Toxicoproteomics: A Parallel Approach to Identifying Biomarkers

Toxicoproteomics is the use of global protein expression technologies to better understand environmental and genetic factors, both in episodes of acute exposure to toxicants and in the long-term development of disease. Integrating transcript, protein, and toxicology data is a major objective of the field of toxicogenomics. Toward that goal, the NCT is pursuing a strategy of conducting parallel DNA microarray and proteomic analyses on the same tissues from each toxicogenomics study. This parallel approach combines the high level of gene discovery of microarray analysis with the utility of proteomics in exploiting post-translational modifications of target proteins affected by toxicants to identify biomarkers.

The Proteomics Program within the NCT consists of an intramural program, a proteomics resource contract, extramural awards in proteomics, and Small Business Innovation Research awards. The close association of the Proteomics Program with the NIEHS Microarray Center and interactions with the National Toxicology Program also provide unique opportunities for integrating genomics, proteomics, and toxicology into joint studies.

Methods

A primary goal of the intramural program is the discovery of biomarkers in organs and serum that reflect toxicant exposure or environmental disease, using both established and emerging proteomics technologies. For example, two-dimensional gel electrophoresis and MS/MS are being used for global protein separation and protein identification. SELDI is being investigated for potential use in serum biomarker development and classification of control and toxicant-exposed experimental animals. Strong capabilities have been built in capillary and nanoscale LC separations and in the use of MS for the identification and fine structure characterization of protein samples digested by enzymes. There is also an emphasis on LC/MS/MS approaches to differential protein expression, such as the use of isotope-coded affinity tags and characterization of post-translational modifications. Additional capabilities include MALDI/TOF/MS, MALDI/TOF/TOF MS/MS, nanoscale LC/ESI/Q-Tof MS/MS, N- and C-terminal protein microsequencing, several capillary HPLCs, a proteomics laboratory information management system, and a National Center for Biotechnology Information database with search capabilities that is housed on campus. Efforts in this area are coordinated by two research groups, the NCT Proteomics Group and the Mass Spectrometry Group in the NIEHS Laboratory of Structural Biology.

Investigations

Researchers in the Proteomics Program are particularly interested in investigating protein phosphorylation as a key post-translational event in pathway signaling and in protein degradation. New fluorescent phosphorylation sensor dyes, IMAC techniques, and site-specific phosphopeptide biotinylation are three new procedures being used to detect and capture large numbers of phosphorylated proteins and peptides simultaneously. Further, MALDI/MS and LC/MS/MS techniques are being used to identify changes in phosphopeptides altered by exposure to toxicants.

Investigation of cellular and subcellular proteomes is another area in which NCT researchers are exploiting toxicoproteomics. Although most tissues are amenable to RNA extraction for generation of cDNA and hybridization to thousands of genes arrayed on DNA chips, protein analysis often involves reductive procedures whereby the complex structure of organs is lost. NCT researchers are retaining a certain level of organization by isolating specialized cell types within the organ and fractionating organs into subcellular components and organelles. Because liver tissue can be analyzed whole or as subcellular fractions, these techniques are being used to investigate liver toxicity.

Although the liver consists primarily of hepatic parenchymal cells, several nonparenchymal cells—including stellate cells, pit cells, Kupffer cells, biliary cells, and endothelial cells—also are important for liver function. These cells may be targets for toxicity and may increase in number in some toxic and pathologic conditions to significantly alter the hepatic protein profile. Isolation of these cells in their resting and active states may reveal protein profiles contributing to liver toxicity that would have been very difficult to observe in a mixed population of cells dominated by hepatocytes.

Fractionation of subcellular organelles is a means to enrich for structurally meaningful subcellular units of the liver in a way that is not possible by protein analysis of whole liver homogenates or even by RNA isolation and transcript analysis. Enriched fractions of nuclei, mitochondria, endoplasmic reticulum, plasma membranes, and cytosol can provide insight into the potential effects of a toxicant on a particular site within subcellular structures or upon protein trafficking or signaling pathways.

Proteomics platforms appear particularly well suited for biomarker development in blood because the appearance of new polypeptides in the serum proteome may reveal early-stage disease and organ toxicity. In the biofluids produced by each organ, serum and plasma proteomes of blood can uniquely reveal signs of specific organ toxicity or pathology from the peptides and proteins passively leaked or actively secreted during dysfunction. In addition, blood carries the nutrients, intermediary metabolites, amino acids, peptides, proteins, and enzymes necessary for organ health, adaptation, and repair of organ toxicity. Such blood-transported biomaterials may arise from other body tissues as well as from alimentary absorption.

Analysis of serum or plasma alone presents a challenge in separation and detection of informative proteins due to the adundance of other serum proteins such as albumin, immunoglobulins, and transferrin. NCT researchers are using immunoaffinity and ligand-affinity columns to remove such numerous and noninformative serum constituents—which comprise almost 90% of serum—to allow for separation and identification of the remaining 10% of informative serum proteins.

Strategies

NCT researchers in the Proteomics Program are in the process of developing and refining strategies for integrating toxicogenomics and toxicoproteomics data.

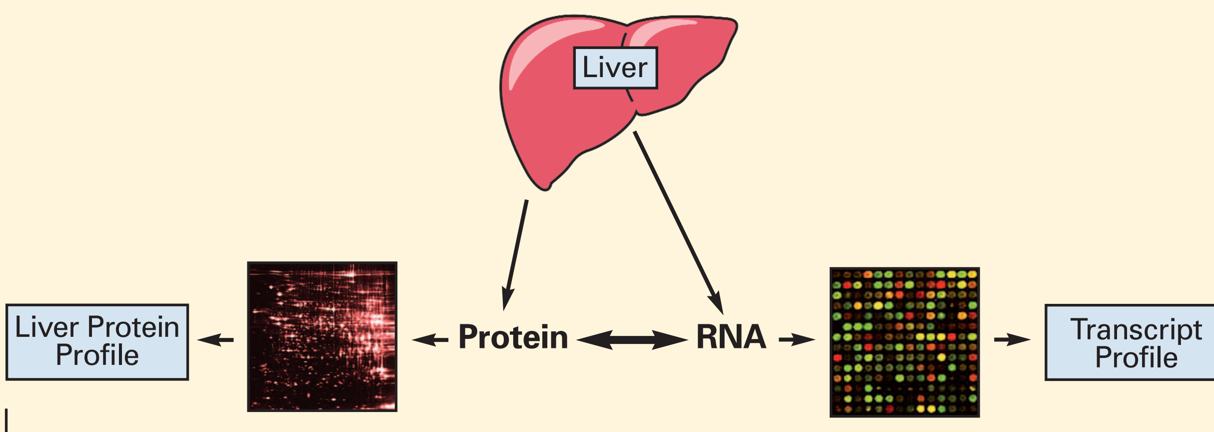
Often the first analysis is a comparison of altered genes from a DNA microarray of liver with the profile of altered liver proteins after two-dimensional gel separation and MS identification of proteins. Annotated gene products and proteins and their accession numbers, assigned by GenBank, are searched for concordance to identify unknown products. A second analysis can involve determining the affected pathways that contain common altered gene transcripts and proteins representing pathway activation or repression. A third analysis involves comparison of proteins altered post-translationally after toxicant exposure with specific gene

transcript changes in kinases, proteases, phosphorylases, conjugating enzymes, and other enzymes that target specific proteins for modification. This can help to identify gene expression.

Another analysis can be performed by kinetically profiling protein changes in the serum proteome over the course of pretoxic, toxic, and recovery stages of chemical exposure. Such changes in the serum proteome represent the dynamic relationship between target organ toxicity and blood during toxicity response, repair, and recovery.

Studies conducted with various hepatotoxicant chemicals will examine the hypothesis that gene and protein profiles can distinguish toxicants acting at different areas of the functional liver lobule. Toxicoproteomics will eventually link the protein expression patterns of multiple organ systems involved in toxicant action and should contribute to a better understanding of mechanisms of toxicity to produce useful biomarkers of environmental disease. **–B. Alex Merrick and Kenneth B. Tomer**

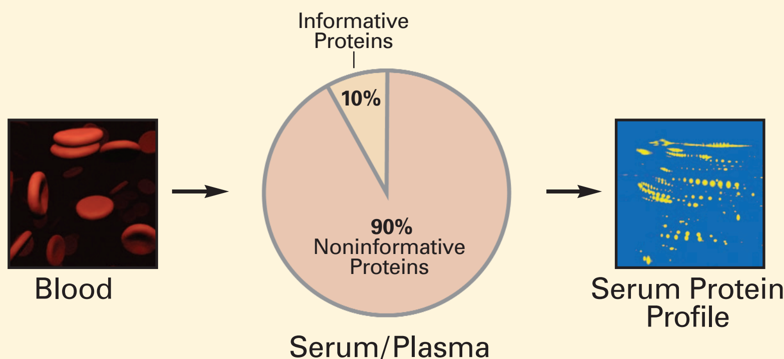
Strategies for Analysis of Toxicoproteomic Data



Analysis 1. Comparison of gene alterations in toxicant-exposed animals obtained from DNA microarray analysis of liver with the profile of altered liver proteins obtained through two-dimensional gel separation and MS identification of proteins. Annotated gene products and proteins and their accession numbers are searched in GenBank to identify unknown products.

Analysis 2. Comparison of DNA microarray analysis of common altered gene transcripts and profile of altered proteins to determine pathway activation or repression.

Analysis 3. Comparison of post-translational modification of proteins with gene transcript changes in kinases, proteases, phosphorylases, conjugating enzymes, and other enzymes to identify gene expression.



Analysis 4. Separation and identification of informative serum or plasma proteins in blood from other biofluids for biomarkers of disease and organ toxicity.

B. Alex Merrick, Matt Ray/EHP