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Review

Biology of atherosclerotic plaques: What we are learning from proteomic analysis

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Abstract

Atherosclerotic plaque rupture triggers the onset of cardiovascular complications such as myocardial infarction and stroke, which represent the main cause of death in western countries. Atherogenesis is a complex process characterized by lipid retention, proteolytic injury and a chronic inflammatory response. The resulting pathological vascular remodeling involves inflammatory cell recruitment, fibrosis, smooth muscle cell proliferation, neovascularization and intraplaque hemorrhage. However, the cellular and molecular mechanisms underlying cardiovascular dysfunction remain widely unknown. The development of differential proteomics allows the identification of novel proteins whose association with the genesis of atherosclerotic plaques is at present unforeseen in the light of available data. Moreover, different strategies have been used to discover new potential biomarkers which could be related to cardiovascular risk. The multi-factorial nature of cardiovascular diseases necessitates the use of biomarkers for early detection, for monitoring the response to therapy and to predict clinical outcome. In this review, we summarize the different proteomic approaches and recent findings that will help us to understand the mechanisms implicated in the pathogenesis of atherothrombosis.
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Keywords: Proteomics; Atherosclerosis

1. Introduction

Atherosclerosis and its complications still represent the major cause of death in developed countries. It has been appreciated for decades that this disease is linked to hypercholesterolemia and the accumulation of inflammatory cells in the artery wall, although the exact mechanisms underlying this process remain unclear. Rupture of an atherosclerotic plaque may result in the occlusion of an artery downstream or in the formation of a thrombus over the atherosclerotic lesion itself, causing myocardial infarction, stroke or peripheral vascular disease [1].

Classical approaches target candidate genes or proteins as being potentially involved in the pathology and aim to test hypotheses based on previously reported data. Differential proteomics can be defined as a qualitative and quantitative comparison of proteomes characterizing various experimental or (patho-)physiological conditions. Non-physiological changes in protein expression levels often reflect the presence of disease, and such proteins are excellent markers for diagnostic, prognostic and therapeutic purposes. The emergence of new proteomic techniques allows the simultaneous evaluation of the abundance of hundreds of proteins. In this review, we will summarize the strategies and results of proteomic

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studies on vascular cells, atherosclerotic plaques and plasma.

2. Sampling

Differential proteomic methods consist of separating polypeptide species based on their physical and chemical properties for subsequent evaluation of their relative abundance comparing two or more experimental conditions. The limitations of this technique are that (a) the sample must be prepared in a way compatible with the separation and quantification techniques and (b) it is extremely difficult to analyse all the proteins or peptides present in a complex mixture simultaneously. This is due to different ranges of expression levels and different behavior of proteins during the separation process. The most abundant proteins will rapidly saturate the signal whereas low abundance species will not be detected. Most of the time, samples need to be processed prior to analysis (Fig. 1).

The sampling is a critical step in differential proteomic analysis and is directly related to the question raised and should be addressed before starting any kind of sample collection and proteomic analysis. For example, if the aim of the study is to discover circulating biomarkers which could be present in plasma or serum, either tissue/cell secretome or plasma/serum will directly be investigated. In contrast, if the objective of the study is to understand the mechanisms of atherogenesis, analysis of cell or tissue extracts will be preferred (Fig. 2).

2.1. Cell and tissue culture

Proteomic analysis can be performed on vascular cells. However, primary culture of vascular cells involves changes in phenotype associated with the procedure of cell extraction and culture. For example, proteases that can be used to dissociate VSMCs from an arterial wall will disrupt all cell–cell and cell–extracellular matrix interactions, leading to the induction of expression of adhesion molecules and neosynthesis of extracellular matrix (ECM) when the cells are plated in culture dishes. Exploring the proteome of cultured cells will probably provide information on the intrinsic pathological potential of the cells and their capacity to respond to a particular stimulus rather than on the original proteome expressed within the arterial wall before culture-induced phenotype adaptation.

Proteomic analysis can also be performed directly on tissue samples. However, this approach is difficult since atherosclerotic plaques are very heterogeneous (in lipid content, presence of intraplaque hemorrhage, fibrosis, calcification, etc.). Moreover, differential proteomics is based on the comparison between at least two conditions and this implies that proteomes from atherosclerotic arteries must be compared with those of the corresponding healthy arteries, often difficult to obtain in similar conditions.

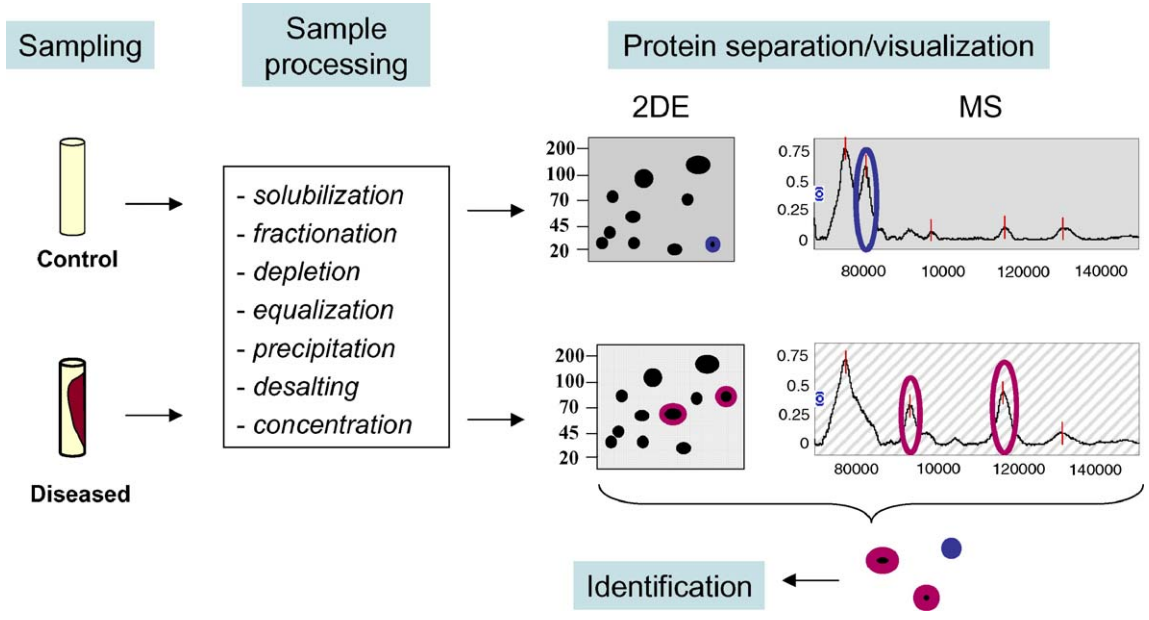

Fig. 1. From the sample to the identification of differently expressed proteins. Before proteomic analysis, samples need to be processed in order to be compatible with the separation techniques (low salt conditions, non-charged detergents), concentrated or "equalized" to reach detectable levels of proteins (even under-represented proteins) and finally, fractionated to be able to detect a maximum of polypeptides. Separation methods can be carried out using chemical and physical properties of the proteins such as isoelectric point, chromatographic affinities and molecular mass. Two-dimensional electrophoresis (2DE) and direct mass spectrometry (MS) of proteins are the most commonly used techniques to separate proteins from a complex mixture. After visualization and quantification, differentially abundant proteins are identified by peptide MS.

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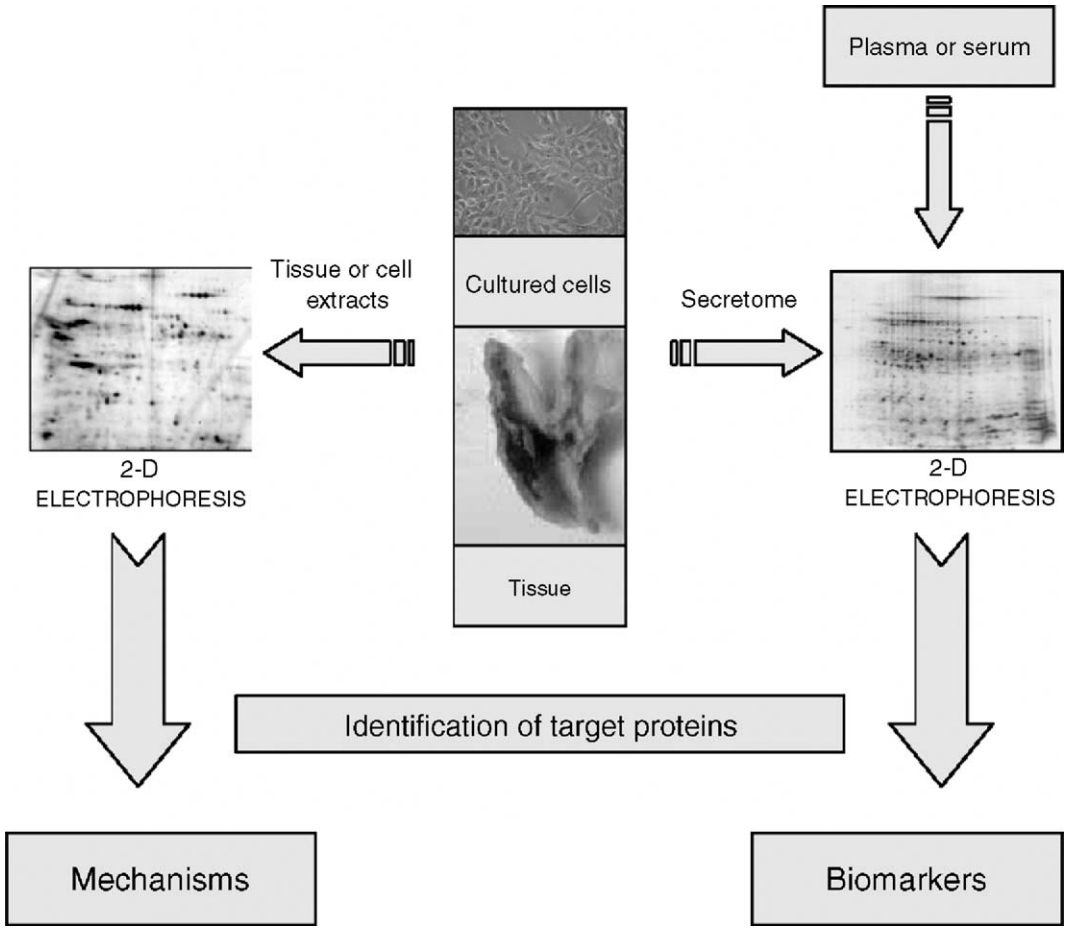

Fig. 2. Different biological issues require different strategies. To determine novel biomarkers of disease, plasma/serum samples or tissue/cell secretome should be studied. To establish mechanisms implicated in the disease, tissue or cell extracts should be analyzed.

Tissues can be homogenized and subsequently analyzed; however, the information provided by this approach will likely be limited to abundant constitutive proteins. Atherosclerotic tissue could also be laser-microdissected, which would allow one to compare different areas of the plaque such as necrotic core, shoulders/fibrous cap, or media, providing valuable spatial information. Interestingly, Tuomisto et al. used this technique to isolate macrophage-rich shoulder areas and then gene expression profiles were analyzed by cDNA array [2]. However, no proteomic studies of laser-microdissected cells from atherosclerotic plaques have been published. Such studies could add complementary information to that obtained by DNA chips, analyzing whether or not there is a correlation between genes, proteins expressed, and their post-translational modifications.

Imaging mass spectrometry (MS) is a new technology aimed at determining protein profiles directly from tissue sections [3] (see below for MS principle). This approach provides information on the spatial distribution of proteins in the tissue. Protein profiles can be compared between normal and diseased zones in the same, or in different tissue sections. Imaging MS can be used to correlate changes in protein abundance in known histological regions within the tissue. This technique bears potential applications in biomarker discovery with additional information on their tissue localization. It is currently used for mapping proteins present in tissue sections of various tumors [4] and could also be used to analyze proteins present in different zones of atherosclerotic plaques.

For both tissues and cells, proteomic analysis can be performed on either protein extract or conditioned medium. Focusing on conditioned medium will allow one to target more specifically secreted/released proteins provided that culture conditions do not induce necrosis. This latter approach can be used to discover circulating biomarkers.

2.2. Blood collection

The blood compartment is easily accessible to proteomic analysis in order to discover biomarkers that could be used in prognosis and diagnosis or to evaluate the efficiency of a treatment in a particular disease. Blood may reflect directly or indirectly a cardiovascular pathological state and the proteome of circulating cells or of plasma may be modified accordingly. Blood sampling must be carried out when it is

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most appropriate, depending on the issue to be addressed. For example, if the objective is to discover markers of coronary atherosclerosis, the blood should not be drawn a few hours after revascularization since stenting likely induces significant changes in the blood proteome that could mask markers of atherosclerosis found in basal conditions.

2.2.1. Serum or plasma?

Plasma is obtained by collecting blood in an anticoagulant solution and subsequent centrifugation. In contrast, serum is obtained after coagulation, a process which involves the activation of proteases in cascade leading to the formation of a clot containing activated leukocytes and platelets. These activated blood cells can release many proteins and proteases [5], which, in addition to coagulating proteases, will drastically affect the serum proteome. This must be taken into account in order to normalize blood sampling (type of anticoagulant, time of clotting for serum, centrifugation speed, etc.).

2.2.2. Circulating cells

The same attention must be paid when isolating circulating cells, since many cells, such as polymorphonuclear neutrophils or platelets, may be activated during this process (centrifugation, temperature, pipetting, etc.). The purity of the preparation is also critical as the proteome of contaminating cells may interfere significantly with the proteome of interest.

3. Different proteomic approaches

An overview of the main proteomic strategies currently available is given in Fig. 3. The objective of differential proteomics is to separate, visualize and quantify the proteins present in a complex mixture in order to reveal the

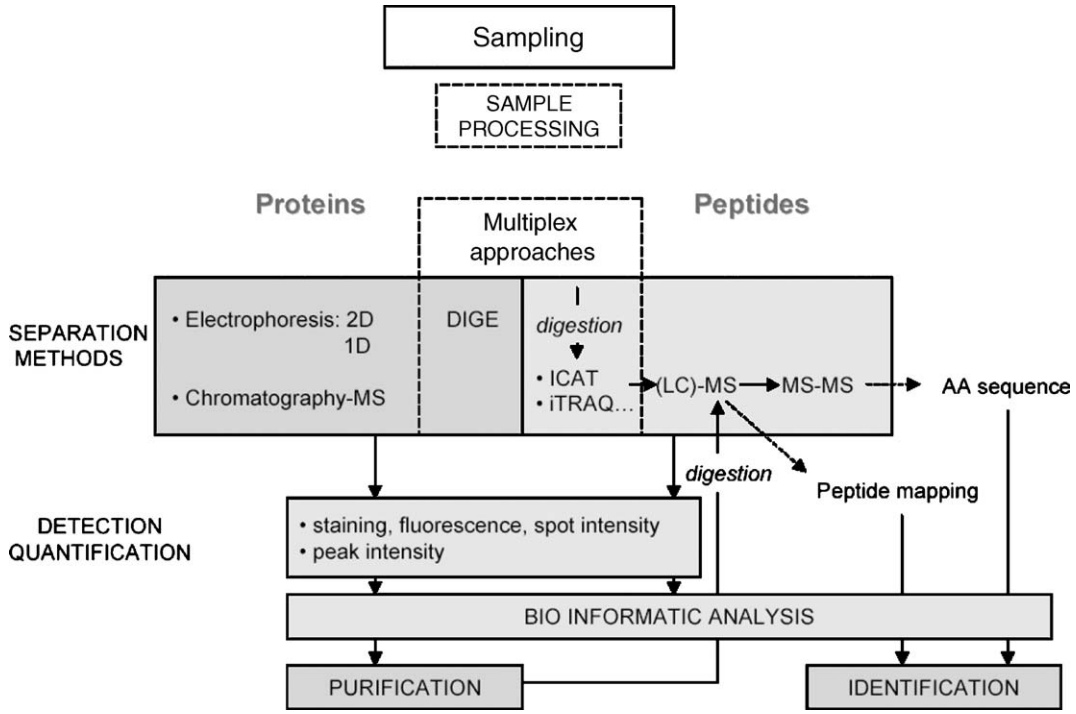

Fig. 3. Overview of the main differential proteomic strategies currently available. Crude samples may be used or processing may be necessary in order to enrich or fractionate the proteome prior to proteomic analysis. In some conditions, abundant proteins, potentially masking the deep proteome, may be removed or "equalized" to highlight under-represented proteins. The different steps comprise protein/peptide separation methods, their detection and quantification, and a bioinformatics analysis potentially leading to final identification. Proteins can be separated either by electrophoresis (one- or two-dimensions) or directly by mass spectrometry (MS). Detection methods for proteins use non-specific protein staining such as Coomassie blue, silver staining or fluorescent stains. Quantification is performed either by densitometry or using fluorescence detectors, the spot intensity being considered to be proportional to the quantity of protein present. For mass spectrometry analysis of proteins, the relative intensity of the peaks of interest is used for quantification. Bioinformatic tools allow targeting spots or peaks of interest that requires identification. Whereas spots can be directly excised and digested for MS analysis, potential markers detected by SELDI-TOF MS require purification before digestion and subsequent MS(-MS) analysis. Other strategies for differential proteomics can be used, consisting of direct quantification of peptides by MS (ICAT: isotope-coded affinity tag) or MS-MS (iTRAQ) (Applied Biosystems). Proteins from two or more conditions are labeled differently, digested and submitted to MS(-MS) analysis. This multiplex approach combining various samples in one tube before analysis is possible by differentiating peptides of a particular condition by a specific tag. This can also be done by using proteins tagged with different fluorochromes and mixed before electrophoresis in a same gel (DIGE: Differential Gel Electrophoresis). Final identification of proteins of interest is reached in most cases by combining peptide fingerprinting (peptide mapping) and amino-acid sequence information obtained by MS(–MS).

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differences of protein expression between two or more experimental conditions.

3.1. Sample processing

Solubilization of proteins is a critical step which must be compatible with the analytical techniques, often requiring that protein charge is not modified for fractionation based on chromatographic properties or for separation according to the isoelectric point of the polypeptidic species. Non-ionic detergents such as CHAPS and chaotropic agents such as urea/thiourea are often used for protein solubilization. When the amount of protein is too low or the sample contains salts which might interfere with separation techniques, precipitation [6] or concentration (dialysis, centrifugation devices) may be required. The most abundant proteins present in a complex sample such as plasma or serum can be removed by immunocapture methods. Up to 20 major plasma proteins can be retained by immunoaffinity columns and the resulting flow-through allows analysis of 10–50 times more material than non-treated samples. Low abundance proteins are highlighted but those associated with the removed protein fraction are also eliminated. A recently reported method of "equalization" was shown to detect trace proteins in serum. This methodology is based on a solid-phase ligand library of large diversity which retains almost all polypeptide/proteins with a limited capacity of binding for abundant proteins and allows analysis of large samples of serum/plasma [7].

3.2. Separation and visualization

The most widely used technique of proteomic protein separation is two-dimensional electrophoresis (2-DE). It is an orthogonal mass/charge analysis consisting of separating proteins in the first dimension by isoelectric focusing (separation according to their isoelectric point) followed by SDS–polyacrylamide gel electrophoresis in the second dimension (separation by the relative molecular mass) [8]. Commercially available immobilized pH gradients and solubilizing cocktails provide good technical reproducibility and make this approach accessible to non-specialist scientists.

The visualization of proteins after separation should also be well standardized and can be achieved by non-specific protein staining. The most commonly used methods are Coomassie blue or the more sensitive silver staining for which a regular scanner is needed for capturing images of the gels. Detection of proteins by fluorescent stains is even more sensitive and provides a good dynamic range, but appropriate equipment is necessary for capture. Whatever staining method is used, it must be compatible with mass spectrometry (MS), an indispensable step in protein identification strategies currently used. The main technical drawback of 2-DE is the comparison of various gels corresponding to individual samples treated separately (migration, staining). This can be overcome by multiplex approaches such as the 2D-DIGE technique (Differential Gel Electrophoresis) based on differential fluorescent tagging of proteins prior to separation (one sample, one fluorochrome) [9]. The samples are then pooled and separated by 2-DE in a single gel. This technique allows easy comparison of two or more samples: i.e., same conditions of migration, easier spot matching. Although 2-DE is the most popular protein separation technique, other separation methods exist, most of them coupled to MS, such as reversed-phase high-performance liquid chromatography [10], capillary zone electrophoresis [11], affinity chromatography [12], among others. Proteins are thus separated directly by time-of-flight mass spectrometry (TOF-MS) after fractionation. The time for the polypeptide/protein to reach a detector is directly proportional to its mass/charge (m/z) ratio. For example, the surface-enhanced laser desorption/ionization (SELDI) TOF-MS system is based on a first step of retention chromatography using chemically or biochemically predefined ProteinChip Arrays as capture surfaces for proteins, which are then analyzed by MS [13]. This technology, complementary to 2D-electrophoresis, is particularly well suited for analysis of small (<30 kDa) and potentially under-represented proteins as it enriches sub-proteomes according to their chromatographic affinities. A similar approach, consisting of fractionation of the proteome prior to MS analysis, can be performed using beads which allow targeting specific subproteomes (i.e., ClinProt which targets the glycoproteome or large proteins). The resulting mass spectra are then compared and the intensity of each peak, which depends on the amount of protein and its ability to fly, permits detection of potential polypeptides or proteins with differential abundance in the various experimental conditions (Fig. 1).

3.3. Identification

After separation and detection, proteins of interest isolated from acrylamide gels or purified by chromatography must be identified. The most commonly used method to identify proteins is MS-TOF following digestion by sequence specific proteases or chemical reagents [14]. In MALDI-TOF, ionization is achieved by crystallizing the sample with organic compounds (matrix) subjected to a laser pulse that vaporizes the peptides. Whatever the method of ionization used (matrix-assisted laser desorption ionization or electro-spray ionization, ESI), the specific digestion of a particular protein provides a peptide mass fingerprint which can be compared with in silico digestion of known or predicted proteins from nucleotide sequence databases [15]. However, this process of identification of proteins has several drawbacks. When a small number of peptides from the protein of interest is obtained, it will result in a poor sequence coverage. Peptide mass obtained without reasonable accuracy could lead to incorrect protein identification. Furthermore, proteins which have not been previously identified are absent from protein databases. Finally, tandem mass spectrometry (MS–MS) permits to obtain elements of

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amino-acid sequence (AA) of the protein of interest. Fragmentation of selected peptides is obtained by collisions with a neutral gas (He, Ar, N_2, etc.) and the AA sequence is deduced from mass determination of fragment ions. Combined to peptide mapping obtained by MS, information on AA sequence gives a high probability of the correct identification of the protein.

3.4. Multiple peptide analysis

Some recently developed approaches separate and quantify directly peptides obtained by digestion proceeding from various experimental conditions. Isotope-coded affinity tag (ICAT) technology is based on differential sample tagging (cysteines are tagged with linkers either with eight ^1H or with eight ^2H) and allows relative quantification of two protein populations by comparing peak intensities of peptides with molecular mass that differ by 8 Da [9]. After protein labeling, the two samples are combined, digested and purified before MS analysis. Alternatively, metabolic-labeling can be performed by incorporation of a heavy versus normal amino-acid allowing discrimination between peptides obtained in two different experimental conditions (SILAC: Stable-isotope labeling in cell culture). A recent technology called iTRAQ permits the comparison of up to 4 conditions in one MS–MS lecture. This technique is based on differential amine labeling with isobaric tags which release 114–117 Da reporters in MS–MS conditions, allowing one to compare the intensities of the peaks corresponding to peptides obtained by digestion of proteins resulting from up to 4 experimental conditions [16].

4. Advantages and disadvantages of proteomic technologies

The different proteomic technologies are complementary in their applicability but all suffer from a limited dynamic range for protein detection. 2-DE allows separation of thousands of polypeptide species per gel; however, the main drawbacks of this technique are its poor resolution for extreme masses and low throughput (one sample per gel), that it requires a large amount of starting sample (>50 μg of protein), and that it is also difficult to automate. These disadvantages are shared with 2D-DIGE, although the number of samples processed per gel is superior (up to three samples). In order to identify the different target proteins, liquid chromatography MS–MS permits the direct identification of several hundred proteins per sample by MS–MS of peptides; however, only one sample is analysed per run, which is very time-consuming. Moreover, detection by MS–MS is often not comprehensive, complicating the comparison between samples. With the ICAT technique, proteins are directly identified by MS–MS peptides and relative quantification is performed. However, only two samples per run can be analysed and only cysteine-containing peptides can be studied. iTRAQ permits the direct identification of biomarkers by MS–MS peptides and provides absolute quantification when the quantity of a reference labelled peptide is known. The limitations of this technique is that only 4 samples per run are analysed and MS–MS of all peptides in a sample is necessary, added to the high sample complexity and limited resolution of liquid chromatography. Finally, MALDI-TOF MS permits analysis of more than hundreds of samples per plate but a lot of starting material for fractionation is needed. SELDI-TOF permits the comparative analysis of 96 samples per bioprocessor and whole samples can be applied directly, thus diminishing the quantity of starting material required. However, SELDI has less resolution than MALDI. Both techniques are unsuitable for high-molecular-weight proteins (>100 kDa). In conclusion, the optimisation of results derived from proteomic analysis will depend on the correct choice of technique.

5. Proteomic analysis from vascular cells

The proteome of the different cellular elements of normal and pathological arteries is largely unknown. In a first step, the application of proteomic strategies to vascular cells in physiological conditions could allow the identification of proteins which should be further studied to understand their cellular functions.

5.1. Physiological conditions

Brunnel et al. have reported the protein expression map of primary cultures of human umbilical vascular endothelial cells (HUVECs) [17]. They identified 53 proteins in quiescent HUVECs. Beside cytoskeletal proteins (actin, tubulin, tropomyosin and vimentin), various other proteins were identified, especially implicated in the regulation of apoptosis and senescence, as well as other proteins involved in coagulation, antigen presentation and enzymatic capabilities.

In addition, a protein expression map of VSMCs from human saphenous vein was also reported [18]. Extracts from these veins were analyzed by 2-DE and about 130 proteins were identified. Furthermore, a 2-D reference map for human VSMCs of internal mammary arteries obtained from patients undergoing coronary artery bypass has recently been published, identifying 83 intracellular proteins [19].

This information represents a useful database to assess the effect of various proatherogenic stimuli on the expression of these novel proteins, or to compare their basal levels to those of vascular cells under pathological conditions.

5.2. Pathophysiological conditions

Atherosclerosis is a multifactorial disease in which hypertension, diabetes, hyperlipidemia and other risk factors are involved. However, the mechanisms underlying plaque formation and progression are still largely unknown. The

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comparison of proteomes of basal versus stimulated cells or healthy versus pathological cells could provide novel information about the mechanisms of atherogenesis. In Table 1, we summarize some of the proteins identified by differential proteomics performed on cells involved in atherogenesis.

Atherosclerotic plaque development is favored by local hemodynamic factors such as low wall shear stress and/or elevated circumferential wall tension. In this context, changes in the expression pattern of proteins of VSMCs exposed to hemodynamic stress were analyzed, identifying proteins responsible for capping the barbed end of actin filaments [20]. In addition, another member of the gelsolin family (capG) was identified when studying protein changes in aortic endothelial cells exposed to oscillatory and/or laminar flow [21].

Proliferation and migration of VSMCs are considered to be key events in the pathogenesis of atherosclerosis. To identify changes in specific proteins associated with either hyperplastic or hypertrophic growth, 2DE was performed on extracts from quiescent rat aortic VSMCs or VSMCs exposed to growth factors [22]. Among the different identified proteins upregulated by growth factors, there were mediators of protein folding and of protein synthesis.

Inflammation has been widely related to atherosclerosis. Cytokines such as tumor necrosis factor alpha (TNFα) are involved in different processes which are critical for the initiation and progression of vascular lesions. In a recent study, VSMCs were treated with TNFα in the presence/absence of alpha lipoic acid (ALA) [23]. Using 2-DE and MS, the authors identified proteins that were upregulated by TNFα and subsequently down-regulated in the presence of ALA (e.g., plasminogen activator inhibitor-2, fetal liver LKB-interacting protein, among others) or that were down-regulated by TNFα and up-regulated in the presence of ALA (e.g., Rho GDP dissociation inhibitor alpha). In addition, different studies have reported alterations of the proteome of monocytes under proinflammatory conditions. Verhoeckx et al. compared the proteome of human monocytic cells in basal conditions to that observed after their differentiation into macrophages by addition of

Table 1
Proteomics studies related with atherosclerosis

From cultured cells

Endothelial cells exposed to laminar flow [21]	*VSMCs exposed to hemodynamic stress* [20]	*VSMCs exposed to growth factors* [22]	*VSMCs exposed to cytokines* [23]	*Monocytes exposed to PMA* [24]	*Monocytes exposed to LPS* [25]
CapG (gelsolin family)	Gelsolin	↑ HSP60, HSP70, ↑ Protein disulfide isomerase	↑ Plasminogen activator inhibitor-2	γ-INF inducible lysosomal thiol reductase	↓ Integrin α-IIB ↓Protein disulfide isomerase
	HSP27	↑ Elongation factor EF-1β	↑ Liver LKB-interacting protein	Cathepsin D	↑ Macrophage capping protein
	CapZ	↑ Vimentin	↓ Rho GDP dissociation inhibitor-α	Adipocyte-fatty acid binding protein	↑ Superoxide dismutase and Catalase
		↑ Actin			↓Platelet-activating factor acetylhydrolase

From secretomes of cultured cells

From VSMCs [19]	*VSMCs exposed to oxidative stress* [36]	*Monocytes exposed to ox-LDL vs. LDL* [38]
Metalliproteinase-1; Collagenase	↑ HSP90; Cyclophilin B	↑ Cathepsins D,L,S; Proteoglycans; Paraoxonase
PAI-1; Peroxiredoxin 1		↓ Apolipoprotein D; Cathepsin H; Cofilin 1

From atherosclerotic tissue			From tissue secretome
Coronary plaques vs. healthy arteries [31]	*Advanced vs stable atherosclerotic plaques* [32]	*Carotid plaques vs. mammary arteries* [33]	*Normal vs. pathological artery* [6,39]
↑ Ferritin light chain	↑ α_1-antitrypsin	↑ Apoptosis-linked gene-2	↑ HSP27; Cathepsin D; Enolase 1; Serum Amyloid P Component; Transthyretin

From serum/plasma
From ACS vs stable patients [46]
↑ ATT; Apolipoprotein AI; Gamma-chain of Fibrinogen; Heavy Chain of Immunoglobulin D; Albumin

ACS: acute coronary syndrome; HSP: heat shock protein; VSMCs: vascular smooth muscle cells; PMA: phorbol myristate acetate; LDL: low-density lipoprotein; LPS: lipopolisaccharide.

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phorbol 12-myristate 13-acetate (PMA) [24]. From a total of 226 differentially expressed proteins, gamma interferon inducible lysosomal thiol reductase, cathepsin D and adipocyte-fatty acid binding protein were confirmed to be good differentiation markers for macrophage maturation as well as of peripheral blood-derived macrophages. Furthermore, Gadgil et al. have reported the protein expression profile of monocytes primed by lipopolysaccharide (LPS) [25]. Interestingly, many of these altered proteins have some functions in inflammation.

Although these studies are technically easy to perform, proteomic information obtained from cell culture experiments may not entirely reflect the complex molecular events that take place in an atherosclerotic lesion. A more interesting approach is the use of atherosclerotic vascular tissues.

6. Proteomic analysis from tissues

In contrast with experiments performed on vascular cells, interpretation of a proteomic or transcriptomic analysis of vascular pathological tissue is difficult due to the heterogeneous cell composition of atherosclerotic plaques. Whereas VSMCs predominate in normal vessels, advanced lesions contain a complex gruel of inflammatory and red blood cell debris, plasma and cell modified lipids and proteins.

Initially, gene expression profiles of whole human atherosclerotic plaques were compared with undiseased arterial wall. These studies revealed differential expression of a wide array of genes, the majority of which are involved in foam cell formation, inflammation, apoptosis, and thrombosis [26,27]. Suppression subtractive hybridization (which can isolate low abundant sequences that could not be detected by microarrays) was then used to identify genes differentially expressed in stable versus ruptured human atherosclerotic lesions, potentially involved in plaque instability [28]. Moreover, in a recent study using coronary atherosclerotic plaques, a novel approach based on connectivity (integrated network of gene interactions), has been proposed to expand the microarray technology in order to analyse the complex biology of atherosclerosis [29]. However, there is no direct correlation between mRNA and protein levels and the expression of mRNA does not reveal the activity of proteins, which are the final effector molecules.

Different studies have analyzed protein extracts from human atherosclerotic plaques by 2DE (Table 1). In the first study, tissue extracts from fatty streak lesions were compared with healthy segments of human aorta and only changes in proteins of plasma origin were detected [30]. Comparing coronary atheroclerotic plaques with normal coronary arteries, You et al. demonstrated an increment in the expression of ferritin light chain, a protein implicated in the storage of iron in cells, in coronary arteries of subjects with coronary artery diseases (CAD) [31]. More recently, Donners et al. examined the differences between advanced, but stable, human atherosclerotic plaques and plaques containing a thrombus [32]. This analysis revealed the expression of six isoforms of α_1-antitrypsin (ATT) in advanced plaques, one of which was uniquely expressed in thrombus-containing plaques. Upregulation of ATT in thrombus-containing lesions could act as a defense mechanism because the anti-elastase activity of ATT could provide a mechanism by which ATT may protect against atherosclerosis. By an alternative approach, Martinet et al. have screened tissue lysates from human carotid atherosclerotic samples and healthy mammary arteries using a western array composed of 823 monoclonal antibodies [33]. They reported seven proteins with >5-fold relative expression difference, one of which was the apoptosis-linked gene 2 (ALG-2), a positive mediator of apoptotic cell death. Since apoptosis is implicated in atherosclerotic plaque instability, the decreased levels observed for ALG-2 could suggest a new survival mechanism in human atherosclerotic plaques.

Finally, proteomics can also help us to understand both the beneficial and/or the deleterious effects of drugs in the treatment of disease, as well as the potential mechanisms of action. For example, a proteomic approach has been used to understand the protein changes associated with the reduction in intima/media ratio when rabbits were treated with rapamycin following balloon catheter injury [34]. 2D electrophoresis analysis of the neointima showed increased ECM synthesis following angioplasty in vehicle treated animals, which was reduced by rapamycin administration.

7. Proteomic analysis from secretomes

The secretome includes all proteins that are secreted or released by cells or tissue in the extracellular compartment. The characterization of the secretome of vascular cells implicated in the atherosclerotic plaque development is of interest as it may lead to the detection of novel biomarkers that could be related with cardiovascular diseases (Table 1). Recently, the secretome of arterial VSMCs in culture has been reported, identifying 18 different proteins that are secreted by VSMCs in culture [19]. These proteins are involved in a wide range of biological functions such as proteolysis [matrix metalloproteinase 1 (MMP-1), collagenase], regulation of fibrinolysis [plasminogen activator protein 1(PAI-1)] and defense mechanisms (peroxiredoxin 1). Interestingly, changes in the expression of these proteins had been previously related with atherosclerosis. Indeed, a local excess of MMP-1 in the fibrous cap could lead to a weakening of ECM and elevated levels of PAI-1 have been also found in diseased aorta [35].

Oxidative stress is involved in the mechanisms underlying plaque formation and progression. The application of chromatography and ESI-MS/MS techniques to study the proteins secreted by VSMCs in response to oxidative stress led to the identification of HSP90 and cyclophilin B [36]. It is relevant that secreted cyclophilins have been reported to be involved in regulating cell growth and inflammatory responses.

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The secretome of human macrophages, including 38 different proteins has recently been reported [37]. Again, secreted proteins were involved in multiple biological functions. In addition, a study was recently conducted using an in vitro "foam cell" model based on the stimulation of differentiated THP1 cells with oxidized low-density lipoproteins (oxLDL) as compared to LDL [38]. Analysis of proteins contained in cell supernatants (secretomes) allowed the identification of 59 proteins whose expression was increased or decreased following treatment with oxLDL, as compared to LDL. Some of these proteins were previously known for their participation in atherosclerosis.

We have recently reported an approach for the study of the atherosclerotic plaque secretomes in the search for potential biomarkers of atherosclerosis [6]. Our strategy is to compare the secretome from normal and pathological arterial tissue using a differential proteomic approach to identify new biological markers potentially released by the arterial wall into the plasma. Incubation of endarterectomy samples versus control endarteries in a protein-free culture medium allowed us to harvest separately the proteins released from pathological and healthy areas, and avoided any interference with abundant plasma proteins during proteomic analysis. Using this approach, we have identified a number of proteins that are differentially released by normal or injured vessels [6,39] which belong to different functional groups such as metabolic enzymes, structural proteins, protein transporters, transcription factors, antioxidants and stress defense proteins. In particular, we have demonstrated that in comparison with healthy mammary arteries, atherosclerotic plaques release less HSP27 [39], which was further confirmed by Western blot and ELISA. To confirm our hypothesis that plasma content can reflect arterial wall secretion, we showed that circulating concentrations of HSP27 were decreased in subjects with atherosclerosis, indicating that this protein could be a novel biomarker of atherosclerosis in relation to healthy subjects (Fig. 4).

Besides the use of identified proteins as surrogate prognostic markers of cardiovascular pathologies, this tissue approach also allows the exploration of their biological significance, providing evidence of new metabolic pathways within the diseased tissue and novel therapeutic targets. Since VSMCs are the main source of HSP27 in normal as well as in atheromatous arterial walls [40], we hypothesized that the observed decrease in HSP27 plasma levels could be due to degradation by proteolytic activities, which are proportional to the degree of plaque complexity. We have observed that when soluble HSP27 was co-incubated with different purified serine proteases and with conditioned media of vulnerable plaques (type V), HSP27 was degraded

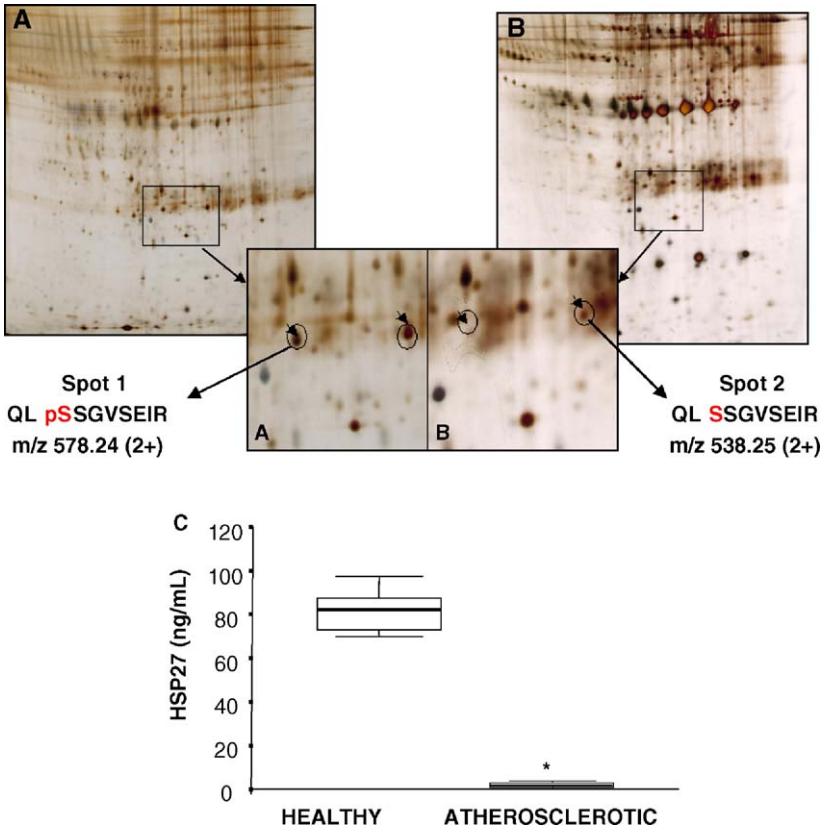

Fig. 4. HSP27 secretion: from arterial wall to plasma. 2D gels of secretomes from control endartery (A) and complicated plaque (B). Circles show 2 spots corresponding to HSP27, corresponding to different phosphorylation states. (C) Plasma levels of atherosclerotic patients ($n=28$) and controls ($n=12$) (*$p<0.0001$). Adapted from Ref. [40], with permission from *Circulation*.

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in the co-incubation medium, providing evidence for the existence of proteolytic activities within the plaque [41]. Therefore, HSP27 appears to be a sensitive biomarker in atheroma, highly down-regulated in patients' plasma, providing evidence for the existence of proteolytic activities in pathological arterial walls.

8. Proteomic analysis from plasma

The plasma proteome is the most interesting from a clinical point of view since it can provide information on the physiological state of the different tissues in the body. This is one of the reasons why great efforts are being made to fully characterize the human plasma proteome in physio- and pathophysiological conditions. However, the complexity of the human plasma proteome makes its analysis difficult. Analysis of crude plasma or serum does not provide information on thousands of proteins and peptides that are masked by the more abundant proteins. Nine major proteins represent 90% of the plasma proteome whereas about 12 other proteins account for an additional 9%. The challenge is thus to reach the "deep proteome" comprising the remaining 1% of the plasma proteome [42]. Targeted analysis can be performed, for example, by focusing on the lipoprotein proteome. Lipoproteins are involved in atherogenesis. However, the molecular mechanisms by which lipoproteins participate in the genesis and progression of atherosclerotic plaques are not fully elucidated. Furthermore, lipoproteins may serve as vehicles for specific proteins that need to be identified. For that reason, Karlsson et al. have recently used a proteomic approach to study the composition of human LDL and HDL [43,44]. In addition to previously known proteins (such as Apo B-100 or Apo A-1), serum amyloid A-IV, calgranulin A and lysozyme C have been identified in LDL particles. Moreover, changes in the protein composition of HDL during infection and inflammation have been recently studied using a proteomic approach. Apo A-IV and apo A-V were identified in HDL isolated from mice injected with endotoxin [45]. The use of proteomic approaches to study lipoproteins has revealed new proteins that should be further investigated to establish their potential role in atherosclerosis.

The complete analysis of the plasma proteome is tempting but difficult due to the presence of very high concentrations of some proteins such as albumin and immunoglobulins (in the order of g/L). An example of proteomic analysis directly performed on crude plasma (11 acute coronary patients versus 8 patients with stable angina) has been published recently [46]. Five major plasma proteins were found to be differentially expressed in patient's plasma: ATT, apolipoprotein AI, gamma-chain of fibrinogen, heavy chain of immunoglobulin D and albumin. However, the use of crude plasma for the discovery of biomarkers by differential proteomics provides only limited information.

As described in the Section 3.1, several methods can be used to eliminate proteins present in abundance which interfere with proteomic analysis. Chromatographic plasma fractionation needs high-throughput proteomics since a single sample generates many fractions. Abundant proteins can also be removed by immuno-affinity columns before proteomic analysis. However, the proteins or peptides which have an affinity for these proteins need to be analyzed separately with the same problem of major proteins masking under-represented ones.

9. Conclusions

Large-scale technologies are powerful tools for unraveling pathways of complex diseases such as atherosclerosis. However, caution must be exercised with regard to the experimental design and interpretation of results. In microarray experiments, there is a significant amount of "biological noise" that can distort the interpretation of the data [45]. In proteomic experiments, separation of samples should be undertaken in order to obtain high reproducibility. For the process of identification, a large number of peptides from the protein of interest should be obtained, in order to achieve high sequence coverage and correct protein identification. Finally, to avoid misinterpretation of these high-throughput technologies, the expression of a subset of genes/proteins should be validated with other techniques, such as real-time PCR, Western blot, ELISA, and/or immunohistochemistry. In relation to the identification of novel potential biomarkers, controlled clinical trials in well-defined clinical populations will be necessary to evaluate their predictive value and to study their potential biological significance in relation to the pathology. In this respect, a multimarker approach has been suggested to provide higher sensitivity and specificity for cardiovascular disease than is afforded with single markers [47].

The potential application of microarrays or proteomics in clinical practice will require significant efforts to standardize the manufacturing techniques, assay protocols and analytical methods used to interpret the data. In this respect, the use of DNA chips or proteomic technologies has been recently applied to the diagnosis of some types of cancer [48,49] since they allow easy determination of profiles characteristic for different conditions. However, the use of these types of techniques as potential screening tools requires the analysis of a large number of samples with a high degree of reproducibility and applying bioinformatics to parse the data into a diagnostic readout. There is still a great need for development of novel methods capable of detecting more genes/proteins and discovering new biomarkers which possess high predictive and prognostic characteristics that can be translated into clinical practice. In the future, genomic/proteomic analysis will identify novel patterns of biomarkers, which, along with traditional risk factors, will help to target

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vulnerable patients and monitor the beneficial effects of pharmacological agents.

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