Tapping an unexploited repository: Carnoy's fixed cell pellets for proteomic biomarker research in leukemia

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Abstract. For chromosomal analysis in tumor genetics, cells from blood and bone marrow are prepared and preserved virtually indefinitely in Carnoy's fixative (methanol/acetic acid). Numerous samples are stored unvalued in hospitals and institutes worldwide. We developed a method to analyze proteins from even a small amount of these cells by mass spectrometry using affinity chromatographic surfaces (SELDI), and demonstrated the application of proteomic biomarker research in cases of acute myeloid leukemia.

Introduction

Acute myeloid leukemia (AML) is characterized by specific cytogenetic aberrations. This leads to the assumption that the proteome also differs in its specific expression patterns and post-translational modifications (1,2). Therefore, protein-based biomarkers in blood or bone marrow cells have great potential as new diagnostic markers in hematological neoplasms. They should be able to serve as early predictive markers at initial diagnosis, help to monitor disease stage throughout therapy and allow the prediction of disease outcome.

For proteomics, blood or bone marrow can be divided into extracellular and intracellular components. The extracellular proteome consists of high-abundant serum-specific proteins such as albumin and of proteins released from cells as a result of damage, cell death or abnormal secretion. Over 10,000 different proteins with high variability have been estimated to be commonly present in plasma; their concentrations range over at least 15 orders of magnitude, including many low-abundant proteins (3). This high range hampers the analysis of low-abundant peptides or proteins. Fractionated plasma can be used for enhanced perfomance. In the case of the proteomic analysis of leukemias, plasma or serum offers the advantage of being usable irrespective of the percentage of blasts. The intracellular proteome, especially of leukemic cells, should be more characteristic and therefore more suitable for the identification of biomarkers for diagnosis, stratification and prognosis. Here, the number of blasts might be an important factor.

Until now, few studies using either plasma or cells have been performed. Albitar *et al* (4) analyzed plasma from acute lymphoblastic leukemia (ALL) patients with surface enhanced laser desorpotion/ionization (SELDI) technology and found a high prognosis for recurrence. They reported that peripheral blood plasma is adequate for the prediction of clinical behavior in patients with ALL, irrespective of the percentage of bone marrow blasts. Bone marrow cells were analyzed by different groups mainly with two-dimensional gel electrophoresis, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS), SELDI and tandem MS. Balkhi *et al* (5) identified significant differences in the proteome and post-translational modification (PTM) of peptides between specific cytogenetic groups [e.g. PRKAC for 11q23 and JUN and MYC for inv(16)]. In two other studies, proteomic differences between leukemia subtypes were found (6,7). Lopez-Pedrera *et al* (8) identified significantly altered proteins including suppressors and signal transduction mediators, which are associated with malignancy.

A well-established tool for protein profiling is SELDI technology, which can be used for the analysis of serum and cells (9). SELDI utilizes chips with affinity surface coatings to specifically retain proteins based on their physicochemical characteristics prior to TOF MS analysis. Crude protein lysates or catapulted specimens can be applied directly onto the chip surface. The desired proteins are retained on the chromatographic surface and contaminants, such as buffer salts or detergents, are washed off. Therefore, this technique is quantitative and more sensitive compared with MALDI systems (10,11). In exchange, the resolution is lower, but this can be disregarded as the instrument is not primarily used for the exact measurement of peptides but rather for the comparison of two different protein lysates (e.g. normal vs. tumor tissue). SELDI can be used to complement 2-DE for biomarker research since small proteins as well as proteins with an extreme pI are detectable. For SELDI-MS, only a small amount of cells is needed, making the technology ideal for small cell numbers or microdissected tissue samples (12,13).

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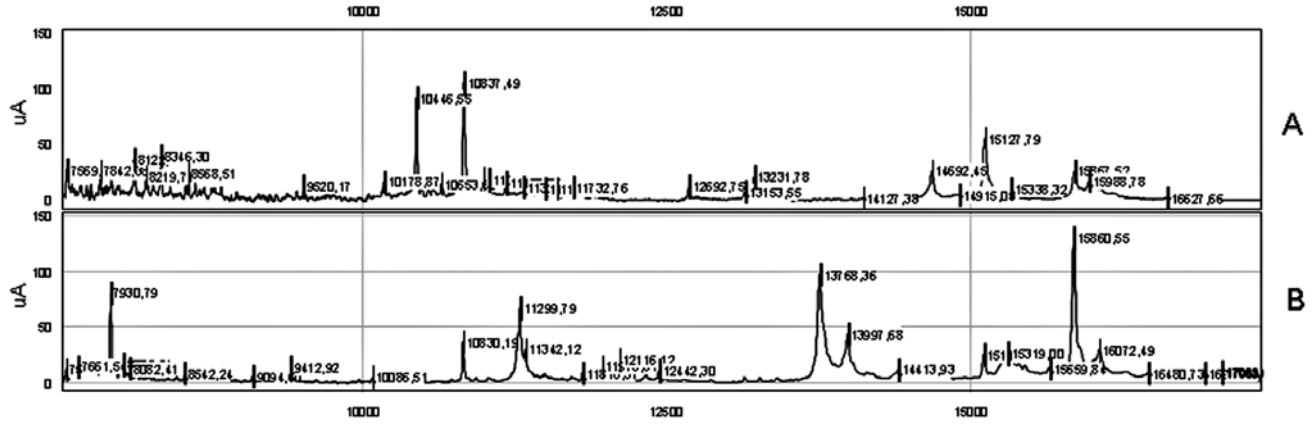

Figure 1. Representative spectra of a cell lysate from native (A) and acetic acid/methanol-fixed (B) leukemic cells.

In contrast to standard protein isolation protocols, we developed a method including cell preparation steps, activation of ProteinChip arrays and the direct application of cells.

Materials and methods

After cultivation, bone marrow cells were processed by routine cytogenetic preparation [hypotonic treatment and fixation with methanol/glacial acetic acid (1:3 v:v)] and frozen at -20˚C as described elsewhere (14). As a control, fresh unfixed cells were obtained by density gradient centrifugation and stored at -80˚C. For successful analysis, we used IMAC 30 ProteinChip arrays (BioRad), but other surfaces could be used as well. Five microliters of 0.1 M Ni-sulfate were applied twice to the spots and incubated for 15 min in a humidity chamber, then washed away with water. Five microliters of binding buffer (0.5 M NaCl) were incubated for 5 min and removed. Two microliters each of the fixed and untreated cells (containing ~10,000 cells) were applied to one spot and dried at room temperature for 10 min. Two microliters of lysis buffer (100 mmol/l Na-phosphate, pH 7.5, 5 mmol/l EDTA, 2 mmol/l $MgCl_2$, 3 mmol/l 2-ß-mercaptoethanol, 1 ml CHAPS, 500 μmol/l leupeptin and 1 mmol/l phenylmethylsulfonyl fluoride) were added, and the chip was incubated for 10 min in a humidity chamber. Binding buffer (2 μl) was applied, incubated for 20 min and removed. The spots were washed three times with the same buffer and twice with aqua demin. After drying at room temperature, 0.5 μl sinapinic acid (saturated solution in 0.5% trifluoroacetic acid/50% acetonitrile) was added twice and dried on air.

We performed mass analysis using a ProteinChip Reader [series 4000; Ciphergen (now BioRad)] according to an automated data collection protocol. Spectra were normalized with total ion current and cluster analysis of the detected signals. We selected signals between 2.5 and 20 kDa for the low range exhibiting a signal-to-noise ratio of at least 10, and analyzed them using the Mann-Whitney U-test for non-parametric data sets (CiphergenExpress; version 3.0).

Results and Discussion

The above protocol was applied to analyze the difference between native and methanol/glacial acetic acid fixed bone marrow cells after cultivation. As expected, due to the different treatment of the cells, the protein patterns showed significant differences, especially in the lower molecular weight range (Fig. 1). Nevertheless, the intensity and number of detected peaks were similar for both sample sets, demonstrating fixed cells to be adequate for the proteomic analysis of leukemias. The virtual value is based on the fact that numerous samples of these fixed cells are stored untapped in hospitals and institutes worldwide. Additionally, fixed cells seem to be robust and can be stored almost indefinitely, as analyses of 12-year-old fixed cell pellets resulted in spectra of equal quality (data not shown).

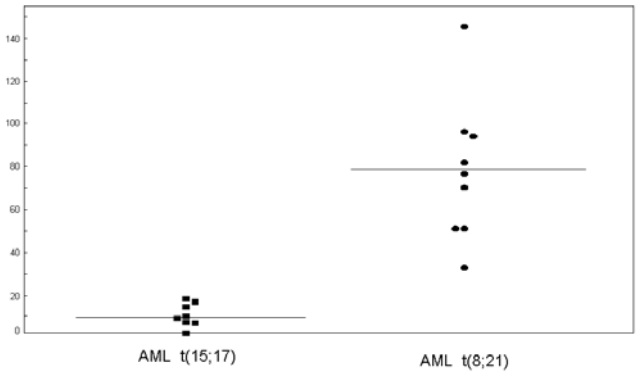

Figure 2. Distribution of the intensity values of a protein peak (10845 Da) of cells with t(15;17) and t(8;21). P=5.3x10^{-4}. This peak corresponds well to a protein identified in a previous study as S100A8 (15).

Having determined that fixed cells are a valuable source for diagnostics, we analyzed in a proof of principle study whether it is possible to differentiate between subgroups of AML. We chose two subgroups with the cytogenetic translocation t(15;17) and t(8;21), respectively. The t(15;17) translocation was chosen because it is pathognomonic for promyelocytic leukemia (FAB subtype M3) and therefore a well-defined subgroup. The translocation t(8;21) with blasts mostly in M2 and M1 is also considered a characteristic subgroup of AML. Eight samples with t(15;17) and 9 samples with t(8;21), independent of the number of blasts, were analyzed on IMAC protein arrays as described above. Twelve

protein peaks were found differentially expressed ($P<0.05$). One of these peaks, with $P=5.3x10^{-4}$, is shown in Fig. 2.

In conclusion, the presented technique allows for the analysis of a small amount of methanol/acetic acid-fixed cells from leukemia patients, which is not possible using standard protocol. We further demonstrated that methanol/acetic acid-fixed cells can be used to differentiate between even subgroups of AML. The high sensitivity and the possibility of directly applying fixed cells, lyse and wash are due to the affinity chromatographic chip surface, which allows for the removal of all contaminants. Further studies are necessary to corroborate these results and to confirm that this technique can be useful for retrospective studies leading to proteomic-based diagnostics and therapy for leukemia.

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