Therapeutic potential of the plasma proteome

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Current Opinion in Molecular Therapeutics 2003 5(3):250-257
 ISSN 1464-8431

Plasma contains numerous and diverse proteins with existing and potential therapeutic value. Plasma has been used clinically as both a source of purified derivatives for treating diseases such as hemophilia, and as a diagnostic medium. Recent research directed towards mining plasma's true potential takes advantage of state-of-the-art proteomic analytical methods to develop multi-protein, disease-specific biomarker panels to improve the reliability and specificity of diagnostics. Recombinant production and chromatographic purification methods are increasing the yield and safety of traditional plasma derivatives. Emerging cell-based technologies are being applied to discover novel protein activities and identify epitope-specific antibodies that may have clinical promise.

Keywords Biomarkers, diagnostics, plasma derivatives, plasma proteins, proteomics, therapeutic antibodies

Introduction

Plasma is a remarkably rich and diverse medium, containing millions of antibodies and thousands of other proteins with a multitude of biological activities ranging from coagulation/complement activation and modulation of receptor-mediated signal transduction to modulation of pathological conditions, including autoimmune diseases and cancer. For decades, plasma has been used as a diagnostic medium and plasma proteins have been purified for therapeutic use since the mid-1940s. Yet, the number of proteins identified in plasma is fewer than one thousand and the number used therapeutically or measured as diagnostics is fewer than two hundred. The most tantalizing question in plasma proteomics today is how to tap into the therapeutic potential of these millions of plasma entities. This review focuses on advances over the past two years that are attempting to develop the true potential of plasma for novel diagnostics and new plasma-derived products, and emerging discovery technologies.

The plasma proteome encompasses all of the proteins that are present in a plasma sample. The actual make-up of plasma varies with age, health, time of day and even posture, with the concentration of the various proteins covering a range of ten orders of magnitude. According to Anderson [1••], there may be more than 500,000 different proteins in plasma, including splice variants, proteolytic fragments and post-translational modifications. These protein modifications can result in vastly different activities than demonstrated by their parent proteins. For example, the anti-angiogenic factors angiostatin and endostatin are proteolytic fragments of plasminogen and collagen XVIII, respectively [2,3]. There are potentially 50,000 proteins whose physiological site of activity is plasma. These proteins include those of the coagulation or complement cascades and are most frequently used therapeutically for replacement of genetic deficiencies, eg, clinical use of concentrated Factor VIII (FVIII) for the treatment of hemophilia A. Other proteins, such as cytokines and hormones, are synthesized in one location (generally the liver) and are active elsewhere. These proteins are detected as they move from their site of synthesis to their physiological destination. Alterations in their serum levels are often used to diagnose disease. In addition, proteins that are secreted or spill into the plasma by virtue of their release from tumors or from broken cells in injured tissue are also used for diagnostic purposes. Finally, the most diverse and potentially powerful category of plasma proteins are the antibodies that make up the immune repertoire; there are estimated to be between 1 and 10 million different antibodies in an individual. Immunoglobulin (Ig) from up to 60,000 different donors is pooled and purified to produce a commercial preparation of intravenous Ig of vast variety.

The Human Proteome Organization (HUPO), formed in 2001, has recognized the complexity and importance of the plasma proteome [4]. In a project chaired by Gilbert M Omenn, HUPO is working with a number of international groups to develop reproducible methods that can be used to catalog the plasma proteome, and with the American Red Cross (ARC) to establish a reference plasma source for the development of improved therapeutics.

Diagnostics

Plasma is an ideal tissue for diagnostics because it serves as a repository of proteins that are associated with deranged protein expression and tumor growth, and is easy and relatively painless to sample. Of the 300+ proteins currently identified in plasma, over 117 are the subject of FDA-approved diagnostic tests, including measurement of prostate-specific antigen (PSA) levels to detect prostate cancer. Cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglyceride levels have been associated with the risk of developing cardiovascular disease (CVD). Evidence has been accumulating that the inflammation marker C-reactive protein (CRP) is associated with increased susceptibility to CVD, even in patients with LDL levels within recommended ranges [5]. Recently, plasma levels of the angiogenic growth factors vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) have been associated with acute coronary

syndromes and prognosis [6]. High levels of HGF were correlated with improved prognosis, while the opposite was true for increased VEGF levels. This correlation has been reported to outweigh the correlation between CVD and CRP [6]. The combination of several single markers, eg, LDL and CRP, may help construct individualized patient treatment plans [7]. Cytokine levels have also been associated with the presence of disease and evidence of treatment success for multiple myeloma [8].

Most current diagnostics employ single markers; however, a more extensive set of related markers may provide more precise diagnostic and prognostic information, and aid in tailoring treatment to an individual patient. Petricoin, Liotta, and colleagues have reported the presence of plasma diagnostic markers for a number of organ-confined cancers. These investigators use the Ciphergen C16 hydrophobic chip system (Ciphergen Biosystems Inc) to perform surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) analysis of plasma samples from patients and healthy control volunteers. The separation analysis is coupled with artificial intelligence to develop a panel of disease-related markers. The method uses a genetic algorithm and cluster analysis to 'train' the program to find and refine proteomic patterns that are specific to diseased and control samples. Using this method the investigators have apparently been able to identify 50 ovarian cancer samples from all stages of disease with 100% accuracy, and to differentiate lack of disease or benign ovarian cysts from tumors with 95% accuracy [9••]; this led to a predictive value of 94% accuracy. Standard screens that use cancer antigen 125 (CA125) levels only have a predictive value of 35%. Interestingly, patients with benign gynecological disease or non-gynecological inflammatory disorder were neither positive nor negative for these markers and fell into a new cluster. Thus, this method differentiates several disease states from control (non-diseased) states, and may have particular value in screening for early-stage disease in high-risk women.

A similar method was used to develop a diagnostic cluster for men with and without histopathological evidence of prostate cancer [10]. The algorithm that was used to analyze mass spectra was able to correctly identify 95% of the patients with cancer from a blinded panel of 228 samples. Although the algorithm identified more than 25% of patients with benign hyperplasia as having cancer when prostate-specific antigen (PSA) levels were elevated (≥ 4 ng/ml), more than 10% of these 'false-positive' patients developed prostate cancer during a five-year follow-up. All of the disease biomarkers identified in these studies have not yet been identified and may be neither causative nor a direct result of the disease process; their reproducible association with a particular disease state is more important than the proteins' actual identity. However, this also means that they may not translate into therapeutic targets.

In addition to the cancer markers described above, autoantibodies have been associated with disease [11]. Recently, antibodies from prostate cancer patients have been screened on phage display peptide libraries for sequences that bind tumor-associated antibodies [12••]. IgG was affinity purified from control serum using protein G, and normal antigen peptides were subtracted from a phage library. The subtracted library was then exposed to protein G-purified IgG from patients' sera. This positive selection led to the discovery of a peptide with the consensus sequence NXS/TDKS/T. Antibodies that recognize this peptide were present in 6 to 7% of control and organ-contained disease, 29% of patients with metastatic, androgen-dependent disease, and 76% of patients with metastatic, androgen-independent disease; the presence of antibodies that recognize this peptide in serum was correlated with negative clinical outcome in these patients. The antigen mimicked by the peptide was identified, using biochemical methods and a BLAST search, as heat shock protein GRP78, which is highly expressed in bone marrow metastases. Although the false-negative rate is significant for a diagnostic test, this and similar methods can clearly be used to identify tumor antigens that could contribute significantly in multi-marker diagnostic panels.

These new methods have demonstrated significant capabilities to expand the power of diagnostics at an investigational scale. However, substantial technical issues may impact the ability to translate these proteomic advances into clinically useful diagnostics (reviewed in [1••]). The range of normal levels of proteins can vary dramatically in a population; thus, individuals will have to serve as their own controls. In addition, the robustness and reproducibility of some methods are challenging. For example, two-dimensional gels are often not reproducible from laboratory to laboratory, which has created impediments to database development. Moreover, while proteomic panels of biomarkers have the potential to improve the specificity of disease diagnosis for a single individual, these sophisticated analyses (eg, by SELDI methods) may require the establishment of a few centralized laboratories with the requisite instrumentation and expertise; thus to some extent impeding community-based screening. Localized centers also introduce the issues of sample transportation, storage, consistency of handling, and standardization of methods, issues which may be more critical in relation to advanced proteomics methods than for existing standardized laboratory tests. Finally, multivariate proteomic panels are accompanied by both high cost and hurdles to regulatory acceptance. Thus, single marker or panels of markers have the benefits of regulatory acceptance, lower cost, robustness, reproducibility, and ease and standardization of use. These will probably remain the first level of health screening in the near future, reserving the use of proteomic panels to confirmatory testing, screening of small, high-risk patient pools, or developing an individualized treatment plan.

Therapeutic antibodies

For pure diversity of the proteome, no group of proteins competes with the human immune repertoire. Oncley first developed methods to purify γ-globulins from plasma [13] to produce a preparation that could be administered intramuscularly to treat primary immune deficiency.

Intravenous Ig (ivIg) is currently the most sought after plasma derivative. It is approved for treating immune deficiencies, but is also used off-label for many indications, including multiple sclerosis and myasthenia gravis [14•]. The identities of the active antibodies in ivIg preparations,

which are derived from pools of thousands of donors, are completely uncharacterized. Specialized immune globulin preparations called 'hyperimmunes' are produced from individuals whose plasma is enriched for therapeutically useful antibodies. These include hyperimmunes for hepatitis B virus, rabies, rhesus factor incompatibility and tetanus. Smallpox and anthrax hyperimmune preparations are considered by the FDA to be among the most desirable plasma derivatives to be prepared for future emergency [15]. These can be used to combat reactions to smallpox vaccination and exposure to anthrax.

The ivIg production methods remove IgM and IgA. IgM is a predominantly pentameric Ig that is present in both membrane-bound and secreted forms, and constitutes approximately 5% of the total serum Ig. IgM is the first antibody expressed in the primary immune response and is an efficient activator of complement; therefore, some forms of IgM may have a role in inducing death of tumor cells or pathogens. IgA comprises 10 to 15% of the secreted Igs and does not induce inflammatory processes but instead binds to pathogens to prevent their uptake, an activity that may have important future therapeutic use. Several monoclonal IgM and IgA antibodies have been shown to be therapeutically active in preclinical models [16], thereby supporting the contention that endogenous IgM and IgA or isolated, epitope-specific antibodies should be considered further for development as therapeutic products.

There is a vast potential reservoir of epitope-specific therapeutic antibodies in plasma. These can be used as passive vaccines directly to kill a target, with or without complement fixation or by the alteration or stimulation of cellular pathways, or as active vaccines to induce an immune response. The recent success of humanized and chimeric monoclonal antibodies for cancer treatment, eg, Herceptin for the treatment of Her2+ breast tumors and rituximab for B-cell lymphoma, supports the once controversial premise that purified, epitope-specific antibodies can be clinically effective therapeutics [17].

Such specific therapeutic antibodies clearly exist in both disease-state and healthy plasma. A common complication of FVIII treatment is the development of inhibitory antibodies against FVIII. In studying these inhibitors, Gilles *et al* discovered that healthy patients also have antibodies against FVIII [18••]. Interestingly, this activity was only detectable when the antibodies had been purified from plasma or serum, apparently separating them from anti-idiotypic antibodies that recognize the inhibitors and suppress their activity. Moreover, anti-idiotypic antibodies that mimic several endogenous antigens, including FVIII and double-stranded DNA (implicated in systemic lupus erythematosus), have been detected in commercial ivIg preparations [19,20]. Thus, plasma from healthy individuals may indeed contain a surprising variety of therapeutic antibodies that can be purified and used to treat disease, as well as an instructive and potentially useful repertoire of anti-idiotypic Ig structural mimics.

Autoimmune antibodies can have diverse activities, including antitumor effects. Dudley *et al* have demonstrated one of the first successful immunotherapy treatments, in which patients with advanced metastatic melanoma were treated with activated CD4+/CD8+ tumor-infiltrating lymphocytes (TIL) plus infusions of interleukin-2 [21]. Several patients who demonstrated tumor regression or disease stabilization also developed vitiligo or uveitis (another autoimmune disease) and antimelanocyte antibodies were detected in the tumors. Thus, it would appear that the TIL cells produced antibodies with activity against both normal and malignant tissue. Autoimmune vitiligo is a disease characterized by unpigmented patches on the skin and has a worldwide incidence of 1%. Vitiligo is associated with the presence of antimelanocyte auto-antibodies in patients' sera. Plasma from vitiligo patients has activity against melanoma cells *in vitro*, and there is some evidence that the fairly common group of melanoma patients who also develop depigmentation tend to have a better prognosis than patients who do not develop this disease [22,23]. Fishman and Schoenberg investigated the activity of antibodies in the serum and purified IgG fractions of vitiligo patients. The serum from vitiligo patients had activity against both mouse and human melanoma cells in culture. Injection of purified IgGs was associated with a significant decrease in the number of metastatic foci in the lungs of a mouse model that was injected with melanoma cells [24]. These data suggest that, as the general health of vitiligo patients is not compromised and they are not deferred from donating blood, there may be a substantial reservoir of antimelanoma antibodies in healthy blood donor pools.

Plasma derivatives

The therapeutic use of plasma proteins outside diagnostics is a multi-billion dollar international business. A handful of international plasma collectors and fractionators, including the ARC, Baxter Healthcare, Bayer, CSL/ZLB, Aventis Behring and others, process over 20 million liters of plasma each year in the US alone to produce approximately 20 plasma proteins as licensed therapeutic products [14•,25,26]. Complete plasma itself has been, and continues to be used as a therapeutic for treating coagulation deficiencies. In addition to plasma transfusion, a technique called plasma exchange (the replacement of a portion of a patient's plasma with donor plasma) is used to treat thrombotic thrombocytopenic purpura [27,28], myasthenia gravis [29] and multiple sclerosis [30]. Although the mechanism by which plasma exchange works is unknown, it is thought to remove deleterious antibodies and other proteins.

The history of plasma fractionation began with the need to meet the requirements of the US military for albumin as a plasma expander in the second world war. Cohn first developed a robust method for fractionating plasma to produce albumin on a large-scale [31•], which was expanded by Oncley to produce Igs [13]. Although yields are limited, these fractionation methods remain the primary methods of producing plasma derivatives today. Furthermore, the cold alcohol precipitation that is the backbone of the process inactivates many plasma proteins, effectively limiting the number of novel proteins that can be purified via these standard methods. More modern methods of ion exchange and affinity chromatography are used to purify a few plasma proteins. While being technically feasible, there are significant impediments to their widespread adoption within existing plasma fractionation

facilities, including the significant cost to re-license products purified by new methods [32].

Purified plasma proteins have served primarily as replacement therapy for treating genetic deficiencies (outlined in Table 1) [14•]. Most recent research and development efforts in the plasma industry have centered on technological improvements to increase yield, purity and safety of current products, as exemplified in the history of FVIII production. Before plasma fractionation, the only treatment for hemophilia A was blood and plasma infusion to replace the missing clotting factors. Treatment was revolutionized with the discovery by Pool and Shannon that freezing and thawing of plasma produced a cryoprecipitate (cryo) that was rich in clotting factor [33]. Cryo was replaced with purified FVIII developed by Brinkhous and Shanbroom [34] in 1968. Current efforts include the development of recombinant and transgenic production to improve yields, and a genetically engineered FVIII with a prolonged *in vivo* half-life [35]. While concentrated FVIII has vastly improved the lives of people with hemophilia, its cost has prevented it from reaching patients in most of the developing world.

Viral safety of plasma products has been one of the industry's primary focuses since its inception and continues to be a top priority today. Currently, FVIII concentrate (like other plasma derivatives) is produced from pools of thousands of donors. An appalling result of the pooling was that, in the early years of the AIDS crisis, donations from HIV-positive individuals could, and probably did contaminate an entire batch of product. HIV contamination of FVIII devastated the hemophilia community in the 1980s and led to the development of critical improvements in viral safety through the development of novel viral inactivation methods [36]. Recombinant and transgenic expression methods to produce plasma derivatives have also been considered. Fibrinogen has been produced in the milk of transgenic cows and methods to express plasma proteins in urine have been developed [37]. α_1 Protease inhibitor (API) has been produced in transgenic sheep [38] and in yeast cultures for the treatment of atopic dermatitis by ProMetic Biosciences. In the future, many new proteins originally identified in plasma may be produced primarily by recombinant methods. While these methods have their advantages, there have been production problems for FVIII leading to product shortages and obliging some recombinant FVIII recipients to use plasma-derived product [25]. Furthermore, the emergence of mad cow disease and variant Creutzfeld-Jakob disease has illuminated the dangers associated with transgenics and transmission of zoonotic diseases. Finally, achieving the required purity of these products from animal tissue can be challenging and cost prohibitive in some species.

There are a number of plasma proteins that have recently been introduced or are currently in development (Table 2). Plasmin is a product that has recently re-emerged as a treatment for acute thrombotic events. Part of the endogenous fibrinolysis cascade, plasmin is derived by cleavage of the zymogen plasminogen (an inherent component of fibrin clots) by tissue plasminogen activator (tPA). Plasmin cleaves the fibrin fibers, leading to clot dissolution. Plasmin was previously investigated as a thrombolytic therapy but was found to be ineffective when

Table 1. Licensed plasma derivatives.

Protein	Indication
Plasma	Coagulation deficiency
Albumin (Plasma protein fraction)	Plasma expander in shock, burns
α_1 Protease inhibitor	Chronic emphysema, anesthetic apnea, cocaine overdose
Antithrombin III	Antithrombin III deficiency
C1 inhibitor	Hereditary angioedema (edema formation)
Factor VIII	Hemophilia A
Factor IX	Hemophilia B
Factor XI	Hemophilia B
Factor XIII	Factor XIII deficiency
von Willebrand Factor	von Willebrand disease
Prothrombin complex	Thrombin deficiency
Fibrinogen	Tissue sealant component
Thrombin	Tissue sealant component
Hyperimmunes	
Anti-D	Rhesus incompatibility
CMV	CMV infection for immunosuppressed patients
Hepatitis B	Hepatitis B prophylaxis
Measles	Measles prophylaxis and treatment
Pertussis	Pertussis treatment
Rabies	Rabies treatment
RSV	RSV treatment in infants
Staphylococcus aureus	*S aureus* infection in immunosuppressed patients
Tetanus	Tetanus prophylaxis and treatment
Varicella zoster	Chicken pox prophylaxis and treatment
WinRho S/D	Immune thrombocytopenic purpura
	Primary and secondary immune deficiency, ITP
ivIg	Sepsis
Activated Protein C	Tissue sealant component

Information shown in this table is derived from references [14•,25,26]. **CMV** cytomegalovirus, **ITP** immune thrombocytopenic purpura, **RSV** respiratory syncytial virus.

Table 2. Plasma therapeutics.

Therapeutic	Indication
Plasma proteins [24]	
Apotransferrin	Iron overdose
Butyrylcholinesterase	Cocaine overdose, organophosphate nerve poisoning
Paraoxonase	Cardiovascular disease, organophosphate nerve poisoning
HDL	Sepsis
Fibrin glue	Local drug delivery
Hemostatic dressing	Severe arterial hemorrhage
Fibronectin	Wound healing
Mannan-binding lectin	Recurrent infection, mannan-binding lectin deficiency
Plasmin	Myocardial infarction, thrombosis
Diagnostics [7,9••,10]	
Single marker	
C-reactive protein	Cardiovascular disease
HGF, VEGF, FGF	Cardiovascular disease
Panels	
Prostate cancer	Identification, decisions on biopsy
Ovarian cancer	Early identification
Antibodies [15,24]	
ivlg	Multiple sclerosis, myasthenia gravis, Parkinson's disease, diabetic neuropathies
Anti-idiotype Ab	Cancer vaccines, autoimmune disease
Smallpox hyperimmune	Smallpox reactions
Anthrax hyperimmune	Anthrax infection

Ab antibody, **FGF** fibroblast growth factor, **HDL** high-density lipoprotein, **HGF** hepatocyte growth factor, **Ivlg** intravenous immunoglobulin, **VEGF** vascular endothelial growth factor.

delivered systemically due to its rapid and irreversible inactivation by antiplasmin [39]. The use of tPA is currently approved for treating myocardial infarction (MI), peripheral vascular occlusion and stroke; however, there are significant drawbacks to its use. Plasminogen activators (PAs) are only effective if the clot contains sufficient plasminogen, which diffuses out of the clot with time. Moreover, up to 9% of patients treated with tPA suffer from intracranial hemorrhage (ICH) due to the activity of tPA in areas beyond the thrombosis being treated. Recombinant and mutated PAs have not proven to be more effective or safer; therefore, plasmin is being reconsidered as a potential thrombolytic agent. Recent studies in animal models have demonstrated that plasmin is effective when delivered directly to the thrombus by catheter and does not cause ICH [40]. Plasmin is as effective as tPA in clots containing plasminogen and more effective if plasminogen was depleted from the clot [41].

Another plasma protein of considerable interest is paraoxonase (PON1), which has been implicated in CVD. It has been reported that low levels of PON1 correlate with increased risk of CVD. PON1 circulates as a complex with HDL and there is evidence in human epidemiological and animal models that decreased levels of PON1 increase susceptibility to atherosclerosis [42,43]. PON1 also has organophosphatase activity that can inactivate and potentially protect against organophosphate (OP) nerve gas poisons such as sarin and soman [44], an activity of much interest to the military. Butyrylcholinesterase also protects against OP poisoning by scavenging OPs in plasma and protecting acetylcholinesterase activity [45], as well as being a potential treatment for cocaine overdose [46]. Other protein products in development include mannan-binding lectin (MBL) which is a part of the innate immune system. MBL is involved in microorganism destruction through its ability to bind to surface mannan groups in conjunction with serine proteases, thereby activating complement. Congenital MBL deficiencies lead to recurrent infections, and uses of MBL may include treatment of primary or secondary immune deficiency, cystic fibrosis, rheumatoid arthritis and recurrent miscarriage. Apotransferrin is being developed as a free iron scavenger for use when normal iron utilization is compromised, eg, in leukemia patients following chemotherapy [25]. A recombinant form of activated protein C (APC, Xigris), part of the endogenous anticoagulant pathway, was licensed in 2001 and is the only approved treatment for severe sepsis in the most vulnerable patients.

Innovative uses of existing products are also in development. The ARC is developing a hemostatic dressing composed of freeze-dried fibrinogen and thrombin that is capable of stopping arterial hemorrhages [47]. Additionally, fibrin glue/fibrin sealant, a liquid mixture of fibrinogen and thrombin, can be used as a delivery matrix to be supplemented with proteins or drugs like antibiotics for local, controlled, therapeutic delivery [48,49]. Hubbell and colleagues have demonstrated a significant advance in controlled delivery of proteins from fibrin matrices by taking advantage of the endogenous Factor XIII transglutaminase activity in fibrin sealants to cross-link proteins into fibrin sealant clots. This method was able to significantly improve *in vitro* nerve regeneration compared with simple addition of nerve growth factors to fibrin sealant [50•].

Novel approaches

In 1939, Cohn reported that "The methods would appear to be at hand...for the study of all proteins. There is no theoretical obstacle to the isolation of all the protein constituents of any given tissue, or to their characterization

as chemical substances, and to the study of their interactions as biological components." [51].

The complexity of plasma is the source of both its promise to the clinic and its challenge to analysis. The desire to understand its constituents is longstanding. However, despite Cohn's optimism, there remain substantial practical and technological obstacles to the identification of all proteins in plasma. Significant advances in analytical methods [52,53] have increased the sensitivity and throughput of protein identification, but can do little to illuminate the functions of these newly identified molecules. Furthermore, the vast dynamic range of protein concentration in plasma, from albumin at 40 mg/ml to cytokines at < 1 ng/ml, essentially prohibits its complete analysis. Proteins can be separated, eg, by charge, size and isoelectric point, but there are currently no methods to separate proteins by abundance. Generally, proteomic analyses serially fractionate plasma in an attempt to isolate the rarest proteins. These fractionation methods are not as effective in practice as in theory [54], although certain affinity ligands effectively remove the most abundant proteins (albumin and Igs). This subtraction results in the concomitant removal of proteins bound to the subtracted species, and thus must be used with care in an analytical context. Importantly, the removal of Ig eliminates antibodies from activity screens. One potential use of the plasma proteomic information generated may be to provide valuable information on the effects of potential therapeutic drugs during the early phase of clinical trials. Global changes in protein expression can be detected in arrays and may help to indicate therapeutic as well as toxic and immunological effects of drug candidates in advance of defined clinical endpoints.

Nevertheless, several inventive methods for characterizing both global protein expression and function have recently been developed to identify protein complexes, sites of functional domains, interaction partners and classes of enzyme activities [55-57]. These have mostly been applied in yeast or tissue proteomics [58] and include chromophore-assisted laser inactivation (CALI), in which specific regions of a protein are inactivated and the resulting effect on its activity are interpreted to identify amino acids involved in a functional domain [59]. These and other methods may be useful for exploring some of the activities in plasma, eg, protein kinase activities and protein-protein interactions, provided that the albumin and Igs do not interfere (reviewed in [60••,61••]).

Many of these activity-based assays described above require at least some knowledge of one of the proteins involved and may also require chemical modification, ie, tagging of at least one of the members of the complex, which is impossible in complex mixtures of proteins such as donated human plasma. According to Phizicky *et al*, the 'broad but shallow' proteomic data generated by these methods needs to be integrated with the study of particular biological problems to develop a deeper understanding of the roles of the myriad of discovered proteins [61••]. In view of the complex composition of plasma and the nature of its source, additional methods that can probe unfractionated plasma for clinically relevant activities are needed [61••].

An approach being pursued by the ARC and Automated Cell Inc, uses combinatorial affinity ligands to separate, concentrate and purify essentially all of the proteins in plasma according to their three-dimensional structure without pre-fractionation. These are assayed *en masse* in disease-relevant, cell-based and biochemical assays in conjunction with sophisticated imaging, analysis and data mining techniques [62,63]. These methods do not require prior knowledge of the identity of the active factor or of the ligand on which it is identified. While this method does not attempt to identify all of the proteins in plasma, it does enable the identification of wholly novel protein activities and epitope-specific antibodies with a desired activity. These methods have been used to purify physiological protein complexes from plasma and to discover factors that support cell growth [62]. The emerging power of mass spectrometry can be applied to such newly discovered factors to determine their molecular identity without interference from the abundant proteins.

It remains to be seen how these discoveries will translate into viable therapeutics. Current alcohol-based plasma fractionation methods inactivate many proteins; consequently, new, chromatography-based purification methods using novel affinity ligands will be required for efficient purification of trace proteins or epitope-specific antibodies. While recombinant methods theoretically have unlimited yields, in practice, production of these proteins has sometimes been an issue, eg, in FVIII supply and production of endostatin and angiostatin [64]. Gene therapy has been suggested as a way to circumvent these difficulties and to deliver proteins; this approach has successfully achieved therapeutic levels of endostatin in mice [65•]. Gene therapy may one day be a viable option for delivering genes required for replacement therapy or inducing expression of novel therapeutic proteins.

Conclusion

Realizing the true, vast therapeutic potential of plasma remains elusive and is largely still restricted to measuring levels of proteins and hormones as disease diagnostics. A key effort currently focuses on the development of multiprotein panels of biomarkers that should enable refined description of disease presence, stage, prognosis, and aid in the design of individualized treatment. A second research area focuses on the identification of antibodies associated with disease to identify tumor-associated antigens and tumor-related antibodies for diagnosis.

Plasma itself has also been used as a therapeutic and as a source of plasma-derived proteins for various therapeutic indications, especially congenital deficiencies. A handful of new plasma derivatives are being developed by the plasma industry. In addition, modern methods of production, including recombinant methods, will improve availability.

The major challenge to developing the potential of plasma is the identification of relevant biological activities. Proteins exist in plasma covering a tremendous concentration range (10^{10}). Moreover, their activity is influenced by association as complexes, post-translational modifications including glycosylation, proteolytic digestion and conformational changes. This precludes their global identification and characterization by current analytical techniques. Furthermore, much of the clinical potential in plasma lies in

the immune repertoire, which is completely inaccessible to identification by mass spectrometry. Despite the number of proteins present, only approximately 20 are currently used clinically. New methods of characterization are required that are able to identify the least abundant proteins in unfractionated plasma. This will enable the comprehensive description of the plasma proteome and will hopefully allow discovery of proteins with novel activities that can be developed into clinical therapeutics.

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