

Proteomics and Cardiovascular Disease

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The description of the human genome has opened new venues for the study and understanding of pathophysiological phenomena. In the 20th century, individual cell components were studied. The 21st century began with a global analysis of cell components. Thanks to the development of new technologies such as DNA chips, or two-dimensional electrophoresis, we can now study the expression of thousands of genes, or the proteins they encode, in a few hours.

Genomics has opened the way for proteomics. Improved knowledge of genes does not provide information about cell functions, because any cell expresses all genes simultaneously. Instead, there is selective gene expression depending on the cell type and the stimuli to which it is exposed. The result of this is the proteome, an ensemble of proteins that are responsible for cell functions at any given moment, which are the object of the study of proteomics.

The description of the proteome of cardiac cells has begun and some new proteins have been found to be dysregulated in different cardiomyopathies. These proteins are involved either in energy production or in the stress response, or belong to the cell proteasome or cytoskeleton. They may be potential risk markers or new therapeutic targets in the future. In this sense, chemogenomics is a new methodology for the development of new drugs using genomic and proteomic data.

Key words: *Proteomics. Genomics. Two-dimensional electrophoresis. Mass spectrometry. Cardiovascular diseases. Dilated cardiomyopathy. Proteins.*

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Proteómica y enfermedad cardiovascular

Con la descripción del genoma humano se ha abierto una nueva manera de estudiar y entender los fenómenos fisiopatológicos. El siglo xx nos permitió conocer multitud de componentes de la célula de modo individual. Sin embargo, el siglo xxi se inicia con un análisis global de los componentes celulares. Gracias al desarrollo de diversas tecnologías, como los chips de ADN, o la electroforesis bidimensional, entre otros, ahora puede estudiarse la expresión de miles de genes, o de las proteínas que codifican, en pocas horas.

Además, la genómica ha dado paso a la proteómica. La mera enumeración de los genes no informa de las funciones celulares, pues ninguna célula los expresa todos simultáneamente, sino que, dependiendo del tipo celular y de los estímulos que reciba, expresará una parte variable de su genoma. El resultado será el proteoma, es decir, un conjunto de proteínas que sí son las responsables de las funciones celulares en cada momento, y que es el objeto de estudio de la proteómica.

En proteómica cardiovascular ha comenzado a describirse el proteoma de las células cardíacas y algunas proteínas nuevas, no identificadas previamente, que están alteradas en distintas miocardiopatías. Estas proteínas están implicadas en la producción de energía, en respuesta al estrés, o pertenecen al proteasoma o al citoesqueleto y pueden ser potenciales marcadores de riesgo y constituir nuevas dianas terapéuticas en el futuro. La quimiogenómica aparece como una reciente metodología que posibilita generar nuevos fármacos a partir de los datos genómicos y proteómicos.

Palabras clave: *Proteómica. Genómica. Electroforesis bidimensional. Espectrometría de masas. Enfermedad cardiovascular. Miocardiopatía dilatada. Proteínas.*

INTRODUCTION

The 20th century was characterized by great advances in the understanding of many components of the cell, the description of receptors, transcription factors, genes involved with various basic (cell division, apoptosis) and pathological (oncogenes, susceptibility

genes) processes, but all in isolation. The 21st century began with a quantum leap of enormous magnitude, the impact of which we still do not appreciate at the present time. Currently we are able to study the entire spectrum of cell or tissue components, basically referred to as genes and proteins, in a much shorter amount of time than that needed to understand a single component only a few years ago.¹⁻³

How was such an important leap made possible? First, due to the documentation of the complete genome of various organisms (from bacteria to the mouse), and more importantly, of the human genome. We now have a total list of the genes that define the human species and it is an invaluable source for understanding of physiological and pathological cell functions. The availability of the genome and of numerous genomic and protein databases are essential for a new perspective on the cell and on living organisms.⁴ Second, the diffusion and accessibility of numerous navigators and simple informatics programs have allowed acquisition of information from genomic and protein databases. It is currently possible to trace a complete genome to find a particular protein or gene sequence in a few seconds. These searches are integrated with other programs that, in turn, predict the function of the proteins identified, their interactions with other proteins, etc.⁵ Third, the development of DNA chips, which contain thousands of cDNA sequences or oligonucleotides in a slide like those used in optic microscopy (2x5 cm in size), allows detection of the expression of thousands of genes simultaneously in a few hours. We can observe «the complete system», the total group of genes expressed. The information contained in these thousands of data, however, challenges our capacity to interpret them intuitively, and therefore we need new tools that allow us to integrate such enormous groups of data. This process involves understanding systems rather than individual components, relating these systems, and giving a biological twist to such complexity. Finally, the appearance and development of the proteomic, or functional genomic, has begun to reveal the identify and function of the thousands of proteins encoded by genes, which are the true molecules that carry out the functions of the cell.⁷

From genomics to proteomics

While genomics is the study of groups of genes (genome) of an organism, proteomics is the study of the proteins (proteome) that a genome might express (Figure 1). If the expression of thousands of genes of an organism can be analyzed through the use of DNA chips, why are proteomics necessary? First, because for each gene, various, rather than one unique, protein is often generated. Alternative splicing, reading the codons in different phases or variations of the stop codons generates different proteins.⁸ Second, because

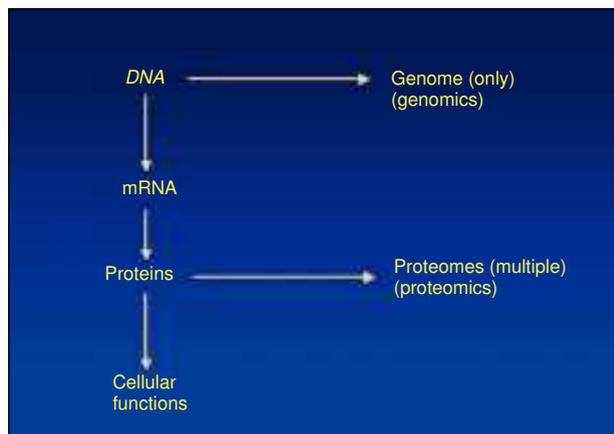


Fig. 1. General chart of the relationship between genomics and proteomics and the reason for their study. Functional genomics attempts to determine the function of each gene, the great majority of which are performed by the proteins, which are the true working molecules of the cell.

the proteins are modified post-translationally, by means of phosphorylation, glycosilation, prenylation, acetylation, and sulphation, among others, and because there are more than 200 types of modifications described. These modifications affect the structure, locations, function, and exchange, and affect activation and regulation functions, response to the environment, etc., and also are critical for control of the protein degradation processes. For example, phosphorylation of various proteins leads to their conjugation with ubiquitin that will cause the breakdown of the protein by proteasome 26S. Therefore, many proteins are present in multiple molecular forms (Figure 2). This essential information can only be determined by studying proteins and not genes. Third, there is no direct correlation between the levels of RNA expression (transcription) and protein expression.⁹ A short half-life messenger RNA (mRNA) can generate a long half-life protein, and vice versa. When the protein is present in the cell (and can be detected) the mRNA already cannot be detected. The expression of mRNA does not reveal the activity of the protein that it encodes or its possible combinations or interactions with other proteins that generate new functions, etc.¹⁰ Finally, it must be taken into account that the targets of 90% of drugs are proteins.¹¹

The proteome is a photograph of protein expression

Each of our cells contains the complete genome of a human being. Nevertheless, not all genes are expressed in all cells. Cellular differentiation to generate distinct cell lineage and tissues is due to a varying expression of genes. Thus, although all cells express some common genes whose proteins provide essential

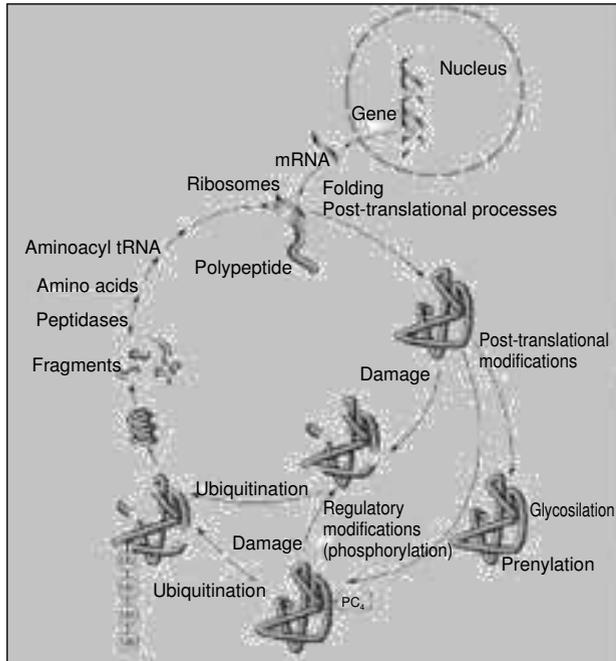


Fig. 2. A protein can present in various molecular forms that are present simultaneously present during its life cycle, causing great complexity in the proteome (8).

functions (glucose catabolism, DNA synthesis, etc.), each type of cell will selectively express those that encode the proteins necessary to perform the functions specific to that cell. The proteome of any cell, at any given moment, represents only a fraction of all the possible proteins that the genome can express. This does not mean that the proteome is simpler than the

genome, but rather the contrary. Proteins that are expressed or modified vary as a function of circumstances, environmental attacks, drug action, energy requirements, etc. (Figure 3). In contrast to a certain static quality that the genome possesses, the proteome is a dynamic group of changing proteins. Therefore, only 1 genome exists while a multitude of proteomes exist, and it is the proteome that faithfully reflects the state of the cell at any given time.^{12,13}

When the total number of genes in diverse organisms was known, it became clear that the complexity of an organism and the number of genes that it possesses is not a simple relationship. It may be surprising that the human genome only has double the number of genes that a worm has; nevertheless, biological complexity does not come simply from the number of genes involved. The number of domains or structural units of proteins that human genes can generate is greater than that of inferior organisms, which in combination generates a larger number of proteins with more complex regulation and multiple functions. The complexity of the human organism probably comes from the diversity of proteomes that we can generate, plus the number of genes that make up our genome.¹⁴

PROTEOMICS TOOLS

Although proteomics is conceptually simple, in practice it is complex. The classic method, common in proteomics, is to separate and measure the proteins in a sample (cell, tissue, or fluid) by 2-dimensional (2-D) electrophoresis¹⁵ or multidimensional chromatography¹⁶ (also by capillary electrophoresis, but use of this

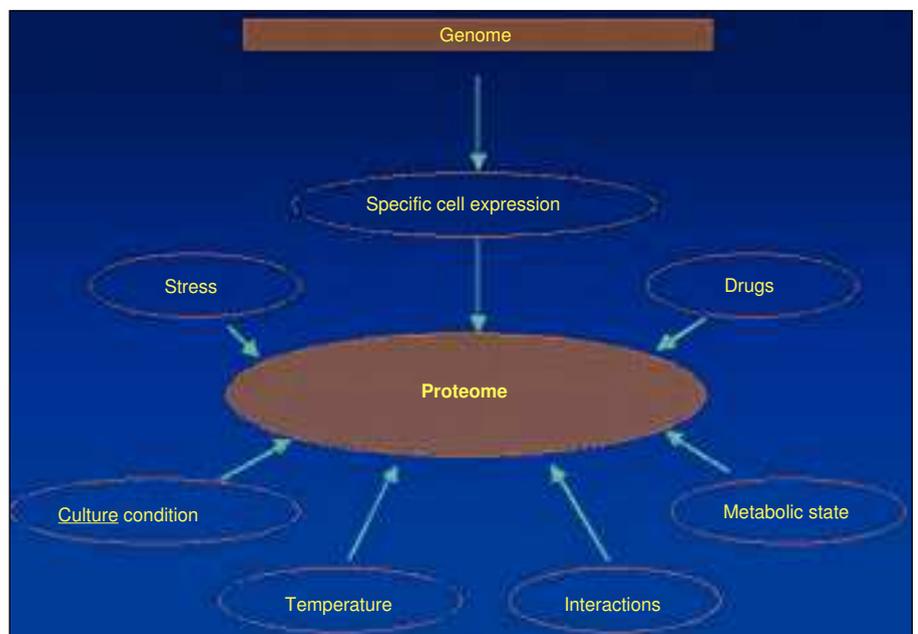


Fig. 3. The expression of the proteins in each cell is influenced by numerous environmental conditions, pharmacological treatments, etc., rendering the proteome changeable and dynamic, reflecting a particular situation at any given time.

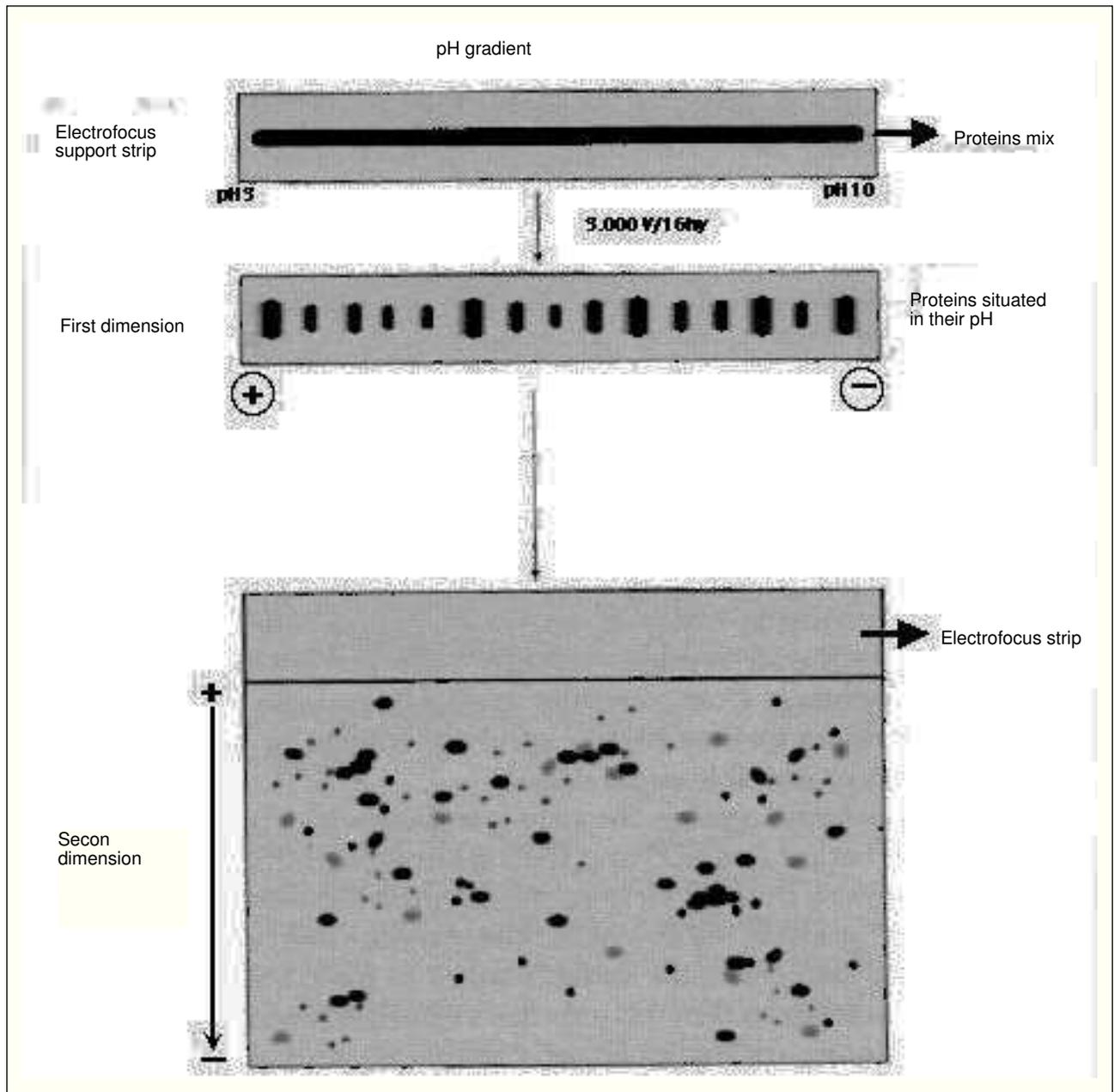


Fig. 4. Graphic of the process of 2-D electrophoresis. The sample proteins (tissue, cells) are applied to an electrofocus support or strip and separate according to the isoelectric point upon application of an electrical field. The strip is then placed on a polyacrylamide gel and the proteins separate according to molecular size, in a second electrophoresis, upon application of an electrical field perpendicular to the first. Once tinted, a 2-D gel is obtained (see Figure 8 with an actual gel).

method is less common),¹⁷ and then identify each of the proteins by mass spectrometry (MS).¹⁸ Mere measurement does not reflect the functional state of the protein, or its location or possible interactions with other molecules; therefore, proteomics involves many other techniques (characterization of the post-translational modifications by MS, structural analysis by X-ray diffraction or magnetic resonance, intracellular location by confocal microscopy, etc.) which completes the description of the proteome.^{8,14}

Two-Dimensional electrophoresis

2-D electrophoresis is a protein separation technique that consists of a succession of 2 different electrophoresis procedures performed on the same sample.¹⁵ The tissue or cells that are to be analyzed are solubilized on buffers that contain dissociating and detergent agents that facilitate the later separation of each individual protein. The first step is an electrofocusing, in which the proteins separate according to their isoelec-

tric point (pI) along a pH gradient (for example, pH 3 to 10), and the second is a polyacrylamide gel electrophoresis in the presence of a detergent (PAGE-SDS) in which the separation occurs according to the molecular mass of the proteins (Figure 4).

These techniques have been available for 25 years; nevertheless, only recently have supports for electrofocus with stable, reproducible, and commercially available pH gradients been obtainable. At present such supports exist (they are small, 0.5×18 cm strips) with a variety of gradients, extended (pH 3 to 10) or expanded (pH 4 to 5, 5 to 6, etc.).¹⁹ The resulting 2-dimensional (2-D) gels are stained with specific protein colorants (Coomassie Blue, silver staining, fluorescent compounds, etc.) and they are scanned and digitized via imaging programs (Melanie, PDQuest); the group of proteins in a sample is obtained in a 20×20-cm gel. Under regular conditions a cell sample that contains 100 to 300 µg of total protein generates 1000 to 2000 stains (proteins) and under certain conditions (larger gels) in specialized laboratories, up to 10 000 stains.²⁰ The proteins that are present in very low concentrations or with particular characteristics (very basic proteins or very hydrophobic membrane proteins) are difficult to detect. The use of expanded gels or prior fractioning of the samples (obtaining membranes or mitochondria, cytoskeleton, etc.) to study specific proteins circumvents these problems and considerably increases detection levels.

Liquid multidimensional chromatography

High performance liquid chromatography (HPLC) is another option for the separation and measurement of proteins. This is an analytical technique that separates molecules according to the type of support (chromatographic column) that is used (ionic change, reverse phase, affinity, etc.). Tandem HPLC, analogous to the 2-D technique, combines 2 different types of chromatography by connecting 2 columns (Figure 5). Usually the first is an ionic exchange column (separated by charge) and the second is reverse phase (separation by hydrophobicity).¹⁶ Although this system allows the separation of mixes of proteins, it is used, for best results, for the separation of peptides. Therefore, once a tissue of cell sample is solubilized, the group of proteins is directed with a protease (usually trypsin), and it produces a very complex mix of peptides (thousands) which separate in HPLC. At present these chromatographs use capillary columns (75 to 100 µm internal diameter and 5 to 10 cm long) which allows for quick analysis of a very small sample (picomoles, femtomoles, or even less). Thus, for example, protein peptides can be identified in very low concentrations in the cell that are normally not detected in 2-D gels. In addition, HPLC can be connected directly to a mass spectrometer, which permits automatic identification

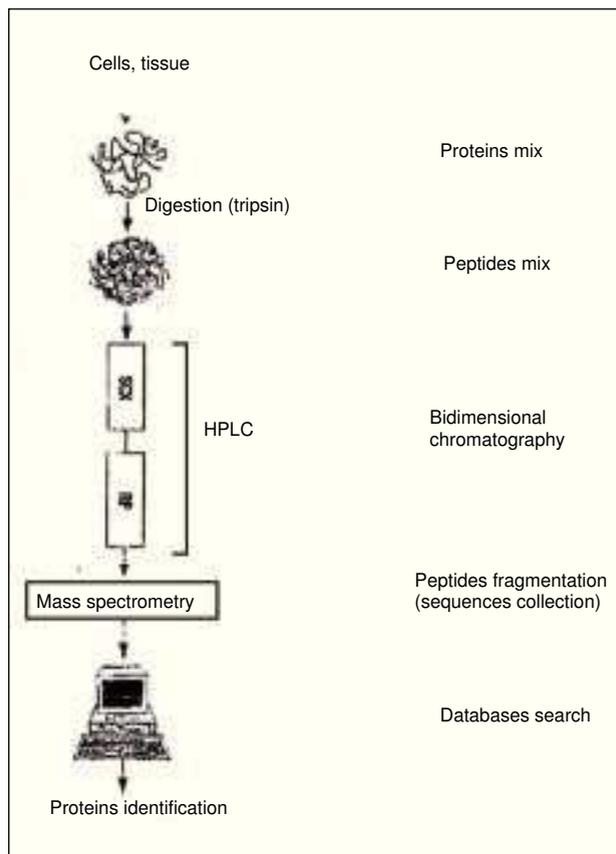


Fig. 5. Chart of the separation by multidimensional chromatography of the peptide group that is a product of the digestion of the proteins in a sample (biopsy, cells). SCX indicates chromatographic column 1 (separation by charge); RP, column 2 (separation by hydrophobicity). The eluted peptides are analyzed by mass spectrometry, usually by an electrospray system (ESI) (see Figure 7), and their sequence is obtained. With this, the search programs (for example, SE-Quest) in the databases identify the protein from which the peptide comes.

of thousands of peptides – and therefore of the proteins from which they originate – even as they are eluting from the HPLC.²¹

Mass spectrometry

Once the proteins are separated and measured by 2-D or HPLC they must be identified. This is carried out with mass spectrometry (MS), an analytical technique that determines in highly sensitive and precise manner the molecular masses of chemical or biological complexes. In addition, mass structural data may be deduced and the complexes may be identified.¹⁸ MS requires the conversion of the complexes (in our case, proteins and peptides) into gas-phase ions, using various procedures. The ions separate according to the ratio of their mass (*m*) to their electrical charge (*z*) (*m/z*), using a mass analyzer, and captured with highly sensitive detectors.¹⁸

In proteomics two mass spectrometers are basically used. The first is the MALDI-TOF (matrix-assisted

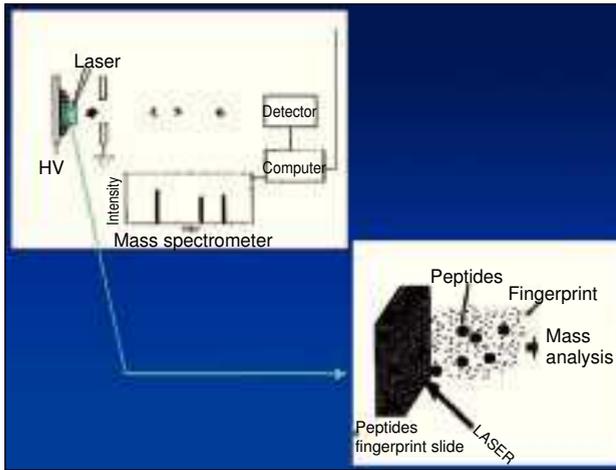


Fig. 6. Analysis by mass spectrometry with MALDI-TOF of the group of peptides generated by digestion of a protein with trypsin. The peptides vaporize with a laser pulse (shown in the upper portion of the figure) and are accelerated with high voltage (HV) toward the flight tube. The computer collects the data from the detector and presents the result or mass spectrum, in which the mass (actually the m/z) of the peptides and their intensity is shown in order from lesser to greater. Each protein generates a characteristic spectrum or peptide fingerprint that permits the identification of the protein in the databases.

laser desorption ionization time of flight)²² (Figure 6). Ionization is achieved by combining the sample (group of peptides) with organic compounds (called matrix) that crystallize and that are subjected to a laser pulse (nanoseconds) that vaporizes the peptides. These are accelerated in an electrical field (20 to 25 kV) and are

sent to a flight tube (3 m), at whose end the detector is located. For a given electrical acceleration voltage (20 to 25 kV), the time of flight (TOF) in microseconds taken to arrive at the detector is proportional to the m/z . Thus, small molecules fly more quickly than large molecules and they are detected in order of increasing mass. The group of peptide masses that originate from a given protein provides a peptide fingerprint that allows comparison with the theoretical masses of the peptides that would be produced upon digestion of the proteins present in the databases. This comparison allows identification of the protein.²³⁻²⁵

The second type of spectrometer vaporizes the sample (peptides, proteins) directly from the liquid phase in which it is dissolved by means of electrospray ionization (ESI) or nebulizer²⁶ (Figure 7) in such a way that the sample is dispersed in microdrops that contain the ionized peptides. This is a mechanism that is similar to the nebulizers used for perfume, with an electrical field being the dispersing force. In addition to determining the peptide mass, with these apparatus (ionic trap, Q-TOF) a peptide of a certain mass can be selected and be broken in a collision chamber in the presence of a gas. The resulting fragments (ion offspring) are sent to the detector and their masses are obtained, from which the sequence of the peptide can be acquired. In addition to the sequence of 1 or more peptides, the search for and identification of the protein in the databases is univocal and is much more reliable than provided by the peptide fingerprint obtained with the MALDI-TOF.²⁷

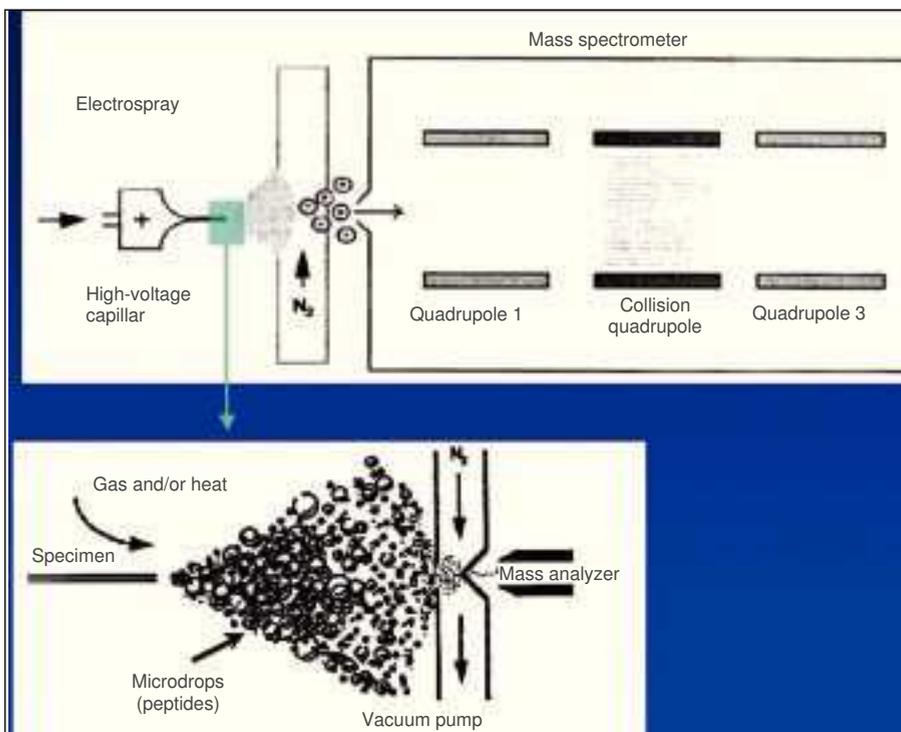
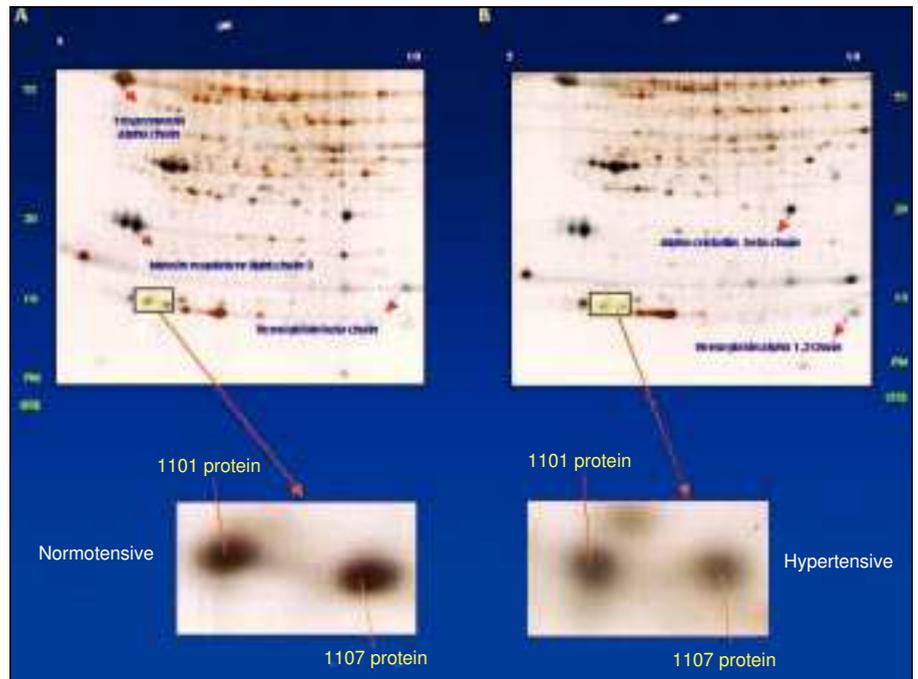


Fig. 7. Graphic of a mass spectrometer (triple-quadrupole type) with an electrospray system (ESI) for vaporizing the sample. The microdrop solvent that the peptides contain is eliminated with an N_2 curtain and the peptides are directed to the mass analyzer by electrical fields. In the collision chamber the peptides can be fragmented and the fragment mass spectrum can be obtained, which allows the peptide sequence to be deduced.

Fig. 8. 2-D gels of normotensive and hypertensive rat heart in which the separated and tinted proteins can be seen. The figure shows only a fraction of the gel. In the upper portion, a widening can be observed in comparison to an area in which the 2 proteins are expressed differently. The figure comes from the authors' laboratory, where a project on cardiovascular proteomics has been in progress for a year. KD indicates kilodaltons; MP, molecular weight.



PROTEOMICS AND CARDIOVASCULAR DISEASE

The various cardiovascular diseases are reflected, collectively or individually, in the proteomes of cardiac muscle and in the diverse components of the cardiovascular system, including smooth muscle cells, endothelial cells, and circulating cells.²⁸⁻³²

For example, the molecular mechanisms of ventricular dysfunction are unknown, but it is logical to assume that significant changes exist in the expression of genes and proteins of the myocardium that characterize this process and effect its development and outcome.²⁸ A detailed study of the proteins and genes responsible could establish the molecular bases of ventricular dysfunction, and could provide new diagnostic markers and result in new treatments. In chronic pathological cases, the abnormal expression of proteins is reflected in altered concentrations of same. Nevertheless, in acute cases, where rapid response does not allow synthesis of proteins, it is reflected in post-translational changes in already existing proteins. Proteomics studies these changes, which can be identified by different experimental approaches. The changes in the group of proteins expressed (protein profile)³³ are detected by comparing the proteins present in a control sample compared with the pathology, and identifying and measuring the differentially expressed proteins. In some cases identification of the proteins is not necessary. For example, in preclinical trials, when one wishes to know the effect of a group of pharmacological agents, it suffices to determine whether the profile of proteic expression varies in a

similar manner with each drug. This allows quick analysis of the differences between various treatments. Post-translational changes must be studied individually for each protein, and once again MS is a great advance for the identification and localization of these changes.²⁵

2-D gels and heart protein databases

An indispensable tool for the study of the cardiac proteome is the establishment of a database that contains the group of proteins of the myocardium. A first attempt has been the creation of databases of 2-D gels with an inventory of the spots (proteins) that have been separated thus far. Some of these were later identified via MS.³⁴⁻⁴³ On images of 2-D gels the protein of interest can be selected (by clicking with the computer mouse) and the available information can be obtained (name, database access number, molecular mass), IP, amino acid sequence, etc.).

The first 2-D gels, which were low resolution and of myocyte proteins, at the beginning of the 90's were able to separate 200 to 250 spots.^{44,45} In 1994, 1500 spots were assigned, according to their position in 2-D gels, using IP and their molecular mass as coordinates.^{39,40} In 1996, separation of more than 3300 spots with high resolution (24×30 cm) 2-D gels was achieved.⁴¹ Currently, under normal conditions, 1500 to 1800 spots have been separated (Figure 8) and more than double that with expanded gels with a single pH unit (pH 5 to 6 along 24 cm) in the first dimension. Table 1 shows the principal 2-D gel databases for cardiac samples and their Internet address. The data-

bases are federated; in other words, a series of requirements must be met, such as allowing remote access, being connected by links, the existence of a general index with unique entries, offering accessibility to each protein from the 2-D gel images, etc.³⁷ Of the total spots (3300) only 10% have been identified in the group of species studied.⁴⁵⁻⁶⁰ This gives an idea of the difficulty in identifying proteins, especially in animal species, such as the rat and dog, in which the genome is barely known and there are a multitude of very similar (paralog) proteins which are difficult to distinguish from each other. Table 2 shows many of the proteins that have been identified in cardiac tissue, especially in the ventricular area. In this process of protein identification, the use of antibodies (immunoblotting) has been a great help.^{38,41,50,53}

Proteic profile of dilated myocardiopathy

Myocardiopathy has been the preferred object of proteomic studies in the cardiovascular field, both in human tissue and in bovine and canine tissue.^{43,45,51,54,61-63} Dilated myocardiopathy (DMC) is the most frequent reason for cardiac transplant, and each year occurs in 5 to 8 of every 100 000 people.⁶⁴ Nevertheless, its etiology is unknown, although it has been related to different factors, particularly genetic ones.^{31,32,65-67} Two-dimensional gels of biopsies from humans with this disease reveal a lower level of expression of 88 proteins compared with the expression profile of samples with ischemic cardiopathy.^{48,49} Notable among these is desmin, ATP synthetase, creatin kinase, light chain myosin, and several members of the heat shock protein family (HSP-60 and 70).⁵⁰⁻⁵² In animal models, canine and especially bovine DCM show great similarities to the humans with the disease. In fact, the proteomic studies of cardiac muscle from the 3 species are very similar, characterized by a lesser expression of a group of proteins. In the canine and bovine models changes can be seen in several mitochondrial proteins implicated in the production of en-

ergy (HSP-70, pyruvate dehydrogenase, triosephosphate isomerase, β -enolase, isocitrate dehydrogenase, etc.) and structural proteins, such as desmin and actin.^{53,54,68}

In the bovine hereditary DCM model there is a notable increase in C-terminal ubiquitin hydrolase,⁶³ which has also been verified in human myocytes from hearts with this disease. The breakdown of proteins by the ubiquitin-proteasome system has a notable role in different cell processes, including the regulation of the cell cycle, transcription, antigen processing, and muscular remodeling. All human hearts with DCM studied show an increased level of expression of different enzymes (E1, E2) of this system. Ubiquitin C-terminal hydrolase quintuples its level in DCM hearts compared with healthy control subjects, a fact that has been confirmed histologically with specific antibodies. This hyper-expression leads to an increase in the ubiquitination of various cardiac proteins that are then seen to reduce their expression. It is thought that this increase in the proteolysis of proteins must be a critical factor that causes a loss of normal cell activity in the myocyte of the DCM heart and, finally, heart failure. Of the 26 abnormally ubiquitinated proteins that have been identified, 16 had previously been identified as proteins with altered levels in human DCM. In fact, these 16 proteins present reduced expression. With this data, the hypothesis has been made that changed or inappropriate conjugation of ubiquitin with key regulatory enzymes during the hypertrophic response may alter the homeostatic processes and constitute a mechanism that contributes to the transition from a state of compensated hypertrophy to a state of decompensated cardiac failure. This group of data has resulted in consideration of the ubiquitin-proteasome route as a new target for therapeutic intervention in heart disease.⁶⁹ When comparing the proteins expressed in the left ventricle of dogs to those in which heart failure has been induced by means of transplantation of a pacemaker with those expressed in control animals, 62

TABLE 1. 2-D gel databases and proteins from the human heart and other species available on the Internet and their access address (URL)

| Tissue | Database | Web address-URL | Institution |
|---|--|--|--|
| Human, ventricle, endothelial cells Rat, ventricle Dog, ventricle | HSC-2D PAGE (last updated 1998) | www.harefield.nthames-nhs.uk/ | Heart Science Centre Harefield Hospital |
| Human, atrium, ventricle | HEART-2D PAGE (last updated 1995) | www.chemic.fu-berlin.de/use/pleiss/ | German Heart Institute Berlin |
| Human, ventricle | HP-2D PAGE (last updated 1997) | www.mdc-berlin.de/~emu/heart/ | Max Delbrück Center for Molecular Medicine (MDC) |
| Rat, ventricle | RAT HEART-2D PAGE (last updated 2001) | www.gelmatching.inf.fu-berlin.de/~pleiss/2d/ | German Heart Institute Berlin |

TABLE 2. Cardiac proteins identified via proteomic studies and their association with various diseases in humans and in different animal models

| Protein, name | Level | Associated disease | Observations | Protein, name | Level | Associated disease | Observations |
|-------------------------------------|-------|--------------------|--------------|--------------------------------------|-------|--------------------|--------------|
| Proteins (cytoskeletal/contraction) | | | | Proteins (energy/metabolism) | | | |
| 1. Desmin | d | MCDp | PKCε | 1. ATPase synthase (α, β) | l | MCDh,d,b | |
| | l | MCDb | | 2. Cytokine C oxidase (VA, VIB) | d | MCDd,b | |
| 2. Actin | l | MCDd | PKCε | 3. Cytochrome b5 | d | MCDd | |
| | | | PTM | 4. Prot. bind. A. Fats | d | MCDd,b | PTM |
| 3. LCM2 (ventr.) | | | TM | 5. 3.2-Transenoi-CoA iso. | D | MCDd | |
| (atrial) | l | HRE | PTM | 6. Dihydroliipoamide dehydrogenase | d | MCDd | |
| 4. LCM1 (atrial) | l | HRE | PTM | 7. Isocitrate dehydrogenase | d | MCDb | |
| | c | MCDh | | 8. Creatinkinase (b, m) | d | MCDb | |
| 5. SERCA 2A | d | MCDd | | 9. OH-metil glut. CoA sin. | D | MCDd | |
| 6. Troponin T2 | | PKCε | | 10. NADh ubiq. oxi. reduc. | | HRE | |
| 7. α-Tropomyosin | | | PKCε | 11. Ubiq. cit. C reduc. | D | MCDb | |
| 8. Prohibitin | | | PKCε | 12. Pyruvate dehydrogenase (E1) | i | MCDd | |
| 9. GM130 | | | PKCε | 13. Trios phosphate isomer. | l | MCDd | PTM |
| 10. Villin | | | PKCε | 14. Phosphoglyc. mut. (musc.) | l | MCDd | |
| 11. Lap2 | | | PKCε | 15. Isocyt. dehydrogenase mitoc. (a) | d | MCDd | |
| 12. Caveolin 3 | | | PKCε | 16. Fac. elon. tu (PreP43) | a | MCDd | |
| 13. Adaptin b | | | PKCε | 17. Per. red. thioredox. | D | MCDb | |
| 14. Heavy C. myosin | | | | 18. Prot. bind. acyl-CoA | l | MCDb | |
| 15. Emerin | | | | 19. Pyruvate dehydrogenase E1-b | | | |
| 16. Lamin A, C | | | | 20. Constitutive HSP-70 | | | |
| 17. Myoglobin | | | | Proteins (stress/HSP) | | | |
| Proteins (signaling) | | | | 1. HSP-72 | n | HSC | |
| 1. PI3 kinase | | | PKCε | 2. HSP-70 inducible | d | MCDd | PKCε |
| 2. PI3 kinase (p170) | | | PKCε | 3. Precursor HSP-70 mit. | D | MCDb | |
| 3. Connexin 43 | | | PKCε | 4. Prot. stress mit. (rel. 70) | l | MCDd | |
| 4. PyK2 kinase | | | PKCε | 5. HSP-60 | d | MCDd | |
| 5. SRC tyrosine kinase | | | PKCε | 6. Prot. matrix mitoc. P1 | d | HRE | PTM |
| 6. LCK tyrosine kinase | | | PKCε | 7. Chaperone cofactor a | l | HRE | |
| 7. BMX. Cytoplasm. | | | PKCε | 8. Crystalline ab | l | MCDh,d | PKCε, PTM |
| 8. PKBa/Akt. Stress | | | PKCε | 9. HSP-27 | l | MCDh | PTM |
| 9. p38MAPK. Stress | | | PKCε | | l | HRE | |
| 10. MAPKA PK2. Stress | | | PKCε | 10. Protein 30-40 kD | n | HSC | |
| 11. JNK1 (p46). Stress | | | PKCε | 11. iNOS | | | PKCε |
| 12. JNK2 (p54). Stress | | | PKCε | 12. eNOS | | | PKCε |
| 13. MEK 1. ERK route | | | PKCε | 13. COX-2 | | | PKCε |
| 14. ERK 1 (p44). Extrac. sig. | | | PKCε | 14. Fac. inducible hypox. | | | PKCε |
| 15. ERK 2 (p42). Extrac. sig. | | | PKCε | 15. Hemoxigenase 1 | | | PKCε |
| 16. RAS. Transduction | | | PKCε | 16. Aldose reductase | | | PKCε |

(continue)

Table 2 includes the proteins described in the databases in Table 1.

HRE indicates hypertrophy induced by phenylephrine (myocytes of neonatal rat in culture); HSP, heat shock proteins; DCM, dilated cardiomyopathy; h, human; b, bovine; d, dog; p, pig; r, rat; LCM, light chain myosin; level i, increase; d, decrease; n, normal value; a, present in the sample and absent in the control; PKCε, proteins associated with protein kinase C; PTM, post-translational modification.

proteins, of a total of 700, were seen to have increased levels.⁶⁰ Of these, 23 identifiable. This study established that heart failure is associated with changes in 3 cell systems: *a*) mitochondrial proteins whose expression is greatly reduced; *b*) glycolytic enzymes whose expression is increased (perhaps to compensate for the loss of ATP synthesis due to mitochondrial involvement), and *c*) changes in cytoskeletal proteins.

Analysis of cardiac subproteomes

This type of experimental approach is concerned with the study of only 1 part of the total proteome, of a specific subproteome. It tends to be used when a molecular mechanism is partially known and some of the involved proteins have been identified, but not all of them. One of the most notable examples was the use of monoclonal antibodies against protein kinase C (PKC),

TABLE 2. Cardiac proteins identified by proteomic studies and their association with various diseases in humans and in different animal models (continued)

| Protein, name | Level | Associated disease | Observations | Protein, name | Level | Associated disease | Observations |
|---|-------|--------------------|--------------|---|-------|--------------------|--------------|
| Other proteins | | | | 36. Phe-4-hydroxylase | d | MCDh | |
| 1. Farnesil di-P far. trans. | | N | MCDd | 37. Phosphofructokinase | d | MCDh | |
| 2. Prec. α 1-antiprotease 1 | | I | MCDb | 38. Transferrin | d | MCDh | |
| 3. Prec. α 1-antiprotease 2 | | d | MCDb | 39. Dihydrophol. Reductase | i | MCDh | PTM |
| 4. Galact. 3(4)-fuc. Transferase | | d | MCDd | 40. Myos. reg. LC2 fragm. | I | MCDh | |
| 5. Receptor Fc II | | I | MCDd | 41. Vimentin i MCDh | | | |
| 6. Isovaleryl-CoA dehydrogenase | | d | MCDb | 42. Act. plasminogen (β) | | | |
| 7. Syndecan 2 | | d | MCDd | 43. Related prot. RAS | | | |
| 8. Allergen Can F1 | | d | MCDd | 44. CK-BB | | | |
| 9. Cystatin c | | d | MCDd | 45. Vinculin | | | |
| 10. Protein rel. RAS | | I | MCDd | 46. Glutation-S-transferase | | | |
| 11. Ubiq. C term. hydrol. | | I | MCDb | 47. Prot. acid glial fibr. | | | |
| 12. Nucleoside di-P cin. A | | I | HRE | 48. 6-Phosphofructocinase | | | |
| 13. Inosin 5'-P dehydrogenase | | d | MCDd | 49. Factor A of C-Arg binding | | | |
| 14. Superoxide dismt. mit. | | | | 50. F1 ATPase (subun. β) | | | |
| 15. β -Enolase | | d | MCDb | 51. Keratin 1-cytoskeletal | | | |
| 16. Homol. prot. 19160 | | | | 52. HSP-71 | | | |
| 17. F1 ATPase | | I | MCDb | 53. NADH ubiq. red oxide | | | |
| 18. Seroalbumin (HSA) | | | | 54. Glucose regul. protein | | | |
| 19. Creatinkinase cad. M | | | | 55. 3-Oxoacil-CoA thiolase | | | |
| 20. Creatinkinase sarcom. | | | | 56. Phospholipase C α | | | |
| 21. Prot. rab. union GTP | | | | 57. Carbonic anhydr. (erythro.) | | | |
| 22. Glyceraldehyde-P dehydrogenase | | | | 58. Carbonic anhydr. (musc.) | | | |
| 23. Apolipoprotein A-I | | | | 59. Porine | | | |
| 24. β -Hexosaminidase (prec.) | | | | 60. Collagen α | | | |
| 25. Endothelin 3 (prec.) | | | | 61. Phosphoglycerate kinase | | | |
| 26. α 1-antitripsin (family) | | | | 62. β -Ketothiolase | | | |
| 27. Hemoglobin (cad. α , β) | | | | 63. Fructose-bis-P-aldolase | | | |
| 28. Malate dehydrogenase | | | | 64. MER5 protein | | | |
| 29. HSP-90 | | | | 65. Phosphatidiletanolamine binding protein | | | |
| 30. Aconitate hidratase | | d | MCDh | 66. Transthyretin (prealbumin) | | | |
| 31. Carbonic anhydrase | | d | MCDh | 67. Enoil-CoA hydratase (peroxis.) | | | |
| 32. Haptoglobin | | d | MCDh | 68. Precursor enoil-CoA hydrat. (mitocond.) | | | |
| 33. Lactate dehydrogenase | | d | MCDh | 69. ES1 (P2) | | | |
| 34. LCM1 ventricular | | d | MCDh | 70. Flavoprot. transferase electro. (precursor) | | | |
| 35. Myosin cad. reg. LC2 | | d | MCDh | 71. 3-Cetoacil-CoA thiolase (mitocond.) | | | |

for co-immunoprecipitation of all those proteins involved in the signal cascade of PKC, analyzing them by 2-D, and identifying them by MS. With this method, it was possible to identify 36 proteins that are physically associated with PKC during myocardial «preconditioning». Data had been accumulated that showed that activation of PKC was an essential step in developing the cardioprotective effect of ischemic preconditioning, but its molecular mechanism was unknown. Proteomic analysis has proven the involvement of structural proteins and proteins from the cytoskeleton (α -actin, prohibitin, villin, desmin, lap-2, etc.), signaling proteins (SRC tyrosine kinase, LCK tyrosine kinase, JNK1, JNK2, ERK1, ERK2, etc.) and stress response proteins (iNOS, eNOS, COX-2, HSP-70, HSP-27, crystalline $\alpha\beta$, etc.) in this phenomenon.

In addition, it was proven that cardioprotection mediated by PKC is associated not only with the previously mentioned altered concentrations of proteins, but also with post-translational changes in 23 of the 36 proteins studied, although 70 were not studied. Nevertheless, a later study was able to demonstrate the presence of myosin light chain-1 phosphorylation in rabbit ventricular myocytes when preconditioning is induced pharmacologically with adenosine.⁷¹ Studies with PKC have led to the formulation of the hypothesis of the «signaling model» as a means of explaining, at the molecular level, cardioprotection mediated by this isoform of PKC.⁷²

A similar experimental method has been to use with rabbit smooth muscle cells to identify 15 proteins that bind to phosphatase1, an enzyme that dephosphory-

lates residual Ser and Thr in proteins activated by phosphorylation. These proteins control key cell processes, such as metabolism and muscle contraction, or the cell cycle and genetic expression.⁷³ Similarly, the group of proteins in the cytoplasm or those that form the myofilaments are subproteomes that have begun to be studied in animal models (pigs) by HPLC. In hearts in which heart failure is induced by ligation of the coronary arteries, 2 proteins notably change their expression — troponin T and heavy chain myosin.⁷⁴ Very recent studies have begun to look at the mitochondrial proteome in normal mice and knockout mice for key proteins, such as mitochondrial superoxide dismutase. Mice deficient in this protein present with a phenotype characteristic of DCM, with an additional group of changed proteins that are still in the process of being identified.⁷⁵ Similar studies have performed that analyze the mitochondrial proteins of transgenic mice deficient in creatinase enzyme⁷⁶ and the proteins of the nuclear membrane in human DCM.⁷⁷

CURRENT PROBLEMS AND FUTURE PERSPECTIVES

The aim of cardiovascular proteomics is to discover the proteins of the myocyte and of the rest of the cell types in this system and to identify their function, as well as to be able to understand their role in pathological processes. At the present time a great deal of data is being accumulated (Table 2) with new proteins that were not previously implicated in pathological processes, although physiopathological integration of such data still does not exist. Moreover, study has not been done of the proteomes of the cells involved in such a highly prevalent diseases such as atherosclerosis.⁷⁸ Knowledge of the proteins of the cells that integrate atherosclerotic plaque and of circulating cells, such as monocytes and platelets, which interact with them, could provide a new molecular view of this process. In this way, it is probable that several of these proteins could become new therapeutic targets in the immediate future. Moreover, variations in the proteic pattern in serum or in circulating cells in this disease would probably have a much greater prognostic value than the isolated analysis of a single protein, as is the case with C-reactive protein.⁷⁰ The recent appearance of chemogenomics, which integrates bioinformatics and combinational chemistry, is shaping up as a potent methodology for generating new drugs based on proteomic and genomic data.⁸⁰

In spite of the considerable technical advances made in recent years, there are still several hurdles. The most fundamental is probably the identification of proteins, which continues to be a slow process. The appearance of new highly automatic spectrometers, such as the so-called TOF-TOF spectrometers,

which provide masses with great accuracy and which generate peptide sequences, as well as the introduction of new Internet searches based on complex algorithms, may speed up and improve these processes in the near future. The protein databases are increasingly complex and contain fewer errors, which will result in greater reliability in protein identification. The measurement of proteins in 2-D gels is another aspect that needs to be improved. New fluorescent probes have been described for labeling proteins in control samples (in green) and pathological samples (in red) which would facilitate comparison of the levels of protein expression when both samples are applied in a single gel and the detection of proteins at each wave length (DIGE method).⁸¹ Similar methods have been described, such as isotope-coded affinity tags (ICAT),^{82,83} which are applied when protein separation is performed by HPLC. The study of different subproteomes will provide an enormous increase in the number of proteins known at the present time, and of the connections and interactions in a multitude of intracellular systems, signaling, regulation, localization, etc. Therefore, in spite of the problems involved, proteomics has an enormous potential for the future.⁸⁴

One of the elements that may shed more light on cardiovascular disease in the near future is the comparison of genomic data by DNA chips⁸⁵⁻⁹⁶ with proteomics, analyzing whether there is a correlation or not between genes, proteins expressed, and their post-translational modifications. The first comparative data are beginning to become available,⁹⁷ but description of these is outside the scope of this review.

GLOSSARY

Proteome: A term derived from combining the words *protein* and *genome* that refers to the group of proteins expressed (that are synthesized and present) by the genome of a cell (or tissue, plasma, physiological liquid, etc.) at a given moment under specific conditions. Since proteins are the molecules that perform the majority of cell functions, the proteome reflects, at the molecular level, the actual situation (normal or pathological) of the cell. Knowing a proteome may lead to establishing the identify of the proteins that make it up, its post-translational modifications, its interactions, localization, activity, and 3-D structure, among others. The purpose of proteomics is the study of the different proteomes. There are numerous international initiatives for the study of human and animal proteomes.

Proteasome/ubiquitin: Proteins, in the cells, are broken down and replaced by new proteins (by synthesis). The breaking down of proteins in the cytoplasm is carried out by a multimolecular complex of different enzymes, called proteasomes. These are made up of 28

subunits, organized into 4 groups of 7, in such a way that a central cavity is created among them, in which the catalytic centers are located that will break down the proteins. These are introduced into the central cavity and are fragmented (proteolysis) into small peptides. Ubiquitin is a small protein that is essential in the degradation process. It binds to the proteins to be destroyed, forming a chain, or poly-ubiquitin, that is the signal the proteasome recognizes to break down the protein.

Changes in this proteolytic pathway have a central role in several diseases, especially the neurodegenerative diseases. In these situations, aggregates that are rich in ubiquitin (inclusion bodies) accumulate in the cells, which die due to apoptosis. Similar aggregates have been found in hereditary cardiomyopathy.

Transcription/codons: Each double helix DNA chain is made up of units or nucleotides. In a given DNA area (in the cell nucleus) only one of the strands encodes a protein. The process by which the encoded area is copied, by enzymes, to messenger RNA (mRNA, which displaces the cytoplasm) is called transcription. Every 3 nucleotides (triplet) of DNA or mRNA is a codon that encodes for an amino acid. Which amino acid corresponds to which codon constitutes the genetic code. Proteins are synthesized binding the amino acids that are encoded for each triplet or codon (translation process). In this way, the codon sequence produces the amino acid sequence. The structure (and function) of each protein depends on its sequence.

Post-translational modifications: Once a protein is synthesized as a chain of amino acids, it undergoes a series of later modifications that principally consist in the binding of different types of molecules to some of its amino acids: the addition of sugars or glycosylation, of lipids (palmitoylation, prenylation, etc.), or of phosphate groups or phosphorylation, among others. These modifications produce intracellular localization, activation or inactivation, interaction with other molecules, and half-life, among others, and constitute an essential element in protein function. After modification, it is not possible to know apart from the genome how a protein is in its final form, without having to directly study the protein itself.

Knockout mice: Gene function has been traditionally studied by observing the effects of spontaneous situations on an organism, and more recently by analyzing the effects of mutations induced in cells in culture. Nevertheless, one of the best systems for finding out the functions of a particular gene is to create a mutant mouse that does not express the gene to be studied. Knock-out mice have a specifically modified gene of interest (and do not express the corresponding protein) and are produced by different genetic techniques. The most common is to inactivate the chosen gene in embryonic mother cells (*in vitro*), via homologous re-

combination and later introducing these cells into mouse blastocytes, which are implanted into female mice. In a portion of the mice descending from these mice the inactivated gene is present.

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