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The Platelet Proteome

Lisa Senzel, MD, PhD,
Department of Pathology, State University of New York, Stony Brook, University Hospital, Level 3, Rm 532, Stony Brook, NY 11794-7300, Phone: 631-444-2601, Fax: 631-444-2653

Dmitri V. Gnatenko, PhD, and
Department of Medicine, State University of New York, Stony Brook, Division of Hematology, HSC T15/030, Stony Brook, N.Y. 11794-8151, Phone: 631-444-1260, FAX: 631-444-7530

Wadie F. Bahou, MD
Department of Medicine, State University of New York, Stony Brook, Division of Hematology, HSC T15/030, Stony Brook, N.Y. 11794-8151, Phone: 631-444-2059, FAX: 631-444-7530

Lisa Senzel: lsenzel@notes.cc.sunysb.edu; Dmitri V. Gnatenko: dgnatenko@notes.cc.sunysb.edu; Wadie F. Bahou: wbahou@notes.cc.sunysb.edu

Abstract

Purpose of review—The proteome is the pool of proteins expressed at a given time and circumstance. The word “proteomics” summarizes several technologies for visualization, quantitation and identification of these proteins. Recent advances in these techniques are helping to elucidate platelet processes which are relevant to bleeding and clotting disorders, transfusion medicine and regulation of angiogenesis.

Recent findings—Over 1100 platelet proteins have been identified using proteomic techniques. Various subproteomes have been characterized, including platelet releasates (the “secretome”), alpha and dense granules, membrane and cytoskeletal proteins, platelet-derived microparticles, and the platelet “phosphoproteome”. Proteomic data about platelets have become increasingly available in integrated databases.

Summary—Proteomic experiments in resting and activated platelets have identified novel signaling pathways and secreted proteins which may represent therapeutic targets, as well as potential cancer biomarkers.

Keywords

proteomics; platelets; mass spectrometry; releasate

Introduction

The primary function of platelets is to stop hemorrhage after tissue trauma and vascular injury. Platelets act not only through the immediate release of a variety of lipid and protein mediators but also through signal-dependent pre-mRNA splicing and the translation of constitutively expressed mRNA[1**]. These post-transcriptional pathways are potential targets for molecular intervention in atherothrombosis[2]. Proteomic experiments can provide data about localization, interactions, posttranslational modifications, and activation states of gene products.

Contact author: Lisa Senzel.

Proteomic Techniques: A Brief Introduction

Proteomic experiments begin with a protein mixture which is digested to a peptide mixture, either in a gel or in solution. Two-dimensional electrophoresis (2-DE) has been available since the 1970s, and was used in the 1990s to construct early maps of platelet proteins. Newer gel-based methods include differential in-gel electrophoresis (DIGE), which allows for quantitation and comparison of samples from different proteomic states. In DIGE, samples are differentially labeled with fluorescent dyes and simultaneously analyzed in a single gel. Alternatively, protein spots may be excised from a gel, digested, and analyzed by mass spectrometry (MS).

Gel-free methods, which use in-solution proteolytic digestion, can detect proteins not well represented by 2-DE, such as transmembrane and basic proteins. Multidimensional protein identification technology (MudPIT) employs ion-exchange and reverse-phase liquid chromatography (LC) for peptide separation[3]. Mass spectrometry (MS) has the capacity to identify proteins in a high throughput manner, using bioinformatics approaches linked to protein sequence databases. The two main mass-spectrometric options are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Both MALDI and a related method, surface-enhanced laser desorption/ionization (SELDI), employ a matrix to ionize proteins; SELDI uses protein chips with chromatographic surfaces. LC-MS/MS is the method now chosen by most proteomics researchers[4]. Isotope affinity tagging techniques help to increase the sensitivity of detection of smaller peptide fragments and provide quantitative information about protein concentrations. Isotope coded affinity tags (ICAT) selectively label cysteine residues of peptide fragments following tryptic digest of the protein sample; this technique increases the depth of protein coverage but is limited by selective labeling. Isotope tags for relative and absolute quantitation (iTRAQ) label every tryptic fragment, thus allowing for measurement of absolute changes in protein composition[5]. Figure 1 summarizes the various proteomic approaches which have been used to study platelets.

As discussed in the next sections, proteomic data have been published for resting and activated platelets, platelet alpha granules, platelet dense granules, platelet membranes, and platelet-derived microparticles.

The Platelet Releasate: Activation-Dependent Changes

Quiescent platelets display minimal translational activity. Platelet activation leads to the rapid translation of pre-existing mRNA, with the release or derivation of platelet-secreted proteins, cytokines, exosomes, and microparticles. Using thrombin-stimulated platelets, a combination of MALDI-TOF and MudPIT identified more than 300 released proteins[6]. Several of the secreted proteins have been identified in atherosclerotic lesions but are absent in normal vasculature; these include secretogranin III, cyclophilin A, and calumenin. In another study, the use of 2-DE, MALDI-TOF and LC-ESI MS/MS to study platelet dense granules led to the identification of an extracellular role for 14-3-3 zeta protein in atherosclerosis[7*]. Such secreted proteins are potential targets for future drug development, given their extracellular localization, without the risk of bleeding that complicates direct inhibition of platelet activation.

Piersma *et al.*[8] obtained an activated platelet releasate proteome using high resolution, high mass accuracy hybrid ion-trap Fourier transform mass spectrometry. This group combined 1-D gel electrophoresis and LC-MS/MS in a strategy known as GeLC-MS/MS. They identified nearly 400 proteins previously reported in platelet alpha granules and microparticles, along with over 300 novel proteins. Only 5% of the proteins overlapped with previously reported platelet releasate datasets[6;9*]. Proteins smaller than 8 kDa were not detected, due to the limitations of the 10% acrylamide gel and use of a cut-off filter to concentrate the samples.

DellaCorte *et al.*[10] combined 2D-DIGE and MALDI-TOF and identified 36 proteins differentially regulated in the platelet releasate following thrombin activation. They detected proteins ranging between 220 kDa and 15 kDa. Coppinger *et al.*[9] compared platelet releasates induced by ADP, collagen and TRAP stimulation in the presence and absence of aspirin using GeLC-MS/MS. An overall decrease in protein expression was observed in the presence of aspirin, and the secretion profiles were agonist-dependent. This group also employed cytokine antibody arrays to quantitate platelet-derived cytokines in the presence and absence of aspirin.

Greening *et al.*[11] co-identified 41 proteins in both non-activated platelets and plasma, and concluded that analyses of the human plasma proteome must take into account the contribution of platelet-derived proteins. This is because some *ex vivo* platelet activation occurs during blood collection, and platelet-derived microparticles circulate in normal plasma.

Proteome of Platelet Microparticles

Microparticles (MP) are small membrane vesicles that are released from cells upon activation or during apoptosis. The platelet microparticle proteome has been characterized[12], and appears to have a composition distinct from the plasma proteome. This could be significant because while plasma-derived MPs imply cellular activation and possible damage, platelet-derived MPs appear to be present in healthy individuals. Smalley *et al.*[13] compared the proteomic profiles of plasma-derived and platelet-derived MPs using two methods, spectral count analysis and isotope-coded affinity tag (ICAT) labeling of proteins. Proteins present only in the plasma MPs included several associated with apoptosis, iron transport, complement components, and the coagulation process.

Signaling Pathways: Platelet Membrane Proteome and Phosphoproteome

Novel membrane proteins that signal during platelet aggregation have been identified using LC-MS/MS and other chromatographic techniques[14-17]. Some of these proteins become phosphorylated on tyrosine or serine residues on platelet aggregation. Maguire *et al.*[18] profiled platelet proteins associated with detergent-resistant membrane lipid rafts. Lipid rafts may act as concentrating platforms that co-cluster receptors and signaling molecules, thus coordinating platelet activation and secretion. Proteins recruited upon von Willebrand factor activation included platelet GPIbα, glucose transporter 14 and C-terminal LIM protein 36.

Garcia *et al.* identified 41 proteins which were differentially phosphorylated following stimulation by thrombin receptor activating peptide (TRAP)[19]. These included a novel protein, the adapter downstream of tyrosine kinase 2 (Dok-2), which may play a role in thrombus formation[20]. The G6b-B protein, which contains an immunoreceptor tyrosine-based inhibitory motif, may play a role in limiting platelet activation[17]. Zahedi *et al.*[21*] noted the limitations of gel-based proteomic techniques for the detection of hydrophobic, alkaline, very small or large proteins, or proteins with less abundant phosphorylation sites. The investigators adopted a two-pronged chromatographic strategy coupled with nano-LC-MS/MS, and identified 278 phosphorylated proteins in resting platelets. Their finding that GP1bα is phosphorylated on a protein kinase A/protein kinaseG consensus site may be important for the regulation of the GPIb-IX-V signaling pathway.

Platelet Storage Lesion

The platelet storage lesion refers to the *in vitro* changes that occur when donated platelets are stored for several days before transfusion to a recipient. These include loss of disk shape, increased release of alpha-granules and cytosolic proteins, increased procoagulant activity, and altered glycoprotein expression[22*]. To characterize the platelet storage lesion in more detail, investigators used 2-DE[23], differential in-gel electrophoresis (DIGE)[22;24], isotope-coded

affinity tagging (ICAT)[22], and isotope tagging for relative and absolute quantitation (iTRAQ)[22]. As expected, the gel-free methods were better than in-gel methods at detecting low-abundance molecules and hydrophobic proteins. Both beta-actin and septin2 were found to be altered by storage in separate studies, highlighting the importance of cytoskeletal reorganization and apoptosis in the platelet storage lesion. These approaches hold promise for finding biomarkers that might be useful in judging platelet quality[25*].

Platelet Proteome and Disease

Recently, proteomic techniques identified a diagnosis of Quebec Platelet Disorder in a family with severe bleeding problems of unknown origin[26**]. While clinical features were not clearly suggestive of the disorder, LC-MS/MS revealed reduced amounts of alpha granule proteins which led to the correct diagnosis. This work relied on the earlier proteomic characterization of platelet alpha granules by Maynard *et al.*[27*].

Investigators have examined the effects of drugs, illnesses and biological variation on platelet protein profiles. An ELISA-based study of carbonyl groups and 3-nitrotyrosine residues in platelet proteins suggested that in patients with schizophrenia, reactive oxygen species and reactive nitrogen species may stimulate oxidative and nitrative modifications of platelet proteins[28]. Another group, using 2-DE, found that treatment with an antihypertensive drug modified the platelet protein profile of hypertensive patients [29]. Winkler *et al.*[30] cautioned that clinical studies of platelet proteomes should employ an analytical method that can detect small quantitative differences, such as DIGE.

Cervi *et al.*[31**] compared the platelet proteome of mice injected with tumor cells compared with saline-injected controls using SELDI-TOF. They identified platelet factor 4 (PF4) as a biomarker for a variety of tumor types, even though plasma levels of PF4 did not change. Next, they demonstrated that increases in platelet PF4 can be measured using the more accessible method of ELISA. Thus, PF4 in platelets (but not plasma) represents both a potentially useful cancer biomarker and a potential target for early cancer therapy. In a different SELDI-TOF study[32**], the platelet (but not plasma) concentrations of angiogenesis regulatory proteins were modified by and reflected the presence of tumors in mice. These differences were attributed to the ability of platelets to selectively take up angiogenesis regulators in cancer bearing hosts. The authors suggested that the “platelet angiogenesis proteome” could be used for early detection of tumor establishment or recurrence.

Integrating Transcriptomic and Proteomic Studies

The degree of correlation between platelet proteomic and transcriptomic data in the end points of detection and identification varies by study[11;33**;34]. Some investigators have found that about one third of platelet proteins identified by proteomic methods are not reflected in the transcriptome[11;35]. The discordance may be due to i) the limited mRNA stability of these genes, ii) failure of microarray analysis to detect very low levels of RNA, iii) the occurrence of proteins which may be synthesized in megakaryocytes, after which mRNA is degraded, and iv) the fact that some proteins may be taken up from plasma or from other cells rather than synthesized in megakaryocytes or platelets[36].

Conversely, mRNA transcripts identified by transcriptomic methods sometimes are not detected by proteomic techniques. When proteins are not identified, this may be due to the failure of current proteomic methods to identify proteins with certain structural or biochemical characteristics, and/or to lack of translation of mRNA. To capture and concentrate the “low-abundance” proteome, Guerrier *et al.*[37**] used a combinatorial ligand library composed of millions of diverse hexapeptide baits. This approach led to the addition of 147 proteins to the

list of over 1100 proteins previously identified in the platelet proteome. In contrast, platelets contain approximately 1,600-3,000 individual transcripts[38].

Activity Based Proteomic Profiling of Platelets

Activity based proteomics is a functional proteomics technology where molecular probes are used to target a select group of functionally related proteins. Wong *et al.*[39*] applied such a strategy to differentially profile the nucleotide-binding proteome of active and resting platelets. The investigators performed affinity chromatography using immobilized nucleotides and employed LC-MS/MS to identify the recovered proteins. Nearly all of the differentially expressed nucleotide binding proteins were associated with the cytoskeleton. The dynamic range of the study was negatively affected by abundant nucleotide-binding cytoskeletal proteins such as actin and myosin. Future work should include better removal of cytoskeletal proteins from platelet lysates or alternative strategies to enhance the detection of low abundance proteins.

Data Resources for the Platelet Proteome

Proteomic data about platelets have become increasingly available in various databases. The Human Proteome Organization (HUPO) Proteomics Standards Initiative has produced a document, known as the minimum information about a proteomics experiment (MIAPE), which enumerates several integrated databases[40]. For example, the SWISS-PROT Web page has published the platelet proteome on a 2D gel (http://ca.expasy.org). The Reactome web site (http://www.reactome.org) includes information about the complex pathways which operate in platelets[41]. The Human Protein Reference Database (http://www.hprd.org) includes features such as PhosphoMotif Finder and Human Proteinpedia[42]. Dittrich *et al.*[33**] created a central resource for the platelet proteome, interactome and phosphorylation state known as PlateletWeb (http://plateletweb.bioapps.biozentrum.uni-wuerzburg.de). This “virtual platelet” knowledge base also includes a characterization of the platelet protein kinase repertoire (kinome).

Conclusions and Future Perspectives

Proteomic approaches have uncovered novel signaling pathways and secreted proteins involved in platelet activation and inhibition. Proteomic discoveries have identified potential therapeutic targets in atherothrombosis, quality markers in platelets stored for transfusion, diagnostic approaches to bleeding dyscrasias, and biomarkers for early detection of cancer.

Acknowledgments

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Abbreviations

MS	Mass spectrometry
LC	Liquid chromatography
MP	Microparticles
MudPIT	Multidimensional protein identification technology
ICAT	isotope-coded affinity tag
iTRAQ	isotope tagging for relative and absolute quantitation
2-DE	Two-dimensional electrophoresis
DIGE	Differential in-gel electrophoresis
ELISA	Enzyme-linked immunosorbent assay
MALDI-TOF	Matrix-assisted laser desorption/ionization – Time of flight
SELDI	Surface-enhanced laser desorption/ionization

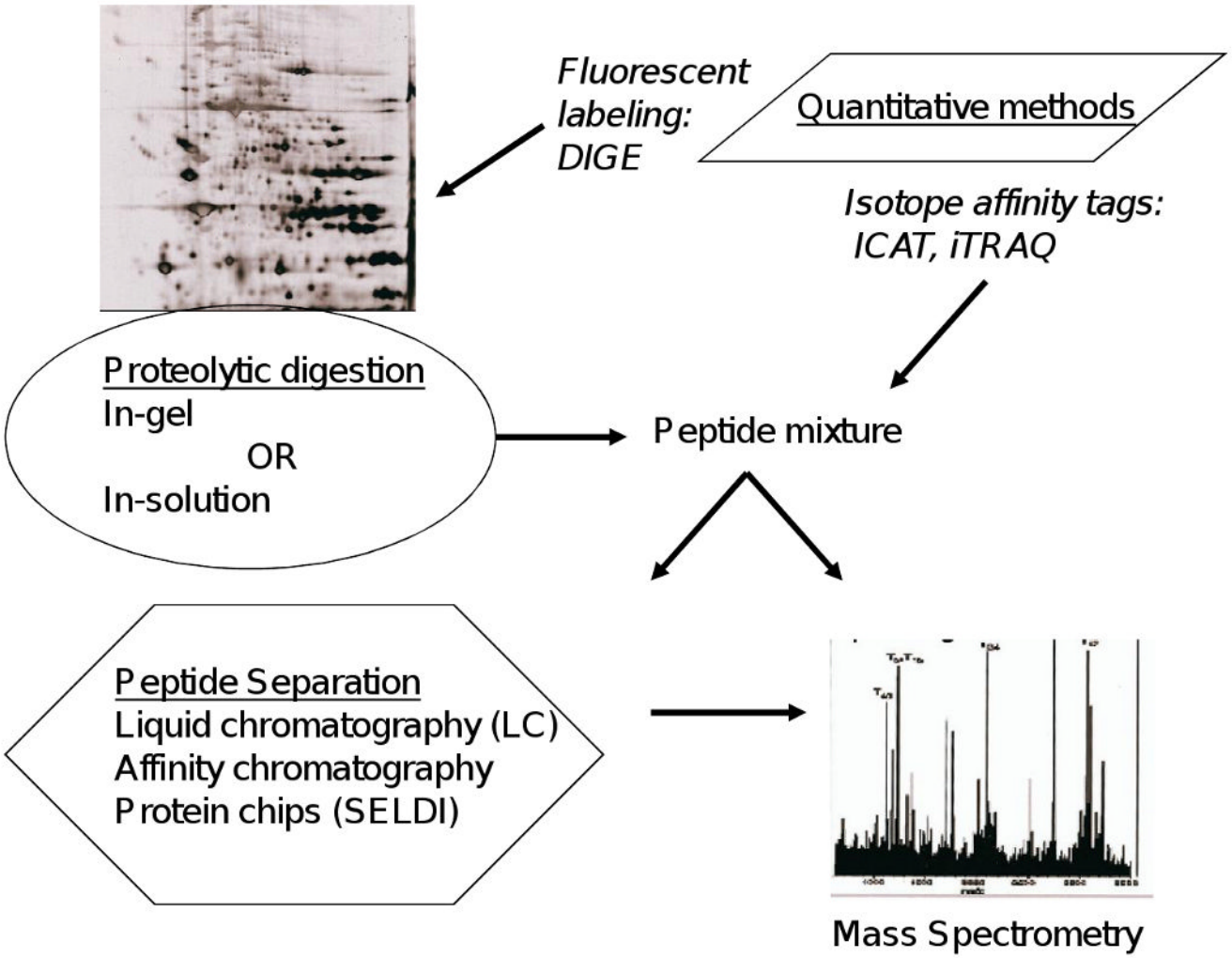

Figure 1. Overview of proteomic techniques

Proteolytic digestion is performed either in a gel or in solution to produce peptides for mass spectrometry (MS) analysis. Differential in-gel electrophoresis (DIGE) employs fluorescent labeling of peptides. Isotope affinity tags can be used in gel-free proteomic experiments to improve peptide quantitation. Various chromatographic methods are available for peptide separation and selective enrichment of proteins. SELDI uses ProteinChip arrays to segregate protein populations. Proteins are identified by database searches following MS analysis. ICAT - isotope-coded affinity tag, iTRAQ - isotope tagging for relative and absolute quantitation, SELDI - Surface-enhanced laser desorption/ionization