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REVIEW

The role of proteomics in toxicology: identification of biomarkers of toxicity by protein expression analysis

SANDY KENNEDY*

Oxford GlycoSciences (UK) Ltd, The Forum, 86 Milton Park, Abingdon, Oxfordshire OX14 4RY, UK

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Proteomics, i.e. the high throughput separation, display and identification of proteins, has the potential to be a powerful tool in drug development. It could increase the predictability of early drug development and identify non-invasive biomarkers of toxicity or efficacy. This review provides an introduction to modern proteomics, with particular reference to applications in toxicology. A literature search was carried out to identify studies in two broad classes: screening/predictive toxicology, and mechanistic toxicology. The strengths and limitations of current methods and the likely impact of techniques in drug development are also considered. Proteomics can increase the speed and sensitivity of toxicological screening by identifying protein markers of toxicity. Proteomics studies have already provided insights into the mechanisms of action of a wide range of substances, from metals to peroxisome proliferators. Current limitations involving speed of throughput are being overcome by increasing automation and the development of new techniques. The isotope-coded affinity tag (ICAT) method appears particularly promising. The application of proteomics to drug development has given rise to the new field of pharmacoproteomics. New associations between proteins and toxicopathological effects are constantly being identified, and major progress is on the horizon as we move into the post-genomic era.

Keywords: proteomics, nephrotoxicity, hepatotoxicity, cardiotoxicity, carcinogenesis, screening, biomarkers.

Abbreviations: 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; AFP, α-fetoprotein; AMAP, 3-acetamidophenol; ATPase, adenosine triphosphatase; CsA, cyclosporin A; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; FGF-2, fibroblast growth factor-2; GGT, γ-glutamyl transferase; GSTP1, glutathione S-transferase P1; HMG-CoA, hydroxymethylglutarate coenzyme A; HPLC, high performance liquid chromatography; ICAT, isotope-coded affinity tag; ISB, Institute for Systems Biology; mRNA, messenger RNA; MS, mass spectrometry; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NAG, *N*-acetyl glucosaminidase; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; NMR, nuclear magnetic resonance; OGS, Oxford GlycoSciences Plc; PAN, puromycin aminonucleoside; PPAR, peroxisome proliferator activated receptor; SAR, structure–activity relationship; SHE, Syrian hamster embryo.

Introduction

Proteomics – 'the high throughput separation, display and identification of proteins' (Anderson and Anderson 1998, Blackstock and Weir 1999) – is a technology with the potential to change our approach to safety assessment in

* Corresponding author: Sandy Kennedy, Oxford GlycoSciences (UK) Ltd, The Forum, 86 Milton Park, Abingdon, Oxfordshire OX14 4RY, UK. Tel: (+44) 1235 208000; Fax: (+44) 1235 207626; e-mail: sandy.kennedy@ogs.co.uk

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animals and humans, both in the laboratory and in the environment. The full range of proteins to be found in a biological sample (the proteome) can be identified. This allows an individual protein or group of proteins to be associated with a disease/toxicity and their subsequent use as biomarkers of that entity. It is now possible to analyse proteins using high throughput, automated techniques ranging from the digital imaging of the protein pattern on a two-dimensional polyacrylamide electrophoresis gel, through robotic protein excision from the gel, to protein identification by mass spectrometry.

A particular advantage of proteomics is that not only tissues but also body fluids can be assayed to investigate the molecular correlates of disease and drug action. This is possible because many proteins, unlike mRNA, are secreted in profiles that vary predictably with physiological state. As a result, proteomic analysis can be carried out in large numbers of patients on the basis of simple blood or urine tests, rather than by the much more hazardous and expensive approach of taking biopsy samples.

The proteomic evaluation of body fluids can be of particular value in the search for non-invasive biomarkers. This capability is enhanced by the ability to remove high abundance proteins such as albumin, immunoglobulin G, haptoglobulin and transferrin from the sample. An immunoaffinity-based enrichment technique is carried out that reveals hundreds of proteins in the gel that would previously have been masked from detection.

The applications of proteomics in toxicology can be divided into two broad and overlapping classes: investigative studies, and screening or predictive toxicology. Investigative studies may help to identify new molecular targets for toxicants or provide novel insights into mechanisms of action. The belief that a specific group or class of compounds will induce specific patterns of protein expression changes provides a basis for the application of proteomics to predictive toxicology. Such patterns or fingerprints could be used, for example, to screen novel compounds and to study structure–activity relationships (SARs) within a group.

When using any novel technology, scientific rigour is paramount. It is crucial to have a scientifically designed protocol based on good toxicological principles for the generation of samples. Similarly, there is a need for a comprehensive analysis of the results. Finally, it is important that the proteomics approach compares favourably with more conventional techniques. This paper reviews a number of studies using proteomics in toxicology and considers the strengths, limitations and likely future development of this approach.

Proteomics technology

Proteomics and genomics

Proteomics is regarded as a sister technology to genomics and there is a strong synergy between the two approaches at the molecular level (figure 1). Given the static nature of the genome, however, the information about a pathological process that can be derived at the level of gene activity is incomplete. Examination of mRNA provides an added insight into which genes are being translated, but not all mRNA is transcribed into protein, and an abundance of mRNA does not necessarily reflect protein abundance (Anderson and Seilhamer 1997). Once a protein has been assembled from its constituent amino acids, post-translational

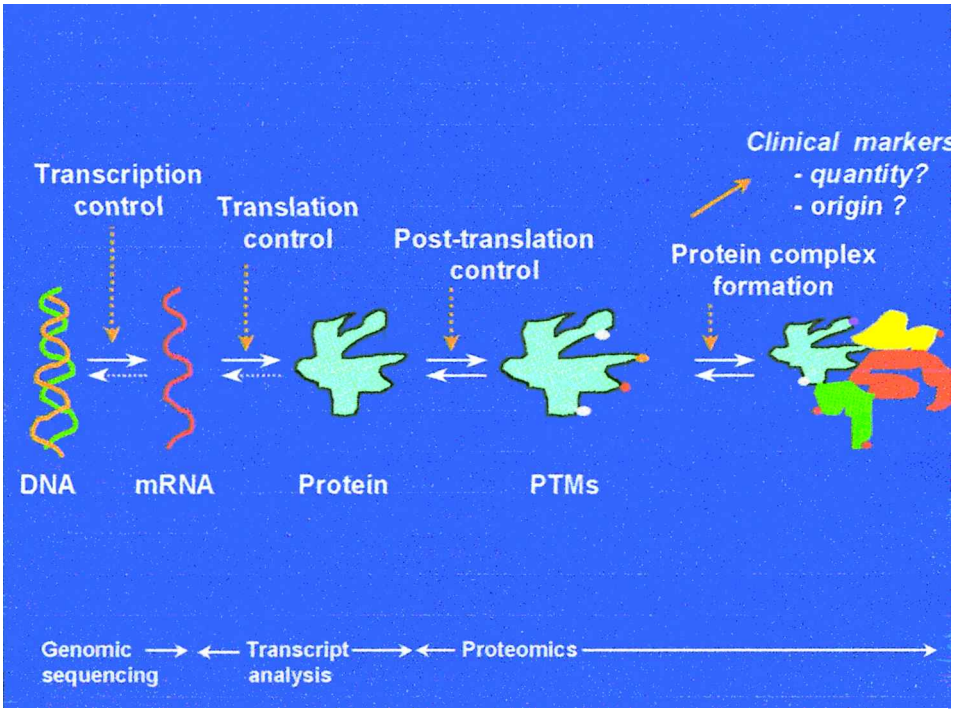

Figure 1. The molecular relationship between proteomics and genomics.

modifications such as glycosylation and phosphorylation can radically alter the protein's function, as can assembly into multi-protein complexes. As proteins are the final effector mechanism both for normal physiology and disease processes, the exact endpoint of altered gene expression can only be determined by direct analysis of proteins.

Another aspect of proteomics, and one that is completely distinct from genomics, is that protein expression can be determined in body fluids in the absence of cellular material. Changes in the protein patterns in serum or cerebrospinal fluid may be used as markers of disease processes such as tumour burden or pathways in degenerative central nervous system disease.

Protein expression analysis

In contemporary proteomics (figure 2), samples of fluids or tissues are firstly separated, normally by high-resolution two-dimensional polyacrylamide gel electrophoresis (2D PAGE). In 2D PAGE, proteins are separated first by electric charge and second by molecular weight (figure 3). This technique is not new. However, the development of a rigid gel separation medium bound to a non-interfering glass support has overcome a major barrier to the development of high throughput proteomics, namely the difficulty of reliably manipulating the fragile separation medium. Another major step forward is the ability to remove highly abundant proteins from samples before separation. This prevents them from obscuring low abundance proteins that migrate to a similar spot on the gel.

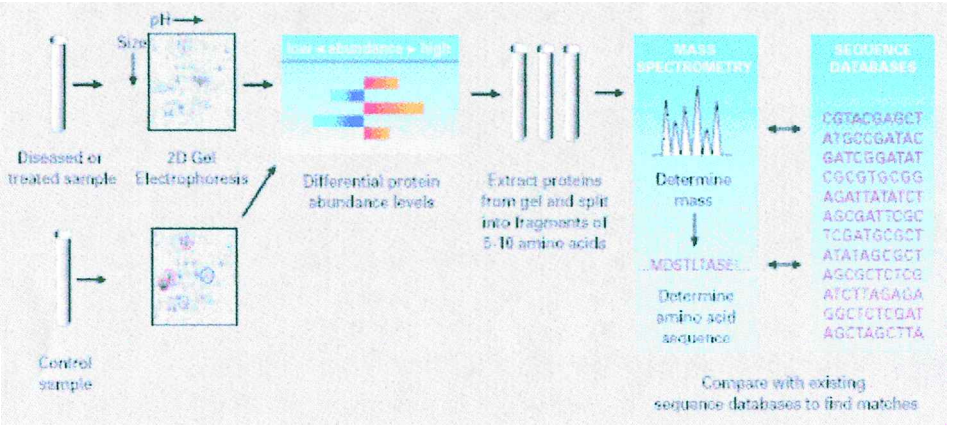

Figure 2. Principal stages of proteomic analysis.

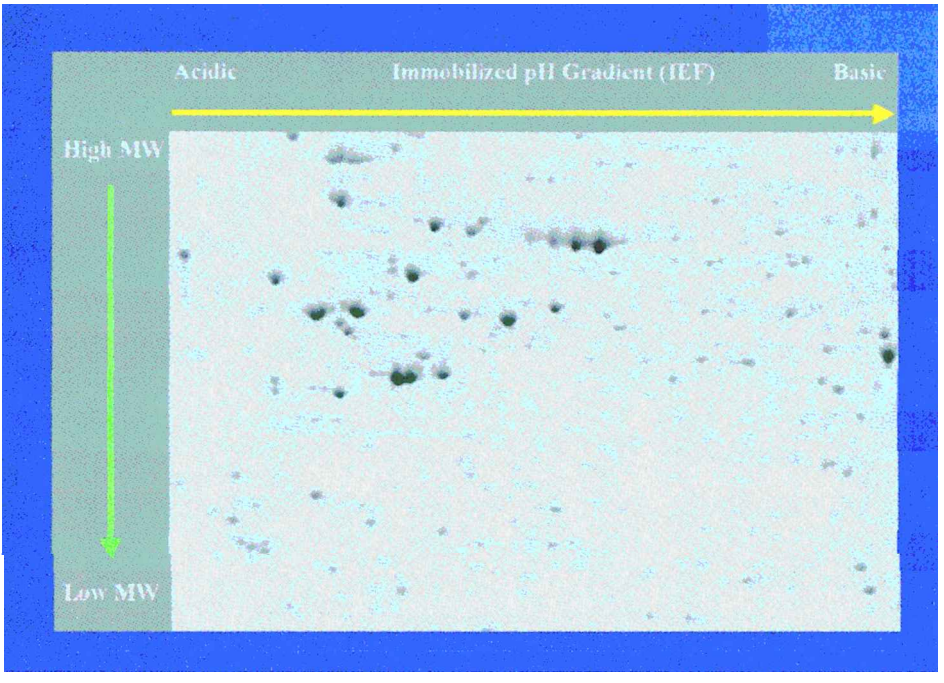

Figure 3. Typical results of 2D PAGE. Proteins are separated by charge and molecular weight (MW). IEF, isoelectric focus.

Conventional proteomics is restricted to the analysis of proteins with a molecular mass > 10 kDa. Technology has recently been developed for the analysis of peptides and small proteins with a molecular mass of 0.5–15 kDa. This approach has been termed 'peptidomics' (Schulz-Knappe *et al.* 2001).

Once the proteins have been separated, their distribution on the gel is analysed to create a digital map of the proteome. Proteins are first stained using either standard techniques (silver or Coomassie blue staining) or the more sensitive techniques developed by specialist proteomics companies (table 1). Silver staining

Table 1. Comparison of protein staining methods.

	Limit of detection	Protein modification	MS compatible
Coomassie blue	100 ng	No	Yes
Silver stain	1.0 ng	Yes	No
Fluorescent dye	0.1 ng	No	Yes

MS, mass spectrometry.

renders mass spectrometry not feasible due to the covalent binding of the proteins to the silver.

Mapping, quantification and analysis of the stained proteins involves the use of specialized software programs such as Melanie 3 (developed by the Swiss Institute of Bioinformatics: see www.expasy.ch). Leading proteomics companies have developed their own very powerful image analysis platforms, with the aim of integrating the various stages of proteomic analysis. Examples are the Kepler system (Large Scale Proteomics, Rockville, Maryland, USA), the Rosetta™ bioinformatics tools developed by Oxford GlycoSciences (OGS) and LifeExpress (a joint project between OGS and InCyte Genomics, Palo Alto, California, USA).

Since for a standardized 2D PAGE system any given protein always migrates to the same location, it will eventually prove possible to identify the individual proteins on gels simply according to their position. At present, however, the vast majority of proteins are unidentified and must be characterized structurally. Once specific proteins of interest have been selected, they are excised from the gel, fragmented and subjected to analysis by mass spectrometry (MS). Bioinformatics methods allow MS to be coupled to peptide sequence searching. Peptide mass data obtained by MS are used by software programs to search partial gene sequence, genomic and protein sequence databases, comparing what would be expected if the protein in the database was subjected to the same pattern of fragmentation. Once a reliable match is found, the database sequence can be used to identify the protein and predict its full sequence.

MS and the associated bioinformatics techniques are undergoing rapid development. One important improvement is tandem MS, in which proteins are subjected to successive phases of fragmentation and mass analysis. Tandem MS obtains direct sequence information from a number of peptide fragments. Used in combination with peptide mass analysis, this method can reduce the risk of ambiguity arising when MS data match more than one protein sequence.

A recent state-of-the-art review of 2D PAGE (Celis and Gromov 1999) concludes that there are various aspects of the technology that can still be improved, including resolution, sample preparation and detection. Given these limitations, other techniques are likely to become more important in the future. Capillary electrophoresis and high performance liquid chromatography (HPLC) may in future be used to separate proteins. Antibody-based techniques, involving antibodies binding selectively to specific proteins, are likely to be useful for both the separation and identification of proteins. This kind of approach may be combined with the 'protein chips' developed by Biovation (Aberdeen, UK) and Ciphergen (Palo Alto, USA). Antibodies or ligands attached to chips could in theory be used to separate and identify proteins without the need to use 2D PAGE or MS.

A particularly promising recent development is the isotope-coded affinity tag (ICAT) method (Gygi *et al.* 1999) pioneered by Dr Ruedi Aebersold and colleagues at the Institute for Systems Biology (ISB) in Seattle, USA. The method uses a reagent made up of three elements: a thiol-specific reactive group, a linker that can incorporate stable isotopes, and an affinity tag for isolating ICAT reagent-labelled peptides. Peptides are separated by microcapillary HPLC and analysed by tandem MS. OGS is working with the ISB to develop an industrial proteomics platform based on the ICAT method.

The importance of bioinformatics

It will be clear from this brief introduction that bioinformatics is essential to the success of proteomics. The 2D PAGE stage requires image analysis software capable of analysing the pattern of protein spots on the gel and identifying differences between gels. As mentioned above, protein characterization is critically dependent on the existence of protein and genomic databases and the software programs used to extract data from them. In turn, proteomics studies contribute to protein sequence databases and help to locate relevant information in genomics databases.

A brief account of the Rosetta bioinformatics system developed by OGS further illustrates the contribution of bioinformatics to proteomics. Proprietary algorithms within Rosetta determine the amino acid sequences of peptide fragments from their mass and charge and an analysis of their MS fingerprints. The sequences in the sample are automatically compared with protein sequences in both publicly available and internal protein databases. Using algorithms within Rosetta, it is also possible to reverse translate amino acid sequences to produce an anticipated expressed mRNA/DNA sequence. This sequence can be scanned against sequences in genomic databases to identify the gene fragments that correspond to the original amino acid sequence. Using this gene fragment information, the full gene sequence encoding a protein of interest can be found and used to confirm the amino acid sequence of the complete protein.

Automation

Automation has also been important in making recent advances in proteomics feasible. 2D gel electrophoresis is highly labour intensive but this does not cause bottlenecks in the analytical process because large numbers of gels can be processed in parallel. Automation of subsequent steps in the procedure results in substantial increases in efficiency (table 2), and these types of throughput have been further industrialized and enhanced by companies such as OGS.

There is an increasing ability not only to automate individual steps but also to integrate them, using robotics, into high throughput industrial systems. The addition of bioinformatics software has allowed leading proteomics companies to make most stages of analysis fully automatic. This leaves researchers free to concentrate on the data generated by the analysis rather than on the complexities of the analytical process itself.

The ICAT method currently under development (see above) is designed to combine labelling, separation and analysis of peptides into a single automated procedure. It is already possible to scan more than 1200 peptide pairs in an hour

Table 2. Effect of automation on proteomic analysis (adapted from Lopez 1999).

Process	Manual throughput	Automated/semi-automated throughput
2D PAGE	10 gels/day 1500–3000 spots/gel 15000–30000 spots/day	Not applicable
2D gel staining (silver)	5 gels/day	10 gels/day
Image capture and analysis	5 gels/day	50 gels/day
Spot picking	100 spots/day	100 spots/hour 800 spots/day
Digestion and peptide extraction	100 spots/day	200 spots/day
MS analysis and database search	20 spots/day	200–1000 spots/day

(Gygi *et al.* 1999). This procedure is likely to represent a major advance in the automated quantitative analysis of the proteome.

Sample preparation

There have been important recent developments in the preparation of cells and tissues for proteomic analysis. Fractionation of samples into different cellular constituents, for example membrane or cytoskeletal components, enables different preparation techniques to be used for different protein types and increases the total number of proteins that can be resolved. For example, Godovac-Zimmermann *et al.* (1999) were able to isolate fibroblast membrane fractions and study patterns of post-translational modification of membrane receptors following stimulation. The development by companies such as OGS and Proteomix (San Diego, USA) of proprietary techniques for removing highly abundant proteins from samples of body fluids helps the identification of relatively rare proteins that might be useful as biomarkers.

The heterogeneous nature of tissue samples, and the presence of healthy and diseased tissue in close proximity, often complicates the interpretation of proteomic studies. Recently, a number of different techniques have been used in preparing pure cell samples for proteomic studies. Tietz *et al.* (1998) used mechanical disruption, enzymatic digestion, gradient centrifugation and immunopurification to isolate liver cell populations highly enriched in cholangiocytes, which represent only 2–3% of liver cells by number and an even smaller fraction by mass. They then used proteomic techniques to establish a 2D gel database of cholangiocyte-specific proteins. Page *et al.* (1999) were able to separate normal human luminal and myoepithelial breast cells using a double affinity cell sorting and Dynabead immunomagnetic technique. Their subsequent proteomic study was the most extensive to date of the normal human breast, providing a basis for future comparisons with purified breast cancer cells.

It is not known whether immunological manipulations influence the protein expression profile. Techniques to isolate pure cell populations without such manipulations have been described by a number of groups. Banks *et al.* (1999) carried out a preliminary study using laser capture microdissection to select cells for analysis. In this technique, retrieval of selected cells is achieved by activation of a transfer film placed in contact with a tissue section, using a laser beam 30 or 60 μm in diameter. The laser beam is focused using an inverted microscope. The

precise area of film targeted by the laser bonds to the tissue beneath it and these cells can then be lifted free of the surrounding tissue. The technique was successfully used in studies of normal and malignant renal tissue and will be a valuable adjunct to proteomic studies in the future.

Finally, Nishizawa *et al.* (1999) developed a novel method for isolating photoreceptors from bovine retina involving dissection of the eyeball and transfer of the photoreceptor layer onto a nitrocellulose membrane. These authors were able to identify four specific proteins among the population of proteins expressed more abundantly in the photoreceptors than in the rest of the retina.

Applications of proteomics in toxicology

Screening/predictive toxicology

The use of proteomics in screening and predictive toxicology has two principal applications: establishing relationships between toxic effects and protein molecular markers, i.e. identifying toxicological biomarkers, and recognition of patterns, e.g. class effects and SARs. In addition, proteomics offers several potential practical benefits. It should be possible to screen for toxic effects more rapidly with the advent of the newer proteomic methodologies than with conventional methods, resulting in savings in resources. The highly sensitive analytical techniques used in proteomics can potentially detect toxic effects at lower doses than methods such as histology and clinical chemistry. Proteomics has already been applied in a variety of different settings.

Liver. Liver function testing is an important and routine part of toxicological evaluation. In an early study, Anderson *et al.* (1996a) used proteomic techniques to develop a prototype database (Molecular Effects Database^{TM}) describing xenobiotic effects on protein expression in rodent liver. Using this database, it was possible to detect, classify and characterize a broad range of liver toxicity mechanisms. The prototype database contained data on the liver effects of 43 compounds. Observed effects ranged from very broad (e.g. sex steroids affecting expression of 45% of all liver proteins) to very specific (e.g. inhibitors affecting only a single enzyme). The key conclusion of this study was that most compounds tested cause observable changes in liver protein abundance; in most cases the protein changes revealed consistent SARs and the specific proteins involved, where identified, were directly related to known or plausible molecular mechanisms.

However, this database was based on information at limited doses and time points that were the same for each toxin, which may not always be relevant for differing mechanisms of toxicity.

The development of specific databases of this type, if they are founded on sound scientific principles, has considerable implications for predictive toxicology. The extent and mechanism of liver toxicity of a new compound could be studied by comparing its effects on the proteome with those of known compounds already stored in a database.

Changes in the pattern of expression of stress proteins were considered to be of value in screening for chemical toxicities by Witzmann *et al.* (1995a, b, 1996). Using 2D gel electrophoresis, this group identified a set of specific heat-shock and

glucose-regulated proteins whose expression in rodent liver and kidney was highly conserved and constitutive. They then compared the effect of *in vivo* exposure to perfluoro-*n*-octanoic acid and perfluoro-*n*-decanoic acid on the expression of those proteins. They reported that the stress response to perfluorocarboxylic acids is tissue-, toxicant-, and stress protein class-specific and dose-related. They suggested that because nearly all of the proteins studied were constitutively expressed at detectable levels in both liver and kidney, then the 2D electrophoresis stress protein pattern could be suitable for future toxicological screening.

Kidney. The kidney is a target of many xenobiotics and there are marked differences in structure and function between its different regions. Witzmann *et al.* (1998) carried out a preliminary proteomic study, which demonstrated the molecular heterogeneity of the rat kidney cortex and medulla. A total of 127 proteins were identified, which differed significantly ($p < 0.001$) in abundance between the two regions. Of these, 26 were unique to the cortex and four to the medulla. Several proteins, including glutathione S-transferase P1 (GSTP1), aflatoxin B1 aldehyde reductase and stress proteins, were identified as potential biomarkers for renal toxicity studies.

Our own studies of gentamicin renal toxicity (Kennedy 2001) have also provided insights that might be used for toxicological screening. Structural proteins such as cytokeratins were identified in the urine of gentamicin-treated rats and could be of value as non-specific indicators of renal damage. We also identified a potential specific marker of gentamicin toxicity that was overexpressed in treated samples compared with control samples. This work is discussed in more detail below.

Carcinogens. There is an 80–90% concordance between the potential of a compound to cause morphological transformation in Syrian hamster embryo (SHE) cells and its carcinogenic activity *in vivo*. Isfort and colleagues (Isfort *et al.* 1992, Asquith *et al.* 1999) have produced a two-dimensional map of SHE cell proteins. The map was originally based on comparisons with other maps and the identities of some proteins were subsequently confirmed by direct sequencing. The updated map will allow the identification of proteins whose expression changes during transformation and thus assist in evaluating the biochemical mechanisms of morphological transformation in SHE cells. Identification of changes in the proteome associated with transformation could also assist in screening novel compounds for carcinogenic activity.

A series of studies (reviewed in Jungblut *et al.* 1999) have identified proteins associated with a preneoplastic or neoplastic state in rat colorectal cancer and hepatoma. In particular, calgranulin B is upregulated in colorectal cancer. This result is relevant to human cancer because elevated levels of calprotectin (a heterodimeric protein composed of calgranulin A and B) are found in stool samples from patients with gastrointestinal cancer. Murphy *et al.* (2001) compared the proteomic profiles of histologically normal colonic mucosa with samples of hypoplastic polyps and colon adenocarcinoma in order to define a 'normal' colon at the molecular level. Again, identification of changes from the normal state could be valuable in identifying potential carcinogens.

In another rat model, hepatoma-derived aldose reductase-like protein was shown to be expressed in the liver during chemically induced carcinogenesis.

This protein is normally expressed in the embryonic liver but not in the adult. Expression was specific to preneoplastic and neoplastic tissue and was not found in surrounding normal tissue. A human homologue of the rat hepatoma-derived aldose reductase-like protein has been identified in human hepatocarcinomas (Cao *et al.* 1998).

Johnson *et al.* (1998) analysed sera from patients with hepatoma with a view to identifying human hepatoma-specific proteins. Proteins were considered potentially specific for the cancer if there was a significant ($p < 0.05$) difference between tumour and control sera (from normal subjects and patients with cirrhosis). α-Fetoprotein (AFP) was identified as a specific protein in the cancer patients' sera. Concentrations of AFP greater than 500 ng/ml are almost diagnostic of primary hepatocellular carcinoma and 80% of patients have AFP above the upper limit of normal (10 ng/ml). In addition, a glycoform previously characterized as hepatoma-specific AFP was identified and its glycan structure elucidated. Several other hepatoma-specific proteins were detected and three of them were identified as precursors of serotransferrin, fibrinogen β chain and haptoglobin-1. This work suggests that proteome analysis may permit the detection and structural elucidation of potential serum tumour markers. Such markers might be used in carcinogenicity testing as well as having clinical applications.

The US National Cancer Institute has developed a protein expression database covering 60 human cancer cell lines, including melanomas, leukaemias and cancers of breast, prostate, lung, colon, ovary, kidney and central nervous system origin (Myers *et al.* 1997, Weinstein *et al.* 1997). This database has been used to study the relationships between protein expression and cell growth inhibitory activity for a large number of potential anticancer compounds (Myers *et al.* 1997).

There have been a number of investigations into the hepatocarcinogen methapyrilene. It was a widely used antihistamine until it was found to produce hepatocellular carcinoma and cholangiocarcinoma in Fischer 344 rats. The structurally similar antihistamine pyrilamine was found to be marginally or non-carcinogenic. In a study using the peroxisome proliferator Wy-14,643 as a positive control, Cunningham *et al.* (1995) used 2D gel electrophoresis and found specific mitochondrial protein charge shifts associated with high dose methapyrilene treatment that were not observed in animals treated with Wy-14,643 or pyrilamine. The authors considered that the protein changes could be of predictive value for assessing the carcinogenicity of chemicals, and that a chemical that does not produce a large number of protein changes could be considered safer than a similar chemical that produces many changes. Earlier reports from other groups (Anderson *et al.* 1992, Richardson *et al.* 1993, 1994) concurred with the protein changes noted by Cunningham *et al.* (1995), illustrating the reproducibility of protein expression changes between studies.

Peroxisome proliferators are non-genotoxic carcinogens in rodent liver. Chevalier *et al.* (2000) found 32 proteins with altered expression when rat primary hepatocyte cultures were exposed to the peroxisome proliferator nafenopin. These proteins included muscarinic acetylcholine receptor 3, intermediate filament vimentin and the β subunit of ATP synthase. Identification of these non-peroxisomal targets provides opportunities to establish toxicological markers to facilitate early identification of non-genotoxic carcinogens.

Finally, Vercoutter-Edouart *et al.* (2001) studied changes in protein synthesis induced by fibroblast growth factor-2 (FGF-2) in MCF-7 human breast cancer

cells. They demonstrated that the heat shock protein HSP90 is required in order to obtain mitogenic stimulation by FGF-2, and concluded that proteomic analysis is valuable for identifying potential markers or therapeutic targets related to cancer growth.

Environmental toxicology. In a recent series of papers, Witzmann and colleagues have used proteomics to study the toxicological implications of exposure to jet fuel vapour (JP-8) in rats (Witzmann *et al*. 1999a, 2000a, b). Their results indicate a significant but comparatively moderate effect of exposure on protein expression in the lungs, kidney and liver. The data suggest some possible risk to humans exposed to JP-8, such as air force or airline personnel. A search for non-invasive biomarkers in humans exposed to JP-8 is proposed. It is likely that in future other environmental substances will be screened for possible low level toxic effects using proteomics.

Mechanistic toxicology

Proteomics, especially when combined with conventional methods such as histopathology and clinical chemistry offers the prospect of new insights into toxic mechanisms. Such insights allow recognition of effects that may be species-specific, giving a more accurate assessment of likely human toxicity. Furthermore, understanding the mechanisms of toxicity of therapeutic drugs may enable selection of derivatives with lower toxicity. I will consider how proteomics has been used to elucidate toxic mechanisms in a number of different systems.

Liver. As mentioned above, the majority of drugs have measurable effects on the protein expression profile of the liver. A few examples of how proteomics has contributed to understanding toxic effects in the liver will be considered.

In a study by Arce *et al*. (1998), the use of proteomics gave insights into the molecular mechanisms of both the pharmacological action of SDZ PGU 693, a hypoglycaemic agent, and the toxic response to the drug in the liver. SDZ PGU 693 acts as a hypoglycaemic agent by stimulating glucose utilization in insulin-sensitive peripheral tissues, such as skeletal muscle and fat. In a 28 day study, the compound induced hepatocellular hypertrophy in Wistar rats at a dose of 300 mg/kg/day. When liver samples were subjected to proteomic analysis, significant treatment-related changes ($p < 0.001$) were found in 29 liver proteins. Major increases were observed in several microsomal proteins, including reduced nicotinamide adenine dinucleotide phosphate (NADPH), cytochrome P450 reductase, cytochrome b5 and serine protease inhibitor. The change in the cytochrome-related enzymes, both known cofactors of the P450 enzyme system, strongly suggests that SDZ PGU 693 induces microsomal proliferation and induction of the P450 enzyme system. These effects are thought to be associated with the toxic effects of SDZ PGU 693.

Decreases were observed in a series of mitochondrial proteins, such as F_1 adenosine triphosphatase (ATPase) δ subunit and ornithine aminotransferase precursor, as well as several cytosolic proteins, including the liver fatty acid binding protein, aryltransferase and the senescence marker protein-30. The changes in F_1 ATPase and liver fatty acid binding protein together suggest a

downregulation of mitochondrial liver fatty acid metabolism, probably reflecting the pharmacological action of the compound.

The peroxisome proliferator activated receptor (PPAR) transcription factors PPARα and PPARγ are potential targets for treatment of dyslipidaemia in humans. However, activation of PPARs in rodent livers induces proliferation of peroxisomes and long-term treatment with peroxisome proliferators is associated with the production of hepatic neoplasms. Edvardsson *et al.* (1999) followed up an earlier study, which used a proteomics approach to characterize the effects of PPARα agonists in livers from lean healthy mice (Anderson *et al.* 1996b). They investigated the effects of a PPARα agonist in obese diabetic (ob/ob) mice. These mice, which have highly elevated levels of plasma triglycerides, glucose and insulin, were treated for 7 days with Wy-14,643 (180 μmol kg^{-1} day^{-1}), a well-characterized selective PPARα agonist. Treatment was associated with significant therapeutic effects on triglycerides and glucose levels. The liver protein compositions were investigated by high resolution 2D PAGE, which showed that Wy-14,643 produced upregulation of at least 16 liver protein spots, 14 of which were found to be components of the peroxisomal fatty acid metabolism system. These results are consistent with the notion that Wy-14,643 induces peroxisomal fatty acid β-oxidation. The upregulation of this metabolic pathway may be responsible for the therapeutic effect of Wy-14,643 on levels of triglycerides and glucose in plasma.

In this study proteomic analysis confirmed the mechanism of action of PPARα agonists and provided a basis for studying the therapeutic and toxic effects of other PPAR agonists. This type of study is important to use as a basis for separating an exaggerated pharmacological effect from that due to toxicity. Giometti *et al.* (1991) also looked at a number of peroxisome proliferators in mice in relation to their liver protein expression. They considered that it was possible to find changes specific to a particular treatment as well as more generalized effects, providing the opportunity to identify patterns of protein expression that are indicators of biological response to different types of cellular insult.

Paracetamol (acetaminophen) in overdose causes acute hepatotoxicity in rodents and humans. Fountoulakis *et al.* (2000) investigated the mechanism of this toxicity using proteomics. Using a 2D protein database for mouse liver, comprising 256 different gene products, they found that expression of some 35 proteins was altered following treatment with paracetamol (100 or 300 mg kg^{-1}) or its non-toxic regioisomer 3-acetamidophenol (AMAP) (300 mg kg^{-1}). Many of the proteins affected were either known targets for covalent modification by *N*-acetyl-*p*-benzoquinoneimine (NAPQI) or involved in the regulation of mechanisms that are believed to drive paracetamol-induced toxicity. Most of the changes caused by AMAP were in a subset of the proteins modified by paracetamol. This is an example of the use of proteomic evaluation on the background of the already known features of a molecule's mechanism of action. In an *in vitro* study by Bruno *et al.* (1998), mouse hepatocytes were exposed to a hepatotoxic concentration of paracetamol. The cells were radiolabelled with ^{32}P-orthophosphoric acid and cell extracts were analysed by 2D gel electrophoresis and autoradiography. Paracetamol selectively increased the phosphorylation state of some proteins and decreased the phosphorylation state of another. These changes were paralleled by a decline in protein phosphatase activity. The physiological consequences of protein phosphatase inactivation could be significant in paracetamol overdose since these enzymes

are involved in the dephosphorylation of regulatory proteins that control many cell functions. Proteomics is increasingly showing the complexity of many toxic mechanisms by revealing effects on enzymes that act as 'gatekeepers' to subsequent physiological pathways.

The application of proteomics to the analysis of existing drugs and the pathways that they regulate is as yet relatively unexplored. Lovastatin is a lipid-lowering agent that inhibits hydroxymethylglutarate coenzyme A (HMG-CoA) reductase, a key regulatory enzyme in cholesterol biosynthesis. When male F344 rats were treated with lovastatin (1.6 or 150 mg/kg/day) for 7 days, 36 liver proteins were significantly ($p < 0.001$) altered by treatment (Steiner *et al.* 2000). In addition to the expected changes in enzymes affecting lipid and carbohydrate metabolism, lovastatin treatment was associated with signs of toxicity. Changes were observed in a heterogeneous set of cellular stress proteins involved in cytoskeletal structure, calcium homeostasis, protease inhibition, cell signalling and apoptosis. This work indicates the potential to detect possible subtle toxic effects that would not be picked up by conventional methods.

The limitations of current proteomics technology are illustrated by a study of hepatomegaly induced by a substituted pyrimidine derivative (Newsholme *et al.* 2000). Liver weights of drug-treated rats were 37% higher than those of controls. Significant ($p < 0.001$) quantitative changes in at least 17 liver proteins were detected by 2D PAGE. Specific proteins affected by drug treatment were identified, but the authors encountered difficulties in attempting to gain mechanistic insights from their data. These difficulties were considered to be due to limitations of the available bioinformatics platform for rodent hepatic proteins and limited knowledge of specific protein functionality.

Kidney. The protocol for our study (Kennedy 2001) of gentamicin renal toxicity was tailored to the known features of this toxicity. Male rats (10 per treatment group plus 20 in a control group) were exposed to 7 days of treatment with intravenous gentamicin sulphate followed by a 14 day recovery period. The doses used were 0, 0.1, 1.0, 10.0, 40.0 and 60.0 mg/kg/day. Blood and urine samples were taken after 2, 3 and 8 days.

The urine biochemistry results from this study at 60 mg/kg/day were consistent with the known renal toxicity of gentamicin, with raised levels of *N*-acetyl glucosaminidase (NAG) and γ-glutamyl transferase (GGT) at days 2, 3 and 8. No significant changes were apparent in the serum at any dose or time, or in the urine at 40 mg/kg/day or lower. There was evidence of mild histological lesions in the proximal tubule epithelial cells of the kidney at 60 mg/kg/day after 7 days' treatment. These were characterized by a loss of the brush border, indicative of an early degenerative change. After a 14 day treatment-free phase there was evidence of regeneration of the proximal tubule epithelium in animals who had received 40 and 60 mg/kg/day. However, the overall severity of the change was very minor, only involving a small proportion of the kidney cortex.

Proteomic evaluation of kidney cortex samples revealed treatment-related changes in expression in a wide range of protein compartments, including those involved in amino acid metabolism, the urea cycle, oxidative stress, and mitochondrial and structural proteins. Some of the protein changes seen were unexpected and may be involved in specific aspects of gentamicin toxicity.

Examination of the serum samples from rats that had received 40 mg/kg/day for 7 days revealed a protein that was consistently overexpressed in all the treated samples when compared with the control samples. This protein is involved in the activation of the alternate pathway of complement and also binds to human renal proximal tubule epithelial cells. Since it may be involved in the mechanism of gentamicin nephrotoxicity, further characterization is being conducted to establish the biological significance of this finding. Overexpression of this marker was found at a dose lower than that at which changes were seen by routine clinical pathology (although only in the urine, no changes where seen in the serum) and was seen as early as day 3 of treatment. The marker had returned to control levels of expression by the end of the treatment withdrawal phase.

The sensitivity of proteomics technology was evident in the detection of changes in protein expression at lower doses and at earlier time points than could be detected by conventional methods.

The importance of combining proteomics with other techniques was emphasized by Cutler *et al*. (1999) in their study of puromycin aminonucleoside (PAN) glomerular toxicity in rats. PAN produces changes in rats resembling those of human minimal change nephropathy and it has been widely used in the investigation of the mechanism contributing to proteinuria in human glomerular disease. In this study, rats were given a single dose of PAN and urinary proteins were analysed during nephrotoxicity and subsequent recovery. 2D PAGE of urine allowed a more detailed understanding of the nature and progression of the proteinuria associated with glomerular toxicity. The proteinuria was detected at a considerably earlier time point (32 h) than has typically been reported previously. Changes in the nuclear magnetic resonance (NMR) profile were observed from 120 to 240 h. The maximal effect, when the protein content and 2D PAGE profile of the urine was similar to that of plasma, was thought to reflect both glomerular and tubular damage, i.e. impairment of both protein filtration and reabsorption. Therefore the authors considered that 2D PAGE has the potential to define relatively early markers. In addition, urinary HPLC and high resolution proton NMR spectroscopy were used to detect toxin-induced changes in a number of metabolites. The data provided by the various techniques showed a high degree of consistency and complementarity. The combination of these techniques has the potential to provide significantly more mechanistic information than is readily provided by traditional clinical chemistry.

The clinical use of the immunosuppressant cyclosporin A (CsA) is limited by adverse effects, of which renal impairment is the most important. The mechanistic basis of this toxicity is not completely understood. In the first of two studies (Steiner *et al*. 1996), 2D PAGE was performed on rat kidney homogenates from CsA-treated rats. Seventeen rat kidney proteins showed significant abundance changes in CsA-treated rats compared with controls. Among those, seven showed at least a two-fold change. The protein with the most marked decrease was identified by peptide mapping and microsequencing as calbindin-D 28 kDa, a cytosolic calcium-binding protein not previously described in connection with CsA renal toxicity. In the second study (Aicher *et al*. 1998), monoclonal anti-calbindin antibody was used to perform enzyme-linked immunosorbent assays (ELISAs) and immunohistochemistry with tissue from rat, dog, monkey and man. The results suggested a close correlation between calbindin decrease and nephrotoxicity. Human renal biopsy samples from CsA-treated patients showed

a decrease in calbindin-D 28 kDa expression similar to that found in the rat. Rats and humans both show CsA-mediated renal toxicity, unlike monkeys and dogs, which do not show toxicity or changes in calbindin. The discovery of the role of calbindin-D 28 kDa in CsA toxicity had not been reported previously, indicating that proteomic methods can provide essential information in mechanistic toxicology.

Lead is a potent neuro- and nephrotoxin and renal carcinogen in rats. Lead exposure induces increases in the activities of specific detoxification enzymes in distinct kidney cell types preceding irreversible renal damage. The preferential susceptibility of the renal cortex to lead effects is clear, but the effects on the medullary region are not. Witzmann *et al.* (1999b) set out to look for regional protein expression differences between lead-exposed rats and controls and to identify regionally distinct markers of lead toxicity. Male Sprague-Dawley rats were injected daily with lead acetate 114 mg kg for 3 days and killed on day 4. Control rats were injected with saline. Following analysis by 2D PAGE, 727 protein spots were resolved for the cortex and 716 for the medulla. The abundance of 76 proteins (10%) in the cortex and 13 (2%) in the medulla was significantly ($p < 0.001$) changed (increased or decreased) by lead exposure. Eleven of the proteins were identified conclusively.

Several of the cortical proteins altered by lead were unchanged in the medulla, while others underwent similar but lesser alterations. The largest changes observed were in 2-microglobulin (down 90% in cortex and not detectable in medulla), aldol reductase (detectable in cortex only following lead administration and increased more than 20-fold in medulla following lead administration), GSTP1 (which increased six-fold in the cortex after lead treatment) and aflatoxin B1 aldehyde reductase (two-fold increase in both cortex and medulla). Lead administration also altered the post-translational modification of GSTP1 in the renal cortex.

An important mechanistic finding was that there was a nearly two-fold decrease in the calcium-binding protein calbindin and the calmodulin-dependent protein phosphatase calcineurin, presumably reflecting a disturbance of calcium homeostasis in the renal cortex. The authors concluded that their findings reflect the complexity of lead's nephrotoxic effects and support the application of proteomics in mechanistic studies as well as biomarker development in toxicology.

Heart. In a study by Holt and Sistare (2000), rats were treated with the anticancer drug doxorubicin. This is known to cause dose-related cardiotoxicity against which metal chelation by ICRF-87 provides significant chemoprotection (Herman and Ferrans 1981). Rats received 1 mg kg per week doxorubicin for 7 weeks with or without co-administration of ICRF-187 and serum samples were taken for proteomic analysis (figure 4). This analysis revealed 34 potential markers of toxicity with changes in protein expression, either an increase or decrease of up to 28-fold. In the groups co-administered with ICRF-187, 29 of these proteins had returned to control levels and five had partially returned to control levels (figure 5). The proteins were from a range of classes, including lipid metabolism (liposome formation), immune surveillance (complement fixation), wound healing (scar formation, protease inhibition) and antioxidant metabolism (metal scavenging). These findings were considered consistent with the chemoprotective effect of ICRF-187 on doxorubicin-induced cardiotoxicity.

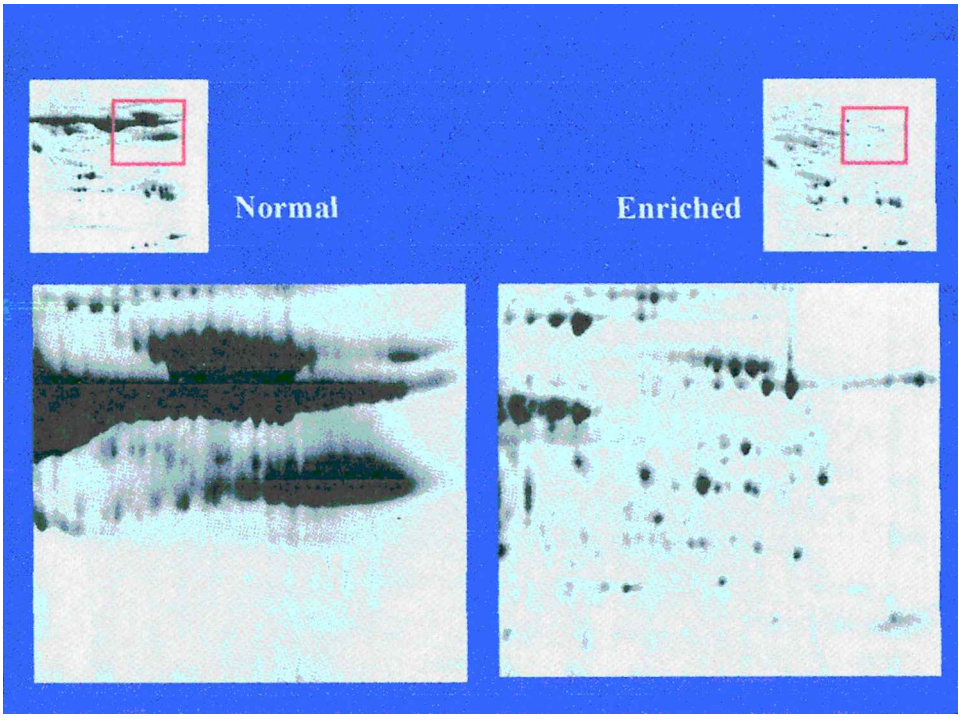

Figure 4. 2D PAGE of serum proteins from doxorubicin-treated rats showing a normal view (left) and an enriched view (right) in which an immunoaffinity enrichment protocol was applied to enhance the detection of low abundance proteins.

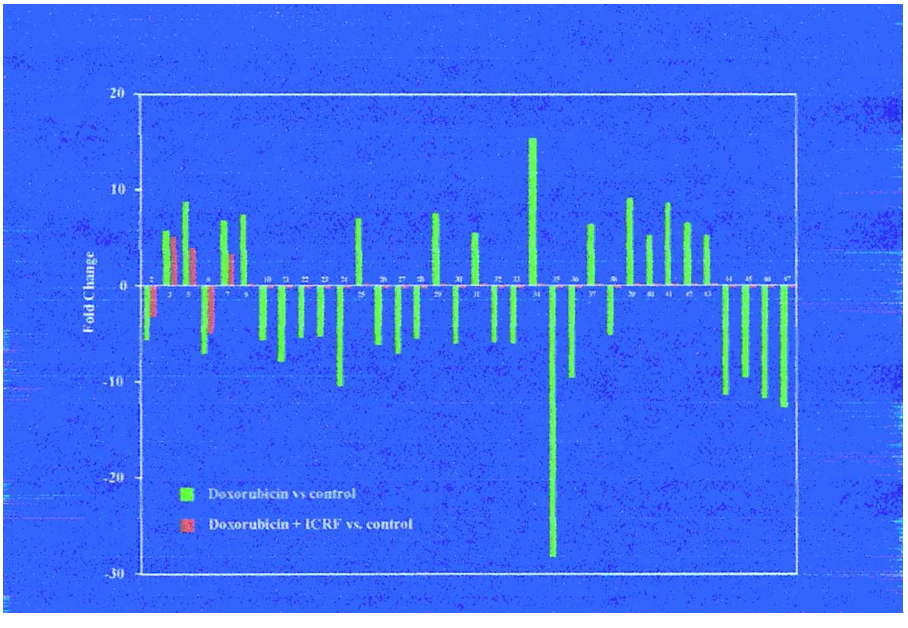

Figure 5. Proteomic analysis revealed 34 potential biomarkers of doxorubicin toxicity in rats (green). Levels of 29 of these proteins were normal in rats treated with doxorubicin and the chemoprotectant ICRF-187 (red).

Carcinogens. Two recent studies have followed up the work of Anderson *et al*. (1996b) and Edvardsson *et al*. (1999) on peroxisome proliferators, with an emphasis on the role of these compounds in carcinogenesis. Chevalier *et al*. (2000) exposed rat primary hepatocytes to epidermal growth factor (EGF) and the peroxisome proliferator nafenopin, both of which induce DNA replication but apparently through different signalling pathways. Proteins showing an altered expression pattern in response to EGF differed from those showing altered expression in response to nafenopin. Specific protein targets for nafenopin were identified.

Further insights into the mechanisms of the anti-apoptotic action of peroxisome proliferators were provided by another study by the same group (Macdonald *et al*. 2000). Hepatocytes from wild-type and PPAR(-null mice were treated with peroxisome proliferators. Eighteen protein spots showing differential expression in treated wild-type hepatocytes were identified. These included not only proteins involved in lipid metabolism, but also the ATP synthase – subunit and the anti-apoptotic glucose-regulated protein 94.

Other studies. In addition to the studies discussed above, proteomics has recently been applied to other problems in mechanistic toxicology. Vido *et al*. (2001) analysed the response of yeast cells to acute cadmium stress and identified 54 induced and 43 repressed proteins. The cellular thiol redox systems, glutathione and thioredoxin were found to be essential for cellular defence against cadmium toxicity. The studies of jet fuel vapour exposure by Witzmann and colleagues (1999a, 2000a, b) have already been briefly discussed. These authors reported effects of the vapour in the lungs that were related to impaired protein synthesis, toxic/metabolic stress and detoxification systems, ultrastructural integrity and functional responses to CO2 handling, acid/base homeostasis and fluid secretion (Witzmann *et al*. 1999a).

The pattern of protein expression and phosphorylation after an apoptotic stimulus was studied in two systems by Robaye *et al*. (1994). Bovine aortic endothelial cells were induced to undergo apoptotic cell death by a combination of a cytokine (tumour necrosis factor) and an inhibitor of protein synthesis such as cyclohexamide. 2D gel electrophoresis of proteins from such cells revealed specific proteolysis of distinct proteins, some at an early stage of apoptosis and some at a later stage. The authors considered that these proteins might have anti-apoptotic properties.

In a clinical application of proteomics, Voss *et al*. (2001) showed that in patients with B-cell chronic lymphocytic leukaemia, shorter survival times were associated with changed levels of redox enzymes, heat shock protein 27 and protein disulphide isomerase. It is considered that these molecules may be involved in the mechanism of cancer cell resistance to cytotoxic drugs. Moller *et al*. (2001) used proteomics to identify a number of proteins that were upregulated following the treatment of pancreatic carcinoma cells with daunorubicin, some in a dose-dependent manner. However, reverse transcriptase polymerase chain reaction studies did not reveal significant alterations in specific mRNA levels.

As can be seen from the above examples, a body of data is building that suggests that protein expression analysis has a value in elucidating mechanisms of toxicity. However, such data should always be 'validated' for its biological

plausibility and repeatability in order to establish that the protein changes are indeed linked to the chemical agent in question.

Proteomics and the future of drug development

In addition to toxicology studies, proteomics has a wide range of potential applications at both the early and later stages of drug development. The application of proteomics to the pharmaceutical industry has been defined as 'pharmacoproteomics' (Moyses 1999). Table 3 summarizes the main potential applications of this new discipline.

Proteomics, in combination with genomics, will allow researchers to obtain a precise molecular description of a biological sample and to identify differences associated with a disease or administration of a particular drug. This is already leading to the generation of new pharmacological targets. For example, OGS has discovered five antigens that might be targets for breast cancer immunotherapy.

We have seen that automation and integration of separate processes are central to proteomics. The use of this technology promises to reduce significantly the investment of time and money required to develop new drugs. In particular, proteomics will allow rapid selection of the most promising candidates for further development, as well as allowing candidates to be eliminated immediately because of insufficient efficacy and/or excessive toxicity.

In the sister field of toxicogenomics, similar applications are being sought (Pennie *et al.* 2000). These authors also urge that sound interpretation and the appropriate toxicological skills and experience are brought to bear in these new areas of data generation to ensure that toxicologically relevant changes are distinguished from those that are of no concern. This reinforces the message that has been repeated throughout this paper to apply the same principles to proteomic data.

Clinically, proteomics is being applied to the development of disease-specific biomarkers that may be useful as patient selection criteria and clinical markers for clinical trials. Doherty *et al.* (1998) identified plasma proteins whose expression

Table 3. Potential applications of pharmacoproteomics (Moyses 1999).

Preclinical applications
Target selection
Drug discovery
Target validation
Lead candidate selection
Drug modes of action
Toxicology
No observed effect level
Screening
Mechanism of action
Clinical applications
Diagnostics
Markers of response
Inclusion/exclusion criteria
Patient subtyping
Post-launch tailoring of therapy to proteotype
Post-launch differentiation of competitors

altered after an acute inflammatory reaction (parenteral typhoid vaccination) and showed that the general pattern of response was similar to that seen in chronic rheumatoid arthritis. Two anti-inflammatory drugs, tenidap and piroxicam, had markedly different effects on acute phase proteins in rheumatoid arthritis patients. It may eventually be possible to subtype individuals according to their protein profile and use this information to predict responses (therapeutic and/or adverse effects) to particular treatments.

Conclusions

Proteomic evaluation will be a rich source of biomarker identification for toxicity, efficacy of drugs or exposure to xenobiotics in humans or wildlife. Protein biomarkers can be investigated in biological samples acquired using non-invasive methodology and the markers are representative of the final secreted protein. Once a biomarker protein or group of proteins is identified, standard methods such as immunoassays can be used for screening.

The techniques of proteomics will make a considerable contribution not only to research but also to regulatory toxicology. However, proteomics methods are likely to complement rather than replace older methods of testing for regulatory purposes in the short term. The great potential is that protein biomarkers will be identified that will improve the predictivity of the drug development process and, in particular, provide that valuable commodity, the preclinical/clinical bridge. This will give more assurance to the interpretation of data from preclinical studies and their predictivity for effects on humans.

As the techniques of proteomics develop and more studies are carried out, new proteins and protein associations will inevitably be identified. This will lead to an increase in scientific complexity, but this should not deter us from moving forward. Applying a rigorous scientific approach will help ensure the validity of our interpretations and the end result is likely to be a step change in our scientific understanding.

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