Proteomic analysis of differential protein expression in human atherosclerotic plaque progression

Marjo MPC Donners,1 Monique J Verluyten,1 Freek G Bouwman,2 Edwin CM Mariman,2 Bart Devreese,3 Frank Vanrobaeys,1 Jozef van Beeumen,3 Luc HJM van den Akker,4 Mat JAP Daemen1 and Sylvia Heeneman1*

1Department of Pathology, Cardiovascular Research Institute Maastricht (CARIM), University of Maastricht, The Netherlands
2Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), University of Maastricht, The Netherlands
3Laboratory of Protein Biochemistry and Protein Engineering, Ghent University, Belgium
4Department of Surgery, Maasland Hospital, Sittard, The Netherlands

*Correspondence to:
Sylvia Heeneman, Department of Pathology, Cardiovascular Research Institute Maastricht (CARIM), University of Maastricht, PO Box 616, 6200 MD Maastricht, The Netherlands. E-mail: sheen@lpat.azm.nl

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Abstract

In this study, differential protein expression was assessed during human atherosclerotic plaque progression. A multifaceted approach was used in which differential protein expression was studied by two-dimensional (2D) gel electrophoresis and validated in individual patients using western blotting and immunohistochemistry. 2D profiles of whole-mount advanced stable lesions were compared to those of plaques containing a thrombus. Mass spectrometry analysis identified vinexin-β and α1-antitrypsin (AAT) in the same spot that was differentially expressed in plaques with a thrombus. Immunohistochemistry and western blotting showed limited expression of both vinexin-β and AAT in early lesions, whereas high expression of both proteins was found in advanced lesions. Differential expression of vinexin-β in lesions with a thrombus compared to stable plaques could not be confirmed, indicating the importance of validation of proteomic analysis. For AAT, western blotting of 2D gels revealed expression of six isoforms in advanced plaques, one of which was confirmed to be solely expressed in thrombus-containing plaques. In conclusion, vinexin-β is expressed in advanced human atherosclerotic plaques, but differential expression of this protein in lesions with a thrombus versus stable plaques could not be confirmed. However, this analysis revealed expression of six isoforms of AAT in advanced plaques, one of which was uniquely expressed in thrombus-containing plaques.

Keywords: atherosclerosis; proteomics; 2D gel electrophoresis; human; vinexin-β; α1-antitrypsin

Introduction

Rupture of an atherosclerotic plaque is the predominant cause of acute coronary syndromes and peripheral vascular disease [1]. The underlying molecular mechanisms of plaque rupture have been extensively studied, but are still not fully known. Gene expression studies have identified many genes to be either upregulated or downregulated in human atherosclerosis [2,3]. However, protein expression patterns do not always reflect differential gene expression patterns [4]. Furthermore, protein functions can also be influenced by post-translational modifications [5].

Two-dimensional (2D) gel electrophoresis is a powerful tool to describe changes in protein expression and modification, and has already been used to study other disease mechanisms [6]. Recently, You et al reported a proteomics study to identify proteins associated with the development of coronary artery disease [7]. In the present study, 2D gel electrophoresis was used to examine the differences between advanced, but stable, human atherosclerotic plaques and plaques containing a thrombus. Expression of six isoforms of α1-antitrypsin (AAT) was revealed in advanced plaques, one of which was shown to be unique to plaques containing a thrombus.

Methods

Tissue harvesting and sample preparation

Human carotid plaque specimens were obtained from patients undergoing endarterectomy (Department of Vascular Surgery, Maasland Hospital, Sittard) according to the guidelines of the Medical Ethics Committee of the Academic Hospital and University of Maastricht. The plaques were divided immediately on ice into 3 mm thick parallel slices, which were alternately either fixed in 10% phosphate-buffered formalin (pH 7.4) for histological analysis or snap-frozen in liquid nitrogen for protein isolation. Protein lysates were isolated after crushing the tissue in liquid nitrogen with a mortar, and then were
extracted with 40 mM Tris–lysis buffer containing 1 mmol/l sodium orthovanadate; 1 mmol/l diithiothreitol; 10 mmol/l soy trypsin inhibitor; 2 mmol/l benzamidine hydrochloride; 10 mmol/l leupeptin; 10 mol/l DNase and 10 mol/l RNase.

Plaques were classified according to Virmani et al [8]. Stable lesions were characterized by an intact fibrous cap containing either a well-formed necrotic core, calcification or fibrous tissue. Lesions containing a thrombus included ruptured plaques or those with intraplaque haemorrhage.

Two-dimensional gel electrophoresis
Protein samples were separated first by their isoelectric point by means of isoelectric focusing. 300 µg of protein was loaded per 24 cm Immobiline pH gradient strip with a linear pH range of 4–7 by in-gel rehydration under low voltage (30 V) overnight at 20°C on IPG-Phor (Amersham Biosciences). The voltage was then raised step-wise and proteins were focused for 15 h at 8000 V. After focusing, proteins were separated by their molecular weight (MW) on 12% acrylamide gels (using an Etten-DALT). Subsequently, gels were stained using Sypro® Ruby Protein gel stain (Bio-Rad) and image analysis was performed using PDQuest 2D gel analysis (Bio-Rad). Differentially expressed spots were excised from the gel using a spot cutter (Bio-Rad). Excised spots were then processed on a MassPREP digestion robot (Waters) according to the manufacturer’s protocol. Proteins were digested using porcine sequencing-grade modified trypsin (6 ng/µl, Promega).

Mass spectrometry
1.5 µl of each peptide mixture was mixed with matrix and spotted on a target plate of a MALDI linear reflectron mass spectrometer (Waters). Acquisition mass range was 900–3000 Da. The instrument was calibrated on six to eight reference masses from a tryptic digest of alcohol dehydrogenase. In addition, a maximum mass accuracy. Proteins were further identified by MALDI-TOF/TOF (4700 proteomics analyser, Applied Biosystems) and nano liquid chromatography (LC)-MS/MS on a Q-Trap LC-MS/MS analyser (Applied Biosystems).

For MALDI-TOF/TOF, 1 µl of tryptic digest was mixed with matrix and spotted on the target. For nano LC-MS/MS, peptide mixtures were separated on an Ultimate nano LC system (LC-Packings Dionex) as described by Devreese et al [9]. Database searches were established using Mascot (www.matrixscience.com) and the NCBI protein database.

Validation of differential spots
Western blotting Protein lysates were either separated on one-dimensional (1D) 12% SDS-PAGE gels or by 2D gel electrophoresis as described above. After transfer to nitrocellulose, membranes were blotted with a polyclonal goat-anti-AAT or goat-anti-vinexin-β (Santa Cruz, USA) and scanned. Densitometric analysis was performed using Quantity One software (Bio-Rad).

Immunohistochemistry Immunohistochemistry was performed according to regular protocols using the same antibodies.

Statistical analysis
Data are expressed as mean ± SEM. Probability values of <0.05 (Mann–Whitney U-test) were considered significant.

Results
Differential protein expression in human atherosclerosis
Extracts from five individual stable plaques and six lesions with a thrombus (all male patients 49–78 years of age) were used in triplicate. In the pH range 4–7 approximately 800 spots could be detected (Figure 1A). The central area, containing 300–350 spots, was used for image analysis. First, the three replicate gels from each lesion were compared to create a reference gel, containing all spots present in at least two of the three replicate gels. Subsequently, reference gels of patients with the same lesion types were compared to create a master reference gel for stable plaques and one for thrombus-containing lesions. These master reference gels contained all the spots that were present in at least three of the five stable lesions or in at least four of the six thrombus-containing plaques. Finally, the master reference gel of stable lesions was compared to the master reference gel of thrombus-containing lesions. This revealed 71 unique spots for stable plaques and 29 unique spots for thrombus-containing plaques. Figure 1(B and C) shows the master reference gels of the stable and thrombus-containing lesions.

Protein identification
Vinexin-β and α1-antitrypsin (AAT) were identified in the same spot that was unique to thrombus-containing plaques (Figure 2). Another differentially expressed spot, unique to stable plaques, contained four different proteins, identified as angiotensin I, lactoferrin, lipocalin-1 and a proline-rich lacrimal protein (Table 1). Other differentially expressed spots yielded good mass spectra, but could not be unequivocally identified.

Expression of vinexin-β in human atherosclerotic plaques
Differential expression of vinexin-β in advanced stable atherosclerotic versus thrombus-containing lesions
Proteomic analysis of human atherosclerosis

Figure 1. Illustration of a 2D gel from human thrombus-containing plaques stained with Sypro Ruby (A). The box indicates the analysed area. Panels B and C show reference gels of advanced, stable plaques versus thrombus-containing lesions, respectively. Arrows indicate some spots unique for thrombus-containing lesions; circles indicate some spots only expressed in stable plaques. The upper left arrow indicates the spot identified by MS to contain both vinexin-β and AAT

Table 1. Identification of 2D spots by MALDI-TOF MS and MS/MS. Molecular weight (MW) and isoelectric point (pI) are derived from the 2D gels. Spots depicted as unknown are either proteins that could not be unequivocally identified or are just not known in the databases

<table>
<thead>
<tr>
<th>Mw</th>
<th>pI</th>
<th>Staining</th>
<th>Expression</th>
<th>MS identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>~50 kDa</td>
<td>~4.4</td>
<td>Sypro Ruby</td>
<td>Lesions with a thrombus</td>
<td>AAT</td>
</tr>
<tr>
<td>~50 kDa</td>
<td>~5.5</td>
<td>Sypro Ruby</td>
<td>Advanced stable</td>
<td>Vinexin-β</td>
</tr>
<tr>
<td>~33 kDa</td>
<td>~5.4</td>
<td>Sypro Ruby</td>
<td>Lesions with a thrombus</td>
<td>Angiotensin I</td>
</tr>
<tr>
<td>~28 kDa</td>
<td>~5.5</td>
<td>Sypro Ruby</td>
<td>Advanced stable</td>
<td>Lactoferrin</td>
</tr>
<tr>
<td>~25 kDa</td>
<td>~5.7</td>
<td>Sypro Ruby</td>
<td>Lesions with a thrombus</td>
<td>Lipocalin-I</td>
</tr>
<tr>
<td>~19 kDa</td>
<td>~4.8</td>
<td>Sypro Ruby</td>
<td>Advanced stable</td>
<td>Proline-rich lacrimal protein</td>
</tr>
<tr>
<td>~15 kDa</td>
<td>~5.9</td>
<td>Sypro Ruby</td>
<td>Lesions with a thrombus</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

could not be confirmed by western blotting (Figure 3A, B). Immunohistochemistry of human atherosclerotic plaques showed minimal expression of vinexin-β in early lesions (Figure 3C). In advanced stable and thrombus-containing lesions, vinexin-β was expressed by macrophages, smooth muscle cells (SMC) and endothelium, although no differences were found between these lesion types (Figure 3D, E).

Expression of AAT in human atherosclerotic plaques
Western blotting of a 2D gel confirmed the presence of AAT in the specific spot and showed expression of six isoforms of AAT in human atherosclerotic plaques (Figure 4A). Western blotting of 1D SDS-PAGE gels of advanced stable versus thrombus-containing lesions showed increased expression of the lower isoforms of AAT (relative to isoforms of higher molecular weight) in thrombus-containing plaques compared to stable lesions, although not significantly (p = 0.068, Figure 4B, C). The antibody used cannot discriminate between the several isoforms. However, Western blotting of 2D gels of individual human atherosclerotic plaques confirmed differential expression of one specific isoform of AAT (Figure 4A).

Immunohistochemistry was performed to examine expression of AAT (all isoforms) further in human atherosclerotic plaques. AAT protein expression was nearly absent in the early stages of atherosclerosis and was highly expressed in advanced stable and thrombus-containing plaques (Figure 4D, E, F). AAT was found to be expressed by several (but not all) macrophages, endothelial cells (Figure 4G, H) and in neutrophils (Figure 4I).

Discussion
In this study, we used 2D gel electrophoresis to explore protein expression profiles of whole-mount human atherosclerotic tissue during the progression from an advanced stable to a ruptured lesion. We have chosen to study differential protein expression instead of gene expression, since mRNA expression does not always reflect protein expression levels. In yeast, it was shown that protein levels varied by more than 20-fold, when mRNA levels were invariant, and vice versa [4]. We identified spots that were (differentially) expressed in human atherosclerotic plaques (Table 1). Of note, we found two proteins in the same differential
Figure 2. Enlargements of Sypro Ruby-stained 2D gels showing differential expression of a 2D spot (indicated by arrows) upregulated in thrombus-containing human atherosclerotic plaques (A) compared to stable lesions (B). Panels C and D show mass spectra and MS/MS spectra, respectively, of this differentially expressed spot. Using the peptide/ion masses in the spectra and the NCBI database, this spot was identified to contain both vinexin-β (C) and AAT (D).

spot unique for thrombus-containing plaques. Another spot, unique for advanced stable plaques, contained four different proteins. This indicates co-migration of proteins of approximately the same molecular weight and pI on the 2D gel, a phenomenon also described in the literature [10]. This co-migration of proteins in 2D gels emphasizes the importance of validation of 2D gel electrophoresis results by additional methods.

This is the first study that identifies vinexin-β as a protein expressed in advanced atherosclerotic plaques, albeit at equal levels in stable and thrombus-containing plaques. Vinexin is a vinculin-binding protein present in focal adhesions [11]. We found vinexin-β expression in advanced (but not early) human atherosclerotic lesions, especially in macrophages, SMC and endothelium. However, no differences in vinexin-β expression were found between stable and thrombus-containing lesions using western blotting and immunohistochemistry. Validation of differential protein expression in thrombus-containing compared to stable lesions therefore revealed differential expression of vinexin-β to
Proteomic analysis of human atherosclerosis

Figure 3. Vinexin-β protein expression. Panels A and B: 1D western blot (A) and densitometric analysis (B) of advanced stable plaques and thrombus-containing lesions. Panels C, D, and E: immunohistochemical staining of an early plaque (C), an advanced stable plaque (D), and a thrombus-containing lesion (E). L indicates the lumen of the carotid artery, T marks thrombus.

be a false-positive result, due to overlap with another protein in the same spot.

This protein was identified as α₁-antitrypsin (AAT), which is an acute-phase protein expressed by the liver during inflammation. AAT is a serine protease inhibitor (serpin) and the endogenous inhibitor of neutrophil elastase. Stastny et al also showed AAT expression in human fibro-fatty lesions [12]. Both neutrophil elastase and AAT have been shown to be expressed by macrophages and neutrophils [13] (Figure 4).

The presence of AAT in atherosclerotic plaques was suggested to enhance fibrosis by its inhibitory effects on collagenases and elastases [14]. Upregulation of AAT in thrombus-containing lesions could therefore act as a counter-regulatory mechanism. On the other hand, the anti-elastase activity of AAT could provide a mechanism by which AAT may protect against atherosclerosis, possibly by prohibiting invasion of inflammatory cells. Western blotting of 2D gels showed six isoforms of AAT, and only one of these isoforms was upregulated in thrombus-containing plaques. It is known that AAT can be modified, giving rise to several non-inhibitory forms of AAT [15]. Lack of anti-elastase activity of AAT may promote invasion and subsequently accumulation of inflammatory cells, rendering the plaque more vulnerable to rupture. Indeed, cleavage of AAT yields a carboxyl terminal fragment, which has been described in human atherosclerotic plaques and which can regulate inflammatory transcription factors and lipid accumulation in monocytes [16]. Furthermore, AAT is known to be a glycosylated protein and this glycosylation can affect the stability, secretion and perhaps inhibitory function of AAT. Variations in AAT glycosylation have indeed been associated with (inflammatory) diseases such as arthritis and Crohn’s disease [17,18]. Using a fluorescent glycoprotein detection kit (GlycoProfile III, Sigma-Aldrich, USA) we confirmed that the six AAT isoforms expressed in advanced human atherosclerotic plaques were indeed glycosylated forms (data not shown). However, the exact type of glycosylation variant of AAT in thrombus-containing lesions and its implications in human atherosclerotic plaque rupture remain to be determined.

In conclusion, we have used a multifaceted approach to identify and validate differentially expressed proteins in human atherosclerotic plaque progression. Vinexin-β was shown to be a false-positive result, probably because of co-migration of proteins. One of the proteins that proved to be differentially expressed in human atherosclerotic plaque progression was α₁-antitrypsin, which was validated by western blotting and immunohistochemistry. We showed expression of six isoforms in advanced atherosclerotic plaques, one of which was unique to lesions with a thrombus.

Acknowledgements

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Figure 4. AAT protein expression. Panel A: 2D western blotting of advanced stable plaques versus thrombus-containing lesions showing several isoforms of AAT. The arrows indicate the spot that is differentially expressed in atherosclerotic thrombus-containing plaques. Panel B shows a 1D blot of five thrombus-containing lesions versus five stable plaques. Panels D, E, and F: immunohistochemistry of AAT in an early plaque (D), an advanced stable plaque (E), and a thrombus-containing lesion (F). Panels G, H and I: AAT expression in endothelial cells, macrophages and neutrophils respectively, marked by arrows. L and T as in Figure 4.

References


