Studying heart disease using the proteomic approach

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The pathogenic mechanisms underlying cardiac dysfunction in heart disease are still largely unknown. It is likely, though, that significant alterations in myocardial gene and protein expression underlie these disease processes and determine their progression and outcome. Most molecular studies of cardiac dysfunction have been carried out on specific cellular systems. However, the application of the proteomic approach to the study of heart disease has made it possible to characterize global alterations in protein expression. This promises new insights into the cellular mechanisms involved in cardiac dysfunction and is likely to result in the discovery of novel diagnostic markers and new therapeutic opportunities.

It is still less than four years ago that the first complete genome sequence, that of the bacterium *Haemophilus influenzae*, was published[1]. Since then, the genomes of 18 other microorganisms have been completed while many others are in progress. The status of these genome sequencing initiatives can be viewed via the Internet at http://www.tigr.org/tdb/mdb/mdb.html. Until recently, the genome of only one eukaryotic organism, the yeast *Saccharomyces cerevisiae*, had been completed. However, just before the end of 1998, the complete genome sequence for the first multicellular organism, the nematode *Caenorhabditis elegans*, was published[2]. Significant progress is being made for several other species, with a current target date for completion of the human genome of 2005. In the post-genome era, this wealth of information will provide an invaluable resource for understanding cellular function in health and disease.

Gene expression

Genome sequences can be used to predict the corresponding gene products. However, it is clear from the currently completed genomes that it is usually impossible to attribute functions to up to 40% of the structural genes within a particular organism. Moreover, it is apparent that the paradigm of one gene encoding a single protein is no longer tenable because of processes such as alternative mRNA splicing, RNA editing and post-translational protein modification. Thus, the functional complexity of an organism far exceeds that indicated by its genome sequence alone. This problem can only be addressed through direct studies of gene expression either at the mRNA or protein level. Powerful techniques have been developed for the rapid screening of mRNA expression, but it has become apparent that there is often a poor correlation between mRNA abundance and the quantity of the corresponding functional protein present within a cell[3,4]. In addition, extensive co- and post-translational modification events can result in a diversity of protein products from a single open reading frame. It has been estimated that the average number of proteins per gene is one or two in bacteria, three in yeast and from three to more than six in humans[5].

Proteomics

The level of complexity resulting from co- and post-translational modification events can only be dissected and understood through qualitative and quantitative studies of gene expression at the level of the functional proteins themselves. This interface between protein biochemistry and molecular biology has become known as 'proteomics'. The term 'proteome', defined as the PROTEin complement

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of the genOME', was first coined by Wilkins working as part of a collaborative team at Macquarie (Australia) and Sydney Universities (Australia) in 1995 (Refs 6,7). Currently complete proteome analysis is being undertaken only for relatively simple organisms such as *Mycoplasma genitalium*[6], *H. influenzae*[8], *Spiroplasma melliferum*[9], *Escherichia coli*[10,11] and yeast[12,13]. Characterizing the complete proteome of more complex organisms, including man, would be a formidable and perhaps impossible task using currently available technology. However, proteomics can be used in a narrower context to define patterns of protein expression in particular cells and tissues. This information can then be used to characterize functional cellular processes, such as those involved in development, during the cell cycle, in apoptosis, in response to pharmaceutical intervention, or extracellular stimuli and toxic agents, and in disease.

Drug discovery and target validation

The aim of rational drug development is often to develop new compounds that are able to up- or down-regulate a specific activity involved in disease pathogenesis or in treatment-associated toxicity. It is now apparent that most drugs operate at the level of the protein and that proteins that can be regulated by a drug provide insights into mechanisms of drug action[14]. The proteomic approach clearly has great potential to provide global, holistic analysis of changes in protein expression in response to drug treatment. Modern techniques of HTS are resulting in the rapid discovery of novel, highly active compounds. However, this process does not generally depend on prior knowledge of their structure–activity relationships so that, for many lead compounds, there is no obvious rationale for their activity based on their structure. Proteomics will have an important role both in validating existing drug targets and in providing insights into the pharmacological mechanisms of new drugs derived using HTS. Moreover, it is likely that proteomics will be important in the identification of novel sites of therapeutic action through characterization of complex biochemical events by analysis of pleiotropic alterations in protein expression.

The technology

The main steps involved in proteomics will now be described briefly. Further details can be found in two recently published books[15,16].

Protein separation

The first requirement for proteome analysis is the separation of complex mixtures of proteins obtained from whole cells, tissues and organisms. Currently, the best method for separating complex protein mixtures is two-dimensional polyacrylamide gel electrophoresis (2-DE). This method was developed almost 25 years ago[17,18] and uses a combination of a first-dimension separation by isoelectric focusing (IEF) under denaturing conditions with a second-dimension separation using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). This orthogonal combination of charge separation (isoelectric point, pI) with size separation (relative molecular mass, M_r) results in the sample proteins being distributed across the two-dimensional gel profile (Fig. 1).

For proteome analysis, it is essential that 2-DE should generate reproducible high-resolution protein separations. Until recently, this was a major problem largely because of the nature of the synthetic carrier ampholytes used to generate

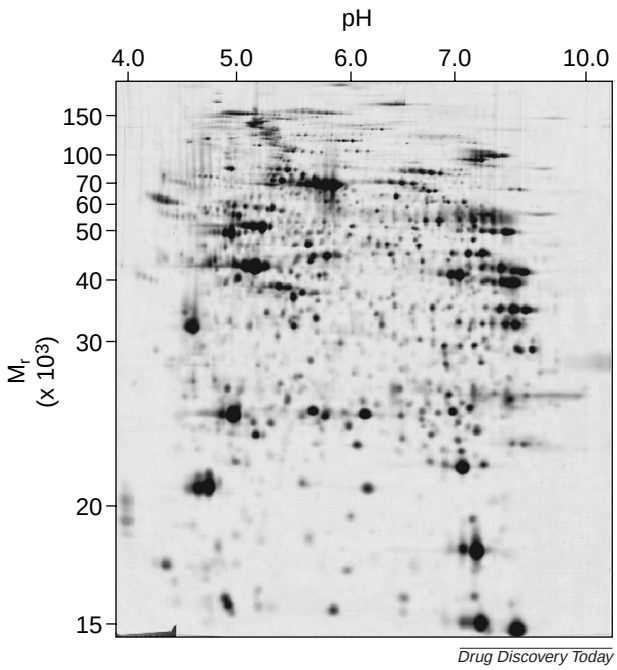

Figure 1. *A two-dimensional electrophoresis (2-DE) separation of 80 μg of heart (ventricle) proteins. An 18 cm non-linear pH 3–10 immobilized pH gradients (IPG) isoelectric focusing (IEF) gel was used in the first dimension. The second dimension was a 21 cm 12% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) gel. The proteins were detected by silver staining. The scale at the top indicates the non-linear pH range of the first-dimension IPG strip from which the apparent isoelectric points (pI) of the separated proteins can be estimated. The M_r (relative molecular mass) scale can be used to estimate the molecular weights of the separated proteins.*

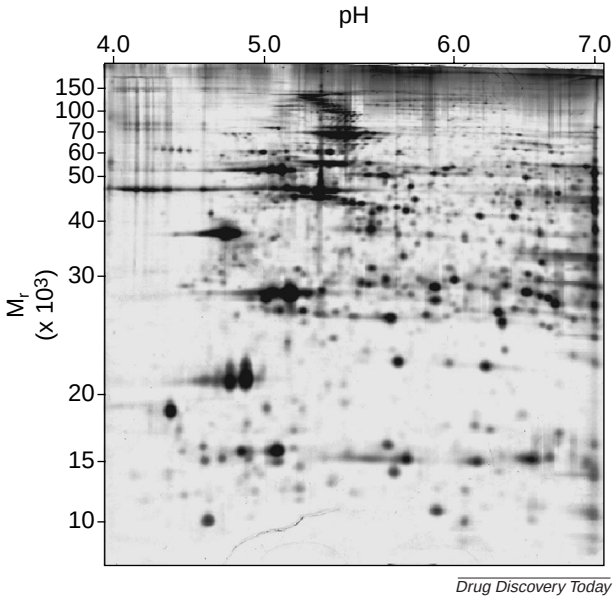

Figure 2. *A two-dimensional electrophoresis (2-DE) separation of 80 μg of heart (ventricle) proteins. An 18 cm linear pH 4–7 immobilized pH gradients (IPG) isoelectric focusing (IEF) gel was used in the first dimension. The second dimension was a 21 cm 12% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) gel. The proteins were detected by silver staining. The scale at the top indicates the linear pH range of the first-dimension IPG strip from which the apparent isoelectric points (pI) of the separated proteins can be estimated. The M_r (relative molecular mass) scale can be used to estimate the molecular weights of the separated proteins.*

the pH gradients required for IEF (for a detailed review, see Ref. 19). This problem has been overcome using Immobiline reagents (Amersham Pharmacia Biotech, Amersham, UK) to generate immobilized pH gradients (IPG)[20]. Early attempts to apply the IPG technology to 2-DE separations encountered many problems, but these have now been overcome so that IPG IEF is the current method of choice for the first-dimension of 2-DE (Ref. 21). IPG IEF is performed on individual gel strips, 3–5 mm wide, cast on a plastic support. After steady-state IEF, the strips are equilibrated and applied to the surface of either vertical or horizontal slab SDS-PAGE gels[21]. Interlaboratory studies of heart, barley and yeast proteins have demonstrated that excellent reproducibility can be achieved using this method[22,23].

Sample preparation is a vital parameter in proteomic studies, involving solubilization, denaturation and reduction to break all the non-covalent interactions between the sample proteins[19]. However, this solubilization process must be compatible with the first-dimension separation by IEF, precluding the use of efficient but highly charged detergents such as SDS. The sample lysis buffer used for 2-DE typically contains 7–9 M urea, a non-ionic (such as NP-40) or zwitterionic detergent (such as CHAPS), and a reducing agent [such as dithiothreitol (DTT)]. Unfortunately, this 'standard' solution is not ideal for all proteins and a major remaining challenge in proteomics is the development of appropriate methods for the solubilization of particular classes of proteins such as membrane and membrane-associated proteins. This has resulted in the development of new reagents such as thiourea, sulfobetaine surfactants and alternative reducing agents (e.g. tributyl phosphine) that provide improved protein solubilization for proteomics[24].

The ability of 2-DE to separate complex protein mixtures is dependent on gel size. Approximately 2,000 proteins can generally be separated from cell and tissue extracts using the current 'standard' combination of 18 cm IPG strips with SDS-PAGE gels of 22 cm length. Very large format gels exceeding 30 cm in each dimension are capable of separating as many as 10,000 proteins[25]. However, the problem associated with handling such large gels precludes their routine use. By contrast, only a few hundred proteins can be separated using small gel formats (e.g. 7 cm square), but the speed with which such 2-DE separations can be performed makes them useful for screening purposes. Separation also depends on the pH gradient used for the first-dimension separation and, fortunately, the IPG method enables considerable flexibility. Wide pH (3–10) gradients can be used to provide an overview of the protein diversity in a sample (Fig. 1), while narrow-range IPG strips covering a pH of 1–3 improve the resolution of this particular separation 'window' (Fig. 2). An additional advantage of narrow-range IPGs is that they tolerate higher protein loading for micro-preparative purposes. It has recently been proposed that using IPG strips with narrow, overlapping pH ranges, enables the resulting 2-DE gels to be viewed side-by-side to form the equivalent of proteomic 'contigs'[26]. Very basic gradients up to pH 12 are now available, and these can be used to separate very basic cellular components such as nuclear and ribosomal proteins[27].

Protein detection

Silver staining is currently the most popular method of protein detection with a sensitivity of approximately 0.1 ng protein per spot. More sensitive fluorescent dyes are currently being developed and these have the additional advantages of superior linearity and dynamic range compared with silver staining. Radiolabelling, if applicable,

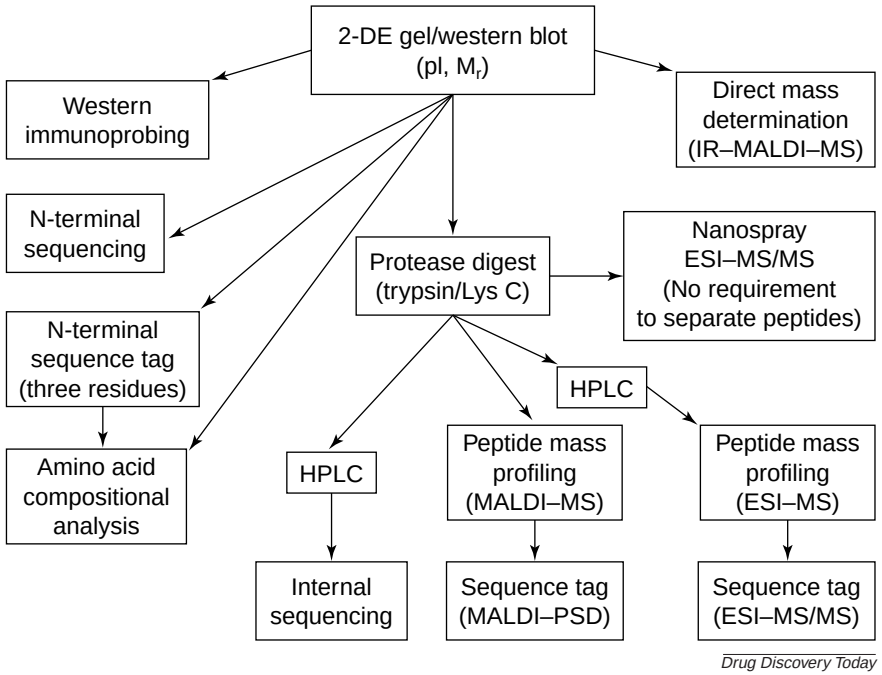

Figure 3. *Methods used for the identification and characterization of proteins separated by 2-DE. Abbreviations: 2-DE, two-dimensional electrophoresis; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; IR, infrared; M_r, relative molecular mass; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; pI, isoelectric point; PSD, post-source decay.*

can also achieve very high sensitivity, while western immunoblotting techniques make it possible to detect as few as 1,000 molecules per cell[28].

Qualitative and quantitative analysis

A range of devices, including modified document scanners, laser densitometers, CCD (charge-coupled device) cameras, and fluorescent and phosphor imagers, are available for the acquisition of 2-DE gel images. Specialized, dedicated software[29] running on desktop workstations enable the derivation of qualitative and quantitative information from individual 2-DE gels, the matching of protein separation profiles from numerous 2-DE gels and the construction of comprehensive databases of quantitative protein expression for cells, tissues and whole organisms.

Protein identification and characterization

2-DE provides information on the abundance, pI and M_r of the separated proteins, but gives no direct clues as to their identities or functions. Fortunately a variety of sensitive methods have become available for the identification and characterization of proteins separated by 2-DE (Fig. 3). Conventional methods include western immunoblotting, microsequencing by automated Edman degradation[30] and amino acid compositional analysis[31].

Over the past few years, mass spectrometry (MS) methods have become increasingly important for the analysis of proteins and peptides because of their very high sensitivity and capacity for high-sample throughput[32,33]. This was made possible by the development of matrix-assisted laser desorption (MALDI) and electrospray (ESI) ionization methods that are capable of ionizing very large molecules with little or no fragmentation. A variety of analyzers are then used to determine molecular mass. MALDI sources are usually coupled to time-of-flight (TOF), while ESI sources can be coupled to analyzers such as quadropole, ion-trap and hybrid quadropole time-of-flight (Q-TOF).

The primary tool for MS identification of proteins in proteomic studies is peptide mass fingerprinting (PMF). This technique is based on the finding that the set of peptide masses obtained by MS analysis of a digested protein (e.g. using trypsin) provides a characteristic mass profile or 'fingerprint' of that protein (Fig. 4). This mass profile is then compared with possible peptide masses either calculated from theoretical *in silico* digestion of known proteins or predicted from nucleotide sequence databases. Several algorithms have been implemented to facilitate this process and their output is a list of putative identities ranked in order of probability (Table 1).

PMF works very effectively for species whose genomes are completely sequenced, but is not so reliable for organisms whose genomes have not been completed. This can be a significant problem for proteomic studies of some animal models of heart disease, for example those involving rats, dogs, pigs and cows. In these instances, the reliability of PMF can be improved by adopting an orthogonal approach combined with amino acid compositional analysis[34].

However, if it is impossible to assign an unequivocal identity to a protein based on PMF, it is then essential to obtain peptide sequence information. This can be generated by conventional automated chemical Edman microsequencing,

but is most readily accomplished using tandem mass spectrometry (MS/MS). MS/MS takes advantage of two-stage MS instruments, either MALDI–MS with post-source decay (PSD) or ESI–MS/MS triple-quadropole, ion-trap or Q-TOF machines, to induce fragmentation of peptide bonds.

The first of these methods, termed peptide sequence tagging, is based on interpretation of a portion of the ESI–MS/MS or PSD–MALDI–MS fragmentation data to generate a short partial sequence or 'tag'. This is used in combination with the mass of the intact parent peptide ion and provides a significant quantity of additional information for the homology search[35]. The second approach is based on the use of the database searching algorithm, SEQUEST (Ref. 36), to match uninterpreted experimental MS/MS spectra with predicted fragment patterns generated *in silico* from sequences in protein and nucleotide databases. These techniques are capable of very high sensitivity. Using a nano-electrospray ion source that enables spraying times of more than 30 min from approximately 1 μl of sample, sensitivities in the low femtomole range can be achieved, enabling the sequencing of protein spots from silver-stained 2-DE gels containing down to 5 ng protein[37].

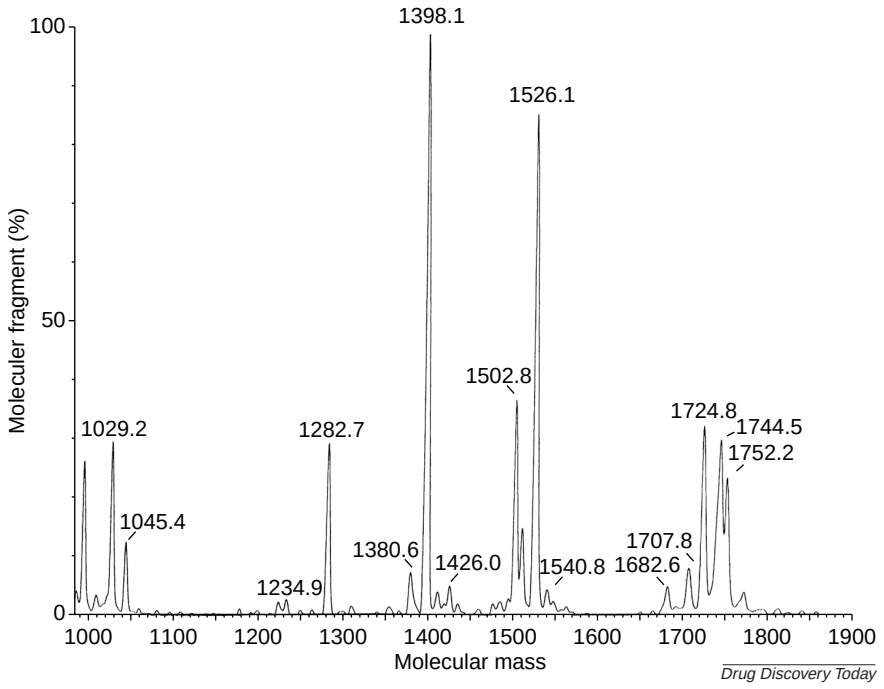

Figure 4. *Peptide mass profiling of a silver stained (modified Pharmacia kit) spot from a two-dimensional electrophoresis (2-DE) separation of human heart (ventricle) proteins. The protein was digested* in situ *within the gel with trypsin. Mass spectrometry (MS) spectrum of the resulting tryptic peptides analyzed using a Micromass Tofspec MALDI (matrix-assisted laser desorption ionization)–MS in reflectron mode.*

Bioinformatics

Bioinformatics plays a central role in proteomics, with special software packages for quantitative analysis and databasing of electrophoretic separations and a range of bioinformatic tools for identifying proteins based on data from micro-chemical analyses. In addition, special bioinformatic tools have been developed for the further characterization of proteins, ranging from the calculation of their physicochemical properties (e.g. pI, M_r) to the prediction of their potential post-translational modifications and their three-dimensional structure. Most of these tools with their associated databases are available on the Internet through the World Wide Web (WWW), and can be accessed through the ExPASy proteomics server (http://www.expasy.ch/tools/).

2-DE protein databases

It is essential that annotated and curated databases are constructed to store all the data generated by proteomics. It is also important that these databases can be interrogated effectively both within the laboratory and, where possible, by other scientists worldwide. Currently this is best achieved using the WWW. It has been suggested that such 2-DE protein databases should be constructed according to a set of fundamental rules[38]. Databases conforming to these rules are said to be 'federated 2-DE databases', while many of the other databases conform to at least some of the rules. An index to these 2-DE protein databases can be accessed via WORLD-2DPAGE (http://www.expasy.ch/ch2d/ 2d-index.html).

Proteomics of the heart

Heart diseases resulting in heart failure are among the leading causes of morbidity and mortality in the developed world. They can result from either systemic diseases (such as hypertensive heart disease and ischaemic heart disease) or specific heart muscle disease (such as dilated cardiomyopathy). In most cases of heart failure, the present generation of therapeutic treatments can do little more than ameliorate the symptoms so that, in the majority of cases, the only option is cardiac transplantation.

Table 1. Result of peptide mass profiling of the protein spot shown in Figure 4

Rank	Number of masses matched (%)	Protein (MW Da/pI)	Species	SWISS-PROT accession number	Protein name
1	12/14 (85%)	21932.2/5.03	Human	P08590	Myosin light chain 1, slow-twitch muscle B/ventricular isoform (MLC1SB) (alkali)
2	9/14 (64%)	22156.3/5.03	Rat	P16409	Myosin light chain 1, slow-twitch muscle B/ventricular isoform
3	4/14 (28%)	28723.3/5.78	Human	Q06323	Interferon gamma up-regulated I-5111 protein precursor (IGUP I-5111)
3	4/14 (28%)	23592.9/5.42	Human	P20340	RAS-related protein RAB-6
3	4/14 (28%)	23843.5/5.27	Mouse	P07361	Alpha-1-acid glycoprotein 2 precursor (AGP 2), orosomucoid 2 (OMD 2)

The database search was performed using the MS-FIT program on Protein Prospector http://falcon.ludwig.ucl.ac.uk/mshome3.2.htm or http://prospector.ucsf.edu/

The pathogenic mechanisms underlying cardiac dysfunction in heart failure are still largely unknown, but it is likely that significant alterations in myocardial gene and protein expression underlie the disease processes and determine their progression and outcome. A detailed characterization of these changes will further the understanding of the basis of cardiac dysfunction in heart disease and heart failure, and could provide new diagnostic markers and offer new therapeutic opportunities.

Until recently, most molecular studies of cardiac dysfunction have been carried out on specific cellular systems. A recent review of this research describes alterations that have been reported in the expression of contractile proteins, calcium homeostasis and signal transduction[39]. However, researchers in this area have been quick to realize the potential of proteomics to characterize alterations in protein expression in heart disease and heart failure. Such a global approach should provide new insights into the cellular mechanisms involved in cardiac dysfunction.

Heart 2-DE protein databases

An essential tool for proteomics of the heart is the availability of appropriate 2-DE gel protein databases and, to date, three groups have established such databases of human cardiac proteins (Table 2). These databases, known as HSC-2DPAGE (Ref. 40), HEART-2DPAGE (Ref. 41), and HP-2DPAGE (Ref. 42), are accessible through the WWW and conform to the rules for federated 2-DE protein databases[38]. The databases contain information on several hundred cardiac proteins that have been identified by protein chemical methods. In addition, 2-DE protein databases for other mammals, such as the mouse, rat[43], dog[44], pig and cow, are also under construction to support work on animal models of heart disease and heart failure.

Proteomics of dilated cardiomyopathy

Proteomic investigations of human heart disease have so far concentrated on dilated cardiomyopathy (DCM), a disease of unknown aetiology. This is a severe disease characterized by impaired systolic function resulting in heart failure. DCM is almost certainly a multi-aetiological disease with known contributory factors being viral infections, cardiac-specific autoantibodies, toxic agents, genetic factors and sustained alcohol abuse. The combined results of several studies[43–48] has shown that the expression of approximately 100 cardiac proteins is significantly altered in DCM, with the majority of these proteins less abundant in the diseased heart. Many of these proteins have been identified

Table 2. 2-DE protein databases accessible via the World Wide Web

Database	Web address	Organ
HEART-2DPAGE	http://userpage.chemie.fu-berlin.de/~pleiss/dhzb.html	Human heart (ventricle) Human heart (atrium)
HP-2DPAGE	http://www.mdc-berlin.de/~emu/heart/	Human heart (ventricle)
HSC-2DPAGE	http://www.harefield.nthames.nhs.uk/nhli/protein/	Human heart (ventricle) Rat heart (ventricle) Dog heart (ventricle)
RAT HEART-2DPAGE	http://gelmatching.inf.fu-berlin.de/~pleiss/2d/	Rat heart

by chemical methods[45,49–51] and they can be classified into three broad functional classes:

- Cytoskeletal and myofibrillar proteins
- Proteins associated with mitochondria and energy production
- Proteins associated with stress responses.

A major challenge will now be to investigate the contribution of these changes to altered cellular functions resulting in cardiac dysfunction. This will require a more traditional system-based approach. For example, the expression of heat shock protein 27 (HSP27) in the human heart has been examined in detail. A family of nearly 60 HSP27 protein spots has been demonstrated on large-format 2-DE gels and differences in spot intensity between DCM and controls were observed[52].

Proteomics of animal models of heart disease

Investigations of human diseased tissues are, of course, complicated by factors such as the disease stage, tissue heterogeneity, genetic variability and the patient's medical therapy. International cooperation, together with improved bioinformatic tools, will be required to investigate the large number of human samples that will be required to overcome these problems. An alternative approach is to apply proteomics to appropriate models of human disease. Such work is still in its infancy, but a recent proteomic analysis of neonatal rat cardiac myocytes treated with phenylephrine (an *in vitro* model of cardiac hypertrophy) revealed 11 proteins with altered expression profiles following induction of hypertrophy[53]. Of these proteins, five have been identified as isoforms of the myosin light chain, two are involved in stress responses and the other four are associated with mitochondria and energy metabolism.

There are several models of cardiac hypertrophy, heart disease and heart failure in small animals, particularly the rat. Proteomic analysis of these models has so far been limited, such examples being studies of changes in cardiac proteins in response to alcohol[54] and lead[55] toxicity. A complicating factor of these small animal models is that their cardiac physiology and their normal pattern of gene expression (for example isoforms of the major cardiac contractile proteins) is rather different from that in larger mammals such as humans. Recently, two proteomic studies of heart failure in large animals (pacing-induced heart failure in the dog[56,57] and bovine dilated cardiomyopathy[58]) have been published. The results from these studies share similarities with the proteomic analysis of human DCM, with the majority of changes involving reduced protein abundance in the diseased heart.

The identification of the altered canine and bovine proteins has been particularly challenging as these species are currently poorly represented in genomic databases, so that new bioinformatic tools have had to be developed to facilitate cross-species protein identification[59]. As for human disease states, changes have been observed in cytoskeletal proteins and proteins associated with the mitochondria and energy metabolism. For bovine DCM, the most notable change was a sevenfold increase in the enzyme ubiquitin carboxyl-terminal hydrolase. This could result in increased protein ubiquination in the diseased state, leading to proteolysis via the 26S proteasome pathway. Interestingly, it has recently been suggested that inappropriate ubiquination of proteins could contribute to the development of heart failure[60].

Proteomic characterization of cardiac antigens in heart disease and transplantation

Proteomics is used for both the global analysis of alterations in cardiac protein expression in heart disease, and to identify cardiac-specific antigens that elicit antibody responses in heart disease and following cardiac transplantation. In this approach, western blot transfers of 2-DE separations of cardiac proteins are probed with patient serum samples and developed using appropriately conjugated anti-human immunoglobulins. Using this strategy, several cardiac antigens have been identified that are reactive with autoantibodies in dilated cardiomyopathy[61,62] and myocarditis[63]. In addition, cardiac antigens have been characterized that are associated with antibody responses following cardiac transplantation and these might be involved in acute[64] and chronic[65] rejection.

Conclusion

The application of proteomics to the study of heart disease is making it possible to characterize global alterations in protein expression in heart disease. It clearly complements genomics-based and more traditional systems-based approaches and promises new insights into the cellular mechanisms involved in cardiac dysfunction. While such proteomic studies are in their infancy, they are likely to result in the discovery of novel diagnostic markers and provide the basis for the development of new therapeutic approaches.

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