblasts (AML M1/M2). In AML M4 cases PSGL-1 staining

of myeloid cells dislayed a bimodal pattern with a PSGL-1 dim (myeloblast) and PSGL-1 bright (monoblast) population.

d. PSGL-1 copy number on normal myeloblasts was not different from that observed on AML blast cells.

e. Simultaneous measurements were carried out on bone marrow and peripheral blast cells and ABC values was not different using paired comparison in the bone marrow and peripheral blasts.

Calcein assay: preanalytical variables, combined stainings

1. Sample storage

In case of whole bone marrow or peripheral blood samples stored up to 24 hours results were compared to that of freshly obtained materials. Samples stored up to 6 hours still provided comparable MAF values, but the 24 h MAF values were significantly decreased. In these samples minimal PI positivity was obtained, but due to ATP depletion the effectiveness of the efflux deteriorates that leads to higher MFI(Vp-) values. Isolated and calcein loaded mononuclear cells can be stored up to 24 hours at 4°C without significant change in MAF values since efflux pumps are effectively blocked at this temperature that results in constant MFI(Vp-) values.

2. Combination of calcein assay and cell surface staining

In order to investigate only leukemic blast cells, CD45dim cells can be gated and selectively analyzed for calcein efflux. Calcein is a very strong fluorescent dye that causes intensive fluorescence spillover at the FL-2 channel as well. However by applying an appropriate compensation calcein assay can be combined with surface antibody staining by using CD45PerCP or CD45Cy5 conjugates that are detectable at FL-3 channel (>650 nm).

3. Determination of Pgp molecule number by monoclonal antibodies and quantitative flow cytometry

Several studies examined the correlation between MDR activity and Pgp expression. Some of them have found discordant results between the two methods that can be explained by the facts that functional tests measure the activity of more than one transporters while monoclonal antibodies provide information about one protein only (efflux+/Pgp- cases). In addition the transporter may be present in a non-functional form, or the antibody can cross-react with MDR3 that can lead to further discordant results (efflux-/Pgp+cases). Comparing the results of the indirect immunofluorescent assay and MDR activity we found that in a high Pgp expressing cell line (KBV1) 500.000 Pgp molecules/cell correspond to a MAF value of 98, while in the low MDR+ (KB8-5) cell line a MAF value of 80 is obtained at 36.000 Pgp

molecules/cell. This means that MAF values were still high even if Pgp copy number significantly decreased. This is particularly striking in clinical samples where in a non-responder and a responder AML patient MAF values corresponded to the clinical state. However Pgp antigen assay showed minimal change both in the ratio of fluorescence intensity (ΔMFI = MFI of MRK16/MFI of isotype control) or % positives and ABC values were below the lowest calibration point in both cases. In case of clinical samples where MAF values are between 0 and 60 (usually < 40) the results of the quantitative antigen test are in a narrow range at the lower part of the calibration curve. This finding not only relates to the enhanced sensitivity of using MAF values with clinical samples but also refers to the potential importance of MRP1 in addition to Pgp in AML.

Relationship between test results and clinical outcome

1. Comparison of MAF values in AML and ALL patients

Results were in agreement with the published data: de novo MDR is more frequent in AML than in ALL patients. Differences between MAF values of two groups were significant in both centers.

2. Relationship between MAF values and response to remission induction therapy: predictive value of the test

In order to determine the cut-off limits, the 65 patients with AML were divided into two groups on the basis of their response to remission induction chemotherapy. Based on the distribution of MAF values in responders (R) and non-responders (NR) cut-off values were eastablished between the (MAF_R+SEM) and (MAF_{NR} - SEM) values (SEM: standard error of mean). These cut-off limits were 25 and 20 in the Debrecen and Budapest center, respectively. By using the respective cut-off values, patients were divided into MDR positive and MDR negative groups, and the percentage of responders and non-responders in both groups were calculated by pooling AML samples of both centers to determine the positive and negative predictive value of the test. MDR negativity showed a high predictive value for therapy response (72%), while the predictivity of MDR positivity for therapy failure was 69%.

3. Relationship between test results and survival rates.

Only patients with 8 months or longer follow–up periods were included in this investigation. Correlation between MDR activity and long term disease outcome was analyzed using Kaplan-Meier curves. Although the difference between the two groups did not reach a statistically significant level in the log rank test (p=0.07), MDR negative cases displayed a 3-fold increase in 50% survival rate compared to MDR positive cases.

DISCUSSION

The identification of the lineage, the determination of type and maturational stage of the leukemic cells has crucial importance in the diagnostics of hematological malignances since classification of leukemia/lymphoma becomes possible by the exact diagnosis. The most reliable tool for lineage assessment is the application of intracellular markers among them MPO, cyCD3 and cyCD79a are most commonly used for the identification of myeloid, T and B lymphoid lineages. To examine the sensitivity and specificity of the permeabilizing techniques, combination of six commercially available permeabilizing reagent, different monoclonal antibody clones and their fluorescent conjugates were compared. Though significant changes in FSC-SSC signals and increments in autofluorescence were detected in all cases the clear discrimination between the major leukocyte subsets still remained possible.

Based on our results the myeloid, B and T lymphoid cell lines can undoubtedly be identified by the above intracellular markers (MPO, cyCD79a and cyCD3) if one of the permeabilizing kits is used (Fix and Perm, Intrastain, Intraprep, Permeafix) combined with a PE conjugated sensitive monoclonal clone (MPO-7, HM57, UCTH-1).

Though there are several markers that has importance in the identification of leukemia subtypes and differentiation between granulocyte, monocyte and megakaryocyte lineages, it is also important to find further markers that are lineage specific or show variant expression on different cell lines. The CD162 molecule investigated by quantitative flow cytometry seem to be useful in the classification of AML subtypes. Expression of PSGL-1 has already been detected on different cell subsets but there are no data about the quantitative expression of the protein. Significant differences between leukemic myeloblasts and monoblasts were found and there was no overlap between the ABC values of the two populations which refers to the possible role of PSGL-1 determination in leukemia diagnostics: it may aid in differentiating AML M1/M2 and M4/M5 subtypes. It should be used additionally to other markers since CD14 displays positivity only in half of the AML M4 cases and sometimes it can be detected in other subtypes - like AML M2 - as well.

In the clinical oncology there is an increasing need for the detection of drug resistance of malignant cells as an important prognostic factor and it can also indicate the use a reverting agent in addition to chemotherapy. A quantitative functional test, the calcein assay was used in samples derived from 93 acute leukemia patients in a two-center study. Since calcein-AM is

the substrate of both Pgp and MRP1, using verapamil as inhibitor - that blocks both proteins - transport activity of both Pgp and MRP1 is detectable simultaneously. In order to improve the selectivity of the results double staining with CD45 or CD34 markers and analysis of CD45 dim or CD34 positive cells is recommended that provides the exclusion of normal cells from the measurement. In selected cases both MDR activity (MAF value) and Pgp molecule number/cell were determined. The correlation between the results of the two methods was not linear and the immunological detection by itself does not seem to be sensitive enough to differentiate between MDR positive and MDR negative cases. Analyzing 93 samples from acute leukemia patients MDR negativity was highly predictive for a successful clinical response to chemotherapy (72%) and had a prognostic value for better survival while 69% of MDR positive cases detected by calcein assay is predicted to therapy failure and shorter survival in AML.

Our investigations have included the characterization of new methods which contribute to the accurate and exact diagnosis of leukemias and to the evaluation of prognostic factors. Our statements were made in the following areas: (i) optimal combinations of permeabilizing agents, monoclonal antibodies and their fluorochromes in the detection of intracellular antigens (ii) diagnostic significance of PSGL-1 expression in AML subtypes using

quantitative flow cytometry (iii) optimization and clinical utilization of calcein assay used for he detection of multidrug resistance. All of these techniques together with commonly used methods contribute to the complex characterization of leukemic cells and to the success of chemotherapy.

Publications included in the thesis:

I. **Karászi É**, Jakab K, Homolya L, Szakács G, Telek B, Kiss A, Rejtő L, Nahajevszky S, Sarkadi B, Kappelmayer J.: Calcein assay for multidrug resistance reliably predicts therapy response and survival rate in acute myeloid leukaemia. *Br J Haematol* 2001; 112: 308-314 IF: 3,068

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5. Telek B, Rejtő L, Mezei G, **Karászi É,** Kappelmayer J, Balázs M, Kiss A, Ujj Gy, Rák K, Udvardy M.: Molekuláris biológiai vizsgálatok krónikus lymphoid leukémiában *Orvosi Hetilap* 2001; 142: 833-839