The proteomics of proliferation and malignancy: analysis of tumor-specific and proliferation-specific transcription complexes in normal and malignant B cells

Paul B. Romesser, David Perlman, Anupama Sinha, Mark E. McComb, Douglas V. Faller, Catherine E. Costello and Gerald V. Denis

Boston University School of Medicine, Boston, MA

Presented at the 54th ASMS: MP26, slot 600: abstract # 3619

Overview

- **Statement of problem:** A common difficulty arising in proteomic profiling of cancer is that too much information of uncertain etiologic significance can be collected during the screening process. It would be helpful to explore ways of simplifying the datasets with a view to enrichment of the pool of biomarkers for functionally important signals. Here, we pioneer an approach for profiling of lymphomas.

- **Significance:** Lymphomas, which include Hodgkin's disease and NHL, are the fifth most common type of cancer diagnosed and the sixth most common cause of death in the US; 16,000 new NHL cases are diagnosed annually. Patients already treated for NHL are at greatest risk. Significantly, a study of patients monitored intensively for relapse determined that, in 91% of patients, relapse was detected at unscheduled visits for symptomatic disease. Furthermore, standard chemotherapy is effective only 40% of patients. Clearly, new and more effective measures are needed, such as proteomic signatures for early detection of relapse.

- **Proteomic reference maps for B cell states:** Hypothesis: normal proliferating B cells and malignant proliferating B cells share the induction of important proliferative proteins in comparison to non-proliferating controls. However, malignant cells show additional induced protein expression and post-translational modifications unique to their malignant state, over and above those changes associated with proliferation alone. To explore this hypothesis, we generated 2D-PAGE reference maps for comparative MS-proteomic analysis. We used extracts from normal resting B cells, normal proliferating B cells (mitogenically stimulated with anti-IgM+anti-CD40) and malignant Tg proliferating B cells, all derived from syngeneic mice to minimize genetic variation between individuals.

- **Results:** A relatively simple set of protein biomarkers defines proliferating malignant B cells, as distinct from normal proliferating B cells. This approach can be extended to human primary cells from patients with lymphoid malignancy.
Introduction I

- In transgenic mice, constitutive lymphoid expression of the dual bromodomain protein Brd2 causes diffuse large B cell lymphoma, a common form of non-Hodgkin’s lymphoma, in part through constitutive transactivation of the cyclin A locus.

Giemsa staining of peripheral blood smears from symptomatic Em-BRD2 transgenic mice reveals abundant chromocenters indicative of active transcription, and homotypic adhesion. Anemia is apparent; endstage peripheral leukemia is characterized by leukemic infiltration of multiple organs and organ failure.

Introduction II

- In an effort to understand the mechanism underlying Brd2-driven B cell lymphoma and how it differs from proliferating or resting B cells, we undertook a 2D-PAGE-based comparative MS-proteomic analysis of this Brd2-transgenic lymphoma in relationship to proliferating and resting B cells.

- These results confirm and extend our recently published study of Brd2 complexes (Denis et al. (2006) J. Proteome Res. 5: 502 – 511), which defined Brd2-associated proteins that regulate transcription.

- Maps for splenic B cells were constructed. We then performed differential proteomics with 2D gels to select signals of interest that are unique to malignant B cells and are not induced in normal proliferating B cells.

- These proteins were identified with our published methods for tryptic digestion, liquid chromatography and tandem mass spectrometry (LC-MS/MS) and advanced proteomics methods for protein identification. We define a “malignancy-specific” signature for this B cell lymphoma, distinct from the proliferation signature of normal B cells.
Methods I

- Mouse splenic B cells were isolated by MACS-based magnetic bead separation with anti-CD43 negative selection. Cells were stimulated \textit{in vitro} with αlgM+αCD40 for 48 - 72 hours.

- B cell nuclear and cytoplasmic extracts were prepared, yielding protein extracts of normal resting B cells, normal proliferating B cells and malignant Brd2-transgenic proliferating B cells. Importantly, all cells were derived from syngeneic mice, minimizing genetic variation between individuals.

- Cytoplasmic and nuclear soluble extracts were pooled and 300 µg of protein in each case were separated by 2D-PAGE.

- The samples were resolved by 1D 10% SDS-PAGE or by 2D-PAGE using pi 3-10 IPG strips followed by 10% SDS-PAGE in the second dimension.

- The gel was Coomassie stained, and protein bands were excised and diced. The gel pieces were washed extensively to remove any possible impurities such as SDS, salts, or other gel contaminants.

Methods II

- Digested peptides were eluted from the gel pieces, then desalted for MS analyses, using C18 ZipTip™ micro-reversed phase chromatography.

- MALDI-TOF MS was conducted with a Bruker Reflex IV™ TOF mass spectrometer.

- MALDI data analysis was conducted with the software program MoverZ™ (Proteometrics LLC), and peptide mass fingerprint results of database searches were obtained by submission of peak lists to MASCOT™ (Matrix Science).

- On-line capillary LC-MS with automatic tandem mass spectrometry (MS/MS) was performed on a Waters/Micromass QTOF-API-US quadropole-time-of-flight mass spectrometer coupled to a Waters capillary LC system (Waters Corporation).

- LC MS/MS data analysis was performed using the software MassLynx™ and ProteinLynx Globe Server™ 2.2 (Waters Corporation).
We have hypothesized that normal proliferating B cells and malignant proliferating B cells share the induction of important proliferative proteins in comparison to non-mitogenically-stimulated controls. However, malignant cells show additional induced protein expression and post-translational modifications unique to their malignant state, over and above those changes associated with cell proliferation alone. To explore this hypothesis, we generated preliminary 2D reference maps of extracts from normal resting B cells, normal proliferating B cells and malignant Brd2-transgenic proliferating B cells, all derived from syngeneic mice. We have compared these maps and have quantified many proteins that appear to undergo significant changes in expression between states, and well as proteins that appear to be unique to particular states, or have unique patterns of post-translational modification in particular states.
Results

- Additionally, we have compared these maps to those made from samples subjected to further purification by Brd2-immunoaffinity chromatography.

- MS analyses have determined the identity of the proteins that change between states and have so far lead to the positive identification of several important transcription factors and co-activators, among others, which contribute to transcriptional control in both proliferating and lymphomic B cells.

- These factors were associated with general transcription factors, histones and histone modification enzymes, as well as nucleosome remodeling complexes.

- Results illustrate with detail that cancer is more complex than cellular proliferation, rather that it involves additional expression changes in many proteins, several of which are important for control of chromatin status and activated and basal transcription.

- Moreover, in shedding light on the protein mechanics particular to proliferation and carcinogenesis, our results may lead to the identification many novel targets for chemotherapeutic intervention.

B cell Proteome Reference Maps

Activated B cells (300 mg) share many proteins with resting B cells (300 mg), as well as about 50% more, clearly identifiable new proteins that are induced upon stimulation. Most interestingly, malignant cells (300 mg) show even more new spots; these unique proteins are likely candidates for a malignancy-specific signature. We employ well-established protocols for preparation of soluble cell extracts, but potentially useful biomarkers of malignancy under this framework could include poorly extractable proteins such as transmembrane proteins or receptors. We expect that even though potentially valuable information may be lost with the choice to use only soluble extracts, we gain in reproducibility, because of the difficulty of handling membrane proteins for these types of biochemical manipulations.
Nuclear Extract B cell Proteome Reference Maps

2D-PAGE differential protein expression maps of stimulated and lymphomic B cells relative to resting B cells. All experiments were comprised of 300 mg prepared under identical conditions. Several hundred protein IDs were obtained. Interestingly significant overlap of protein expression occurred in all 3 cell lines.

Example of Peptide Mass Fingerprinting (PMF)

Nucleolin

Example II of Peptide Mass Fingerprinting (PMF)

Coronin-1A

Function: May be a crucial component of the cytoskeleton of highly motile cells, functioning both in the invagination of large pieces of plasma membrane, as well as in forming protrusions of the plasma membrane involved in cell locomotion. May play a role in metastatic cancer growth. In mycobacteria-infected cells, its retention on the phagosomal membrane prevents fusion between phagosomes and lysosomes.

Results I

- TRANSCRIPTION
  - Wnt-5b precursor
  - Q8IYX0, Zinc finger protein 679
  - Q91VY9, Zinc finger protein 622
  - Q9P203, BTB/POZ domain-containing protein 7
  - Q9BUG6, Zinc finger protein 495
  - Transcription factor NF-E2 45 kDa subunit
  - Q9BUQ8 Probable ATP-dependent RNA helicase
Results II

- **B CELL – SPECIFIC**
  - Q96JZ2, Hematopoietic SH2 domain-containing protein
  - P49710, Hematopoietic lineage cell-specific protein (LckBP1)
  - O60682, Activated B-cell factor 1 (ABF-1)
  - Q865W7, Interleukin-6 precursor (IL-6)
  - P12399, CTLA-2α protein precursor
  - P06799, Interferon α-7 precursor

Results III

- **SIGNAL TRANSDUCTION**
  - Q99PT1, Rho GDP-dissociation inhibitor 1 (Rho-GDI α)
  - Q5PQN2, Bifunctional apoptosis regulator
  - Q13315, Serine-protein kinase ATM
  - Q9H4B4, Serine/threonine-protein kinase PLK3
  - Q8C0Q9, Rap guanine nucleotide exchange factor 5
  - Q15691, Microtubule-associated protein RP/EB family member 1 (APC-binding protein EB1)
  - Q9NS23, Ras association domain-containing protein 1
Discussion

- Genome-wide transcriptional profiling of resting or proliferating normal B cells and proliferating malignant B cells has suggested that B cells could be characterized by two major axes of gene expression: one group of genes significantly differentially expressed between resting and proliferating normal cells; a “proliferation signature”, and another group of genes differentially expressed along an orthogonal axis unrelated to normal proliferation (a “cancer signature”).

- Transcriptional signatures of lymphomas establish a basis and justification for our work; proteomic signatures of lymphoid malignancy are required in part because messenger RNA-based signals are not a perfect proxy for protein-based signals.

- We identify several regulators of signal transduction, mitosis and transcriptional control, as well as lineage-specific markers, which are expected for B cell malignancies.

- These identified proteins will be validated and form the basis for a tentative pool of malignancy-specific proteomic biomarkers for lymphoma.

Discussion II

- We performed extractions of murine splenic B cells, followed by 2D PAGE separation, then mass spectrometry (MALDI and LC-MS/MS of tryptic peptides) and proteomic identification of potential biomarkers for lymphoid malignancy: a “malignancy proteome”.

- Our results support a central hypothesis: resting and mitogenically activated, normal B cells provide a framework to interpret malignant B cell proliferation.

- We have begun to establish proteomic 2D reference maps for resting, activated and malignant B cells taken from transgenic mouse spleen. Based on these maps, we may conclude that a relatively simple set of protein biomarkers defines proliferating malignant B cells, distinct from proliferating normal B cells.

- This approach greatly simplifies proteomic biomarker discovery for lymphoid malignancy.

- The heuristic should be adaptable and extendable to a wide spectrum of human hematologic malignancies, including myeloid leukemias.
Acknowledgements

- ACS grant RSG-05-072-01
- NIH grants P41-RR10888, S10-RR15942, CA075107, CA102889
- NHLBI contract N01-HV-28178
- Partially funded by the Boston University Undergraduate Research Opportunity Program