

Chapter 10

Discovery and Evaluation of Biomarkers for Atherosclerosis

Differential Proteomics of Plasma and Tissues

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Abstract The usage of biomarkers reflecting atherosclerosis progression is important for preventing serious incidence of cardiovascular events. To elucidate clinically relevant molecular determinants in atherosclerosis, we have taken a comprehensive approach to combine mass spectrometry-based differential proteomics using both clinical and animal model specimens. Clinical plasma samples were collected from patients with acute myocardial infarction (AMI), stable angina (SA), and healthy/low-risk individuals (H-LR). We also obtained plasma and arterial tissue samples from apolipoprotein E-deficient and wild-type mice at various pathognomonic points of age. Cleavable isotope-coded affinity tags were

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used for differential mass spectrometry. Differential proteomics of clinical plasma samples revealed that more than 10 proteins appeared to be upregulated (relative abundance AMI/H-LR or SA/H-LR >1.5) and 5 proteins downregulated (AMI/H-LR or SA/H-LR $<1/1.5$). These trends associated with the disease progression are not always coincident with those of mouse ortholog proteins, suggesting a pathophysiological difference between humans and the mouse model. Among the downregulated proteins, the complement factor D (CFD) showed monotonic decrease that was in good agreement with the enzyme-linked immunosorbent assay. These results suggest that the comprehensive and systematic proteomic approach may be promising in terms of the selection and evaluation of biomarker candidates.

Keywords Proteomic study • Atherosclerosis • Inflammation • apoE-deficient mouse

10.1 Introduction

Atherosclerosis is a chronic inflammatory disease and often causes subsequent thrombotic complication. It is well known as a main etiology of critical diseases such as acute coronary syndrome including myocardial infarction, sudden cardiac death, and stroke [1]. To detect the signs of severe incidences, it is important to accurately evaluate the progression stages of atherosclerosis, vulnerability, and rupture risk of atherosclerotic plaques. Therefore, a variety of approaches to establish novel biomarkers directly reflecting the status of atherosclerosis have been conducted to date [2]. However, due to the complexity of the disease, discovery and clinical validation of such biomarkers are still underway in many institutions.

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Disease proteomics is a profiling technology using mass spectrometry that makes it possible to identify disease-related alteration of protein expression comprehensively in a wide variety of biological specimens including serum or plasma or extracts from tissues of interests [3]. Numerous studies have been conducted to develop biomarkers for early diagnosis, selection of appropriate therapy, and prognosis assessment. A decade of progress to determine changes in disease-related protein expression has achieved accurate and high-throughput quantification of the individual proteins within mixtures by using differential stable isotopic labeling, such as cleavable isotope-coded affinity tags (cICATs) [4]. Either light or heavy cICATs are labeled at the free thiol groups of cysteine residues of which proteins are isolated from two distinct samples (i.e., disease and healthy states). The ratio of protein amount between these states is estimated by comparing MS signal intensity of the corresponding proteolytic peptide fragments [4].

To elucidate clinically relevant molecular determinants in atherosclerotic development, we have taken a comprehensive approach by using mass spectrometry-based differential proteomics. First, we conducted differential human plasma proteomic studies using clinical samples of patients with atherosclerosis-related cardiovascular disease and healthy (or low-risk) volunteers. Second, to validate the results we obtained in the studies, a biomarker candidate, the complement factor D (CFD) level of patients with atherosclerosis-related cardiovascular disease, was determined by carrying out an enzyme-linked immunosorbent assay (ELISA). Then, we tried combining differential proteome of plasma and arterial tissue obtained from both wild-type (WT) and apoED mice at four pathognomonic time points of age. Finally, we compared identified proteins in humans with mouse plasma proteome.

10.2 Materials and Methods

This study was approved by the Ethics Committee of Hokkaido University Hospital (no. 010–0118) and the institutional ethics committees of Hitachi, Ltd. Clinical plasma samples were collected prospectively for 48 acute myocardial infarction (AMI) patients, 73 stable angina (SA) patients, and 69 healthy/low-risk (H-LR) individuals.

Animal care and animal experiments were conducted at Japan SLC, Inc. (Hamamatsu, Japan), under the approval of the company's animal care committee. The apoED (C57BL/6.KOR/StmSlc-Apoeshl) and WT (C57BL/6) mice were fed a high-fat diet from 8 weeks of age. Plasma and arterial tissues were collected from each group of male or female mice at ages of 12, 18, 25, and 35 weeks.

Proteins prepared from plasma or arterial tissue as described above were labeled with isotope-coded affinity tags using the Cleavable ICAT® Reagent Kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions with minor modifications. Tryptic peptides were applied to the strong cation exchange (SCX) column, and the eluted solution was fractionated into 25 fractions. The SCX separated fractions were analyzed using NanoFrontier LD, a liquid chromatograph

mass spectrometry (LC-MS) system (Hitachi High-Technologies Corporation, Tokyo, Japan). Original MS/MS peak lists (PLs) were generated using built-in PL generation software in the mass spectrometer, and further protein/peptide identification and iCAT quantitative analyses were conducted using a custom developed software platform with a relational database.

Plasma CFD levels were measured with the use of ELISA kits according to the manufacturer's instructions (Quantikine, DFD00, R&D Systems, Inc., Minneapolis, USA). Plasma levels of known markers including high-sensitive C-reactive protein (hsCRP), amino-terminal pro-brain natriuretic peptide (NT-pro BNP), and adiponectin were also determined (SRL, Inc., Tokyo, Japan).

10.3 Results and Discussion

Atherosclerosis is a chronic inflammatory disease, in which atherosclerotic lesions are formed in blood vessels through complicated molecular processes occurring between the circulating blood stream and lesions. To identify proteins showing changes in expression levels associated with atherosclerotic plaque progression, we

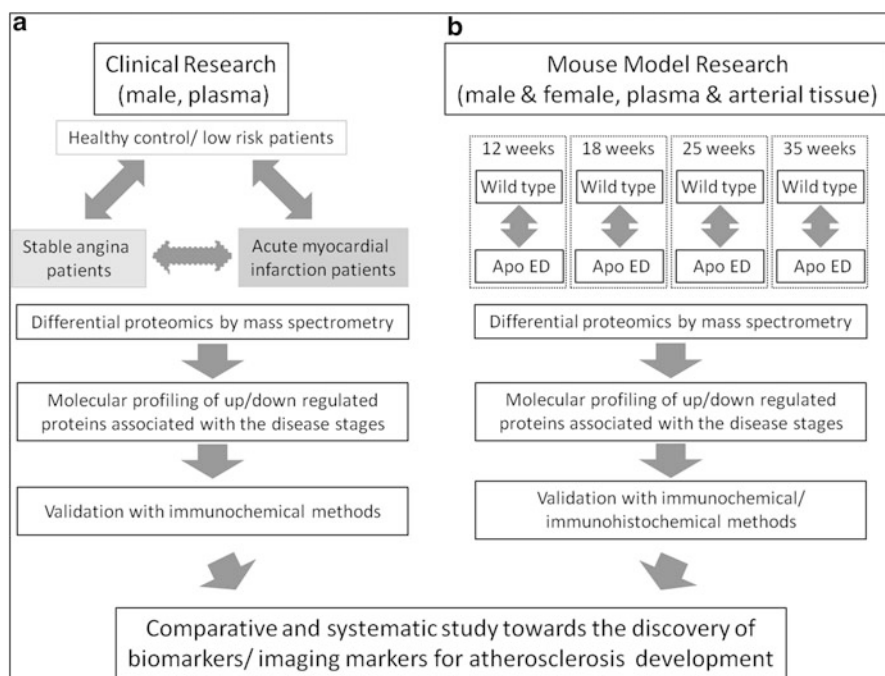


Fig. 10.1 Entire research workflow of stage-dependent, mass spectrometry-based differential proteomics. **(a)** Clinical proteomic study using human plasma samples. **(b)** Animal model proteomic study using plasma and arterial tissue samples from WT and atherosclerotic model mice

conducted mass spectrometry-based proteomic analyses. Figure 10.1 shows an overall picture of the proteomic experiments we have done. Most previous proteomic studies have focused on only one point of the disease. However, these strategies would not be adequate to identify proteins showing serial quantitative change in accordance with progression of atherosclerosis. In this study, we conducted a differential proteome study at several characteristic points of disease stage and compared the results of identified proteins. The proteins we identified that showed changes in plasma level according to the severity of the atherosclerosis-related disease (e.g., ischemic heart disease) or atherosclerosis progression of the mouse model might be diagnostic or predictive biomarker candidates for blood testing. On the other hand, the proteins that showed changes, especially an increase tendency, in arterial tissues might be biomarkers for diagnostic imaging.

10.3.1 Disease Stage-Dependent Differential Proteome in Human Plasma

As shown in Fig. 10.1, we conducted disease stage-dependent differential human plasma proteomic experiments using clinical samples. We selected three clinical conditions, H-LR, SA, and AMI (Table 10.1). Two independent age-matched plasma pools from each group ($n = 7$) were used for the experiments with combinations of H-LR vs. SA, H-LR vs. AMI, and SA vs. AMI. Table 10.2 shows the summary of identified proteins by differential proteomics of clinical plasma samples. We identified about 110 proteins in each trial of differential proteome and found that 8–13 proteins appeared to be upregulated (relative abundance AMI/H-LR or SA/H-LR > 1.5), and 2–8 proteins were downregulated (AMI/H-LR or SA/H-LR $< 1/1.5$). Finally, five identical proteins were identified in common between trials 1 and 2. Three were blood coagulation related, such as von Willebrand factor, and the other two were categorized as structural-integrity-related or immunoglobulin-related proteins. The downregulated proteins identified in common between trials were found to be apolipoproteins involving cholesterol homeostasis, blood coagulation, and CFD.

To validate the results we obtained in the differential proteome experiments as described above, we determined the CFD level of plasma of clinical samples by ELISA as a representative example. We found that CFD levels in plasma of the AMI group were the lowest among the three clinical groups. The statistical

Table 10.1 Description of three clinical groups in human plasma proteome

Description	H-LR	SA	AMI
Group	Healthy and low-risk volunteers	Patients with stable angina	Patients with acute myocardial infarction
Risk and progression stage of atherosclerosis	Low	Moderate/early	High/advanced

Table 10.2 Up- or downregulated proteins detected by differential proteomic analysis in clinical pooled plasma samples

Group		SA/H-LR	AMI/H-LR	AMI/SA
Total number of identified proteins	Trial 1	116	115	110
	Trial 2	106	112	114
Upregulated	Trial 1	8	8	5
	Trial 2	7	8	13
	Common	1	5	2
Downregulated	Trial 1	4	6	8
	Trial 2	7	2	5
	Common	2	1	1

H-LR group of healthy control and low-risk volunteers, *SA* group of patients with stable angina, *AMI* group of patients with acute myocardial infarction, *Upregulated* proteins showed expression ratio >1.5 , *Downregulated* proteins showed expression ratio $<1/1.5$

difference of AMI to H-LR was the most pronounced (H/LR vs. AMI, $p = 2.4 \times 10^{-10}$; H/LR vs. SA, $p = 0.54$). The diminished plasma levels of CFD in AMI patients showed that the results we obtained in our proteomic study were basically verified. Furthermore, the plasma CFD levels showed no correlation with body mass index (BMI) ($r = 0.03$, $P < 0.05$), hsCRP ($r = -0.09$, $P < 0.05$), NT-proBNP ($r = -0.03$, $P < 0.05$), and adiponectin ($r = 0.11$, $P < 0.05$). In recent years, it has been suggested that the activation of the complement system plays a role in atherosclerosis progression [5]. Among the components of the complement pathway, CFD is known as a key regulatory serine protease of the alternative complement pathway. CFD mRNA and protein are abundantly expressed in adipose tissue, and the protein is found at high levels in serum [6]. CFD expression levels in rodent models of obesity are reduced [7]. Our results may suggest some kind of CFD implication in atherosclerosis.

10.3.2 Disease Stage-Dependent Differential Proteome in Atherosclerosis Mouse Model

As shown in Fig. 10.1, we conducted differential proteome experiments using plasma and arterial tissues of WT and apolipoprotein E-deficient (apoED) mice. Table 10.3 shows phenotypic characteristics of atherosclerotic lesions of WT and apoED at each point of age. As we previously demonstrated, on a high-fat diet, early lesions were observed in apoED at 12–18 weeks of age, and advanced lesions were prominent at 25–35 weeks. No atherosclerotic lesions were found in WT mice throughout four points of age [8]. At 25 weeks of age, vulnerable atheromatous lesions were more abundant, but fibroatheromatous lesions were less at 25 weeks than those at 35 weeks [8]. Table 10.4 shows a summary of identified proteins. In plasma, the total number of identified proteins and the ratio of their upregulated

Table 10.3 Description of possible progression stages of atherosclerotic lesion of atherosclerosis mouse model

Age (weeks)		12	18	25	35
Risk and progression stage of atherosclerosis	WT	None	None	None	None
	apoED	Low/early	Low/early	High/advanced	Moderate/advanced
Lesion phenotype	WT	None	None	None	None
	apoED	Monocyte adhesion	Foam cell formation	Vulnerable plaque	Stable plaque

Table 10.4 Summary of up- or downregulated proteins detected in plasma and arterial tissues by differential proteome of mouse model

Age (weeks)	Plasma				Arterial tissue			
	12	18	25	35	12	18	25	35
Total number of identified proteins	146	133	119	124	446	616	659	693
Upregulated (%)	7	14	15	11	8	6	16	16
Downregulated (%)	5	5	11	15	2	5	8	9
No change (%)	88	81	74	74	90	89	76	75

Upregulated expression ratio (apoED/WT) > 1.5, *Downregulated* expression ratio (apoED/WT) < 1/1.5, *No change* 1/1.5 < expression ratio (apoED/WT) < 1.5. This table is modified from reference [9]

proteins (expression ratio, apoED/WT; > 1.5) did not significantly change throughout the time course (Table 10.4). However, the ratio of downregulated proteins (apoED/WT; < 1/1.5) increased on and after 25 weeks of age (Table 10.4). We finally found 100 proteins in the plasma, and 390 proteins in the arterial tissues were detected throughout all four time points: 29 were identified in common between plasma and arterial tissues [data not shown, 9]. Interestingly, we found that disease stage-dependent quantitative variation patterns did not always correspond between plasma and arterial tissues. Furthermore, proteins showed characteristic change in abundance in plasma, and/or arterial tissues were found to be components of inflammation, thrombus formation, and vascular remodeling [9].

10.3.3 Comparison Between Human and Mouse Plasma Proteome

In this study, we conducted disease stage-dependent differential proteome experiments in two identical studies: one for human plasma proteome using clinical samples and one for plasma/arterial tissue proteome using an atherosclerotic mouse model, apoED. Importantly, we found that the changes associated with the disease progression in the amount of identified proteins were not always coincident

with those of mouse ortholog proteins in plasma or arterial tissues determined by proteomic studies (data not shown). In case of CFD, the plasma level of CFD in humans determined in the proteomic study or ELISA decreased in accordance with the severity of the clinical conditions. On the other hand, the plasma level of CFD in mice showed a slight but not significant decreasing tendency determined in differential proteome between WT mice and the apoED mouse model. We preliminarily determined the plasma level of CFD in mice by ELISA and detected no statistical significance between WT mice and apoED throughout all four time points. CD5L, a soluble member of the scavenger receptor cysteine-rich domain superfamily protein, did not show any significant change in abundance as determined by human plasma differential analyses. However, the expression ratios (apoED/WT) of CD5L in mouse plasma were high throughout the four time points [9]. In addition, we found that CD5L accumulated in advanced lesions of apoED in accordance with macrophage infiltration by immunohistochemistry. These results suggest that fundamental mechanisms of atherosclerosis development are similar to each other; however, there would be a pathophysiological difference between humans and the mouse model.

10.3.4 Limitation

We failed to detect several known atherosclerotic plaque-related proteins such as cytokines, monocyte chemoattractant protein-1 (MCP-1), and intercellular adhesion molecule-1(ICAM-1). We assume that there were several technical reasons for this. For example, the practical detection limit in our mass spectrometry system was assumed to be 1–10 ng/mL, which is much higher than the normal concentration of cytokines. We used cICAT labeling reagents, which covalently bind only to cysteine residues of targeted proteins. Therefore, it becomes difficult to detect proteins with trypsin-digested peptides with few or no cysteine residues.

10.4 Conclusion

To identify proteins showing changes in expression levels associated with atherosclerotic plaque progression, we conducted mass spectrometry-based proteomic analyses. We adopted two separate study designs: one for human plasma differential proteome using clinical samples and one for mouse plasma/arterial tissue differential proteome using samples obtained from wild-type mice and an atherosclerotic mouse model apoED. Then several proteins showing quantitative changes in accordance with disease were found, including the complement factor D (CFD). The diminished plasma levels of CFD in acute myocardial infarction patients were verified in an enzyme-linked immunosorbent assay. These results suggest that CFD might be a potential biomarker for atherosclerosis. The comprehensive and

systematic proteomic approach using different states of samples is promising in terms of the selection of biomarker candidates.

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