Proteomics of Acute Coronary Syndromes

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Acute coronary syndromes (ACS), such as unstable angina, acute myocardial infarction, and sudden cardiac death, are commonly associated with the presence of vulnerable plaques in coronary arteries. Rupture or erosion of vulnerable plaques results in the formation of luminal thrombi due to the physical contact between platelets and thrombogenic elements within the atherosclerotic lesions. Considering the socioeconomic burden of ACS, it is imperative that the scientific community achieves a clear understanding of the multifaceted pathophysiology of vulnerable atheroma. The analytical power of modern proteomic technologies could facilitate our understanding of vulnerable plaques and lead to the discovery of novel therapeutic targets and diagnostic biomarkers.

Introduction

The pathologic features of atherosclerotic plaques define their propensity to rupture. Vulnerable atherosclerotic plaques are generally characterized by the presence of a necrotic, foam-cell lipid core separated from the arterial lumen by a thin and disrupted fibrous cap that exhibits a reduction in collagen content. The fibrous cap is infiltrated by activated macrophages and T lymphocytes [1]. In contrast, histologic examination of stable plaques shows reduced macrophage infiltration and a thicker fibrous cap with increased smooth muscle cells (SMCs) [2]. It is important to note that approximately one third of acute luminal thrombi arise from eroded plaques. Compared with ruptured plaques, erosions demonstrate smaller necrotic cores and a proteoglycan-rich fibrous cap with more SMCs and fewer macrophages. The thrombus is usually formed in direct contact with the intima, in an area of absent endothelium [3]. The fibrous cap is largely characterized by its dense extracellular matrix (ECM), a complex network of structural molecules such as fibrillar and nonfibrillar collagens and proteoglycans. The quantity and quality of the ECM in the fibrous cap is of paramount importance in defining plaque stability. Notably, SMCs residing in the fibrous cap are responsible for the synthesis of these macromolecules and for the dynamic ECM remodelling. Hence, it is not surprising that the presence of SMCs in the fibrous cap is believed to stabilize the plaque and prevent it from rupturing via the production and maintenance of ECM [4]. However, the presence of macrophages and T lymphocytes is considered to be deleterious because they produce inflammatory mediators and proteolytic enzymes.

Current Understanding of the Vulnerable Plaque: Beyond Inflammation

Contemporary atherosclerosis research has primarily focused on the inflammatory component of the disease. Excellent studies based on the analysis of human plaques and cell culture studies have shown the presence of activated immune cells, mainly macrophages, T lymphocytes, and mast cells, at the sites of plaque rupture [5]. Their biological activity appears to induce dysregulation of ECM metabolism and, consequently, plaque destabilization. For example, activated T cells secrete interferon-γ, a potent inhibitor of collagen synthesis by SMCs [6], and CD40 ligand, an activator of matrix metalloproteinases (MMPs) in SMCs [7]. Similarly, macrophages secrete proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), which amplify the inflammatory response and upregulate MMP gene expression by SMCs [8]. Activated macrophages also promote SMC death [9••]. Most importantly, however, macrophages produce a wide range of MMPs [10] that have the ability to degrade most ECM components, including collagens I and III and proteoglycan core proteins [11••], resulting in plaque destabilization.

The importance of inflammation on atherosclerosis is supported by findings in mouse models. Hypercholesterolemic apolipoprotein E (apoE) or low-density lipoprotein receptor knockout mice have often been crossed with mice bearing

genetic deletion of elements of innate and adaptive immunity, such as toll-like receptors (TLR2/4), cytokines and their receptors (IL-1/IL-1R, TNF-α/TNF-αR) and chemokines (CCL2/CCR2). These studies have shown that inflammatory mediators are involved in the pathophysiology and progression of the disease in vivo. However, they have failed to address the molecular events leading to plaque destabilization and rupture [9••]. The main limitation of the current approaches is their focus on the investigation of individual factors, which are presumed to be involved in the pathophysiology of plaque rupture and whose biological functions are, at least in part, understood. Given the biological complexity of the vulnerable plaque, research into atherosclerosis will benefit from a more holistic approach aiming at a comprehensive analysis of the microenvironment within atherosclerotic lesions.

Proteomics: A Promising Armamentarium for Research on Atherosclerosis

The complexity of atherosclerotic plaques results from the heterogeneous cell composition and the different proteins secreted by local cells and inflammatory cells infiltrating the tissue. The promise of proteomic techniques is to offer an unbiased platform for the comprehensive analysis of proteins rather than investigating individual molecules. Although mRNA expression is widely considered to be indicative of protein expression, the actual protein levels within the lesion are the net result of protein synthesis and degradation. Although mRNA levels may correlate with protein synthesis, they are not informative on protein degradation. In contrast, the proteomic approach investigates protein levels directly. This is particularly important in the context of atherosclerosis, in which protein degradation is a key determinant of actual protein concentrations. Moreover, proteomics have the potential to reveal posttranslational modifications (eg, phosphorylation, oxidation, glycation, nitrosylation, and so forth), which can be relevant to many human diseases. Undoubtedly, large-scale proteomic studies will facilitate the discovery of novel proteins, biomarkers, or mechanisms of disease [12].

Proteomic methodologies: choice matters

Modern proteomic methodologies and their application to atherosclerosis research have been described in detail in a review published recently in this journal [13]. Thus, this article highlights the main differences in the methodologies currently employed for proteomic analysis. There are two key strategies in contemporary proteomic research.

Separation at the protein level by two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2DE) offers powerful separation of proteins by utilizing two principal physical properties of proteins: charge and molecular mass. In the first dimension, proteins are separated according to their isoelectric point within an immobilized pH gradient, a process called isoelectric focusing (IEF). In the second dimension, sodium dodecyl sulfate polyacrylamide gel electrophoresis is used to further separate the proteins according to their molecular mass. Importantly, 2DE not only allows the separation of proteins from complex biological samples, but it also enables the visualization of posttranslational modifications or isoforms of the same protein as a shift in their isoelectric point or molecular mass [14]. The current gold standard in 2DE is difference in-gel electrophoresis (DIGE) [15]. DIGE is performed by labelling protein samples with distinct fluorescent probes. The different samples can then be co-separated in the same 2DE gel and co-detected by using the overlay of their fluorescent patterns. An internal standard labelled with a third dye is used for normalization. The DIGE technology is particularly useful for quantitative comparisons because it minimizes gel-to-gel variations and substantially improves the accuracy of the quantitative analysis compared with other techniques, such as silver staining. Following separation by 2DE, proteins of interest are excised from the gel and digested by trypsin. The tryptic peptides are then subjected to analysis by mass spectrometry, and there are two major approaches.

The first, matrix-assisted laser desorption ionization time of-flight (MALDI-TOF) mass spectrometry, involves determining the masses of the intact peptides, producing a "peptide mass fingerprint" of a sample. MALDI-TOF, however, is not considered state of the art for reliable protein identification because it does not provide direct evidence for the amino acid sequence of a protein. The second approach, tandem mass spectrometers (MS/MS), not only records the mass of the peptide ions but also induces a random fragmentation process. The masses of these fragment ions can then be matched against a sequence database. In this way, the protein identification can be established more reliably. For analysis, peptides are usually separated by reverse-phase, high-pressure liquid chromatography to which the tandem mass spectrometer is coupled in-line (LC-MS/MS). Modern tandem mass spectrometers offer attomole on-column sensitivity and high mass accuracy. In addition, novel methods for the fragmentation of ionized peptides, such as electron transfer dissociation, enable the preservation of posttranslational modifications such as phosphorylation or glycosylation, which are lost during collision-induced dissociation, which is currently the most commonly used fragmentation method.

Separation at the peptide level by gel-free "shotgun" proteomics

In "shotgun" proteomics, protein mixtures are subjected to enzymatic digestion followed by chromatographic separation and analysis by tandem mass spectrometry. Quantification relies on differential labelling of peptides with stable isotopes. Commonly used methods are stable isotope labelling with amino acids in cell culture (SILAC) or isobaric tags for relative and absolute quantification (iTRAQ). For SILAC, the culture medium is supplemented with a 'heavy' form of the amino acid (i.e. ^{13}C, ^{15}N). The corresponding peptides containing the light and heavy isotopes can be readily differentiated by their mass difference. This technique can reliably detect quantitative

changes, especially in pairwise comparisons; however, complete labelling with stable isotopes in culture is only achieved over five population doublings. By this time, most primary cells are considered too old for further analysis. For iTRAQ, proteins are not a priori labelled in culture, but an isobaric mass tag is introduced to unlabelled proteins after digestion with trypsin. Upon fragmentation, this isobaric mass tag generates low molecular mass reporter ions that differ in size. There are currently four-plex and eight-plex reagents that allow relative quantification of proteins originating from four or eight different samples in a single analysis, respectively. The disadvantage of any shotgun approach is that the number of samples/treatments that can be analyzed is very limited and that the quantification is readily compromised by experimental variation (ie, incomplete labelling or digestion of the analytes). Moreover, shotgun proteomics do not quantify the actual protein level but rely on peptides to estimate protein abundance. Reliable quantification is more readily obtained for highly abundant components for which multiple peptides are detected and accurately quantified. For poorly abundant proteins, reliable quantification may be impossible, especially in complex biological samples. Thus, although the gel-free approach is more sensitive in identifying poorly abundant proteins, it is not necessarily better for quantifying proteins in very complex biological samples or tissues.

Proteomics of atherosclerosis

Few studies have used proteomic approaches to investigate atherosclerotic lesions in human or animal tissue. In one of the first attempts to compare protein expression in atherosclerotic versus normal coronary arteries by 2DE, You et al. [16] reported elevated expression of ferritin light chain in diseased arteries. The authors concluded that ferritin light chain might contribute to coronary atherosclerosis by modulating the oxidation of lipids in the vessel wall [16]. In a more recent study, laser capture microdissection was used to obtain samples from human coronary atherosclerosis [17]. Proteomic analysis of these samples was then carried out by a gel-free LC-MS approach that generated 495 protein identifications with multiple peptides. Although laser capture microdissection enabled the analysis of the protein content from very specific areas of atherosclerotic lesions, quantitative comparisons between diseased and normal tissues remain a challenge, as differences in cellular composition will be largely responsible for the observed changes in protein content. To overcome this limitation, we have performed a proteomic and metabolomic analysis of aortas obtained from apoE knockout ($apoE^{-/-}$) mice. We were particularly interested in the different stages of the disease process and tried to identify alterations of the proteome and metabolome before the onset of atherosclerosis. Our findings revealed that inefficient glucose and energy metabolism coincide with increased oxidative stress in aortas of $apoE^{-/-}$ mice even before lesion development. This was evidenced by oxidation of redox-sensitive proteins, such as peroxiredoxin 6, in aortas of young $apoE^{-/-}$ mice. Moreover, attenuation of lesion formation was associated with replenishment of the vascular energy pool and posttranslational modifications of cytosolic malic enzyme, which provides reducing equivalents for lipid synthesis and glutathione recycling [14]. In an alternative approach, Martin-Ventura et al. [18] utilised 2DE and mass spectrometry to compare the secretome of cultured carotid atherosclerotic endarterectomy specimens with normal arterial specimens. The most significant finding was a reduction of heat shock protein 27 (hsp27) in the culture supernatant of the atherosclerotic plaques. The clinical relevance of this finding was further substantiated by reduced plasma levels of hsp27 in a small cohort of patients undergoing carotid endarterectomy [18]. Interestingly, a very recent 2DE proteomic analysis of stable versus unstable carotid plaque extracts confirmed a significant reduction in stress response proteins including hsp27, hsp20, superoxide dismutase 3, and others in unstable plaques [19]. Larger studies, however, are needed to establish whether small heat shock proteins can be of clinical value as biomarkers for vascular disease.

Proteomics of plasma

Mateos-Caceres et al. [20] reported an analysis of modifications in the plasma proteome during unstable angina and acute myocardial infarction. Using 2DE and mass spectrometry, they identified significant changes in four major plasma proteins: α1-antitrypsin, apoA-1, fibrinogen, and immunoglobulin γ (heavy chain) [20]. In another study, proteomic analysis of serum taken from patients suffering from acute myocardial infarction revealed significant degradation of apoA-1 by utilizing the visualization of apoA-1 cleavage products on 2DE [21]. The authors concluded that apoA-1 degradation was at least in part the result of in vivo proteolytic activity by MMPs. Of course, many more changes in the plasma proteome would be expected, including the release of established markers for myocardial damage such as troponin I and C, creatine kinase, and lactate dehydrogenase. It is also noteworthy that plasma proteomics failed to identify C-reactive protein as the dynamic range of protein concentrations in plasma spans nine orders of magnitude, whereas current proteomic technologies can only resolve four orders of magnitude. 2DE, in particular, will be restricted to the most abundant plasma proteins, limiting its potential use for biomarker discovery. Similarly, in "shotgun" proteomics, the vast number of peptides originating from the most abundant plasma proteins (ie, albumin and immunoglobulins) will mask the less abundant peptides derived from cytokines or growth factors or proteins leaking from diseased tissues. More recently, Barderas et al. [22] analyzed the proteome of cultured monocytes taken from patients suffering from ACS with non–ST-segment elevation. Upon admission, 18 proteins were found to be significantly altered in the proteome of the ACS group when compared with the proteome of monocytes taken from stable patients. Some of these proteins were cathepsin D, HSP60, HSP70, and S100A8. Interestingly, the proteomic pattern of the ACS monocytes changed over time, and 6 months after the acute event it resembled the proteomic profile of monocytes from stable

patients. The main findings of proteomic studies published to date are summarized in Table 1. Similar to proteomics, novel metabolomic technologies allow the sensitive analysis of multiple metabolites from cells or body fluids. The use of metabolomics for the analysis of novel elements involved in cardiovascular pathology is emerging. An example is a recent study by Lewis et al. [23]. The authors used LC-MS to analyze early changes in the plasma metabolite profile from patients with planned myocardial infarction. Interestingly, they found novel changes in plasma levels of pyrimidine metabolites, such as orotic acid, malonic acid, and aminoisobutyric acid. It remains to be seen whether such changes in the metabolomic profile could be used for the very early detection of myocardial infarction [23].

Proteomics on vascular progenitors

Recent evidence suggests that endothelial progenitor cells (EPCs) and smooth muscle progenitor cells (SPCs) are present in bone marrow and circulate in peripheral blood [24•]. EPCs are probably the most widely studied adult progenitor cell type, but several issues regarding their characterization and activity remain unclear. Despite a decade of research, there is still no specific marker for their unambiguous identification, and clinical trials using EPCs as a cell-based therapy following myocardial infarction produced controversial results [25]. Proteomics could improve the characterization of these cell populations (an example is shown in Figure 1). Microarray analysis provided important insights into the possible paracrine effects of EPCs by demonstrating that EPCs secrete high levels of cathepsins [26]. This finding was further substantiated by proteomics, which revealed the presence of additional angiogenic growth factors [27]. Interestingly, the increased proteolytic activity of EPCs would explain why infusion of EPCs in apoE$^{-/-}$ mice increased plaque size and decreased markers indicative of plaque stability [28]. In contrast, recent data on SPCs suggest a more benevolent nature of this cell type. For instance, Zoll et al. [29••] demonstrated that intravenous injection of human SPCs in double knockout apoE/rag2 mice decreased plaque development and promoted a stable plaque phenotype. In contrast, injection of EPCs had no effect. Besides circulating progenitors, resident stem cells in the vasculature have attracted considerable attention. For example, Hu et al have shown that stem cell antigen 1–positive progenitors exist in the vascular adventitia of apoE$^{-/-}$ mice [30]. These resident progenitors migrate from the adventitia to the media during atherosclerotic lesion development. Using 2DE and mass spectrometry, we carefully characterized this cell population and showed that they acquire characteristics of mature SMCs upon incubation with platelet-derived growth factor. Moreover, the phenotype of progenitor-derived SMCs closely resembled apoE$^{-/-}$ rather than wild-type SMCs demonstrating the potential of using an unbiased proteomic approach to assess cellular phenotypes [31].

Proteomics on extracellular matrix

As mentioned previously, the two main determinants of plaque stability are the ECM of the fibrous cap and the presence of SMCs, which are believed to stabilize the plaque and prevent it from rupture. The ECM composition of the fibrous cap has been studied mostly through electron microscopy visualization and histochemical and immunohistochemical staining of few specific ECM molecules in atherectomy specimens [32]. The most prominent components of the fibrous cap are fibrillar collagens, small elastin fibers, and proteoglycans. Other minor elements, such as nonfibrillar collagens, have also been detected in the fibrous cap [33].

Fibrillar collagens type I and III comprise approximately 60% of total protein in the fibrous cap [34]. Type I is largely responsible for tensile strength whereas type III confers elasticity. In contrast to the normal tunica intima, which contains more type III collagen, the fibrous cap is particularly rich in type I, indicating decreased resilience and susceptibility to hemodynamic forces [33]. Nevertheless, the presence of fibrillar collagen in the fibrous cap is generally associated with stability. In contrast, sites of plaque rupture are characterized by a marked reduction in fibrillar collagen and increased collagen proteolysis. The potential role of nonfibrillar collagens in the fibrous cap, or in atherogenesis in general, remains unknown. Type VIII collagen has recently received more attention. It has been shown to accumulate in atherosclerotic lesions and it may play a role in plaque stabilization [35].

Proteoglycans are important macromolecules of the fibrous cap [36]. Together with type I collagen, they comprise the bulk of ECM synthesized by the SMCs in the fibrous cap. Their structure is characterized by a protein core, which is substituted by heavily sulphated glycosaminoglycan (GAG) chains. The amount of GAG chains attached to the protein core is very variable and so is the sulphation pattern of GAGs. These negatively charged structures attract water and confer swelling pressure to the collagen network. An important feature of proteoglycans is that their core protein associates with the large nonsulphated glycosaminoglycan hyaluronic acid to form essential ECM aggregates. The proteoglycans that have been shown to reside in the fibrous cap are versican (substituted exclusively with chondroitin sulphate GAG chains), biglycan and decorin (substituted with dermatan sulphate GAG chains), and perlecan (substituted with heparan sulphate GAG chains).

Very little is known about the role of proteoglycans in vulnerable atherosclerotic lesions. Historically, chondroitin sulphate–rich and dermatan-sulphate rich proteoglycans and hyaluronic acid have been shown to associate strongly with the proatherogenic apoB-containing lipoprotein particles (ie, low-density lipoprotein and very low-density lipoprotein) to form highly aggregated complexes [37]. The presence of these particles is strongly related to atherogenesis, and accumulation of proteoglycans is believed to increase plaque burden [38]. Other studies have shown that in advanced atherosclerotic lesions, the proteoglycan-hyaluronic acid aggregates are smaller and less stable, indicating a possible role in the decreased integrity of the ECM [39]. An interesting study by Kolodgie et

al. [40] showed that at sites of plaque rupture there was severe reduction of versican, biglycan, and hyaluronic acid, together with reduction in type I collagen, indicating extensive ECM degradation. Interestingly, the presence of decorin was unaffected [40]. However, apart from immunostaining, no comprehensive analysis of the extracellular matrix has been performed.

A comparative and unbiased proteomic analysis focused on the ECM components of stable versus unstable atherosclerotic plaques could result in the identification of new connective tissue proteins potentially involved in the formation of atherosclerotic lesions. The presence of proteoglycans, nonfibrillar collagens, and other molecules needs to be assessed in the context of plaque vulnerability. Furthermore, it is also important to investigate whether structural alterations of connective tissue proteins, especially proteolytic processing, participate in the pathology of the vulnerable plaque. For instance, it has been shown that hyaluronic acid degradation products (i.e., tetra- and hexasaccharides) have the ability to act as endogenous TLR-2 and TLR-4 ligands and induce a proinflammatory response in a variety of cell types, including macrophages and endothelial cells [41]. Currently, the pathologic consequences of depolymerized hyaluronic acid are being studied extensively in relation to chronic lung diseases, osteoarthritis, and rheumatoid arthritis. Similarly, the fibronectin splice variant extra type III domain A has been shown to activate T cells and induce MMP-9 expression in human monocytes by activating TLR-4 [42], but no such studies have been conducted in unstable atherosclerotic lesions.

Proteomics on proteolytic products of the ECM

The proteolytic activity of MMPs is a key regulator of ECM integrity. MMPs are a family of 23 proteinases in humans that play a key role in ECM turnover under normal conditions. They are also involved in the increased ECM breakdown observed during disease processes such as atherosclerosis. Several studies have shown increased expression and activity of MMPs, including MMP-1, -2, -3, and -9, in vulnerable areas of atherosclerotic plaques [43]. Other studies have shown increased collagenolysis in vulnerable plaques. Collagenolysis was detected by antibodies raised against type I collagen neoepitopes and was attributed to the proteolytic activities of MMP-1 and -13 [44]. Interestingly, synthesis of active MMP-9 by macrophages and SMCs was demonstrated in human coronary atherectomy specimens taken from patients with unstable angina but not from stable patients [45]. Unfortunately, recent efforts to inhibit MMP activity using synthetic MMP inhibitors in an attempt to improve clinical outcome have been inconclusive [46]. This may be due to the absence of selective MMP inhibitors or due to the complexity of in vivo proteolytic regulation. Indeed, in vivo proteolytic regulation involves the activity of the natural MMP inhibitors, the Tissue Inhibitors of MetalloProteinases (TIMPs). TIMP-1, -2, and -3 collectively inhibit all metalloproteinases with different substrate specificity. The regulation of their activity in the context of plaque vulnerability is poorly understood and one could speculate that the fine balance between MMP-TIMP activity is responsible for plaque stability in some individuals or vulnerability in others. Moreover, MMP transgenic mice have also produced inconsistent results regarding atherogenesis. A number of cathepsins, which are cysteine proteases that can degrade collagen and elastin, have been identified in macrophages in the shoulder region of advanced atheroma [47]. Whether cathepsins participate in the destabilization of vulnerable plaques remains to be determined. Finally, little is known regarding the regulation of proteoglycan degradation. Halpert et al. [48] found that versican is a substrate for MMP-7 and MMP-12 at sites of plaque rupture. A class of metalloproteinases, namely the ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) have recently received attention because they induce very potent and specific proteolysis of the aggrecan core protein. Jonsson-Rylander et al. [49] demonstrated that ADAMTS-1 can degrade versican, but other functions of ADAMTSs in atherosclerosis are still unclear. Proteomics could advance our understanding on the regulation of ECM catabolism. For instance, proteomic analysis of tissue extracts may result in the identification of a wider range of proteinases and inhibitors present in the vulnerable plaque. More importantly, proteomics could identify specific ECM degradation products that might be associated with the activity of specific proteinases in atherosclerotic lesions.

Conclusions

The protein expression profile of cells and tissues defines their biological properties. Instead of investigating a limited and preselected number of proteins, proteomics enable us to analyze global protein expression. In the context of ACS, proteomic analysis of vulnerable plaques can provide novel information regarding the pathologic mechanisms of plaque rupture. The unbiased dataset generated by proteomic techniques may reveal novel biomarkers and therapeutic targets that can be exploited for clinical practice.

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Disclosure

No potential conflicts of interest relevant to this article were reported.

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