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Review

The -omics era: Proteomics and lipidomics in vascular research

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ABSTRACT

A main limitation of the current approaches to atherosclerosis research is the focus on the investigation of individual factors, which are presumed to be involved in the pathophysiology and whose biological functions are, at least in part, understood. These molecules are investigated extensively while others are not studied at all. In comparison to our detailed knowledge about the role of inflammation in atherosclerosis, little is known about extracellular matrix remodelling and the retention of individual lipid species rather than lipid classes in early and advanced atherosclerotic lesions. The recent development of mass spectrometry-based methods and advanced analytical tools are transforming our ability to profile extracellular proteins and lipid species in animal models and clinical specimen with the goal of illuminating pathological processes and discovering new biomarkers.

Contents

1. Introduction

The retention of proatherogenic low-density lipoprotein (LDL) particles on the subendothelial extracellular matrix (ECM) is a hallmark of atherosclerosis [1]. Apolipoprotein B (apoB)-containing lipoprotein particles are trapped in the arterial intima by proteoglycans in atherosclerosis-prone areas and eventually become modified, commonly by aggregation and oxidation [2]. The initial accumulation of proatherogenic lipoproteins initiates an inflammatory response, which results in the release of proteolytic enzymes and induces the dedifferentiation of vascular smooth muscle cells (SMCs) resulting in alterations of their matrix-producing properties [3]. The precise mechanisms responsible for the accumulation of certain matrix components and subsequent lipoprotein retention on the vessel wall are not fully elucidated. Undoubtedly, ECM remodelling contributes to the formation of atherosclerotic lesions and the lipid composition of apolipoproteins influences their binding properties to the matrix. An unbiased discovery approach, which is not limited to known molecules of presumed importance, will be invaluable for the identification of novel, previously unknown mediators of disease. Although descriptive, the detailed examination of atherosclerotic plaques using advanced proteomics and lipidomics techniques can generate novel insights and form the basis for further mechanistic investigations

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on atherogenesis. In this review, we will highlight key studies on atherosclerosis using mass spectrometry with a particular focus on proteomics of the vascular ECM and lipidomics.

2. Proteomics of atherosclerosis

In comparison to the analysis of messenger RNA (mRNA), proteomics offers certain advantages [4]: First, atherosclerosis is a chronic disease and lesions develop over years. While transcript levels can provide information abut cellular activity at the time of harvest, the actual protein content is dependent on the balance of protein synthesis and degradation [5]. This balance is particularly important when studying the ECM and its associated proteins, which build-up over time. Newly synthesised ECM proteins are incorporated into the existing matrix and a snapshot of mRNA expression is less informative than a proteomics screen. Second, proteomics can explore the chemical diversity beyond the genome, such as posttranslational modifications, which are not detectable at the mRNA level [6], but affect protein function and human disease including atherosclerosis. Over the last 10 years, few proteomics studies have been performed on atherosclerotic lesions.

2.1. Proteomics of isolated cells or explant tissues

Most studies focused on the identification of potential biomarkers of human atherosclerosis and used carotid and coronary atheroma or conditioned medium from isolated cells or explant tissues. Some early findings included increased levels of ferritin light-chain in coronary lesions, which was related to lipid oxidation in the vessel wall [7], and a reduction in heat shock protein (Hsp) 20 and 27 and superoxide dismutase in unstable carotid atheromas [8]. Hsp27, a cytoplasmic protein, was decreased in the supernatant of cultured carotid plaques and its plasma levels were reported to be lower in a small cohort of patients undergoing carotid endarterectomy [9]. Given the importance of inflammation in atherosclerosis, Barderas et al. analyzed the cellular proteome of circulating monocytes. Monocytes from patients with acute coronary syndrome showed higher levels of cathepsin D, Hsp60 and 70 and protein S100A8 compared to patients with asymptomatic coronary artery disease [10]. We performed one of the first studies using a combined proteomics and metabolomics approach in an animal model of atherosclerosis, namely apolipoprotein E-deficient (apoE−/−) mice. We studied different stages of the disease process to identify alterations in 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 [11]. A metabolomic comparison of 18-month and 10-week old aortas from chow-fed apoE−/− mice demonstrated a 2-fold rise in choline without significant changes in tissue concentrations of trimethyl amine oxide (TMAO) [11]. Interestingly, TMAO is not an endogenous metabolite but generated from the gut flora upon ingestion of phospholipids. Gut bacteria break down products with high phospholipid content (such as red meat and milk) to betaine and choline, which are then converted to TMAO by liver enzymes. A recent metabolomics screen in plasma identified these functionally related metabolites – betaine, choline, and TMAO – as potential biomarkers of atherosclerosis [12]. Furthermore, choline or TMAO feeding accelerated atherosclerosis in apoE−/− mice [12]. We also studied smooth muscle cells isolated from atherosclerosis-prone apoE−/− aortas. Their proteomics and metabolomics profiles were clearly distinct to wild-type smooth muscle cells, including differences in glucose metabolism and accompanying expression changes of interleukin-6 and insulin-like growth factor binding proteins 3 and 6, which regulate the extracellular distribution and bioavailability of insulin-like growth factor 1 [13]. In an alternative approach, Wu et al. performed a proteomics analysis of the biotinylated endothelium from wild-type or apoE−/− aortas. Differentially expressed proteins included proteins involved in inflammatory responses, angiogenesis and lipid metabolism [14]. Finally, proteomics, metabolomics and immunomics profiles were obtained from microparticles derived from human carotid endarterectomies [15]. The proteomic arm of this study identified membrane proteins confirming that plaque microparticles stem primarily from leukocytes. The metabolomic approach revealed taurine as the most prominent metabolite in plaque-derived microparticles, which serves as a negative feedback after oxidative burst in leukocytes. The immunomic experiment demonstrated that immunoglobulins were present within plaque microparticles and that the portfolio of plaque antibodies was different from circulating antibodies. Thus, the capture of plasma antibodies within atherosclerotic lesions must be highly specific. Surprisingly, certain anti-carbohydrate moiety antibodies recognizing carbohydrate antigens of the ABO blood group and the antigen responsible for hyperacute rejection in xenotransplantation, the Gal-α-(1,3)-Gal linkage, were enriched in atherosclerotic lesions as reviewed elsewhere [16].

2.2. Proteomics of the ECM

Since most proteomics studies on atherosclerosis to date reported predominantly changes in cellular proteins, the extracellular proteome, which is composed of ECM proteins including collagens, proteoglycans and glycoproteins and proteins associated with the ECM such as lipoproteins, growth factors, cytokines and proteinases, is not well explored. During the progression of atherosclerosis, the continuous pathological remodelling of the ECM contributes to the transition of a stable to a vulnerable lesion. Stable lesions exhibit a thicker fibrous cap, enriched in cross-linked fibrillar collagens, types I and III. In contrast, vulnerable plaques are covered by a thin and disrupted fibrous cap with reduced collagen content [4]. A comprehensive characterization of the ECM is essential for the understanding of this disease process. However, the vascular ECM is mainly studied by antibody staining or by transcript analysis. Thus, our understanding of its composition and remodelling in different vascular territories is limited. In one of the very few proteomics studies that targeted the ECM, Talusan et al. isolated proteoglycans from human intimal hyperplasia specimens from either atherosclerosis-prone internal carotid arteries or atherosclerosis-resistant internal thoracic arteries. They observed increased levels of lumican in the atherosclerosis-prone arteries and provided the first evidence for the presence of the cartilage proteoglycan aggrecan in the vasculature [17]. Recently, de Kleijn et al. identified the ECM glycoprotein osteopontin as a potential biomarker of carotid atherosclerosis and showed that its plasma levels are predictive for atherothrombotic events even in other vascular territories [18]. This study proves the concept that tissue proteomics can reveal novel circulating biomarkers. Other examples of extracellular proteins with cardiovascular biomarker potential include leukocyte myeloperoxidase, an enzyme released upon neutrophil activation [19,20], the C-terminal propeptide of procollagen I (PICP) [21], the N-terminal propeptide of procollagen III (PIIINP) [22], matrix metalloproteinase-9 (MMP-9) [23] and the tissue inhibitor of metalloproteinases-1 (TIMP-1) [24].

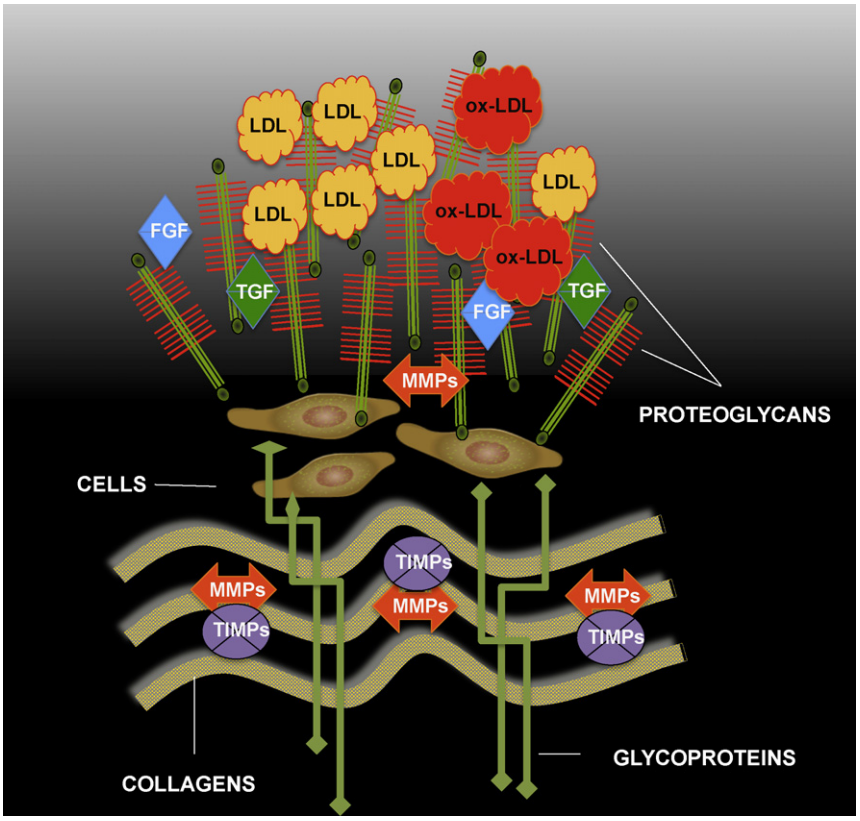

Fig. 1. ECM in atherosclerosis. The bulk of the vascular ECM is synthesised by smooth muscle cells and composed primarily of collagens, proteoglycans and glycoproteins. During the early stages of atherosclerosis, LDL binds to the proteoglycans of the vessel wall, becomes modified, i.e. by oxidation (ox-LDL), and sustains a proinflammatory cascade that is proatherogenic.

2.3. Novel method for the enrichment of vascular ECM

The characterization of extracellular proteins by proteomics is difficult for two reasons: First, most extracellular proteins are scarce and their identification is hampered by the presence of abundant cellular or plasma proteins, and second, matrix proteins are usually cross-linked in tight aggregates and are therefore difficult to extract and solubilize. Moreover, they are subject to extensive post-translational modifications, in particular glycosylation, which alter their molecular mass, charge and electrophoretic properties. These characteristics not only render the proteins difficult to identify by mass spectrometry but they are also responsible for many of the technical problems associated with separation methods such as incomplete isoelectric focusing and poor resolution during electrophoresis. To overcome these shortcomings, we adapted a biochemical subfractionation procedure recently used in cartilage research to extract ECM proteins based on their differential solubility [25] and included an additional decellularization step [26]. First, an ionic buffer selectively solubilizes newly synthesised matrix proteins (the salt-soluble ECM) and facilitates the extraction of degradation products, which are weakly bound on the interstitial matrix and could be released in plasma as biomarkers. Second, the tissue is decellularized using low concentration sodium dodecyl sulfate (SDS). The depletion of cellular proteins allows the identification of scarce proteins in the remaining ECM, which is composed of heavily cross-linked, salt-insoluble protein aggregates including collagens, proteoglycans and glycoproteins. Finally, the mature ECM is solubilized in a strongly denaturing buffer.

Using this extraction methodology, we performed a detailed proteomics comparison of the ECM between healthy human aortas and abdominal aortic aneurysms (AAA). Six glycoproteins were upregulated during ECM remodelling in AAA, including collagen XII, thrombospondin 2, aortic carboxypeptidase-like protein (ACLP), periostin, fibronectin and tenascin [27]. By extracting and preserving the proteolytic fragments within the tissue we showed that these six glycoproteins were also degraded and their fragmentation could be linked to metalloproteinases (MMP)-12 activity. Several studies have shown that MMPs are responsible for plaque destabilization and aneurysm formation, but their vascular targets are not comprehensively characterized. Proteomics can be used to investigate the functional role of MMPs and other proteases in vascular disease by mapping their proteolytic activity on vascular tissues. Finally, numerous other extracellular proteins were identified for the first time in the vasculature, including the glycoproteins podocan, sclerostin and agrin, the uncharacterized retinal pigment epithelium (RPE) spondin and target of Nesh (TARSH). These proteomics findings will form the basis for future investigations exploring the role of novel extracellular proteins in the vasculature (Fig. 1).

3. Lipidomics in atherosclerosis

The retention of apoB-containing lipoprotein particles in the vessel wall results in the formation of fatty streaks, an early event in atherogenesis. ApoB-100, the main protein component of LDL, binds not only to the LDL receptor (LDLR) [28], a key mediator of reverse cholesterol transport from macrophages, but also to vascular proteoglycans [29]. Biglycan and versican, both abundant in the vasculature, are key mediators of lipoprotein binding. They accumulate in atherosclerosis-prone arteries and their glycosylation pattern is affected by proatherogenic stimuli [30]. Other matrix molecules, including collagen, elastin, laminins and fibronectin, have been shown to interact with LDL but their role in primary

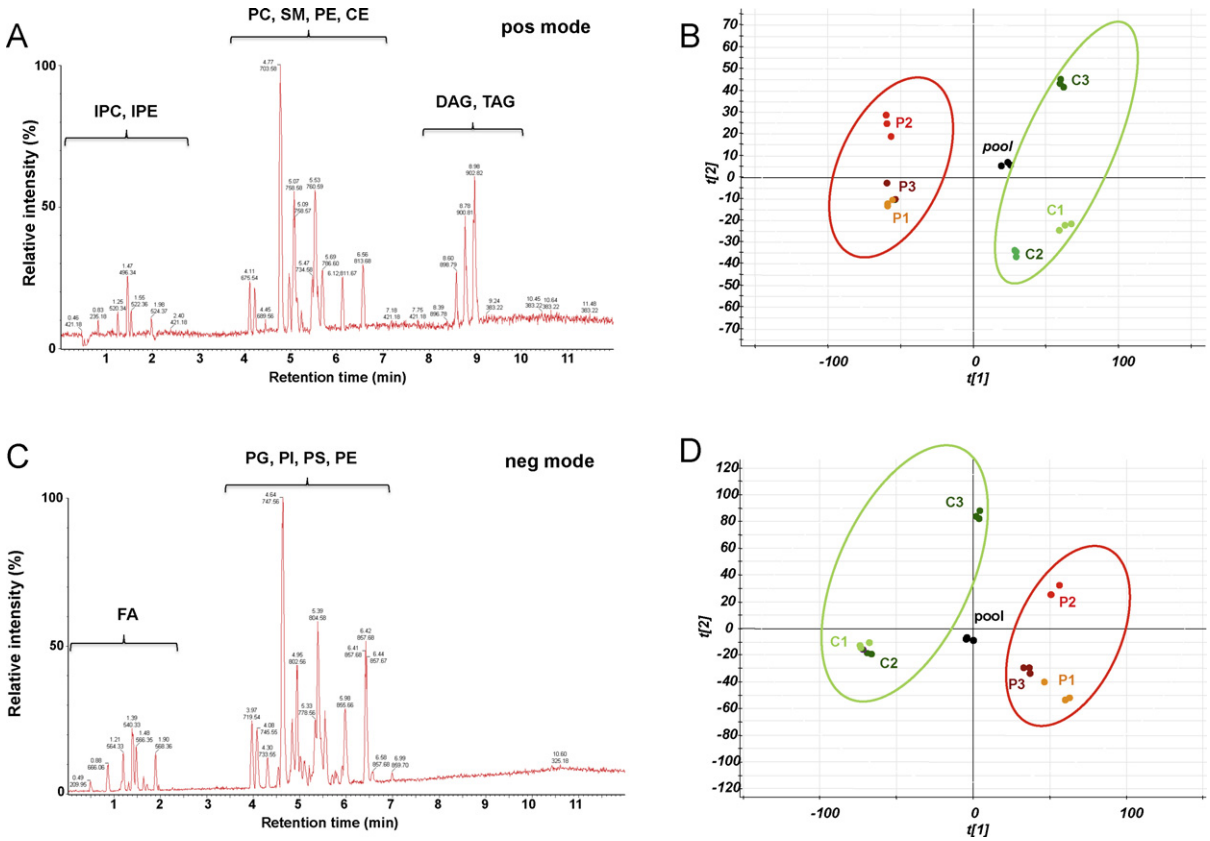

Fig. 2. Lipidomics of atherosclerotic plaques. Lipids were separated by ultra performance reverse phase liquid chromatography on a Waters® ACQUITY UPLC® (HSS T3 Column, 100 mm × 2.1 mm i.d., 1.8 μm particle size, 55 °C, flow rate 400 μL/min, Waters, Milford MA, USA) and analyzed on a quadrupole time-of-flight mass spectrometer (Waters® SYNAPT™ HDMS™ system) in both positive (A) and negative ion mode (C). In positive MS mode, lysophosphatidylcholines (lPCs) and lysophosphatidylethanolamines (lPEs) eluted first; followed by phosphatidylcholines (PCs), sphingomyelin (SMs), phosphatidylethanolamines (PEs) and cholesteryl esters (CEs); diacylglycerols (DAGs) and triacylglycerols (TAGs) had the longest retention times. In negative MS mode, fatty acids (FA) were followed by phosphatidylglycerols (PGs), phosphatidylinositols (PIs), phosphatidylserines (PS) and PEs. The chromatographic peaks corresponding to the different classes were detected as retention time-mass to charge ratio (m/z) pairs and their areas were recorded. Principal component analyses on 629 variables from triplicate analysis (C1, 2, 3 = control 1, 2, 3; P1, 2, 3 = endarterectomy patient 1, 2, 3) demonstrated a clear separation of atherosclerotic plaques and control radial arteries in positive (B) and negative (D) ion mode. The clustering of the technical replicates and the central projection of the pooled sample within the scores plot confirm the reproducibility of the analyses, and the Goodness of Fit test returned a chi-squared of 0.4 and a R-squared value of 0.6.

lipoprotein retention appears to be less important. The interaction of lipoprotein particles with proteoglycans is ionic and involves the interaction of positively charged residues on apoB (especially apoB-100) with the negatively charged glycosaminoglycan (GAG) side-chains of proteoglycans. Additional interactions may exist between the lipid moieties of LDL and the proteoglycan core proteins. *Vice versa*, the binding affinity of LDL relates to its diameter, its apoC-III content as well as the lipid composition of the surface (phospholipids) and the core (cholesteryl esters, triglycerides) [31]. The concentration of cholesteryl esters in particular is a key determinant. Cholesteryl ester enrichment of LDL increases its affinity to GAGs and LDLR, probably mediated by a conformational change of the apoB-100 molecules [32]. Triglyceride enrichment has the opposite effect [33]. Moreover, lipids, glycolipids and lipoproteins bind to toll-like receptors (TLRs) and can initiate intracellular signalling. Recent studies have explored the role of TLRs in atherosclerosis, which act as proatherogenic (TLR2 and 4) [34] and as protective (TLR3) [35] modulators of vascular inflammation. Seimon et al. showed that proatherogenic oxidized phospholipids, oxidized LDL and saturated fatty acids could initiate apoptosis in macrophages through a mechanism requiring both the scavenger receptor and the TLR2 [36]. Moreover, Sun et al. showed that loading of macrophages with free-cholesterol results in the activation of TLRs and the induction of MMPs and cathepsin K [37]. Apart from the well-characterized function of lipid classes in plaque formation, surprisingly little is known about the role of individual lipid species in atherosclerosis. In most studies, atherosclerotic lesions are just visualized by oil-red O staining. While this fat-soluble dye is a reliable read-out of the total lipid-burden in the vasculature, it does not provide detailed information on the lipid composition. Arguably, quantifying the "redness" of an artery or tissue section should not be considered state-of-the-art for assessing the lipid content of atherosclerotic plaques. Mass spectrometry allows a far more comprehensive lipid analysis with the promise of identifying novel biomarkers for atherosclerosis and plaque vulnerability [38]. In analogy to genome or proteome, the "lipidome" comprises the entire spectrum of lipid species in a biological system. "Lipidomics" aims to characterize these lipid species and clarify their biological functions [39]. Thus far, very few studies have applied lipidomics to atherosclerosis. For instance, Hiukka et al. used mass spectrometry to measure the lipid composition of LDL particles isolated from hyperlipidemic diabetic patients and apoB transgenic mice [40]. Other studies targeted individual lipid classes in atherosclerotic plaques [41–44] but no comparisons were performed across different classes. One recent proof-of-concept study identified 26 lipid species from a single human atherosclerotic plaque using desorption electrospray ionization mass spectrometry [45]. To provide a more detailed investigation of the lipid composition in atherosclerotic lesions and search for characteristic lipid signatures of plaque vulnerability, we used shotgun lipidomics [46]. Liquid extraction surface analysis (LESA) from tissue sections was complemented by lipid extraction with chloroform/methanol to compare radial

arteries, endarterectomy samples from symptomatic and asymptomatic patients, and stable versus unstable areas within the same symptomatic lesion [47]. Our analysis resulted in the detection of 150 lipid species from 9 different classes of which 24 were detected in carotid plaques only. Triacylglycerols (TAGs) were detected in both control and plaque samples. In comparison to healthy arteries, carotid plaques showed an increase in the relative amount of cholesteryl esters with linoleic acid at the expense of other polyunsaturated fatty acids like arachidonic acid and eicosapentaenoic acid (Fig. 2). These changes in the relative amount of lipid species could be an indicator for altered substrate availability for inflammatory mediators and mediators of resolution (lipoxins, resolvins, and protectins) [48]. Certain sphingomyelin species were also markedly enriched in atherosclerotic lesions. Importantly, the combination of lipid species across different classes provided a better separation of stable and unstable areas in principal component analysis than species from individual lipid classes, demonstrating the diagnostic potential of this global lipidomics approach [49]. Similarly, a very recent study demonstrated the ability of plasma lipidomics profiling to distinguish patients with stable and unstable coronary artery disease [50].

4. Challenges in mass spectrometry

Mass spectrometry is an evolving technology and the technological advances facilitate the detection and quantification of scarce proteins. Nonetheless, the enrichment of specific subproteomes using differential solubility [27,51] or isolation of cellular organelles [52] will remain important to increase coverage and, at least partially, overcome the inhomogeneity of diseased tissue, one of the major factors affecting sample-to-sample variation. Proteomics is also the method of choice for the identification of post-translational modifications [53], which play an essential role in protein function, i.e. enzymatic activation, binding ability and formation of ECM structures. Again, efficient enrichment is essential to increase the likelihood of identifying modified peptides in complex mixtures. Lipidomics faces similar challenges. While the extraction of lipids is more selective [54,55], new enrichment methods are needed for scarce lipids as well as labile lipid metabolites, that may have important bioactivity [56]. Another pressing issue in lipidomics is data analysis, in particular the lack of automated search engines that can analyze mass spectra obtained from instruments of different vendors. Efforts to overcome this issue are currently underway [57].

5. Conclusions

Proteomics and lipidomics offer an unbiased platform for the investigation of ECM and lipids within atherosclerosis. In combination, these innovative technologies will reveal key differences in proteolytic processes responsible for plaque rupture and advance our understanding of ECM – lipoprotein interactions in atherosclerosis.

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