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Medical Applications of Mass Spectrometry
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Chapter 15

Proteomics of viruses

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1. Introduction

The term "proteome" was first coined in late 1994 by Marc Wilkins at the Siena two-dimensional gel electrophoresis (2DE) meeting and defines the entire protein complement in a given cell, tissue, or organism [1]. In its wider sense, proteomics research also assesses protein activities, modifications and localization, and interactions of proteins in complexes. It relies heavily on technology since it needs to identify proteins and protein complexes in biological samples comprehensively and quantitatively with both high sensitivity and fidelity.

Proteomics is a promising approach for the study of viruses. It allows a better understanding of disease processes and the development of new biomarkers for diagnosis and early detection of disease, and accelerates drug development. Areas of proteomics that are particularly promising include the determination of altered protein expression, not only at the whole-cell or tissue level, but also in subcellular structures and biological fluids; the development of novel biomarkers for diagnosis and early detection of disease; and the identification of new targets for therapeutics and the potential for accelerating drug development through more effective strategies to evaluate therapeutic effect and toxicity.

There is a growing interest in applying proteomics to the study of infectious disease. A complicating factor in therapy for infectious disease is the development of resistance to commonly used drugs, which heightens the need for developing effective new therapies. The availability of the complete sequences of a number of viruses has provided a framework for identifying proteins encoded in these genomes using mass spectrometry (MS). Applying proteomics to the study of viruses allows the characterization of subviral proteomes (e.g., secreted proteins, surface proteins, and immunogenic proteins), the comparative analysis of different stains or physiological states, the identification of proteins related to pathogenicity and host–pathogen interactions, and the evaluation of mechanisms of action of antiviral therapies.

1.1. Highlights for medical professionals

Viral infections cause significant morbidity and disease including cancer, immunosuppression, and death. Often infections are not diagnosed until symptoms appear and, in several cases, this may be years or decades after the initial infection. The ability to diagnose infection or cancer before the appearance of symptoms would be of critical importance for effective treatment. Proteomic analysis of serum has been proposed as a means of diagnosing infectious disease and/or the early diagnosis of cancer. There have been some recent exciting findings in the proteomics of the host or pathogen, and the use of standard mass spectrometric technologies has enabled many physicians and scientists to examine more closely the pathological and biological questions that can only be answered using proteomic approaches.

Therefore, in this chapter we will discuss some recent findings on the proteomics of DNA and RNA viral infections that are associated with clinically important diseases in humans, including human cytomegalovirus (HCMV), herpes simplex virus (HSV), Epstein–Barr virus (EBV), human immunodeficiency virus (HIV), hepatitis B and C (HBV and HCV, respectively), and adenovirus, as well as the coronavirus that causes severe acute respiratory syndrome (SARS).

HCMV is the largest member of the human herpesviruses. After initial infection, HCMV remains in a persistent state with the host [2]. Immunity against the virus controls replication, although intermittent viral shedding can still take place in the seropositive immunocompetent person [2]. As replication of cytomegalovirus in the absence of an effective immune response is central to the pathogenesis of disease, complications are primarily seen in individuals whose immune system is immature or suppressed by drug treatment or coinfection with other pathogens [3]. Estimates of the coding capacity of HCMV range from 160 open reading frames (ORFs) to more than 200 ORFs [4]. Recent studies using MS to determine the viral proteome suggest that the number of viral proteins may be even greater than previous estimates [5]. Analysis of proteins from purified HCMV virion preparations has indicated that the particle contains significantly more viral proteins than the previously known 71 HCMV virion proteins. Twelve of the identified proteins were encoded by known viral ORFs previously not associated with virions, and 12 proteins were from novel viral ORFs [6]. Therefore, new protein markers including HCMV tegument and various cellular structural proteins, enzymes, and chaperones are now serving as biomarkers for HCMV infection and as possible drug targets.

Other herpesvirus members have also been explored for the presence of possible biomarkers. EBV is a ubiquitous member of the herpesvirus family that is associated with a variety of lymphomas and lymphoproliferative diseases [7]. It encodes a multitude of genes that drive proliferation or confer resistance to cell death [8]. Infection of human B lymphocytes with EBV induces proliferative B-lymphoblastoid cell lines (LCLs). Recently, proteomic profiles of three LCLs were analyzed comparatively at the early and the late passages of cell culture. The phosphoprotein stathmin was identified, and expression significantly decreased with immortalization of LCLs [9]. Stathmin is critically important not only for the formation of a normal mitotic spindle upon entry into mitosis but also for the regulation of the function of the mitotic spindle in the later stages of mitosis and for the timely exit from mitosis [9]. In another study using standard matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) methods, 20 EBNA2 target proteins were identified, 11 of which were c-*myc* dependent and therefore most probably associated with proliferation of the host cell [10]. These findings further stress the role of EBV viral proteins, namely EBNA and LMP, in disease pathogenesis. Interestingly, when EBV-infected cells were treated with the drug 5′-azacytidine (AZC)—a demethylating agent that induces the expression of silenced genes, i.e., the p16 tumor suppressor gene—21 polypeptides were down-regulated, while

14 showed increased expression. Many of the induced proteins were involved in energy metabolism, organization of cytoskeletal structures, protein synthesis, or cell viability [11]. Therefore, the effect of drugs that activate silenced tumor suppressor genes and their proteomic profile following treatment is of considerable interest.

Finally, an important herpesvirus to consider is HSV. HSV types 1 and 2 are ubiquitous viruses that cause infections in human populations throughout the world. The clinical manifestations of HSV infections are varied, ranging from asymptomatic to life-threatening illness in neonates and immunocompromised hosts [12,13]. HSV-1 infection induces severe alterations of the host translational apparatus, including the phosphorylation of a few ribosomal proteins and the progressive association of several nonribosomal proteins to ribosomes [14–16]. Using a proteomics approach, it was shown that VP19C, VP26, and ICP27 associated with ribosomal proteins [17]. Specifically, immediate early ICP27 protein associated with the cellular translation initiation factor poly A binding protein (PABP), eukaryotic initiation factor 3 (eIF3), and eukaryotic initiation factor 4G (eIF4G) in infected cells, resulting in the stimulation of translation of certain viral mRNAs and inhibiting host mRNA translation [18]. Another study has shown that approximately 50 cellular and viral proteins associate with the HSV-1 ICP8 single-stranded DNA-binding protein, some of which belong to DNA repair and chromatin family members [19], implying that HSV-1 infection results in control of host cellular DNA replication/repair and gene expression machineries.

Proteomic analyses of RNA viruses with regard to diagnosis and novel biomarker detection are also of considerable interest in the medical community. For instance, SARS is a new infectious disease that first emerged in the Guangdong province, China, in November 2002 [20]. A novel coronavirus was later identified in patients with SARS. The detection of the virus in these patients, its absence in healthy controls or other patients with atypical pneumonia, and the reproduction of a similar disease in a relevant animal model indicated that this coronavirus was the causative agent of SARS (SARS-CoV) [21]. Interestingly, the full genome sequence was determined within weeks of the identification of the virus, but the proteome and biomarkers associated with SARS are slowly forthcoming. In a recent study using a mass spectrometric decision tree classification algorithm, Kang et al. identified four biomarkers determined in the training set that could precisely detect 36 of 37 (sensitivity, 97.3%) acute SARS and 987 of 993 (specificity, 99.4%) non-SARS samples [22]. A reasonably complete proteomic analysis was also performed on four patients with SARS at different times of infection, and a total of 38 differential spots were selected for protein identification. Most of the proteins identified were acute phase proteins, and their presence represented the consequence of a serial cascade of inflammatory reactions initiated by SARS-CoV infection. Of significance was the level of plasma peroxiredoxin II in patients with SARS, which was significantly higher in SARS patients and could be secreted by

T cells [23]. Finally, while pursuing new enzyme targets, another study identified 14 putative ORFs, 12 of which were predicted to be expressed from a nested set of eight subgenomic mRNAs. Distant homologs of cellular RNA processing enzymes were identified in group 2 coronaviruses, with four of them being conserved in SARS-CoV. These newly recognized viral enzymes put the mechanism of coronavirus RNA synthesis in a completely new perspective, which has opened the door for new drug targets for the treatment of SARS [24].

The proteomes of three other RNA viruses including HCV, HBV, and HIV-1 have also been studied. Hepatitis C often progresses to chronic infection in the majority of patients and is an emerging cause of viral hepatitis. Clinically, the infection is generally asymptomatic, but may present with a wide variety of symptoms. Cirrhosis, hepatocellular carcinoma (HCC), cryoglobulinemia, autoantibodies, and glomerulonephritis have been strongly associated with HCV infection [25]. When analyzing proteins that interacted with the HCV protein NS5A, Choi et al. found that the cytoplasmic heat shock protein 27 (HSP27) bound to NS5A was concentrated in the ER [26], where drugs for HCV treatment would have easy access (as opposed to drugs delivered into the nucleus). Chronic infection with HBV is associated with the majority of HCC. Using woodchucks as a model system, HCC induced dramatically higher levels of serum-associated core alpha-1,6-linked fucose, as compared with woodchucks without HCC. The coupling of this model system with 2D gel electrophoresis has permitted the identification of several glycoproteins with altered glycosylation as correlated to cancer prognosis. One such glycoprotein, the golgi protein 73 (GP73), was found to be elevated and hyperfucosylated in animals with HCC [27]. Finally, in an effort to identify useful biomarkers for HBV- or HCV-associated HCC, 60 proteins were identified which exhibited significant changes in expression between nontumorous and tumorous tissues. Among these, 14 proteins were commonly changed in all three of the HCC types, but 46 proteins showed a tendency toward viral marker specificity, suggesting that the pathogenic mechanisms of hepatocarcinogenesis may be different according to the viral etiology of HBV or HCV [28].

Diagnosis and treatment strategies for HCV have become extremely important as one-third of HIV-infected individuals in Europe and the USA are coinfected with HCV [29]. Therefore, defining biomarkers in coinfections after highly active antiretroviral therapy (HAART) is currently the focus of many laboratories. HIV accelerates HCV liver disease especially with the progression of HIV-associated immunodeficiency. With the introduction of pegylated interferon in combination with ribavirin, greatly improved treatment options for patients coinfected with HIV and HCV have become available and have led to sustained virological response rates of up to 40% [30]. Furthermore, recent cohort analyses have shown that immune reconstitution induced by HAART can improve the course of hepatitis C infection leading to a decline in liver-related mortality. However, patients with HCV coinfection are at increased risk of hepatotoxicity from HAART [29].

Owing to the high rates of HIV and HCV coinfections worldwide, new improved biomarkers and treatment strategies and guidelines for the management of coinfection remain a major goal. Biomarkers could include protein fingerprints of HIV-1-infected human monocyte-derived macrophages (MDMs) after viral infection, as well as HCV-infected liver cells. Recently, 58 proteins have already been identified to be up- or down-regulated after HIV-1 infection [31].

1.2. Highlights for chemists

The overall awareness of the importance of proteins and peptides in physiology and pathophysiology has increased dramatically over the last few years. With progress in the analysis of whole genomes, the knowledge base in gene sequence and expression data, useful for protein and peptide analysis, has increased considerably. Therefore, the medical need for relevant biomarkers is enormous. This is particularly true for many viral infections and various types of cancer, where there is a lack of useful and adequate diagnostic markers with high specificity and sensitivity.

However, proteomic and peptide-based techniques have evolved in recent years to simplify the search for biomarkers. Peptide-based technologies provide new opportunities for the detection of low-molecular-weight protein biomarkers (peptides) by MS. Improvements in peptide-based research are based on separation of peptides and/or proteins by their physicochemical properties in combination with mass spectrometric detection and identification using sophisticated bioinformatics tools for data analysis. Therefore, peptide-based technologies offer an opportunity to discover novel biomarkers for diagnosis and management of disease including prognosis, treatment decision, and monitoring response to therapy.

There are a number of critical viral infections that have dominated the research and biomarker landscape. Many of these findings rely on somewhat simple or “off the shelf” technologies that are fairly straightforward to use. Perhaps the simplest of these technologies is the surface-enhanced laser desorption/ionization (SELDI) technology. In a study for SARS detection, Kang et al. developed a mass spectrometric decision tree classification algorithm using SELDI-TOF MS. Serum samples were grouped into acute SARS and non-SARS and healthy control cohorts. Diluted samples were applied to WCX-2 ProteinChip arrays (Ciphergen), and the bound proteins were assessed on a ProteinChip Reader (Model PBS II). The results clearly indicated an impressive accuracy for discriminatory classifiers [22]. Another similar study indicated that nine serum markers significantly increased and three significantly decreased in SARS patients as compared to controls [32].

Another ProteinChip assay used to study HIV-1 infection showed a unique MDM protein fingerprint during HIV-associated dementia (HAD) and HAART. Seven unique protein peaks between 3.0 and 20.0 kDa were found in the HAD MDM samples, all of which were abrogated after HAART [33]. A very similar study using specific proteins produced from monocytes from HAD patients

showed a total of 177 protein peaks from 2 to 80 kDa in 31 MDM lysates. Select protein peaks, at 5028 and 4320 Da, separated HIV-1-infected from HIV-1-seronegative subjects with 100% sensitivity and 80% specificity [34].

However, most viral proteomics studies to date have utilized either 2DE and MALDI-TOF MS or LC/MS/MS. The HIV virion is composed of a lipid bilayer that surrounds the viral capsid (Fig. 1A). In a clever study, Fuchigami et al. [35] studied the HIV-1(LAV-1) particles, which were collected by ultracentrifugation, treated with subtilisin, and then purified by Sepharose CL-4B column chromatography to remove microvesicles. The lysate of the purified HIV-1 particles was subjected to 2DE and stained, and the stained spots were excised and digested with trypsin. The resulting peptide fragments were characterized by MALDI-TOF MS. Twenty-five proteins were identified as proteins inside the virion, and the acid-labile formyl group of an amino terminal proline residue of HIV-1(LAV-1) p24(gag) was determined by MALDI-TOF MS before and after weak-acid treatments (0.6 N hydrochloric acid) and confirmed by postsource decay (PSD) of the *N*-formylated N-terminal tryptic peptide (*N*-formylated Pro(1)-Arg(18)). Interestingly, formylation plays a critical role in the formation of the HIV-1 core for conferring HIV-1 infectivity [35].

More recently, the use of liquid chromatography and tandem MS (LC/MS/MS) has also eased purification and recovery methods. For instance, Varnum and colleagues utilized gel-free two-dimensional capillary LC/MS/MS and Fourier transform ion cyclotron resonance MS to identify and determine the relative abundances of viral and cellular proteins in purified HCMV virions and dense bodies. Analysis of the proteins from purified HCMV virion preparations has indicated that the particle contains significantly more viral proteins than previously known. They identified more than 71 HCMV-encoded proteins and 70 host cellular proteins in HCMV virions, which included cellular structural proteins, enzymes, and chaperones [6]. Another study using LC/MS/MS for the adenovirus type 5 proteome found a total of 11 protein species from 154 peptides, at a sensitivity of 10 copies per virus and a detection limit of 70 fmol for two proteins [36].

Two new methods have been used recently to decipher viral proteomes. A method for proteolytic stable isotope labeling was recently used to provide quantitative and concurrent comparisons between individual proteins from two different proteome pools or their subfractions. Using this technique two ^{18}O atoms were incorporated universally into the carboxyl termini of all tryptic peptides during the proteolytic cleavage of proteins in the first pool. Proteins in the second pool were analogously cleaved with the carboxyl termini of the resulting peptides containing two ^{16}O atoms (i.e., no labeling). The method was used to compare two virus strains, adenovirus types 2 and 5. This shotgun approach for proteomic studies with quantitative capability may be a very powerful tool for comparative proteomic studies of very complex protein mixtures [37]. Finally, the isotope-coded affinity tag (ICAT) procedure has also yielded some very interesting results for a

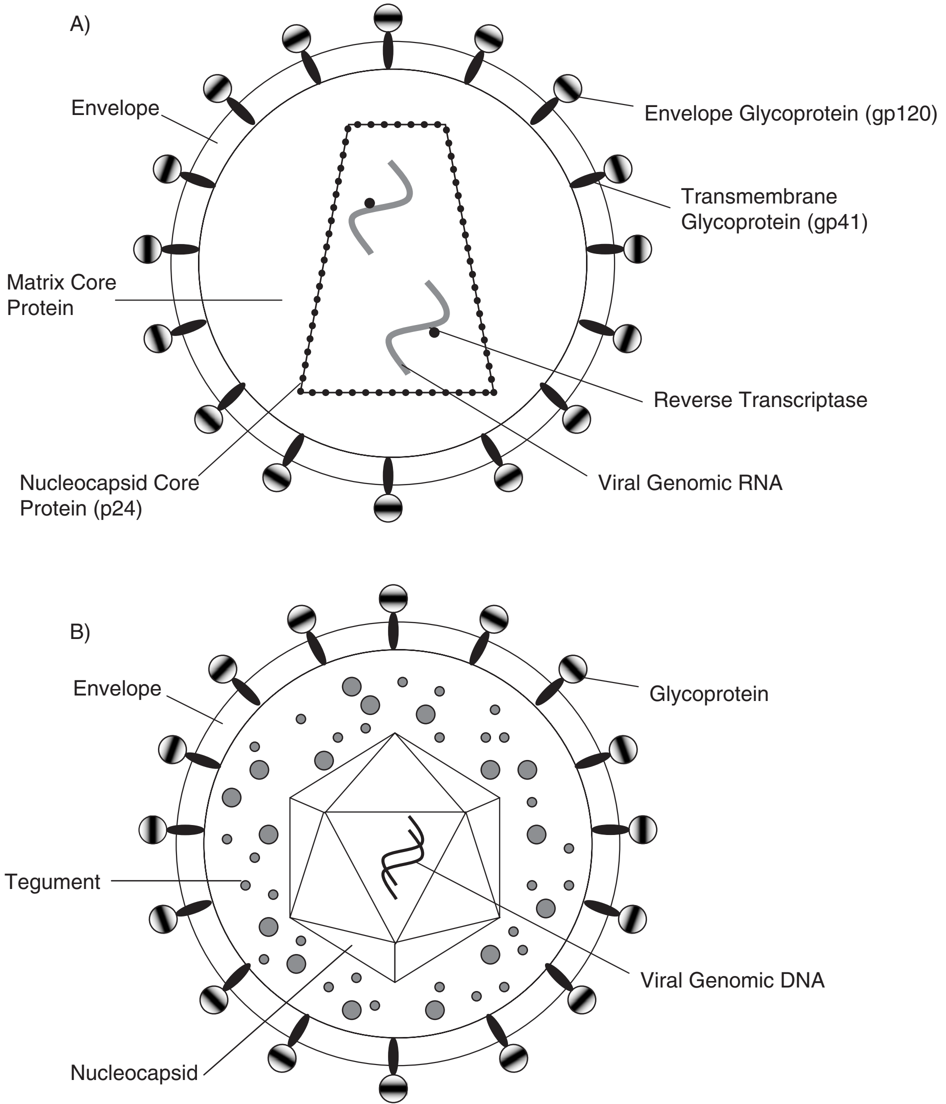

Fig. 1. *Virion structures.* (A) *HIV virion*. The HIV virion is composed of a lipid bilayer membrane (envelope) that surrounds the capsid. Two viral glycoproteins (gp120 and gp41) are part of the envelope and are important for viral binding and entry. The capsid is composed of the matrix core and nucleocapsid (p24) core proteins and surrounds two copies of the viral genomic RNA and reverse transcriptase. (B) *Herpesvirus virion*. The herpesvirus virion is composed of a lipid bilayer membrane (envelope) that surrounds the tegument and the capsid. Viral glycoproteins required for binding to and entering the host cell are imbedded into the envelope. The tegument is an amorphous proteinaceous structure that contains a variety of viral and cellular proteins. The herpesvirus capsid is an icosahedron of 150 hexons and 12 pentons that surrounds the double-stranded DNA genome.

rather complex viral infection setting. For instance, proteins from human liver carcinoma cells, representing transformed liver cells, and cultured primary human fetal hepatocytes (HFH) were extracted and processed for ICAT chromatography. Proteins from hepatitis C virus-infected cells and corresponding control cells were labeled with light and heavy cleavable ICAT reagents, respectively. After the labeled samples were combined, trypsinized, and subjected to cation-exchange and avidin-affinity chromatographies, the resulting cysteine-containing peptides were analyzed by microcapillary LC/MS/MS. Using SEQUEST and other bioinformatics software a total of ~1500 proteins or related protein groups were identified in three subdatasets from uninfected and infected cells [38]. Collectively, these results further emphasize the new targets for biomarkers and drug development for HCV infection.

The described new technologies have collectively added to our arsenal of possible biomarkers when diagnosing various viral infections. However, many of these markers still need to be validated using more rigorous sample methods, biological and biochemical tests, and more sophisticated bioinformatics tools. Bioinformatics tools that have been valuable for viral diagnosis and fast retrieval of DNA or protein sequences include the ORFer program (http://www.proteinstrukturfabrik.de/orfer), 2D proteome database (http://proteome.btc.nus.edu.sg/hccm), the Poxvirus proteomics database (http://contact14.ics.uci.edu/virus/vaccinia.php), and VirGen (http://bioinfo.ernet.in/virgen/virgen.html).

In this chapter, we will explore the importance of proteomics in studying virus–host interactions in several viral systems including HCMV, KSHV, EBV, HSV, HIV-1, HTLV-1, and HCV. We will also describe the methods that have been employed to study viral disease progression using several techniques including 2DE, LC–MS/MS, SELDI, and protein microarrays.

2. Virus–host interactions

Viral proteomics has included the analysis of viral particles to determine all proteins—viral and cellular—that compose the infectious virus, the examination of cellular proteins associated with a single viral protein in the hopes of determining all the functions of that viral protein, or the determination of cellular proteins induced or altered during a particular disease state. Identification of viral proteins requires that the viral genome has been fully sequenced and potential ORFs have been identified. Presently, over 1200 different viral genomes have been sequenced, annotated, and deposited in public sequence databases (GenBank, EMBL, and DDBJ) [39]. Additionally, the National Center for Biotechnology Information (NCBI) has established a Viral Genomes Project to provide standards for viral genomic research [39]. This resource will further the research of virus proteomics. The viral proteome of several herpesviruses, hepatitis C virus, human

T-lymphotropic virus (HTLV), and the HIV have been analyzed and will be reviewed here.

2.1. Proteomics of herpesvirus virions

Viral particles of HCMV and Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 (KSHV/HHV-8) have recently been examined. During the herpesviral replicative cycle, different viral particles are formed. For HCMV, this includes mature, infectious virions, noninfectious enveloped particles, and dense bodies [6]. Similarly for KSHV, only a portion of the produced virus particles is infectious [40]. Therefore, analysis of infectious virions requires their separation from the noninfectious and immature forms. Density ultacentrifugation gradients are typically used to separate the various forms. Each fraction can be analyzed by electron microscopy to determine the level of purity [6,41] or by assaying for viral DNA and an envelope glycoprotein [40].

The herpesviruses are large enveloped DNA viruses (Fig. 1B). The viral particle consists of a lipid envelope, in which are embedded viral glycoproteins important for infection of target cells. The envelope surrounds an amorphous proteinaceous structure called the tegument [42]. The tegument is often composed of viral proteins critical for the initiation of viral gene expression, for example the VP16 protein of HSV [43], as well as other viral and cellular proteins whose functions are unknown. The tegument surrounds the viral capsid, which is composed of a major capsid protein, one or more minor capsid proteins, and viral DNA. Identification of tegument and capsid proteins can be differentiated from the envelope glycoproteins by their differential sensitivity to trypsin and detergents. The tegument and capsid proteins are resistant to trypsin digestion in the absence of detergents. The envelope glycoproteins, however, are sensitive to trypsin digestion whether or not detergents are present. However, only the surface-exposed portions of glycoproteins are sensitive to trypsin in the absence of detergents.

2.1.1. Identification of proteins in HCMV particles

Following gradient purification of virions, LC/MS/MS was used to identify the components of the HCMV virion [6]. The results were verified by coupling high-accuracy mass measurements with LC and FT-ICR (Fourier transform ion cyclotron resonance) MS. Fifty-nine proteins were identified including 12 proteins encoded by known HCMV ORFs not previously known to reside in virions. The classes of proteins identified included capsid proteins, tegument proteins, glycoproteins, and 12 proteins involved in DNA replication and transcription. Additionally, 12 more viral polypeptides were identified that had not been previously characterized [6].

Using the intensities in the FT-ICR spectra, the relative quantities of the virion proteins were determined, indicating that 50% of the virion was composed of

tegument proteins, 30% were capsid proteins, 13% were envelope proteins, while 7% were undefined proteins. These undefined proteins are likely to be cellular proteins that are incorporated into the virion. Host cellular proteins were detected by comparison with peptides predicted from a human-FASTA database. There were 71 cellular proteins identified to be associated with the HCMV virion. They included cytoskeletal proteins, proteins involved in translation control, and several signal transduction proteins [6]. The identification of cellular proteins involved in translation and signal transduction as components of the HCMV virion suggests that these proteins may have a function in the initiation of viral gene expression or inducing an environment that is suitable for HCMV infection.

2.1.2. Identification of proteins in KSHV particles

KSHV has only been fully sequenced in the last 10 years [44], and therefore not much is known about the composition of the virus particle. Nealon et al. [41] used SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting to identify the major capsid and scaffolding proteins as components of isolated virions. They then used ion trap MS to identify three additional components of the virion as ORFs 62, 26, and 65. This study, however, was limited and unable to identify all components of the infectious virus. Zhu et al. used a more comprehensive approach to identify virion components. Extracellular virions were purified by double gradient centrifugation. SDS-PAGE revealed 30–40 protein bands whose identity was determined by in-gel trypsin digestion, followed by LC and MS. Both peptide masses and peptide sequences were produced by tandem MS (MS/MS) and used to determine protein identity. The isolated proteins included five capsid proteins, eight glycoproteins, six tegument proteins, and five other KSHV ORFs. Twenty cellular proteins were also identified and, as seen with HCMV, these included cytoskeletal proteins, signal transduction proteins, as well as heat shock proteins [45]. Similar results were seen in the study by Bechtel et al. [40]. However, fewer proteins were identified in this study as a single 7.5% SDS-PAGE gel was used to separate virion proteins. Zhu et al. [45] used three SDS-PAGE gel—a 4–12% gel, a 3–8% gel to separate proteins larger than 50 kDa, and a 12% gel to separate proteins smaller than 50 kDa. These studies underscore the need for good separation methods to be able to identify all proteins in a virus particle.

2.2. Proteomics of Epstein–Barr virus

The Epstein–Barr virus (EBV) is a B-cell lymphotropic herpesvirus that induces a usually asymptomatic infection and is carried by more than 90% of adults. However, EBV is the causative agent for Burkitt's lymphoma and nasopharyngeal carcinoma and is involved in a number of acquired immunodeficiency syndrome (AIDS)-associated lymphomas. EBV can induce immortalization of B cells *in vitro* to generate lymphoblastoid cell lines (LCLs), a model for the carcinogenic potential

of EBV. LCLs are latently infected with EBV; they maintain the virus as an extra-chromosomal episome, and have limited viral gene expression. The latently expressed proteins are the six EBV nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C, and -LP) and three latent membrane proteins (LMPs 1, 2A, and 2B). Of the latently expressed proteins, EBNA2 and LMP are required for transformation induced by EBV [46]. Characterizations of cellular proteins associated with EBNA2 or proteins differentially expressed in the early stages of the transformation process will be described in the following text. Results of these studies may lead to a better understanding of EBV-mediated transformation and the identification of cellular targets for therapy.

A proteome database of LCLs, before and after transformation, has been developed to identify the cellular mechanisms of virus-induced immortalization [9,47]. 2DE was used to first separate proteins based on their relative charge (pI) and then based on their molecular weight. Differentially expressed proteins were digested and subjected to electrospray ionization MS. Proteins were identified based on their peptide mass fingerprint and amino acid sequences of peptides determined by Edman degradation. There were 32 differentially expressed proteins and 20 were assigned to known proteins. The expression of several proteins involved in proliferation or nucleotide metabolism was increased in the immortalized cells, which may result in the growth stimulation seen in immortalized cells. A database of 2D gel images as well as the identity of the differentially expressed proteins has been made available to the public at www.proteome.jp/2D/. The availability of these images and the identification of the differentially expressed proteins may prove useful to others in their analysis of EBV-infected cells.

EBNA2 is required for transformation of LCLs by EBV [48,49] and induces the expression of c-*myc* [50]. c-*myc* is an oncogene which drives cell proliferation; however, the proliferation program induced by c-*myc* is different than that observed by the expression of EBNA2 [51], suggesting that other cellular proteins and events are induced by EBNA2 to mediate transformation. Furthermore, there is limited information on the cellular targets of EBNA2. To identify the EBNA2-induced changes as c-myc dependent and c-myc independent, EBNA2 and c-*myc*-conditionally expressing cell lines were used and the proteome of each cell line was compared [10]. Proteins were separated by 2D SDS-PAGE and identified by MALDI-TOF MS. In EBNA2 expressing cells, there were 20 differentially expressed proteins; 12 were induced and 8 were repressed. Of the proteins that were induced following EBNA2 expression, several were involved in nucleotide metabolism, protein synthesis, or the control of apoptosis. Many of these proteins were also induced in c-*myc* expressing cells, though six proteins were found to be EBNA2 specific. Two EBNA2-specific proteins were induced (Bid and IgE-HRF) and four were repressed (Annexin IV, γ-actin, GMFγ, and AF103803). Furthermore, analysis of the activation kinetics demonstrated that expression of EBNA2 preceded that of c-*myc*, which was then followed by the expression of Nm23-H1

(a nucleotide diphosphate kinase that may suppress metastasis [52]), indicating that c-*myc* was a direct target of EBNA2 [10].

2.3. Proteomics of herpes simplex virus

Herpes simplex virus (HSV) has been the most extensively studied of the human herpesviruses owing to its ability to easily infect cells *in vitro* to produce infectious virus. As with all herpesviruses, HSV encodes a number of proteins for efficient viral gene expression, viral DNA replication, and the shutoff of cellular gene transcription and translation [53]. These virally expressed proteins do not function in isolation but associate with a variety of cellular and viral proteins. Furthermore, many have exhibited multiple different functions. In an effort to understand the biology of HSV and the function of its proteins, a proteomics approach has been used to study a critical viral transactivator (ICP27), the alteration of the cellular translation machinery, and components of the viral replication complex, which will be reviewed here.

The ICP27 protein is expressed early in infection and is essential for viral replication and expression of certain early genes and virtually all late genes. It is a multifunctional protein that may function with the virion host shutoff (vhs) protein of HSV to repress cellular protein synthesis. This repression serves to direct cellular resources to the synthesis of viral proteins. Using immunoprecipitation of ICP27 from HSV-infected cells followed by SDS-PAGE and MS, several translation initiation factors were identified, including PABP, eIF3, and eIF4G [18]. The interaction of ICP27 with translation initiation factors may recruit these factors to viral mRNA to facilitate translation of viral mRNAs and also to sequester these factors away from the translation of cellular mRNAs.

HSV infection also induces ribosomal changes and it has been hypothesized that these changes may contribute to HSV-mediated translational control of host and viral gene expression [17]. To identify the changes in ribosomes following HSV infection, ribosomes were purified by ultracentrifugation, the proteins were separated by 2D SDS-PAGE, and their identities were determined by MALDI-TOF MS. Seven additional protein spots were found associated with ribosomes following HSV infection, including several viral proteins: VP19C and VP26—components of the viral capsid, and US11—a tegument protein. Three of the seven spots were phosphorylated forms of US11. One nonribosomal protein, PABP, was also found associated with ribosomes. The association of PABP with ribosomes increased following HSV infection [17]. Interestingly, PABP was also found associated with ICP27 in the previous study [18]. Although Greco et al. [17] did not find ICP27 associated with ribosomes, this is likely due to the different separation procedures used. Greco et al. [17] used 2DE and focused solely on basic proteins with a p*I* greater than 8.6 while Fontaine-Rodriguez et al. [18] separated isolated proteins based on molecular weight. Together, these results suggest that ICP27 likely associates with ribosomes in infected cells.

Herpesvirus DNA replication occurs in intranuclear structures called replication compartments [54,55]. HSV encodes seven proteins that participate in viral DNA replication; however, it is not known what cellular proteins are involved in this process. To identify cellular proteins in HSV replication compartments, ICP8, the HSV single-stranded DNA-binding protein, was immunoprecipitated from infected cells and coprecipitating proteins were separated by SDS-PAGE and identified by ion trap MS [19]. Greater than 50 viral and cellular proteins were identified as copurifying with ICP8. The cellular proteins included those that participate in DNA replication/repair/recombination, chromatin remodeling, RNA binding/splicing, and transcription factors. Several of these proteins require DNA binding to associate with ICP8, including several chromatin-remodeling proteins. The roles of a number of interacting cellular proteins are presently unclear and further studies are needed to determine their exact roles in viral DNA replication.

2.4. Proteomics of retroviruses—HIV and HTLV

HIV encodes a critical transcriptional activator, Tat, which directs a cellular transcription factor, pTEFb, to the HIV LTR to mediate transcription elongation [56,57]. However, it has been shown that the viral genome is bound by nucleosomes that inhibit viral gene expression [58,59]. To determine if Tat interacts with additional cellular proteins to further assist viral gene expression, we used Tat peptides linked to biotin to pull down all Tat-associated proteins [60]. Additionally, acetylated and unmodified peptides were also used because acetylation of Tat has been attributed to alternative functions of Tat [61,62]. We found that many more cellular proteins bound to the unmodified Tat, including proteins involved in modification of chromatin structure (CHD2 and p/CAF) and additional transcription factors (TIF1—a TRIM family member—and SCL—a bHLH transcription factor) [60]. These results indicate that Tat influences viral gene expression at various levels and suggests that targeting these specific interactions may be a viable form of treatment of HIV infection and AIDS.

HIV infects several cell types during the course of infection and progression to AIDS. In HIV-infected patients, the virus establishes a persistent infection in cells of the monocyte/macrophage lineage. Monocytes and macrophages are the first line of defense in the immune system: they phagocytose and kill a range of microorganisms. However, little is know about how HIV persists in these cells. To understand how HIV may persist in these cells, Carlson et al. [31] used a “ProteinChip” and SELDI to identify unique protein signatures in HIV-infected monocytes obtained from different donors. Infection of monocytes isolated from humans was used to mimic the virus–host interactions that would occur in an infected individual. The ProteinChips used in the study were used to partially purify samples. One is a weak cation exchange, and the second is a reverse-phase hydrophobic interaction chip. Charged proteins will bind to the cation ion-exchange chip while hydrophobic, i.e.,

membrane-associated, proteins will bind to the reverse-phase chip. Proteins bound to the chip were then analyzed by MS. Each peak represents a protein of a particular mass; however, the nature of the protein in a peak after SELDI MS is unknown. A different, separation technology and MS are needed to determine protein identities. To determine the identities of proteins up- or down-regulated following HIV infection of monocytes, total protein extracts were subjected to trypsin digestion, LC, and tandem MS to determine sequences of tryptic peptides. Sequences were then analyzed against a Protein Bank to determine the identities of the proteins and given a score [31]. The problem with this type of study is that there is no quantitative assessment of the increase or decrease in the protein levels or changes in posttranslational modifications (PTM) following infection.

HAD affects almost one-third of adults infected with HIV [63]. The exact cause of dementia is not known. There is significant neuronal loss but neurons are not infected with HIV [64]. It has been hypothesized that HIV-infected astrocytes are critical in the development of HIV dementia, and that Tat is a contributor to this disease. Extracellular Tat released from astrocytes induces cell death in neurons, though Tat protects astrocytes from cell death [65]. To understand this dichotomy, proteins differentially expressed in Tat-expressing astrocytes were identified [66]. Total protein extracts of Tat-expressing and control cells were separated by 2D gel electrophoresis and identified by MALDI MS. Interestingly, seven proteins were found to be repressed in Tat astrocytes, including Rho GDP dissociation inhibitor and protein phosphatase 2A (PP2A) inhibitor. Many of these proteins have been shown to be involved in the biology of HIV and interact with Tat [66]. Three proteins identified by a slot blot technique were found to be induced, and included HSP70, heme oxygenase, and inducible nitric oxide synthase (iNOS) [66]. Previously published data have demonstrated a correlation between iNOS and the severity of HAD [67,68]; however, the role of the other differentially expressed proteins in astrocyte survival and HIV dementia will require further study.

The human T-cell leukemia virus type 1 (HTLV-1) causes adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [69]. HTLV-1 encodes a transactivator, Tax, that is critical for virus replication and plays a central role in the development of ATL and HAM/TSP [69]. Tax does not bind to DNA directly but functions by interacting with a variety of cellular proteins [69]. Many protein–protein interactions of Tax have been determined by mutational analysis including CREB [70–72] and NF-κB [70,72]. To identify all the cellular proteins that interact with Tax, Wu et al. used chromatography, 2D gel electrophoresis, and mass spectrometric analysis of an HTLV-1-infected cell line (C81) [73]. As Tax functions in both the cytoplasm and the nucleus [70,74], Tax-interacting proteins were identified from both cellular compartments. Some of the cytoplasmic proteins included small GTPases and components of the cytoskeleton while some of the more interesting nuclear proteins included components of the SWI/SNF chromatin remodeling complex [73].

The interaction of Tax with many of the identified cellular proteins may be involved in the ability of Tax to dysregulate cellular functions leading to T-cell transformation and leukemia.

2.5. Proteomics of hepatitis C virus and hepatocellular carcinoma

Hepatocellular carcinoma (HCC) causes approximately one million deaths a year [75]. Two viruses are the main causes of HCC: HBV and HCV [76–78]. Although HBV is the most important cause of HCC, accounting for 80% of HCC cases, an effective vaccine is available [79]. HCV, however, is a major cause of the increasing incidence of HCC in developed countries [80] and no effective vaccine is available. HCC progresses after decades of chronic infection and often is at an advanced stage once it presents clinically [81]. As such, good noninvasive diagnostic markers are needed. This will be discussed further in Section 3. The focus of this section will be on the identification of cellular proteins that are induced following HCV infection or cellular proteins that interact with HCV proteins.

An extensive study by Wirth et al. [82] analyzed normal liver tissue and hepatoma-derived cell lines by 2D gel electrophoresis and N-terminal sequencing and identified a number of proteins that were differentially expressed between normal tissue and hepatoma cell lines. Similar studies have been performed by others as well [83,84]. However, these studies have used cell lines that have been in culture that may not accurately reflect all the changes seen in HCC. Comparison of liver tumor tissue with normal tissue would be ideal; however, tissue heterogeneity is an issue and could confound the results [85]. Until only very recently [86,87], an infectious cell culture model for HCV has not been available. This new model system will allow for the identification of cellular proteins that are induced following HCV infection and further the development of a treatment for HCV.

The HCV genome encodes a large polyprotein that is cleaved to generate 9–10 proteins, including the core and envelope proteins E1 and E2, and the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B [88]. NS5A has been proposed to act as a cofactor in HCV replication [89], as a transcription activator [90], or as an anti-apoptotic factor [91]. To identify the cellular proteins that interacted with NS5A, Choi et al. [26] coimmunoprecipitated NS5A-interacting proteins using antisera against NS5A, separated them via 2D gel electrophoresis, and determined their identity by MS. One cellular protein was found to interact specifically with NS5a, the heat shock protein (HSP) 27, also known as SRP27. Further analysis indicated that HSP27 interacted with the C-terminal domain of NS5A and both proteins were shown to colocalize around the nucleus. The HSPs are induced following cellular stress to protect cells from apoptosis [92]; yet, overexpression of HSP27 did not protect cells from HCV core-induced apoptosis. As HSP27 expression could not be reduced by siRNA, the role of HSP27 in HCV RNA replication could not be determined. However, due to the multifunctional

nature of NS5A, it is surprising that only one protein was found to interact with NS5A. Because an antibody to NS5A was used to coimmunoprecipitate cellular proteins, the antibody used in the study may be interfering with the binding of other cellular proteins. This problem may be overcome by using different antibodies to NS5A or by using a tagged NS5A and affinity chromatography.

3. Diagnostics

Proteomic analysis has provided a unique tool for the identification of diagnostic biomarkers, evaluation of disease progression, and drug development [93,94]. It is also an important approach for clinical diagnostics. In fact, early diagnosis of disease could be possible through the use of unique protein profiles, consisting of a panel of biomarkers that serves as a surrogate marker of disease. Novel diagnostic tests may be generated through proteomic discoveries, and many more proteins can be identified as potential drug targets. These biomarkers are likely to serve multiple purposes, including the assessment of drug efficacy and drug toxicity, and diagnosis. We will review the various methodologies used for viral diagnostics and discuss the advantages and disadvantages of each technique.

3.1. 2DE-MS: SARS, HBV, HCV, and HIV-1

3.1.1. Description

The classical proteomics platforms include 2DE and MS [85]. 2DE is employed to separate proteins in a mixture in the first dimension by their isoelectric points and then in the second dimension by molecular mass. The resulting gel can be stained with a variety of protein dyes to reveal a pattern of spots. In the first dimension, isoelectric focusing (IEF) is performed by using IPG strips which are based on the use of bifunctional immobiline reagents, a series of 10 chemically well-defined acrylamide derivatives that copolymerize with the acrylamide matrix, to generate extremely stable pH gradients forming a series of buffers with different p*K* values between 1 and 13. Subsequently, linear or nonlinear wide (IPG 3–12), medium (IPG 4–7), narrow (IPG 4.5–5.5), and ultra-narrow (IPG 4.9–5.3) pH range IPGs can be cast [95]. We suggest the reader to refer to the review by Gorg et al. for more details about IPG strip rehydration, sample application, and IPG strip equilibration [96]. The second dimension consists of using SDS-PAGE to separate proteins according to their molecular weight. However, the analysis of low-molecular-weight ($<$15 kDa) and high-molecular-weight ($>$150 kDa) proteins is challenging since there is no standard 2DE system that effectively allows separation of proteins over the entire range between 5 and 500 kDa. A common approach is to combine several gels optimized for different molecular weight ranges instead of using a single standard 2DE system.

3.1.2. Application for virus studies

Current methods for the diagnosis of HCC rely on serological markers such as α-fetoprotein (AFP) [97] and certain liver enzymes as well as Des gamma carboxyprothrombin (DCP) [98]. This type of diagnosis lacks the sensitivity to detect HCC at an early stage when therapy can be more effective. To find markers of disease progression, 2DE was employed to resolve and compare proteins present in serum obtained from individuals infected with HBV or HCV and with varying risks for the development of HCC [99,100]. In several studies, proteins expressed at different levels among diseased individuals as compared to those of healthy ones were identified as markers for disease progression as well as proteins with different *N*-glycosylation patterns [99–101]. In another study, 2D-MS was also employed to analyze altered plasma proteins due to SARS-CoV infection. Thirty-eight different plasma proteins from SARS patients were identified, most of which were associated with acute phase proteins [23].

3.1.3. Advantages

One advantage of 2D gels is their resolution since they can resolve as many as 2000 proteins simultaneously and proteins can be detected at greater than 1 ng in one spot [96]. 2DE is currently the only technique that can be routinely applied for parallel quantitative expression profiling of large sets of complex protein mixtures such as whole cell lysates. In addition, 2DE produces a map of intact proteins, which reflects changes in protein expression level, different isoforms, or PTM. In fact, a great advantage of this methodology is its capability to study proteins that have undergone some form of PTM (such as phosphorylation, glycosylation, or limited proteolysis) that can be detected visually on the 2DE gels as they appear as distinct spot trains in the horizontal and/or vertical axis of the 2DE gel. This is in contrast with other methods, including LC-based methods, which perform analysis on peptides, where molecular weight and p*I* information is lost, and stable isotope labeling is required for quantitative analysis [96].

3.1.4. Drawbacks

Although 2D gel electrophoresis is a standard technology, it suffers from several problems that may limit its utility. These include issues with reproducibility, as well as the inability to separate hydrophobic proteins, which are poorly soluble. Although the use of IPG strips increases the reproducibility of 2DE, various problems with 2D separation remain such as streaking, poor focusing, and the variable occurrence of gaps [85]. Although 2DE allows for high resolution of individual spots, a single spot may not correspond to a single protein, since proteins can comigrate as a single spot on a 2D gel [102]. Furthermore, 2DE requires milligram

quantities of protein, reflecting the low sensitivity of this method. To further enhance the utility of 2DE-MS, enrichment of samples for low-abundance proteins by improved methods is required. Enrichment can include prefractionation of samples, as well as more sensitive detection and quantitation methods, or the use of alternative methods including laser capture microdissection [103] for heterogeneous tissues. In most of the studies mentioned previously, the resolution problem was overcome by narrowing the pH range allowing for greater focusing. However, the reduced pH range in IEF can lead to the elimination of a large number of proteins that may be informative. Comparing hundreds of protein spots across gel images taken from a large number of different samples is extremely time-consuming, even with specialized software. For this reason, although 2D electrophoresis is a promising tool, it is not very practical for clinical application. The challenge is to develop this technique into a system capable of automation, high throughput, and high sensitivity.

3.2. LC–MS: HIV-1 and HCV

3.2.1. Description

Multidimensional LC/MS/MS involves solution proteolysis of a complex mixture of proteins, which are then fractionated by high-performance liquid chromatography (HPLC). Peptides are then analyzed by tandem MS consisting of two phases. In the first phase, peptides in each chromatographic fraction are electrosprayed and ionized producing a mass spectrum characteristic of the molecular weight of each peptide in the sample. In the second phase, the first mass analyzer of the instrument is used to select a single $(M + H)^+$ ion from the mixture and to transmit it to a collision chamber, where the peptide undergoes collisions with argon atoms and suffers fragmentation. The resulting fragment ions are then transferred to a second analyzer, which separates them according to mass [104]. The end result is a mass spectrum containing ions characteristic of the sequence of amino acids in the selected peptide. When mixtures are extremely complex, online reverse-phase LC is used to concentrate and separate the peptides before sequencing by MS [105]. An online capillary LC/MS/MS system consists of conventional HPLC pumps, transfer tubing, a precolumn flow splitter, a liquid junction, a reverse-phase microcapillary column, and a tandem mass spectrometer [106].

3.2.2. Application for virus studies

Two studies have used LC/MS/MS to identify differential protein expression in HIV- or HCV-infected cells. In the first study, traditional HPLC (ion exchange and reverse-phase columns) coupled to an ultrasensitive ion trap MS was employed to

identify proteins that were unique to MDM and to identify proteins present in HIV-1-infected MDM lysates by microsequencing [31]. The second study used normal hepatocytes and immortalized human hepatocytes that can be induced to express the entire HCV ORF. The two different cell types were labeled with an isotopically light (^{12}C for stimulated) or heavy (^{13}C, for control cells) reagent called isotope-coded affinity tag (ICAT); the two differentially labeled samples were then combined and digested with trypsin. Digested peptides were separated by strong cation-exchange chromatography, affinity purified with an avidin cartridge, and subjected to LC-ESI-MS/MS. This study led to the identification of 2159 unique proteins that could be used as markers for disease progression.

3.2.3. Advantages

Some of the advantages include automation in sample application, ability to switch columns, and sensitivity, as this method is able to identify proteins at very low levels [107]. In addition, this method has been extensively used for the determination of drugs and hormone levels in human serum [108–111], making it a promising tool for the detection of disease prognosis markers.

3.2.4. Drawbacks

2DE-based proteome analysis provides information about protein abundance at the gel level by comparing staining intensities. However, when peptide mixtures are analyzed directly by LC/MS/MS techniques, the original quantitative information is lost. For this reason, one of the drawbacks of using LC/MS/MS is the dependence on incorporating stable isotope labeling for quantitative proteome analysis involving the addition of a chemically identical form of the analyte(s) containing stable heavy isotopes (e.g., ^{2}H, ^{13}C, ^{15}N, etc.) to the sample.

3.3. SELDI ProteinChip: SARS, HIV, and hepatitis

3.3.1. Description

SELDI-TOF is a proteomic technology that aims at the quantitative analysis of protein mixtures. This technique relies on the use of trapping surfaces that allow differential capture of proteins based on intrinsic properties of the proteins themselves to identify proteins from crude samples without the need for an initial separation step. A small amount of sample can be directly applied to a biochip coated with specific chemical matrices (hydrophobic, cationic, or anionic) or specific biochemical materials, including DNA fragments or purified proteins. Bound proteins can then be analyzed by MS to obtain either the protein fingerprints or the amino acid sequence when interfaced with a tandem MS.

3.3.2. Application for virus studies

The SELDI ProteinChip approach has been employed to study the protein profiles of cells infected with viruses, including severe acute respiratory syndrome coronavirus (SARS-CoV), HIV-1, and chronic hepatitis B virus infection (CHB) [31]. SARS is a viral respiratory illness caused by SARS-CoV. SARS was recognized as a global threat in March 2003, after first appearing in Southern China in November 2002 (http://www.cdc.gov; [20]). Current serological methods used for laboratory diagnosis of SARS fail to guarantee early diagnosis since most are based on the detection of antibodies that are produced 17–20 days after the onset of symptoms. ELISA-based antigen detection tests offer high specificity and reproducibility, but they lack sensitivity. On the contrary, PCR-based methods, including reverse transcription-PCR, lack sensitivity and specificity [112]. For this reason, there is a need to develop a diagnostic methodology that can detect SARS before the onset of the symptoms to allow for specific prevention and treatment measures for SARS. According to recent studies, SELDI-TOF seems to be a promising approach to study the protein profile unique for SARS. Sera from acute SARS patients or from healthy donors were examined to identify serum marker that could distinguish SARS from non-SARS patients. In this study, analysis of spectra accurately classified 36 of 37 (97.3%) SARS specimens and accurately classified 987 of 993 (99.4%) of the controls as non-SARS. In addition, the classification algorithm successfully distinguished acute SARS from other type of infections with very high precision [22]. The same approach was also employed for the discovery of diagnostic proteomic signatures in the sera of patients with CHB having liver fibrosis and cirrhosis. Results show that 30 serum proteomic features formed a unique fingerprint for fibrosis that correlated with the different stages of fibrosis from minimal fibrosis to cirrhosis [66].

In another study that evaluated the protein fingerprints of HIV-1-infected MDM, cell lysates were directly applied on two types of protein chips: weak cation exchange and reverse-phase hydrophobic interaction. After washing to remove the unbound proteins, bound proteins were ionized and their molecular mass/charge ratio was determined using TOF analysis. Analysis of the obtained profiles showed distinct patterns between uninfected and infected MDM [33].

3.3.3. Advantages

The SELDI ProteinChip approach allows for high-throughput protein analysis of crude protein mixtures without the need for a separation step. It is sensitive since it takes advantage of the analytical capacity of MS combined with novel surface chemistry. It can provide a phenotypic fingerprint of complex mixtures. Sample requirements are dramatically reduced, and because this approach employs MS

for its readout, attomolar to femtomolar concentrations of proteins can be detected. Additionally, reproducibility is greater than that of other techniques such as 2D gels; proteins at extreme p*I*s can be identified, a condition that is problematic under normal 2D gel electrophoresis conditions; and finally there is a greater sensitivity and accuracy for low-molecular-weight proteins (<25 kDa) using SELDI, especially below 10 kDa, which is particularly troublesome for 2D gels.

3.3.4. Drawbacks

This method needs a very robust algorithm to ensure specificity of the profile, in that it can distinguish the pattern between disease and healthy individuals with high accuracy, taking into account variations in profiles between healthy individuals as well as persons with a variety of different infections at different time periods in their course of illness. Two additional drawbacks of this approach are the following: (i) The identity of the proteins cannot be discovered and (ii) as the absolute intensity of the peaks is measured in relationship to the most abundant peaks, peaks in low abundance will be masked by the more abundant ones. In addition, this method employs the direct analysis of tissues or biological fluids by MALDI. The main drawbacks of this approach are the preferential detection of proteins with a lower molecular mass and the difficulty in determining the identity of proteins owing to PTM obscuring the correspondence of measured and predicted masses.

3.4. Protein microarray: vaccinia virus

3.4.1. Description

A protein microarray relies on high-throughput amplification of each predicted ORF by using gene-specific primers, followed by *in vivo* homologous recombination into a T7 expression vector. The proteins are expressed in an *Escherichia coli*-based cell-free *in vitro* transcription/translation system. The protein products from the unpurified reactions are printed directly onto nitrocellulose microarrays without further purification [113].

3.4.2. Application for virus studies

This approach was used to determine the complete antigen-specific humoral immune-response profile from infected humans and animals. The vaccinia virus proteome containing 185 individual viral proteins was printed on a chip after cloning and expression. The chips were then used to determine the antibody profile in serum from vaccinia-virus-immunized humans, primates, and mice [113].

3.4.3. Advantages

Once it has been developed and produced, a protein microarray can be a very rapid method (3 days for most of the genes) to comprehensively scan the humoral immune response of vaccinated or infected individuals.

3.4.4. Drawbacks

The generation of a complete proteome is technically challenging. One problem is the amplification of long genes. Furthermore, expression of some proteins in heterologous systems is not efficient. This technique also does not take into account PTM of viral proteins that are expressed in bacteria. Lastly, expression in *E. coli* might lead to folding problems of the protein.

4. Discussion

Proteomic analysis of cellular protein samples began with the development of PAGE [114] and later with the development of two-dimensional gel electrophoresis (2D-PAGE) [115]. These techniques allowed for the separation of proteins based on size (PAGE) or charge and size (2D-PAGE). These methods, however, did not allow for direct identification of these protein bands. Indirect methods such as Western blotting with specific antibodies were required for identification—a slow and laborious process. However, by combining a variety of mass spectrometric methods with PAGE, identification of a larger number of proteins has become possible. These methods have proven invaluable in furthering various avenues of viral research. Proteomic analysis of viruses has included identification of proteins in virus particles, characterization of virus–host protein–protein interactions, and analysis of serum proteins for biomarkers of disease.

One aspect of viral proteomics has been the characterization of virus particles and virally infected cells. Characterization of purified virions has led to the identification of viral proteins that were not originally identified with the virion as well as the identification of cellular proteins associated with the purified virus. For example, analysis of HCMV viral particles identified 12 additional ORFs not previously known to reside in virions as well as the identification of 71 cellular proteins [6]. The importance of these cellular and viral proteins in viral replication or pathogenesis awaits further analysis. Additionally, 12 unique polypeptides were identified that did not correspond to previously identified ORFs [6], illustrating the fact that despite intensive sequence analysis, sequence characteristics of viral promoters and ORFs are still not entirely understood. Analysis of virally infected cells has also led to the characterization of events leading to EBV-induced transformation [9,10,47], identification of cellular proteins induced in HIV-infected

macrophages [31], and identification of cellular proteins that may be involved in AIDS-associated dementia [66].

The characterization of virus–host protein–protein interactions has been intensely studied. Originally most studies have relied on the analysis of the interaction of two proteins or used the yeast two-hybrid system to identify new protein partners of a protein of interest. These studies, however, are quite labor intensive. Furthermore, the yeast two-hybrid system is susceptible to false-positive identifications, cannot be used to identify multiprotein complexes, and typically does not take into account possible PTM that may influence protein binding. Proteomic analysis, however, can be used to identify multiprotein complexes and, when used in the analysis of infected cells, will take into account any PTM that occur in infected cells. Proteomic analysis of infected cells has resulted in the identification of cellular proteins that may mediate HSV IC27-induced repression of cellular protein synthesis [18], and the identification of over 50 cellular and viral proteins in HSV DNA replication [19]. Furthermore, analysis of the HIV Tat and HTLV Tax proteomes identified members of chromatin remodeling complexes as components of these viral transactivator multiprotein complexes [60,73]. Many of these studies will allow for further understanding of virus transcription, replication, and transformation. Additionally, these studies may lead to the identification of unique drug targets. For example, the p-TEFb complex has been shown to be critical for HIV gene expression [56,60,116] and HIV-infected cells are uniquely sensitive to the transcription suppressing effects of the p-TEFb inhibitor flavopiridol [116–118].

A number of viruses are the causative agents of cancer, including EBV, hepatitis B virus, and hepatitis C virus (HCV). HCV is a major cause of the increasing incidence of liver cancer in developed countries [80], though events leading to transformation are not well understood. Until recently [86,87], an infectious cell culture model of HCV has not been available. The lack of a cell culture model has prevented the systematic analysis of changes induced by HCV infection. Alternative approaches to studying HCV transformation have been the comparison of liver tissue and hepatoma-derived cell lines [82–84] and analysis of a single virus (NS5a)–host (HSP27) protein–protein interaction [26]. Further analysis of additional HCV proteins and infected cells will provide additional insights into the nature of this virus and its ability to cause cancer.

One aspect of viral proteomics that is of interest to physicians is the analysis of serum for protein biomarkers of disease. Studies have been performed on patients infected with SARS-CoV, HIV, HCV, HBV, and HIV-1 using a variety of approaches. Some of the methods that have been used are 2D-PAGE followed by MS, LC/MS/MS, SELDI ProteinChips, and protein microarrays. These methods have their advantages and disadvantages. 2D gel electrophoresis allows for resolution of greater than 1000 protein species, can be used for quantitative analysis of expression, and reflects changes in PTM and the identification of isoforms. However, several issues with 2D gel electrophoresis are its lack of reproducibility, the difficulty in detecting hydrophobic proteins, low sensitivity, and the inability

to use a high-throughput method to analyze a large number of samples. LC–MS has the advantages of solubilization of the majority of proteins, automation, ability to switch columns, and sensitivity; however, the ability to quantify changes in protein levels is lost with LC–MS.

The SELDI ProteinChip is unique in that it allows for differential separation of complex protein mixtures based on chemical characteristics such as hydrophobicity or charge, resulting in a decrease in the complexity of the sample analyzed. However, SELDI is considered a soft-ionization method and the results obtained are patterns of protein peaks and not the identification of peptide masses. To ensure the specificity of peak profile for a particular disease state, a robust algorithm is needed. Lastly, protein microarrays have been developed to determine the immune response to a viral infection. The method requires the expression and printing of all ORFs of a pathogen and cross-linking them to a solid support. Protein microarrays would allow for the rapid diagnosis of a particular viral infection. However, expression of a complete proteome is a challenging task. As the proteins are expressed in bacteria, potentially important PTM are lost and proteins may not be properly folded.

Serum is a complex mixture of proteins that is dominated by two proteins—albumin and immunoglobulin (Ig) [119]. The abundance of these proteins means that analysis of serum for potential biomarkers of disease requires either very sensitive methods or separation of albumin and Ig from serum. Several albumin and/or Ig depletion methods have been developed to resolve this issue. Pieper et al. [120] developed a series of chromatographic columns to separate immunoglobulins based on their affinity for proteins A and G as well as columns containing antibodies with specificities for individual proteins such as albumin, fibrinogen, and transferrin. The columns were successful in depleting serum samples of their respective proteins, and use of several columns significantly decreased the complexity of the sample analyzed [120]. Additionally, a mixed-bed column was developed that allowed the simultaneous separation of several proteins, which would allow for automated processing of samples. A similar approach has been developed by Bio-Rad (Affi-Gel Blue) to deplete samples of albumin, enhancing the detection of other proteins in the sample [121]. Affi-Gel Blue has affinity for hydrophobic, aromatic, or sterically active binding sites of protein. Although this product has high affinity for albumin, it may bind other proteins as well, limiting its usefulness. Lastly, Baussant et al. [122] developed a peptide-based approach to deplete albumin. Their approach was based on the fact that although protein G has affinity for the Fc region of IgG, it can also bind albumin less specifically. Baussant et al. modified a peptide of protein G to have a much higher affinity for albumin, which significantly and specifically depleted the serum of albumin; however, other hydrophobic proteins, i.e., apolipoproteins, were also captured [122].

Despite their relatively small size, viruses are fairly complex and encode between a dozen and more than 200 proteins. Many of these proteins are post-translationally modified and interact with other viral and host proteins to function.

Identifying the proteins that are encoded by viruses and the proteins with which they interact will greatly further the understanding of viral replication and pathogenesis and proteomic approaches will greatly facilitate these studies. Lastly, the ability to diagnose cancer or viral infections at early stages will allow for early treatment and reduce the morbidity and mortality associated with these diseases. Proteomic analysis of biological markers in serum should allow for the early non-invasive diagnosis of cancer. Although good reliable methods are available for the analysis of the serum proteome, the abundance of a few proteins, i.e., albumin and Ig, and the low abundance of many other proteins will require methods for separating out the high-abundance proteins and instruments and methods with enough sensitivity to identify proteins at low concentration.

5. Future trends

It is becoming increasingly clear that the field of proteomics may require better and more robust separation methods, sensitive instrumentation, and unbiased bioinformatic tools. 2DE has historically provided a rapid means for separating thousands of proteins from cell and tissue samples in one run. Although this is a powerful research tool and has been enthusiastically applied in many fields of biomedical research, accurate analysis and interpretation of the data have provided many challenges. Several analysis steps are needed to convert the large amount of noisy data obtained with 2DE into reliable and interpretable biological information. The goals of such analysis steps include accurate protein detection and quantification, consistent comparative visualization methods, as well as the identification of differentially expressed proteins between samples run on different gels. To achieve these goals, systematic errors such as geometric distortions between the gels must be corrected by using computer-assisted methods. A wide range of computer software has been developed, but no general consensus exists as a standard for 2DE data analysis protocols.

In search for new diagnostic and therapeutic targets, 2DE has been used to study differential expression of peptides and proteins in various disease entities. However, 2DE usually requires large amounts of starting material, is time-consuming, and reveals only a fraction of the proteins present in a given sample. More recently, the ProteinChip technology coupled with bioinformatics has gained considerable attention. This technique uses SELDI-TOF/MS to screen protein sources for putative disease biomarkers in a spectrum from 2 to 20 kDa. Several studies have provided evidence that ProteinChip technology is capable of detecting early-stage cancer by its unique cancer-specific proteomic fingerprints, with sensitivities and specificities reaching far beyond well-established serum-based tumor markers [123]. However, as in most rapid diagnosis tests, SELDI technology can still not detect the nature of the amino acid biomarkers or their

PTM in a consistent and reproducible manner. Clearly other technologies such as the LC/MS/MS and the LC-FTICR are far more sensitive and better in defining the composition of these biomarkers.

Finally, very recently, much effort has gone into the concept of "Lab-on-a-chip." These chips involve micron-sized channels embedded in glass or silicon chips. Attempts have been made to carry out two-dimensional gel-based experiments on chips. Microchips that are able to carry out microfluidic experiments are being developed (e.g., Nanogen Inc., DiagnoSwiss, Caliper Technologies), which are faster and more accurate than the conventional gel technology. If such technologies were made 2DE compatible then it would offer immense research potential. Especially promising are advancements in detecting low-abundance proteins and PTM.

6. Conclusions

In this chapter, we have discussed the latest new proteomics findings that relate to some of the most important viral infections known to humans. These included HCMV, HSV, EBV, KSHV, HIV, HTLV, HBV, HCV, and SARS infections. In many instances we have seen a mere description of the viral or the infected host cell proteome; however, most of the data to date are descriptive in nature and very few studies have correlated phenotype of the infection to the pathology or drug treatment. Although in some cases investigators have found new enzyme targets as markers (i.e., SARS-CoV), no serious attempts have been made to functionally identify their significance in the pathology of the virus. This is mainly because the field of viral proteomics is at its early stages of development and much confirmatory information would be required from animal or human model studies, which are currently either in progress or will need to be developed in near future. Therefore, a new field of functional viral proteomics is developing in both industrial and academic settings to address issues related to functional biomarkers, drug-resistance viruses, and host/pathogen relations that pertain to disease prognosis, treatment decision, and monitoring response to therapy.

Another challenging consideration is the mixed infections seen in AIDS patients who not only may have varying HIV-1 clade infections (more than seven clades, and close to 1500 genetically distinct HIV-1 genotypes) but also are coinfected with other viruses such as HCV or KSHV. The complication of identifying biomarkers in these patients, or in some instances animal models, has never been properly addressed in the current literature, nor is there enough awareness between various compartments of patient bench to bedside practices. Therefore, a better flow of information using solid epidemiological data followed by better diagnostics for the viral etiology would allow a meaningful identification of the proteome biomarkers seen in these patients. These multiple biomarkers would serve as invaluable tools for multiple drug treatments and better control of mixed infections.

Finally, the issue of frontend purification for the collected test material is perhaps the most important aspect of sample preparation. Currently there are various methods that utilize standard separation techniques to remove most abundant proteins prior to MS, i.e., removal of some 20 high-abundance proteins and better visualization of low-abundance proteins (Sigma-Aldrich kits). However, in most cases the removal of these proteins may in fact compromise the detection of biomarkers or their partners, since in many instances, viral infection leads to overexpression of the most abundant proteins such as the actins, keratins, tubulins, cyclophilins, vimentin, and HSPs among others. Therefore, future attempts at the identification of biomarkers would have to define not only the most high- and low-abundance proteins but also their partners and possible modifications.

Acknowledgments

This work was supported in part by grants from the George Washington University REF funds to Drs. Fatah Kashanchi and Akos Vertes, and NIH grants AI44357, AI43894 and 13969 to Dr. Fatah Kashanchi. We would like to thank Cynthia de la Fuente and Kylene Kehn for their contributions and review of the literature and Lynne Mied for the development of figures.

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