Applications of two-dimensional electrophoresis technology to the study of atherosclerosis

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Abstract

Atherosclerosis is a multifactorial disease in which hypertension, diabetes, hyperlipidemia and other risk factors are thought to play a role. However, the molecular processes underlying plaque formation and progression are not yet completely known. In the last years some researchers applied proteomics technologies for the comprehension of biochemical pathways of atherogenesis and to search new cardiovascular biomarkers to be utilized either as early diagnostic traits or as targets for new drug therapies. Due to its intrinsic complexity, the problem has been approached by different strategies, all of which have some limitations. In this review, we summarize the most common critical experimental variables in two-dimensional electrophoresis-based techniques and recent data obtained by applying proteomic approaches in the study of atherosclerosis.

Introduction

Atherosclerosis is a form of chronic inflammation characterized by the accumulation of lipids and fibrous elements in medium and large arteries. Oxidation of low density lipoproteins (LDL, Lp (a)) infiltered into the subendothelial space induces migration of circulating monocytes and their differentiation into macrophages. Uptake of a large amount of ox-LDL by macrophages leads to foam cell formation and necrosis. Cytokines secreted by T-limphocytes and foam cells attract smooth muscle cells that migrate from the medial portion of the artery, proliferate and secrete new extracellular matrix forming a fibrous cap surrounding the necrotic core. The stability of advanced atherosclerotic lesions may be influenced by the degree of inflammation, extracellular matrix degradation, calcification and neovascularization[1]. The rupture of the atherosclerotic plaque is the predominant underlying process in the pathogenesis of acute coronary syndromes and peripheral vascular disease[2]. Disease etiology is very complex and includes several important environmental and genetic risk factors such as hyperlipidemia, diabetes, and hypertension. Knowledge of the molecular mechanisms underlying this pathology is nowadays a challenge. In this regard, in the last years some researchers have approached these issues by means of proteomics to individuate both

pathways of atherosclerotic process and possible circulating markers for prevention and early diagnosis[3]. Proteomics technologies allow one to evaluate simultaneously patterns and expression levels of hundreds or even thousands proteins, also identifying differentially expressed proteins and post-translational modifications[4]. The technology of choice for these purposes is high-resolution two-dimensional electrophoresis (2-DE) that enables the separation of proteins in the first dimension in relation to their intrinsic charge (isoelectric point) and then, orthogonally, according to their relative mass (Mr)[5,6].

The analyses of the entire proteomes are limited by the high dynamic range of protein abundance in different samples and their heterogeneity with respect to Mr, pI, and hydrophobicity. Cellular proteins range from 10^5-10^6 copies/cell for housekeeping proteins to $<10^2$ copies/cell for receptor molecules with a total of up to 10000 different proteins in eukaryotic cell extracts. Plasma is composed for 90% by only 9 proteins (albumin, IgG, transferrin, haptoglobin etc.), others 12 proteins represent another 9% of total and the remaining 1% is constituted by about 50000 low expressed protein variants called "deep proteome". Plasma protein levels range from 40-50mg/ml for albumin to <10ng/ml for interleukins, chatepsins and peptide hormones. Tissue proteomics is also complicated by the large heterogeneity of cellular components. In this respect, although several standard protocols have been developed, sample preparation for subsequent proteomic analysis must be adapted and optimized in relation to the specimen under study. Due to the high complexity of different proteomes often researchers focalize on specific sub-proteomes by analysing sub-cellular components (such as organelles or membranes), enriched plasma fractions (immuno-subtraction of more abundant proteins), or tissue microdissections[7].

Sample preparation for 2D analysis

Many different procedures for cell lysis and protein extraction could be performed, individually or in combination, according to specific objectives. Hard procedures include freeze-thawing of sample, detergent lysis, sonication and homogenization. Soft procedures are based on enzymatic or osmotic lysis, also useful to obtain intact subcellular fractions. Some substances could interfere with subsequent 2D analysis and must be inactivated or removed. First, proteolytic enzymes present in all specimens must be inactivated, usually by adding a cocktail of protease inhibitors and/or lowering the pH, or denaturated by boiling the sample in SDS containing buffers. Salts are interfering compounds for the isoelectric focusing at concentrations >100mM. Dialysis, gel filtration and protein precipitation with TCA are the most common methods for salt removal. Also nucleic acids and polysaccharides, if presents at interfering concentrations, must be removed since they could give rise to streaky 2D patterns. Protein precipitation with TCA/acetone, treatment with

a mixture of protease-free DNAses and RNAses or with specific glycosidases are common methods. Then, extracted proteins, free of interfering compounds, must be resolubilised in a denaturating buffer containing a chaotrope (7-9 mol/L urea in combination with 2 mol/L thiourea for hydrophobic proteins), zwitterionic or non-ionic detergents (CHAPS, sulfobetaines, Triton-X100 and NP-40 ranging from 1 to 4%) and a reducing agent (DTT, DTE, TBP, TCEP ranging from 2 to 100 mmol/L) for complete disruption of inter- and intra-molecular interactions. Sometimes protease inhibitors are also added to solubilisation buffer. Urea, in aqueous solutions is in equilibrium with ammonium cyanate that can react with protein amino groups so introducing charge artefacts. So, it would be better to avoid heating urea containing solutions over 37 °C. Also temperatures lower than 10 °C must be avoided (urea crystallization). Optimal working temperature is approximately 20 °C. By using all these pre-treatments, extracted proteins result free of interfering compounds and completely unfolded, although maintaining their intrinsic charge.

2D electrophoresis

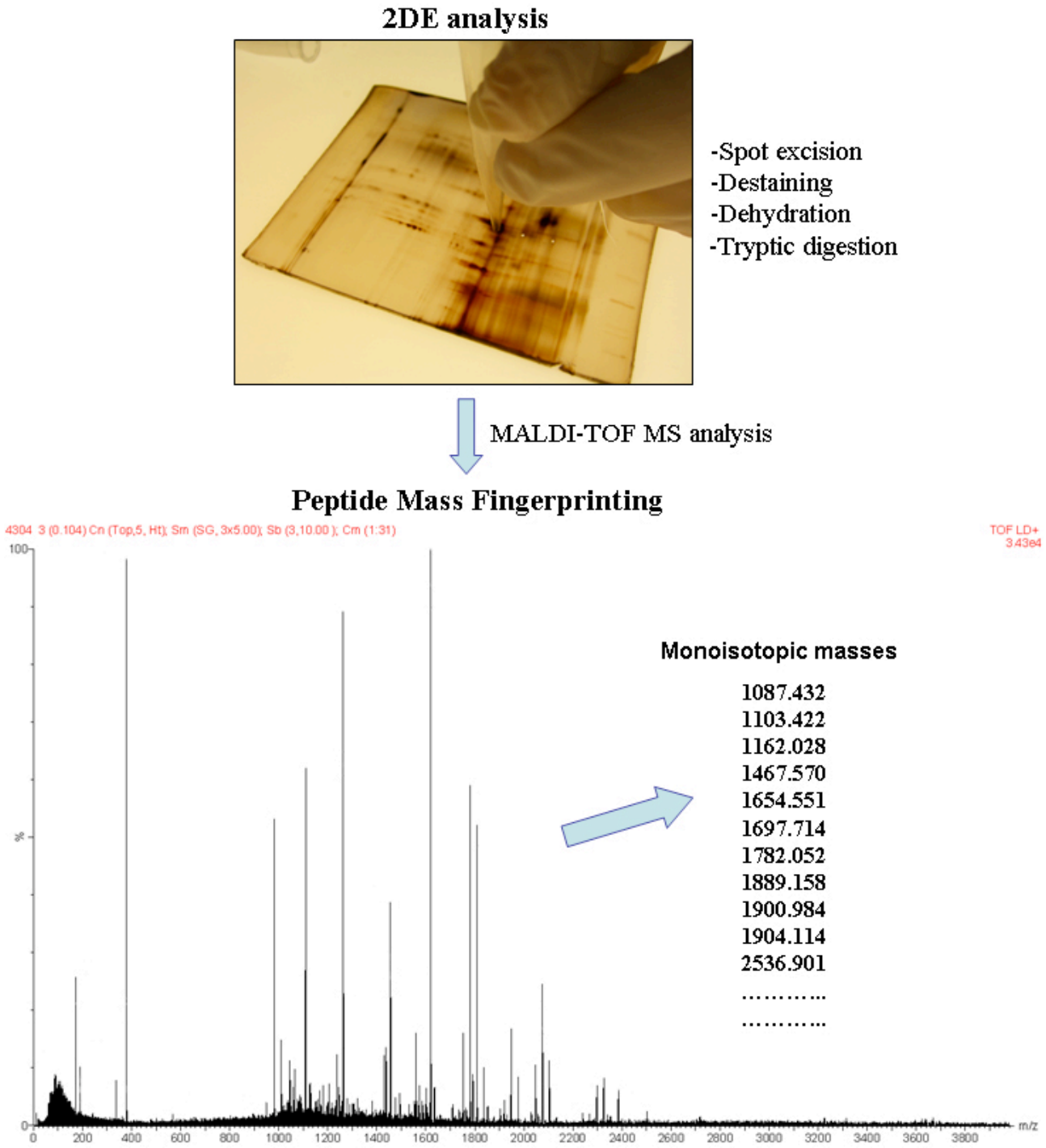
The most widely used approach for evaluate protein patterns and expression levels is based on the method described by O'Farrell, that allowed to resolve more than 1000 different protein spots from *Escherichia coli* by an isoelectric focusing separation based on intrinsic charge of each protein isoform and subsequently an SDS-PAGE orthogonally conducted[8]. Due to the instability of pH gradients generated by pre-focusing polyamino- polycarboxylic acids (carrier ampholytes), especially at basic pH, the method evolved following the introduction of immobilized pH gradient strips (IPG strips), in which pH gradients are generated by copolymerizing, together with acrylamide, ten derivatives containing either a carboxyl or an amino group. This allowed to obtain extremely stable pH gradients over the range 1-13 with a greatest resolution of 0.001 pH units. Sample loading could be carried out by active or passive rehydration of dried strips or by cup-loading. Then proteins are resolved under high voltages in function of their isoelectric point. After focusing two steps of equilibration are needed: first, strips are incubated in a Tris buffer containing 6 mol/L urea, 30% glycerol, and 3% SDS with the addition of 1% DTT, followed by an equilibration in the same buffer without DTT, but with the addition of 2.5% iodoacetamide. These treatments allow to completely solubilise proteins eventually precipitated at their pI, to give a uniform negative charge density to the proteins, because of the anionic detergent SDS, and to reduce and alkylate any protein sulfydryl groups. Proteins are then resolved by SDS-PAGE as spots in function of their M_r and visualized by gel staining. In this respect many procedures are described: Coomassie brillant blue and silver staining, fluorescent dyes, or autoradiography for radioactively labelled proteins, and some others. All these procedures have one or more limitations in relation to

sensitivity, range of linearity, reproducibility and compatibility with subsequent mass spectrometry analyses.

2D electrophoresis is a technology well suitable to also analyse many post-translational modifications (PTM) of proteins, often evidenced by protein pI or M_r shifts, such as protein phosphorylation, glycosylation, acetylation and lipidation. Many of these PTMs could be revealed by western blotting of 2D gels with a specific antibody (e.g. anti phospho-residues antibodies, anti-4-hydroxynonenal adducts antibodies), by using lectins conjugated with specific enzymes (peroxidase, phosphatase etc.) or with fluorescent dye molecules (fluorescein-isothiocyanate) for revealing glycoproteins, or by mass spectrometry. All 2D data obtained must be digitalized by means of image acquisition systems specific for the staining methods used (e.g. acquisition in white light, fluorescence, chemiluminescence or radioactivity) and analysed by means of dedicated softwares (e.g. PD-Quest from Bio-Rad, Image master 2D from GE Healthcare) that allow to evaluate and compare levels of protein expression and, eventually, PTMs between samples in different experimental conditions (pathological vs control specimens, stimulated vs quiescent cells, etc.).

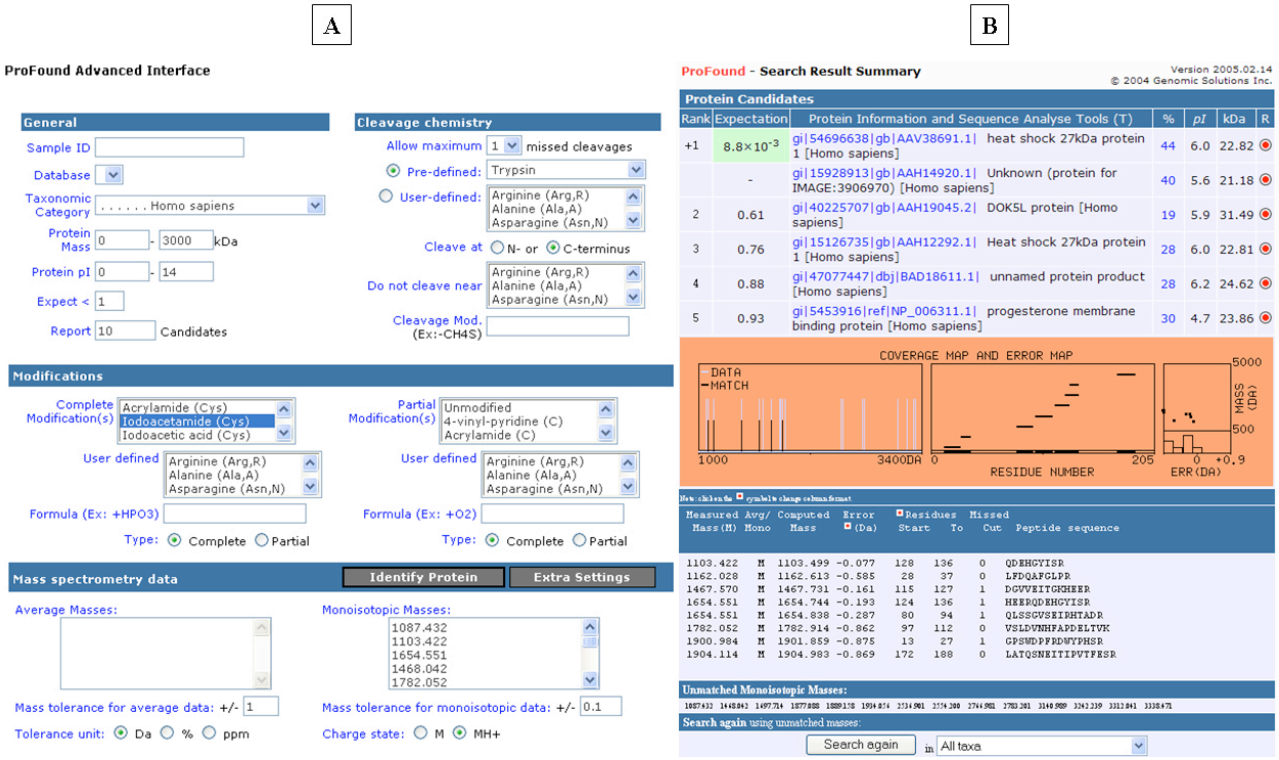
Spot identification

Spots of interest, e.g. differentially expressed or variably modified, could be identified and characterized by means of mass spectrometry analysis. These instruments can perform highly accurate mass measurements and sequencing of peptides ranging from 1000 to 4000 amu (atomic mass unit). Behind this technology there is the high specificity of certain proteolytic enzymes or chemicals to cleave the proteins at specific sites. For example, trypsin the most widely used enzyme cleaves peptide chains at the carboxyl side of the amino acids lysine and arginine, except when they are followed by proline, so producing a pattern of peptides characteristics for every protein (Peptide Mass Fingerprinting). First, proteins must be excised from the gel (stained with a mass compatible protocol) by a scalpel or a sterile tip, destained, dehydrated and air dried. Subsequently, spots are reswollen with a solution containing a sequencing grade modified trypsin resistant to autoproteolysis and incubated over night at 37°C (in-gel digestion). Peptides are then extracted from the gel and analysed by mass spectrometry [figure 1].



In short, the mass spectrometer (MALDI-TOF MS, ESI MS/MS) converts, in the source, components of a peptide mixture to ions, and then analyzes them on the basis of their mass/charge (m/z) ratio. In a MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization-Time of Flight) peptides are mixed with an organic compound (matrix) that absorbs photons when reached by a pulse laser beam and becomes electronically excited. This excess energy is transferred to the peptides, which are then ejected into the gas phase (MALDI). Positively singly-charged peptides are then resolved in the mass analyzer (TOF) respect to m/z and revealed by the detector.

The resulting mass spectra could be compared, by means of specific softwares, free on web (e.g. Profound, MASCOT, ProteinProspector, ALDENTE), to the theoretical masses obtained from *in silico* digestion with the same enzyme of sequences deposited in protein- and nucleotide-sequence databases (NCBI, SWISS-PROT). The search parameters include: taxa, proteolytic enzyme used, protein molecular mass and pI ranges, complete and eventually partial modifications, mass tolerance [figure 2, panel A].



Search result is a list of putative proteins and peptides matched [figure 2, panel B].

Aims of proteomics

A proteome is the entire protein content expressed by a genome. However, it is something of an abstraction because, in the same organism, it is dependent on the time, the cellular type, and the physio-pathological conditions. It includes all splicing variants and isoforms from post-translational modifications. Proteome of cell cultures also reflects the experimental conditions adopted.

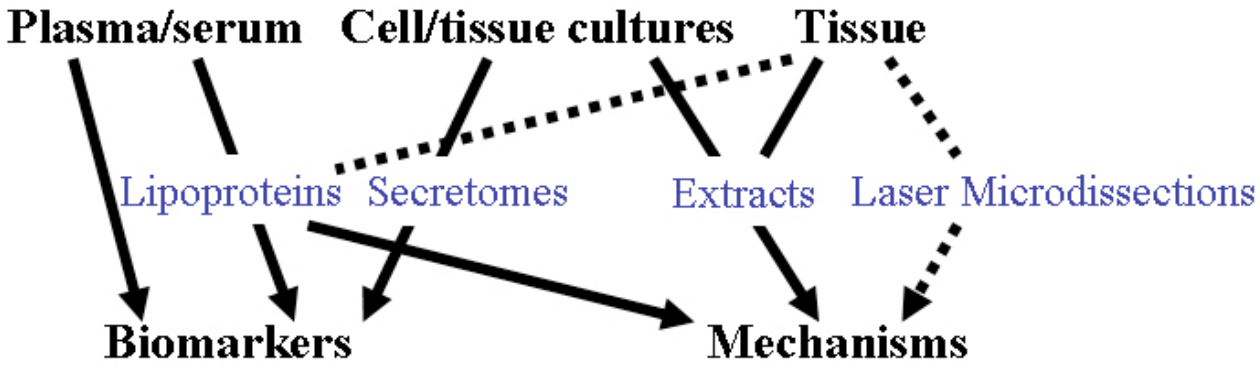
The purpose of *proteome mining* is to identify as many components of the proteome as possible to construct data bases of expressed proteins useful for many biochemical fields of research. A powerful tool in the search of possible markers of pathology or targets for drug therapies is *differential proteomics* that compares protein expression profiles and PTMs of pathological *vs* healthy, stimulated *vs* basal, pharmacologically treated *vs* control samples. Starting materials are often tumoral tissues or cells, but plasma, cerebrospinal fluid and urine are also widely used. Usually, a subset of gene/proteins identified by differential proteomics should be validated by one

or more different methodologies, such as Western blotting, ELISA, immunohistochemistry, and RT-PCR.

Cellular proteins rarely work alone but interact together to form multi-component complexes that carry out specific functions. These functional units can be as simple as dimers or as complex as the transduction signal systems. To find out how cells work it is essential to understand how protein complexes work. In this regard, the first goal is to isolate the protein complexe (e.g. affinity chromatography based strategies, immunoprecipitation approaches) and then to identify its components. These are the main targets of *functional proteomics*.

Proteomics in the study of human atherosclerotic plaques

Cardiovascular diseases are the leading cause of mortality and morbidity in developed countries being atherosclerosis the major contributor. Atherosclerotic lesions result from a variety of pathogenetic processes, including sub-endothelial retention and oxidation of low density lipoproteins and Lp(a), macrophage foam cell formation and death, chronic inflammation, secretion of new extracellular matrix, neovascularization and disruption of the lesion surface. Plaque rupture and thrombosis are the most important clinical complications in the pathogenesis of acute coronary syndromes and peripheral vascular disease. Individuation of specific biochemical pathways for atherogenesis and markers for screening and early therapeutic intervention would be desirable. In the last years some researchers used proteomic technologies on plasma/serum, cell/tissue cultures and tissue extracts with these objectives [figure 3].



Plasma is an useful tool for biomarker searching and for monitoring progression of pathology also in relation to drug therapies. It is easy to draw and differential protein expression patterns could reflect directly or indirectly a cardiovascular disease or other pathological states. However, studies on plasma are complicated by the number of low expressed proteins and the high dynamic range of protein concentration. The HUPO (Human Proteome Organization, http://www.hupo.org/) Plasma Proteome Project pilot phase examined human plasma with various proteomic platforms across multiple laboratories worldwide so identifying 3020 proteins. On the basis of the current

knowledge, the study individuated a subset of proteins showing cardiovascular-related functions (markers of inflammation and/or cardiovascular disease, proteins implicated in coagulation, signalling, growth, differentiation, and vascular remodelling)[9].

In 1995 Williams and Tabas published *the response-to-retention hypothesis* by which early events in atherogenesis are related to a selective retention of low density lipoproteins (LDL and Lp (a)) in the sub-endothelial space by means of specific interactions with some extracellular matrix components[10]. The molecular mechanisms underlying these processes are not completely understood but knowledge of lipoprotein structure, apolipoprotein composition and their post-translational modifications could help in this respect. Sub-proteomic approaches on lipoprotein purified fractions have provided detailed information about the protein composition of LDL, HDL and VLDL also identifying new protein isoforms and proteins not yet described to be associated with these particles[11-14]. The physiological roles of these proteins and their possible implications in the atherosclerotic process deserve further investigations.

Cell cultures allow to study single aspects of the problem in very controlled conditions but with all limitations of an *in vitro* system. The proteome (the intracellular proteins) and the secretome (the proteins released into the cell culture medium) obtained by two dimensional electrophoresis and MS of endothelial cells (HUVEC)[15], smooth muscle cells[16], monocyte-derived macrophages[17] have been reported in literature. The main objectives of these studies are the construction of 2D reference maps and protein databases of human vascular cells that are useful tools for studying protein expression changes in relation to cardiovascular disorders or environmental stimuli. In this regard, differential proteomics studies on cell cultures subjected to different experimental conditions are reported [Table 1].

Table 1. Differential proteomic studies on cultured cells related with atherosclerosis

Cell type	Experimental conditions	Differentially expressed proteins	Known functions	References
HUVECs	senescence	↑ Chatepsin B others	Intracellular proteolysis and extracellular matrix remodelling Cytoskeletal alterations, protein biosynthesis and degradation	18
Aortic ECs	haemodynamic forces	Cap G	Cytoskeletal reorganization, gene transcription, and modulation of the signaling pathways	19
TNFa stimulated SMCs	alpha lipoic acid	↑ eEF, Rho GDI, others ↓ PAI-2, LKB-interacting protein, others	Multiple roles of ALA in the immune/inflammatory process, cell cycle, regulation and expression of cell adhesion molecules in VSMCs	20
SMCs	PKCd-/-	altered expressesion of > 30 proteins	Regulation of glucose and lipid metabolism, cellular redox state, and SMC differentiation	21
SMCs	haemodynamic forces	↑ total HSP27, ↓	Modulation of contractile stress fiber biosynthesis	22

		phospho HSP27, ↓ CapZ		
monocytes	priming with lipopolysaccharide	↑ 12 proteins, ↓ 2 proteins	Modulation of oxidation and inflammation	23
monocytes (secretome)	oxLDL/LDL	↑ 59 proteins, ↓ 17 proteins	Set of candidate biomarkers of atherosclerosis	24
monocytes	oxLDL/LDL	altered expressesion of 93 proteins	Set of proteins associated with macrophage differentiation	25

The interpretation of differential protein expression patterns in atherosclerotic plaques are complicated by the large heterogeneity of vascular cellular components. In this respect, atherosclerotic plaques, besides SMCs and ECs, are composed by inflammatory cells, new-formed extracellular matrix, cellular debris and end-products of lipid and protein oxidation. Such a problem could be partially overcome by the analysis of laser microdissected areas (shoulder region, necrotic core, thrombus containing areas). Another critical point in the *in situ* analysis of protein expression in atherosclerotic plaques is the choice of appropriate control specimens. It would be desirable to utilize artery controls of the same vascular district, in order to minimize intrinsic tissue differences, and from surgical endarterectomy rather than from post-mortem material, to avoid the occurrence of proteolytic modifications prior to analysis. So the histochemical classification of the complicated lesions[26-27] is often necessary.

Some proteomic studies on homogenates of human atherosclerotic plaques have been reported. You et al.[28], by analyzing 10 diseased (stenosed by 80% to 100%) and 7 normal autoptic coronary arteries reported an about 2 fold increase of the ferritin light chain in the pathological specimens. Either pro-oxidant or anti-oxidant roles for this protein in atherosclerosis have been suggested. However, according to the "iron hypothesis", the authors speculated that this increase may contribute to the pathogenesis of coronary artery disease by modulating oxidation of lipids within the vessel wall through the generation of reactive oxygen species.

Donners et al.[29] analyzed 5 stable plaques and 6 lesions with a thrombus from patients undergoing endarterectomy, classified according to Virmani et al.[27,] so revealing, in advanced plaques, the expression of six isoforms of the acute phase protein α1-antitrypsin, one of which was uniquely expressed in thrombus-containing plaques. Recently, Sung et al.[30] analyzed atherosclerotic specimens from 7 patients undergoing aorta bypass surgery and biopsies of the normal aorta tissues from the same patients, as controls. They identified a panel of 27 proteins differentially expressed in the atherosclerotic aorta compared with the normal aorta involved in a number of biological processes, including calcium-mediated processes, migration of vascular smooth muscle cells, matrix metalloproteinase activation and regulation of pro-inflammatory cytokines.

A different approach was adopted by Martin-Ventura et al.[31,32] that analyzed the protein secretion profiles obtained from 35 cultured atherosclerotic plaques and 36 control arteries (24 mammary, 12 radial) in the search of new biological markers potentially released by the arterial wall into the plasma. In particular, they isolated and analyzed in parallel non-complicated and ruptured/thrombosed areas of the carotid plaques. Plaque and control samples were cut in small fragments and incubated in protein-free culture medium during 24 h at 37°C. Proteins secreted in the supernatants were then resolved by 2D electrophoresis and differential image analysis was carried out. They showed that heat shock protein 27 secretion into the culture medium was significantly lower in atherosclerotic plaques than in control arteries, and confirmed the finding by Western blotting analysis. They also evidenced a 20-fold reduction in HSP27 levels in the plasma of patients with carotid stenosis respect to healthy controls so identifying HSP27 as a possible marker of atherosclerosis.

The same research group evaluated the effects of incubation with atorvastatin on the secretomes of cultured atherosclerotic plaques[33]. They identified 24 proteins that were increased and 20 proteins that were decreased in atheroma plaque supernatants compared to controls. Interestingly, the presence of atorvastatin in culture medium reverted secretion of 66% proteins to control values. In this report, authors identified Cathepsin D as a potential target for therapeutical treatment of atherosclerosis.

Recently, we evaluated differential protein expression in 48 immunohistochemical classified stable and unstable plaques obtained from carotid endarterectomy[34]. We analyzed, by a sub-proteomic approach, extracts from finely minced tissues in order to permit an enrichment in both topically expressed and filtered/retained proteins in attempting to simplify an otherwise very complex two dimensional protein pattern. A total of 57 distinct spots corresponding to 33 different proteins were identified in both stable and unstable plaques by peptide mass fingerprinting, most of which were of plasma origin (about 70%). This suggests the existence of an impaired endothelial barrier function independent of plaque type. Compared to stable plaques, unstable ones showed reduced abundance of protective enzymes superoxide dismutase 3 (SOD3) and glutathione s-transferase (GST), small heat shock proteins HSP27 and HSP20, annexin A10, and Rho GDP-dissociation inhibitor and an higher abundance of ferritin light subunit, superoxide dismutase 2 (SOD2) and β-fibrinogen fragment D. These proteins are described to play a role in either oxidative or inflammatory processes and in the formation and progression of the atherosclerotic plaques. The limitation of our findings rests on the fact that we studied the whole plaques that are known to be structurally heterogeneous. In this respect, it should be noticed that, in a single lesion, a variable degree of

histological heterogeneity is present so probably accounting for different gene and protein expression between diverse plaque areas.

Future directions in the study of atherosclerotic plaques by proteomic approaches could be the analysis of laser captured microdissections and the evaluation of protein post-translational modifications, in particular oxidative modifications, in the search of specific biochemical pathways or new markers for diagnostic, prognostic, and therapeutic purposes.

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Legends to figures

Figure 1. Processing of protein spots by peptide mass fingerprinting.

Figure 2. Examples for data base searching. ProFound interface for identification of protein spots adopted in reference 34 (panel A) and obtained results for HSP27 (panel B).

Figure 3. Overview of the main proteomic strategies utilized until now in the study of atherosclerotic disease. Dotted lines are referred to unexplored proteomic approaches.