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Proteomic approach in the search of new cardiovascular biomarkers

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Proteomic approach in the search of new cardiovascular biomarkers. With the increasing incidence of cardiovascular diseases worldwide, specifically atherosclerosis and heart failure, the search for novel biomarkers remains a priority. As opposed to complex diagnostic techniques that may not be suitable to be applied to the wider population, biomarkers are useful for population screening. The search for novel biomarkers is based on knowledge of the molecular and cellular processes that take place in the development of a specific disease. Atherosclerosis and heart failure are characterized by a long period of silent disease progression, allowing early diagnosis and the potential of early therapeutic intervention. The use of the so-called proteomic techniques allows not only protein identification but partial characterization, which includes expression and also post-translational modification of these proteins. This allows for the discovery of previously unknown proteins involved in cardiovascular diseases, including some that may be suitable to be used as biomarkers. However, to approach this issue, we have to overcome difficulties such as tissue heterogeneity (vessel wall or myocardium) and the lack of fresh human samples. We discuss the proteomic study of human plaques, secreted proteins by pathologic and normal vessel wall, and left ventricular hypertrophy as potential sources of new biologic markers of cardiovascular disease.

Cardiovascular diseases (CVD), including myocardial infarction, heart failure, stroke, and peripheral arterial disease, are among the leading causes of morbidity and mortality in Western societies and developing countries. The thrombotic complication of atherosclerosis is one of the higher prevalent processes leading to CVD. In atherosclerosis, hemodynamic, thrombotic, and carbohydrate-lipid metabolic variables merge, leading to a chronic vascular wall inflammatory process [1, 2] that, after decades of asymptomatic progress, may ultimately lead to life-threatening events. The main mechanism involved is the rupture/erosion of an atherosclerotic plaque, allowing the interaction between the lipid core and other highly thrombogenic plaque components with flowing blood. This initiates a platelet-dependent thrombus that may progress to totally occlude the lumen and/or be the source of thromboembolic events [3, 4]. Atherosclerosis of the coronaries, carotids, aortic arch, abdominal aorta, and iliac-femoral arteries leads to the most common clinical manifestations of atherothrombosis: myocardial infarction, unstable angina, stroke, and transient cerebral ischemia, and intermittent claudication and gangrene that jeopardize limb viability [5].

Heart failure prevalence and incidence are increasing worldwide as a consequence mainly of either ischemic or hypertensive heart disease, and also because of specific myocardial diseases such as dilated cardiomyopathy (DCM). Hypertrophic growth accompanies many forms of heart failure, and, in fact, left ventricular hypertrophy is a major predictor of systolic heart failure [6]. Although many mechanisms have been shown to be involved in cardiac dysfunction, little is known about the molecular pathways underlying heart disease.

The understanding that one gene can encode more than a single protein product as a result of transcriptional and translational processes has led to the concept that the functional complexity of an organism far exceeds that indicated by its genome sequence alone. Furthermore, protein maturation, degradation, and post-translational modifications are dynamic processes that can control the amount of functionally active protein within a cell. It is estimated that in humans there are approximately 6 to 7 times more proteins than genes. Proteins also show complexity because of their primary, secondary, tertiary, and quaternary structural elements [7].

In this sense, proteomic analysis has been shown to provide novel information about protein expression in

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different settings, usually by combining bi-dimensional separation of proteins (2-DE) and mass spectrometry (MS) [8]. The application of proteomics provides major opportunities for a more comprehensive understanding of cell and organ function and dysfunction, providing new avenues to elucidate disease mechanisms and to identify new diagnostic biomarkers and therapeutics targets.

KNOWN MARKERS AND RISK FACTORS OF CVD

The outstanding progress in cardiovascular medicine allows for a very high level of success in managing patients with CVD. With the advent of new effective treatments for hypertension and more efficient management of acute myocardial infarction, deaths resulting from stroke and acute coronary syndromes have steadily decreased [9]. However, CVD is still associated with high morbidity and mortality rates [10, 11].

Both atherosclerotic vascular disease and heart failure are characterized by a period of silent disease, with no overt clinical manifestations. It is important to consider that nowadays there are many effective treatments to slow the progress and even stabilize both processes, with demonstrated survival benefits. The potential to know the presence of any of these diseases in previously asymptomatic individuals may permit the use of specific therapeutic approaches and lifestyle modification plans that may alter the natural history of such important diseases in the initial phase. Recent clinical trials on primary and secondary prevention of adverse cardiovascular events have shown this potential, although patient selection in these trials has been limited by the knowledge of the increased risk of adverse events based only on clinical data. The potential for prevention in a more stable population is one of the major challenges in cardiovascular medicine. Although it is well known that classical risk factors are associated with the presence of cardiovascular events, we still need additive prognostic markers to predict this risk more accurately. Although noninvasive imaging techniques are being investigated in this setting, these techniques cannot be applied to a wide population range to the same extent as can the new potential prognostic biomarkers.

The most extensively studied potential biomarker for atherosclerosis to date has been C-reactive protein (CRP). Prospective studies have demonstrated that high CRP levels are associated with an increase of cardiovascular risk in apparently healthy men [12]. In this regard, it is actually accepted that patients with CRP levels lower than 1 mg/L, between 1 and 3 mg/L, or higher than 3 mg/L show low, medium, or high risk, respectively, of developing cardiovascular disease. Although CRP could be used to stratify risk situations, it appears to have moderate predictive value [13], and it has not gained wide acceptance in clinical practice. In addition to CRP, many markers of systemic inflammation have been studied in recent years as potential candidates for risk prediction [14]. In this sense, it has been observed that increased plasma CD40L levels predict a higher risk of cardiovascular events in healthy women [15]. Other markers of CVD are monocyte chemo-attractant protein-1, adhesion molecules, myeloperoxidase, and several interleukins. Nevertheless, none have been consistently demonstrated to add predictive value to the clinical variables used in clinical practice, and, in most cases, there are no commercially available standardized assays [14]. Despite the available biomarkers of cardiac necrosis and ventricular failure, like B-type natriuretic peptides, new specific biomarkers for different stages of the disease or the infarct size are necessary to better predict prognosis in these patients.

PROTEOMICS STUDY OF CVD

Today, it is possible to perform a differential proteomic analysis on a variety of biologic samples, including cells, tissues, or biologic fluids. Atherosclerosis is characterized by focal plaques from which proteins can diffuse in low amounts and be retrieved from plasma. In the context of biomarker discovery, biologic fluids such as plasma or urine seem to be the most logical samples for investigation. Plasma proteomic studies still show important difficulties because of the presence of large amounts of albumin that may mask key potential protein biomarkers. However, recent technologic improvements permit depletion of such abundant plasma proteins and increase the potential of plasma-proteomic analysis to find relevant biomarkers. Another approach consists of the analysis of either vascular cells under pathologic conditions, atherosclerotic plaques, or cardiac tissue, by comparison with healthy cells or tissues.

Proteomic analysis of atherosclerotic plaques

A particular issue in proteomic analysis of vascular pathology is the heterogeneous cellular composition of the vascular wall (endothelial cells, smooth muscle cells, leukocytes, and so forth). The first published proteomic study of human atherosclerosis was performed on tissue extracts, and the only detected changes corresponded to plasma proteins (albumin, immunoglobulin G, Apo-I, and so forth) [16]. Years later, You et al showed an increase in ferritin light chain expression in coronary arteries from 10 patients with coronary artery disease compared with seven healthy controls [17]. However, the proteomic analysis of a tissue probably identifies mainly constitutive proteins and may under-represent potential biomarkers. A different approach was carried out by Martinet et al [18] with Western array technology to identify differentially expressed proteins of potential pathobiological relevance in human atherosclerotic plaques. Tissue lysates from human carotid endarterectomy specimens and non-atherosclerotic mammary arteries were

screened with 823 monoclonal antibodies. They identified seven proteins with a >5-fold relative expression. One of the most interesting downregulated proteins in atherosclerotic plaques was apoptosis-linked gene 2, a recently discovered pro-apoptotic protein that belongs to a Ca^{2+}-binding protein family with EF-hand motifs [19]. Apoptosis has been identified as a prominent feature of advanced human atherosclerotic plaques [20], so these findings may shed light on how macrophage-derived foam cells survive pro-apoptotic signaling in human plaques.

Proteomic analysis of the tissue secretome

An alternative approach for the study of atherosclerotic plaque proteins released by normal and pathologic arterial walls has recently been used by the authors and others [21]. This consists of the analysis of proteins secreted to the media by normal and pathologic explanted arteries. In addition, the incubation of complicated and noncomplicated endarterectomy samples in a protein-free culture medium allowed us to harvest separately the proteins released from these vessels. Two-dimensional electrophoresis of this "secretome" showed that normal artery segments secreted at least 40 different proteins, compared with atherosclerotic vessels that were shown to secrete almost 80 proteins. The protein spots were also higher in the 2-DE gels of artery segments with a ruptured plaque and thrombus [21]. Interestingly, our preliminary data show an increased secretion of protein disulfide isomerase by atherosclerotic plaques, a protein recently considered a major proinflammatory mediator involved in atherosclerosis [22, 23].

Among the differentially secreted proteins, decreased heat shock protein-27 (Hsp27) was identified as a potential marker of atherosclerosis [24]. In this study, when compared with control mammary arteries, Hsp27 release was drastically decreased in atherosclerotic plaques and barely detectable in complicated plaque supernatants. In addition, Hsp27 production by arterial wall correlates negatively with atherosclerotic plaque complexity. Furthermore, we have measured circulating plasma levels of Hsp27 in patients with carotid atherosclerosis (stenosis >70%) and in healthy patients. Circulating Hsp27 levels were decreased 20 fold in patients with carotid atherosclerosis relative to healthy patients [24], indicating that plasma protein content can reflect arterial wall secretion of this protein. These data suggest that low levels of plasma Hsp27 could be a potential marker of atherosclerosis, although the pathologic meaning of this diminution of plasma Hsp27 requires further investigation.

In the same study, two Hsp27 isoforms were identified by matrix-assisted laser desorption/ionization mass spectrometry peptide mapping, corresponding to the nonphosphorylated (isoform 2) and the monophosphorylated state of Hsp27, with Ser82 as the modified residue (isoform 1). Although the exact role of Hsp27 in atherosclerosis is unknown, previous data suggest that Hsp27 could play a protective role. This protein is expressed in endothelial and vascular smooth muscle and is able to bind and stabilize actin microfilaments, favoring the formation of actin stress fibers. Hsp27 has also been implicated in the apoptosis process by downregulating key apoptotic signaling pathways [25]. Finally, Hsp27 could also interfere with the inflammatory response present in atherosclerotic lesions by inhibiting nuclear factor kappa B activation [26]. These examples show how proteomics can be a useful technique to identify not only new proteins involved in the pathogenesis of CVD, but also the specific post-translational modifications associated with the disease process.

Further studies are in progress to fully identify and define the role of these proteins in atherogenesis and their potential role to be novel markers/therapeutic targets of cardiovascular risk.

Proteomic analysis of cardiac diseases

As mentioned above, a particular problem in proteomic analysis of the heart is the diversity of cell types present in the vascular wall. Protein expression patterns of total myocardial lysates are predominately due to the cardiac myocyte proteins, but these samples will also contain lower amounts of proteins derived from other cell types such as fibroblasts and smooth muscle and endothelial cells [27]. Recently, proteomic techniques have been coupled with other research fields, with laser capture microdissection [28]. This powerful combination allows the isolation of specific cell types from tissue sections, which overcomes the problem of tissue heterogeneity [29]. This combination is beginning to be used in the cardiovascular field. However, the difficulties and ethical problems in obtaining human heart samples make it necessary to study heart disease through indirect approaches. The study of heart diseases in established animal models, such as dogs for the DCM or spontaneously hypertensive rats for heart failure or hypertrophied heart, make it possible to identify altered proteins specific to the disease, which will be a potential specific biomarker or new therapeutic target. Using a bovine model of hereditary DCM, it has been found that from more than 1000 resolved proteins, 24 proteins were decreased and 11 were increased in pathologic versus control tissue [30]. The identification by MS of some of the altered proteins allows us to conclude that inappropriate protein ubiquitination plays a major role in DCM [30]. Other authors, using a model of pacing-induced heart failure, showed that heart failure is associated with changes in the protein expression involving mitochondrial energy production, cytoskeletal architecture, and calcium-regulated processes [31, 32]. Particularly, diminished expression of mitochondrial hydroxylmethyl glutaryl coenzyme A synthase was observed, consistent with the hypothesis of reduced mitochondrial energy supply during DCM. They also

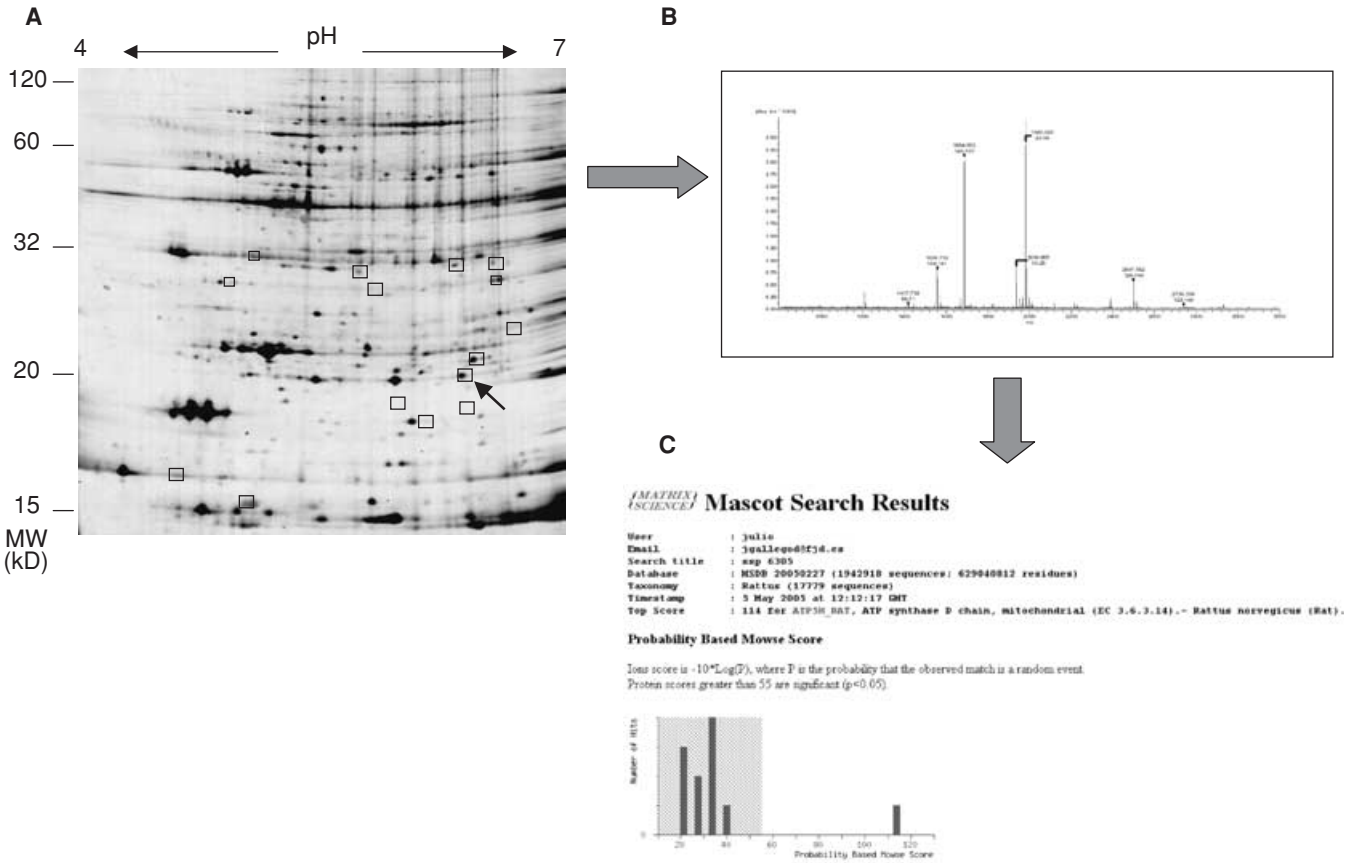

Fig. 1. (*A*) Representative image of 2-DE gel from left ventricular samples, showing 15 altered protein spots (squares) compared with normal heart. As an example, we show the identification of one of these altered proteins (arrow). (*B*) Peptide mass fingerprint spectrum was obtained on a Bruker Ultraflex TOF/TOF MALDI mass spectrometer (Bruker-Daltonics GmbH, Bremen, Germany). (*C*) The measured tryptic peptide masses were transferred through the MS BioTools program (Bruker-Daltonics) as inputs to search the MSBD database using Mascot software (Matrix Science, London, UK). This spot protein was positively identified as mitochondrial adenosine triphosphate synthase D chain.

noted an increased level of several glycolytic enzymes, which is probably a compensatory mechanism to counteract insufficient mitochondrial energy production [31, 32].

The process of myocardial necrosis leads to the release of proteins from dead myocytes into the circulation and hence provides relatively accessible targets to identify biomarkers. The original biomarkers used in myocardial necrosis diagnostics included serum glutamate oxaloacetate transferase, lactate dehydrogenase, and creatine kinase, specifically the MB isoform. Recently, the use of troponin-I and troponin-C increased the sensibility of myocardial necrosis. The clinical utility of these markers has been extensively demonstrated, but there may still be room for improvement. Several animal studies have investigated changes in the protein profile in the myocardium after ischemia-reperfusion injury. Sakai et al identified rat myocardial proteins altered on ischemia by using fluorescence-based two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [33]. Likewise, Schwertz et al have found marked differences in protein expression in a rabbit model of myocardial ischemia-reperfusion [34].

Proteomic analysis of the diseased heart may allow for the identification of markers specific for heart diseases (valvular, congenital, and so forth) of potential clinical interest. In addition, proteomic studies of the heart under different pathologic states will give important information regarding cardiac physiology that may be useful in finding specific therapeutic targets to improve the prognosis of these patients. Of special interest is hypertension-induced left ventricular hypertrophy, in which the diagnosis still relies basically on echocardiographic data. The increasing hypertensive population and the lack of tight blood pressure control in this group of patients make it a condition of special interest for research into a specific marker. This will allow us to closely monitor hypertensive patients and may help to prevent this condition, which, if unrecognized, would progress to overt heart failure. We are studying the proteome of hypertension-induced early left ventricle hypertrophy in a spontaneously hypertensive rat model. Through a proteomic approach from 464 analyzed protein spots, we have found significant differences in 15 cardiac protein spots in a rat model of early left

ventricular hypertrophy. Some of these altered spots have been identified by MS, which belong to contractile proteins (tropomyosin alpha-chain) and oxidative phosphorylation complex (adenosine triphosphate synthase D chain, mitochondrial) (Fig. 1). These findings suggest an inadequate aerobic metabolism in the early-hypertrophy cardiomyocytes.

Although a great deal of research effort is currently underway to find new biologic markers of CVD, it is clear that proteomic analysis is one of the most powerful tools available for this purpose.

CONCLUSION

The use of new techniques, such as proteomic analysis, will permit the analysis of multiple proteins in blood. This approximation will give us the opportunity to explore a lot of possible risk markers and evaluate which are the most appropriate for clinical use. On the other hand, use of new markers probably will serve us to analyze the effect of diverse treatments in different patients and adequate them to the characteristics of each patient. Furthermore, a set of markers (multipanel) could potentially give more information about different levels of the disease and will be of more clinical benefit for us from a prognostic point of view.

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