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Applications of Proteomics in the Study of Inflammatory Bowel Diseases: Current Status and Future Directions with Available Technologies

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Abstract

Inflammatory bowel diseases (IBD) are chronic, heterogeneous, and multi-factorial intestinal inflammatory disorders. Major challenges in IBD research include identification of major pathogenic alterations of genes/proteins as well as effective biomarkers for early diagnosis, prognosis, and prediction of therapeutic response. Since proteins govern cellular structure and biological function, a wide selection of proteomic approaches enables effective characterization of IBD pathogenesis by investigating the dynamic nature of protein expression, cellular and subcellular distribution, post-translational modifications, and interactions at both cellular and subcellular levels. The aims of this review are to 1) highlight the current status of proteomic studies of IBD and 2) introduce the available and emerging proteomic technologies that have potential applications in the study of IBD. These technologies include various mass spectrometry technologies, quantitative proteomics (2D-PAGE, ICAT, SILAC, iTRAQ), protein/antibody arrays, and multi-epitope-ligand cartographie. This review also presents information and methodologies, from sample-selection and enrichment to protein-identification, that are not only essential but also particularly relevant to IBD research. The potential future application of these technologies is expected to have a significant impact on the discovery of novel biomarkers and key pathogenic factors for IBD.

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a prevalent, chronic, inflammatory disorder of the gastrointestinal tract (1). With more than a million diagnosed patients in the US alone, and a prevalence of ~0.2% of the western population, IBD has caused enormous suffering and health-care costs (more than \$1.2 billion total annual US estimated medical costs in 2000) (2,3). It has been thought that IBD pathogenesis is the consequence of an overly aggressive cell-mediated immune response to commensal enteric bacteria in a genetically susceptible host (1,4). Although major advances have enhanced the understanding of the multifactorial influence of genetic, environmental, microbial, and inflammatory determinants on IBD, the etiology of the disease remains elusive (4,5).

Clinically, early diagnosis may allow timely therapeutic intervention to minimize disease progression and cellular/pathologic changes that occur in many patients with IBD (6). Furthermore, intestinal metaplasia via a sequential series of dysplastic events (although still controversial) has been shown to transform into neoplasia and therefore predispose IBD to colorectal carcinoma (7). A delay in diagnosis may therefore squander the window of

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opportunity during which aggressive therapy might alter the long-term course of the disease (8). Therefore, a broad understanding of the biology underlying the disease processes in IBD is necessary to reduce disease related morbidity and mortality. Since biological and functional output of cells is governed primarily by proteins, characterization at the level of the proteome is necessary to resolve the critical changes that occur at different stages of IBD pathogenesis. Proteomic technologies also provide new tools in the identification of novel biomarkers for disease activity, diagnosis, and prognosis.

Current proteomic methodologies are beginning to have a profound impact on the way and capacity by which we profile protein expression and post-translational modifications, functional interactions between proteins, and disease biomarkers (9,10). It is important to note here that, although the applications of proteomic approaches in IBD are still in its infancy, its potential is unlimited. The aims of this review are, in addition to discussing its current status in the study of IBD, to introduce the currently available proteomic technologies to the IBD research community.

I. Proteomic Approaches

Current proteomic methodologies have been classified into three sub-categories: mass spectrometry (MS)-based technologies, array-based technologies and imaging MS [see review (11)]. The most explored area of proteomic applications is the discovery of disease-specific biomarkers in body fluid (such serum, plasma, and urine), tissues, and other biologic samples (9,10,12). Proteins are represented by several hundreds of diverse post-translational modifications (13,14) whose functional state varies depending on their respective modifications, alteration of conformation, transport, and translocation (15). The challenges in proteomics impinge on techniques that require not only accurate protein fractionation, identification, quantification and proteome-bioinformatics, but also careful selection and reproducible processing of tissues/samples to be analyzed. This is illustrated along the representative workflow approach for all proteomic studies (16), which includes: a) sample selection b) protein preparation c) protein separation d) protein identification, and e) proteome-bioinformatics. These continually evolving protein technologies, combined with increasing data-gathering/analyzing capabilities, will undoubtedly enhance our capability to 1) better characterize intestinal “inflammatory proteomes” which are critical in IBD pathogenesis and 2) more efficiently identify protein-based IBD biomarkers.

I.1. Mass spectrometry (MS)

MS, an indispensable core of proteomic technologies, allows highly sensitive and high-throughput identification of proteins/peptides, and the post-translational modifications. MS technologies have been extensively reviewed recently (9,11,13), and therefore details of these technologies will not be the focus of this review. Briefly, a large variation of MS technologies is currently available, evolved from electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) to a new generation of mass analyzers and complex multistage instruments [such as hybrid quadrupole time-of-flight (Q-Q-TOF) and tandem time-of-flight (TOF-TOF) instruments] (9,17). While newer and higher capacity MS technologies (such as LTQ-FT-MS and Orbitrap type of analyzers) are being developed (18,19), the most commonly used MS technologies includes, but are not limited to, MALDI-TOF, SELDI-TOF, MS/MS (tandem MS).

MS technologies have been used in several studies relevant to IBD. Since variants in the bacterial sensor domain of NOD2 are genetically associated with an increased risk for the development of CD, Weichert et al. looked at global protein expression changes after bacterial proteoglycan fragment muramyl dipeptide (MDP) stimulation (20). Differentially regulated proteins were identified by MALDI-TOF-MS and MALDI-MS/MS (20). The

authors showed the role of complex pro-inflammatory program regulated by NOD2 that encompasses the regulation of key genes involved in protein folding, DNA repair, cellular redox homeostasis, and metabolism (observed both under normal growth conditions and after stimulation with MDP). This study demonstrated the significant influence of genetic variations in the NOD2 gene in the pathophysiology of chronic IBD. Additional studies using involved in MS application are reviewed throughout the sections that follow.

I.2. Quantitative/Comparative Proteomics

One of the most significant usages of proteomics is to quantitatively identify proteins changes or compare proteomes between normal and different disease states. Current development of quantitative proteomic approaches has facilitated the MS application to biomarker discovery for various diseases (the word “quantitative” is really a relative term not absolute). The commonly used quantitative proteomic methodologies are 1) **gel-based** 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and 2-dimensional difference gel electrophoresis (2D-DIGE); 2) “**gel-free**” isotope-tagging/labeling technologies, including ICAT (isotope-coded affinity tagging), SILAC (stable isotope-labeling with amino acids in cell culture), proteolytic ^{18}O labeling, and iTRAQ (stable isotope-tagged amine-reactive reagents) [see review (11,21)], and more recently, “label-free” MS-based proteomics (22). In general, gel-free methods can address many of the shortcomings of gel-based approaches, which is tedious and inefficient in resolving proteins that are low abundant, insoluble or large (>200 kDa). Depending on investigators’ selection of biosamples or study models (see **Section II**) or the accessibility to proteomic instrumentation, these technologies are all suitable for identifying IBD biomarkers or differentially expressed proteins in IBD, although, so far, few have been used in the study of IBD.

I.2.1. 2D-PAGE and 2D-DIGE—This topic is discussed in details in **Section V.1.1**.

I.2.2. ICAT—ICAT is one of the most employed chemical isotope labeling methods and the first quantitative proteomic method to be based solely on using MS (11,22,23). By labeling cysteine residues of proteins from two different sources with ^{12}C and ^{13}C , respectively, ICAT allows comparison and relative quantitation of two or more biosamples [see detailed protocol by Haqqani et al (22)].

I.2.3. SILAC—SILAC is an easy, reliable, and robust MS-based proteomic approach (11,24,25). It labels newly synthesized cellular proteins by non-radioisotope stable isotope-containing amino acids via normal metabolic processes (11,24,25). Availability of multiple isotopically distinct forms of labeling amino acids allows comparison of proteomes of either 2 or 3 different cell populations in a single SILAC experiment (25). SILAC is the only quantitative proteomic approach that enables in vivo protein labeling, primarily using tissue culture cells. Thus, it will be a valuable approach to study IBD using cell culture models (see **Section II.4**)

I.2.4. Proteolytic ^{18}O labeling—This approach uses a protease and H_2^{18}O to generate labeled peptides, which are subsequently subjected to MS analysis for protein identification and quantitation. Application of ^{18}O labeling is similar to ICAT.

I.2.5. iTRAQ—iTRAQ technology (Applied Biosystems) is a powerful multiplexing tool for comparing protein abundance across 4 to 8 samples in one experiment (11,26,27). Samples are trypsin digested, and after labeling (isotope tagging), the iTRAQ labeled peptide samples are combined and after SCX fractionation analyzed on nanoLC-MS/MS. Proteins are quantified by comparing abundances of reporter fragment ions [tags 114, 115,

116 & 117 in 4-plex kit, and additional four (113, 118, 119, 121) in 8-plex kit] in MS/MS spectra. When considering its application in IBD proteomics, it is beneficial to analyze two healthy controls and two patient samples in a single iTRAQ experiment. The obvious benefits of doing this are: a) internal check on reproducibility of sample preparation; b) estimate of biological variability; c) increased confidence in values for quantifying proteins; and d) increase in chances of identifying modified peptides. Although there have been no reports so far using iTRAQ in IBD study, we have applied iTRAQ-labeling shotgun 2D LC-MS/MS approach to evaluate the differential expression of small intestinal brush border (BB) proteins between WT and NHERF1 knockout (KO) mice (C57B1/6B strain) (28). We found that, in NHERF1 KO mice (compared to WT), expression of at least 7 transporters and associated proteins altered [such as SLC16A1 & its chaperon basigin, and intracellular Cl channels CIC-1 & CIC-5] (28). The expression of more than a dozen of other proteins such as non-receptor Tyr kinase YK2 & β -catenin, ezrin & cryptdin 6 was also altered in the NHERF1 KO mice (28). Our study therefore demonstrates that iTRAQ technology can easily be applied to the proteomic study of intestinal diseases such as IBD.

I.3. Protein/antibody arrays (chips)

A protein-array consists of proteins, peptides, protein fragments, or antibodies that are immobilized and arrayed on specially coated glass microscope slides (29). The arrayed proteins can then be used to probe and evaluate samples containing proteins or classes of proteins (30). Significant progress has been made in protein arrays over the past 5 years, including the use of functional protein microarrays, and reverse phase protein microarrays (30). Functional protein microarrays are composed of arrays containing full-length functional proteins/domains (such as kinases, cytokines and chemokines) and can be used to study the biochemical activities of a specific subset of proteins or entire proteome in a single experiment (30). Reverse phase protein microarrays are composed of arrays of cell lysates on a nitrocellulose slide, which are then probed with antibodies against the target protein of interest (31). This process allows the detection of post-translation modifications, which are important in the pathogenesis of IBD. Only a limited number of applications of protein array technologies have been reported in IBD research. Kader et al. (32) used antibody-arrays to examine the relative expression of 78 cytokines, growth factors, and soluble receptors from 65 CD pediatric patients and 23 UC pediatric patients. The authors showed that in CD, 4 analytes (PLGF, IL-7, IL-12p40, and TGF- β 1) were significantly elevated in patients in clinical remissions when compared to those with active disease, while in UC only 1 cytokine (IL12p40) showed significance between clinical remissions and active disease. This study (32) suggests that these cytokines are directly involved in intestinal inflammation in IBD, and is in agreement with our data obtained by multiplex ELISA (33,34).

Since most current major IBD biomarkers are IBD-specific antibodies, either anti-intestinal microorganisms or anti-human endogenous proteins (autoantibodies), protein arrays are excellent tools for screening and identifying novel circulating disease-specific antibodies. In an attempt to identify CD- and UC-specific serological biomarkers, we developed a highly reproducible serum antibody screening protocol using high-density yeast protein chips, each containing 5800 unique yeast proteins that cover essentially the entire yeast proteome (35). Screening was done using sera from 45 children (15 with active CD, 15 with active UC, 15 healthy controls). More than 220 IBD-specific antibodies, which were defined as antibodies that were not present in sera of any normal subject, were identified from serum of patients with UC and CD patients, suggesting a hyper-inflammatory immune response (35). ~20% of the 220 unique yeast proteins recognized by IBD-specific antibodies have human homologues. Among IBD-specific antibodies, ~130 were CD-specific and 40 were UC-specific (35). Additional 50 antibodies were identified in at least one of the CD or UC patients studied (35). Another study presented by Vermeulen et al. (36) at DDW 2008

reported a study using commercial human protein arrays to profile serum IBD biomarkers from a very small cohort of subjects (10 UC, 15 CD and 5 healthy controls). 75 proteins were found to react more strongly with IBD sera than those from healthy controls, while reactivity of another 88 proteins was just opposite. One identified antigen, described as an autoantigen in IBD was pleckstrin homology-like domain, family A, member 1 (Phla1). However, validation experiment showed that the discriminative power of this anti-Phla1 for CD vs UC or IBD vs controls was poor. Due to that fact that the numbers and immunoreactivity of detectable antibodies vary greatly among IBD patients and even among healthy individuals, it's necessary to point out that a large cohort of subjects have to be included for initial screening in order to eliminate "false" hits and capture the true hits before further downstream validation.

I.4. Multi-Epitope-Ligand Cartographie (MELC) technology

Multi-Epitope-Ligand Cartographie (MELC) is an ultrasensitive topological proteomics technology analysing proteins at the single cell level. It allows *in situ* protein detection via fluorescence by creating a highly flexible multiplex detection system, and therefore can trace out large scale protein patterns with subcellular resolution, mapping the topological position of many proteins simultaneously in a cell (37). Using this technology, Berndt et al analyzed the expression of 32 different proteins in endoscopic biopsies from patients with CD (n=10), UC (n=10), and control subjects (n=10), and identified distinct immune response profiles in CD vs UC (38). While the number of CD4+CD7 – memory T cells was increased in the mucosa of patients with both CD and UC, CD4+CD25+ T cells were elevated only in UC, but not in CD and healthy controls. In patients with CD, the number of CD3+CD45RA+ naive T cells was markedly increased. The expression of Bax and active caspase-3 or -8 was decreased in activated (but not naive) memory T cells (38). In UC, however, caspase-8 was positive only in CD4+ T cells that coexpressed NF- κ B. Moreover, several proteins exhibited distinct co-localization patterns with NF- κ B in CD vs UC, suggesting potential interplays of NF- κ B with other cellular proteins in the pathogenesis of CD vs UC (38).

II. Sample selection and Processing

Careful selection and processing of samples for analysis are one of the most important aspects of effective and reproducible characterization of biologically important differences in proteomics. This is absolutely vital for meaningful identification and characterization of proteins important for pathogenesis or biomarkers of IBD. Several factors that will be important to consider for the source of the protein includes the degree and specificity of separation, the depth of proteome coverage, the degree of resolution, extent of enrichment, reproducibility, sample availability and throughput, and cost (39). Samples storage conditions should be consistent and in multiple aliquots (when applicable). As a rule of thumb, biosamples (particularly tissue samples) should be stored in liquid nitrogen for long-term storage. Since there are issues of potential cross-contamination between samples that are submersed in liquid nitrogen during storage (40), ideally, new generation of liquid nitrogen vapor-based freezers may be a better choice, as it will prevent indirect contact between storage tubes via liquid nitrogen). -80 °C freezer can be used for short-term (a few months) storage of tissues, and repeated freeze-and-thaw cycles should be avoided for all samples. All these factors must be considered to an extent before initiating IBD proteomics.

II.1. Mucosal Specimens of Intestinal Biopsies and Surgically Resected Intestine

Clinical intestinal tissue samples are the most important source for the effective identification of disease-specific proteins. Intestinal biopsy samples (mucosa) are routinely obtained during endoscopic visits and can be utilized effectively for large-scale proteomic

studies. Surgically resected intestine is another valuable source for obtaining mucosal samples in a much larger quantity. Since these tissue samples contain proteins that may be directly involved in the disease pathogenesis, a dynamic array of disease-specific profiles can be immediately identified. However, two unique features of intestinal mucosal biopsies have to be considered for proteomic analysis of biopsies: 1) intestinal mucosa is enriched in various proteases and therefore controlling protein degradation is always an issue during and post protein extraction, and 2) intestinal mucosa contains multiple populations of cells (such as enterocytes and immune cells, and this heterogeneity is further pronounced in inflamed vs normal mucosa. As a result, when total mucosa is used for analysis of differential protein expression, this heterogeneity will likely lead to over- or under-estimation of the expression of certain proteins. To minimize potential protein degradation, multiple protease inhibitors, particularly phosphoramidon, should be included in protein extraction buffer. We found that a protease inhibitor cocktail (Sigma, Cat # P8340) plus PMSF (1–3 mM) and phosphoramidon (10 µg/ml) could effectively minimize intestinal mucosal protein degradation (41,42). To deal with heterogeneity problem of mucosa, several approaches have been developed to separate enterocytes from lamina propria cells or immune cells (see **Sections III & IV**). Whole biopsies from IBD patients has only been recently used in proteomic studies (see **Section V.1.1**), although no data has been reported using isolated enterocytes or lamina propria cells.

II.2. Biofluids

Biofluids, including serum, plasma, ascites fluid, urine and saliva, enable rapid convenient modalities for biomarker discovery and patient screening that are reflective of immunopathophysiologic changes and therefore are important sources of interest for proteomic analysis (43–45). So far there are only a few reports in using proteomic approaches to profile IBD biomarkers in body fluid (serum/plasma or urine). Using SELDI-TOF-MS (Surface Enhanced Laser Desorption Ionization-TOF-MS), Meuwis et al (46) analyzed protein profiles of 120 serum samples collected from a cohort of 30 CD, 30 UC, and 60 unaffected controls (46). Multivariate analysis generated models that could classify samples with minimum 80% sensitivity and specificity in discriminating groups of patients (46). Using similar approaches, the same group piloted a study of sera from 20 CD patients who showed either response or no response to infliximab (47). In both studies, PF4 (platelet aggregation factor 4) was identified as a potential marker. The intensity level of SELDI peak in which PF4 was identified was inversely associated with infliximab non-responders. However, this association could not be confirmed by ELISA and PF4 did not exhibit any correlation with other disease markers (sCD40L, IL-6 CRP) or clinical indices. Another application of MS-based profiling of serum IBD markers was reported by Nanni et al (48) using MALDI-TOF-MS. The study, which involved a small cohort (15 CD, 26 UC and 22 healthy controls), found that reversed-phase extraction and selection of 20 m/z value gave the best overall predictive value (96.9%). In another study, reported in Digestive Disease Week (DDW) 2008, Subramanian et al (49) analyzed sera from a cohort of 62 UC and 48 CD by SELDI-TOF MS. Biostatistical analysis identified 12 discriminative peaks, with specificity and sensitivity at ~95% and four serum proteins were identified, inter alpha trypsin inhibitor 4, Apolipoprotein C1, platelet activated factor 4 variant, suggesting the utility of serum proteomic profiling in IBD.

II.3. Immune Cells

The hallmark histopathological feature of IBD includes the infiltration of acute and chronic inflammatory cells into the affected intestine. Monocytes/macrophages, neutrophils, and lymphocytes are important cellular mediators in the initiation and perpetuation of this inflammatory response in IBD, and therefore provide important clues into the pathogenesis of the disease (50). The exquisite repertoire of available antibodies against cell-surface

antigens on immune cells provides powerful tools for rapidly isolating and characterizing various surface antigen-specific immune cell populations, commonly done using magnetic-isolation techniques, fractionation and fluorescence-activated cell sorting (51,52) (also see **IV.3. Immunoisolation**). It was the application of proteomics that uncovered the extensive temporal reorganization of the lipid raft proteome following T-cell antigen receptor triggers (53) implying the importance of immune cell proteomics. Loyet et al. utilized MS to profile differential expression of cell surface proteins on primary human T helper (Th1 and Th2) cells from plasma membrane preparations with the stable isotope labeling by amino acids in cell culture (SILAC) approach (54). The authors performed both proteomic and microarray analyses concurrently and found that the greatest difference between Th1 to Th2 ratios were observed for BST2 (bone marrow stromal protein 2) and TRIM (T cell receptor interacting molecule) (54). While the investigation of the proteomic profile of immune cells significantly increased our understanding of immune-mediated signaling (54–56), the proteomic study of isolated immune cells in IBD has not been adequately explored. In one study, Liu et al (57) analyzed the protein differential expression in lymphocytes isolated from mesenteric lymph nodes of rats with TNBS-induced colitis by 2D-PAGE and MS, and identified 26 altered proteins involved in inflammation, apoptosis, metabolism, cell cycle regulation, cell proliferation, and signal transduction.

Blood monocytes have also been used as an immune cell model for studies of various immunological diseases. Recently, several reports were published examining the proteomic profiles of blood monocytes either among healthy subjects or patients (58–60). Proteomic analysis of purified circulating monocytes in CD vs UC vs healthy controls may provide opportunity to identify new IBD biomarkers, although such a study has not been reported. In a study relevant to IBD, Pabst et al (61) recently characterized the changes of proteins in isolated human circulating monocytes that were primed with LPS by 2D gels and ion trap MS. Expression of 12 proteins was altered in response to LPS, including a sharp increase in IL-1 β precursor. Using 2D gel and MS, Pereira et al (62) analyzed protein changes that occur during differentiation of dendritic cells (DCs) from monocytes (Mo) and during the maturation of immature DCs stimulated with LPS (62). 36 differentially expressed proteins were found. Among the largest differences in expression were actin (21-fold in Mo), Rho GDP-dissociation inhibitor 2 (20-fold in Mo), vimentin (eight-fold in immature DCs), lymphocyte-specific protein 1 (12-fold in mature DCs) and thioredoxin (14-fold in mature DCs). These proteins are directly related to either functional or morphological characteristics of DCs.

II.4. Tissue Culture Cell lines

Cell lines have been used extensively as an in vitro model in the study of the modulation of protein expression in IBD (33,63–65). Various intestinal epithelial cell lines (such as Caco-2, HT29 and T84), immortalized T cells, and EBV-transformed human B-cell lines are some of in vitro models for IBD-related studies (33,63–65). At least three proteomic studies that may be relevant to IBD have been reported using colonic epithelial cells. Hardwidge et al (66) analyzed changes of protein expression in Caco-2 cells in response to enteropathogenic *E. coli* (EPEC) using isotope-coded affinity tag (ICAT)- μ L-MS/MS. Although it is unclear if EPEC is involved in pathogenesis of human IBD, mice infected with *C. rodentium*, a mouse homologue of EPEC, showed a mucosal Th1 response and lesions similar to those in murine IBD (67). In another study (68), protein patterns from (35)S-labeled homogenates of human adenocarcinoma DLD-1 cells were compared by 2D-PAGE before and after exposure to interferon-gamma (IFN γ), IL-1 β and IL-6, all of which were implicated in mucosal inflammation. Tryptic in-gel digestion and MS identified differential expression of several cytokine-modulated proteins, including tryptophanyl-tRNA synthetase, indoleamine-2,3-dioxygenase, heterogeneous nuclear ribonucleoprotein

JKTBP, interferon-induced 35 kDa protein, proteasome subunit LMP2, and arginosuccinate synthetase.

II.5 Paraffin-embedded tissues

It was only recently realized that archived formalin-fixed paraffin-embedded (FFPE) tissues can be used as an alternative to frozen tissues for MS-based proteomic analysis [see review and protocols in (69,70)]. Techniques and protocols were developed to reverse (to a significant degree) the formalin-mediated cross-linking, which was once thought irreversible. This provides an exciting new way of using FFPE as resources for those who are interested in proteomic study of IBD, since FFPE intestinal biopsies have been made routinely at the time of endoscopy at research-oriented medical centers from both healthy controls and patients with IBD and other intestinal disorders (such as IBS and infectious diarrhea). In addition, this new proteomic development is particularly valuable since the pathological and clinical information are usually available for the subjects from whom FFPE biopsies are obtained. Although FFPE tissue proteomics is still in the developing/optimization phase, this new approach has led to highly promising results with the proteomic identification and resolution from FFPE tissues being comparable to those from fresh frozen tissues (69,70). It is therefore a highly attractive area for IBD researchers to explore.

III. ENRICHMENT OF SPECIFIC CELL TYPES

Proficient and accurate interpretation of proteomic data depends on the quality and complexity of samples used. Given the masking effect of wide-range of abundant proteins, ideal sample preparation involves enrichment of specific cell type (**Section III**) and/or organelles (see **Section IV**) for analysis of cell-specific proteomes or subcellular proteomes, as well as effective, reproducible solubilization, disaggregation of the proteins and/or to enable the identification of less abundant proteins (see **Section V**) (16,71). Since each sample (serum, cell, tissue) varies individually in their component properties, optimal procedures must be determined empirically for each type of sample.

III.1. *In vivo* and *in vitro* EDTA-mediated epithelial cell detachment

Significant evidence has accumulated that suggests the important role of intestinal epithelial cells in the pathogenesis of IBD and the induction of colonic inflammation (72). Isolation of viable colonic epithelial cells is therefore an important approach for applicable proteomic studies. Methods used include a microbial metalloenzyme-based disperse-collagenase digestion method (72,73), explant-based cultures (74), and more recently, an EDTA-perfusion method (75,76). For example, using the EDTA-perfusion, in combination with MACS-based immuno-subtraction of immune cells with anti-CD3 ϵ , -B220, -CD11b, -CD11c, and -NK1.1 mAbs, Mizoguchi et al obtained highly purified intact epithelial cells (76). Using gene-expression microarray analysis, they examined the expression in purified colonic epithelial cells of the adoptive transfer colitis murine model (76), and identified distinct expressional profile of colonic epithelial cells during acute and recovery phases of colitis and in Th1- and Th2-mediated chronic colitis. This data suggests that the functions of colonic epithelial cells are likely to be differentially regulated during the course of intestinal inflammation, and emphasizes the utility of EDTA-perfusion to isolate colonic epithelial cells for proteomic applications. Since intestinal epithelial cells die rapidly *in vitro* due to loss of anchorage during isolation, the biggest challenge of isolating intestinal epithelial cells is to minimize cell death. An efficient EDTA-mediated enzyme-free isolation technique for human colonic epithelial cells was developed by Grossmann et al (77). The entire isolation process took only 1 h (compared to 2–5 h in previously described methods) and only 2% of cells isolated were apoptotic (compared to 18–84% in previously described

methods) (77). Since the epithelial cells isolated by this method are of high quality (high cell purity and low apoptosis) and no digestive enzymes were added during the process, they should be well suitable for proteomic analysis. Using a similar EDTA-mediated approach previously described (78), we obtained predominantly epithelial cells from mouse small bowel, and then isolated brush borders (BB) from these cells (79). By LC-MS/MS, we performed the first characterization of BB proteome, which contains 570 proteins (79), including 45 transporter proteins. Our data again demonstrated that these epithelial enrichment techniques can be readily applied to proteomic study of GI disorders such as IBD, although so far such a study has not been reported.

III.2. Laser-capture microdissection

As mentioned in **Section II.1**, the intestinal mucosa consists of multiple cell types, each of which contributes its proteome to the whole mucosa. Protocols described in **Sections III.1** are useful for isolation of intestinal epithelial cells, but the cell purity by these chemical or mechanical approaches is always relative. It will be almost certain that some degree of contamination of other cells types will occur. To overcome this problem, techniques capable of isolating pure cell populations are recommended for comparative proteomic analysis of disease and disease-free states. Laser capture microdissection (LCM) technology is the most widely used microtechnology for reliable isolation of pure cell populations from tissue specimens, and has been extensively reviewed (42,80,81). Although application of LCM in IBD proteomics has not been reported, LCM was used to isolate specific cells for PCR analysis of specific gene expression in IBD. Yuki et al. (82) showed that LCM coupled with RT-PCR identified increased receptor-like protein-tyrosine phosphatase (RPTP- β) expression in LCM-isolated colonic epithelial cells, which may stimulate mucosal regeneration during the healing process of colitis. Ryan et al (83) demonstrated by PCR that *Mycobacterium avium* subsp paratuberculosis (Map) DNA was detected in LCM-isolated granulomas in 6 of 15 Crohn's cases, but in 0 of 12 controls. In a separate study (84), the authors also showed that DNA extracted from LCM-isolated granulomas of CD patients detected *E. coli* DNA in 12/15 CD patients and in 1/10 controls. This indicated a tendency for luminal bacteria to colonize inflamed tissue, or the potential of increased uptake of bacterial DNA by gut antigen presenting cells (84). It is obvious that application of LCM in protein analysis is more challenging than that in RNA analysis using PCR since more cells are needed. However, LCM has been extensively used in proteomic profiling of gene expression in various diseases (42,80,81). These studies therefore suggest that LCM is a useful application for sample separation, which when combined with the application of proteomics has great potential in IBD research.

IV. Enrichment of organelles and other subcellular compartments

To reduce the complexity of the cell proteomes and increase the efficiency of protein identification, various techniques have been (or have potentials to be) used in proteomic analysis of specific organelles (such as lipid rafts, lysosome, & endosomes) and/or other subcellular compartments (such as extracellular matrix (ECM) and secreted vesicles, apical vs basolateral membranes) (79,85,86). The simplest subcellular fractionation approach is to use ultra-centrifugation to separate total cellular membranes from cytosol, and/or to further separate subfractionate total membranes into detergent-soluble and detergent insoluble (solubility-based membrane subfractionation; see more in **Section IV.2**), as we previously described (87). More extensive fractionation techniques include, but are not limited to, density gradient fractionation, immuno-isolation, and sequential extraction. A particular benefit of subcellular fractionation is that it allows identification of proteins that are trafficking or translocating between different organelles or between cytosol and specific membranes/organelles as parts of specific signaling/regulation. This is important since total cellular levels of these proteins may remain unchanged in normal vs disease conditions, and

therefore they will not be identified by simply proteomic profiling of whole cell lysate. A good example is the dynamic translocation of Src family kinases, from cytosol to specific membranes (when phosphorylated/activated) or vice versa (when dephosphorylated/inactivated). Src is known to phosphorylate Dectin-1, an innate fungal recognition receptor, and activates the signaling cascade that is involved in IBD pathogenesis (88).

IV.1. Density Gradient Fractionation

Density gradient fractionation, widely used for physical enrichment (rather than purification) of subcellular membrane vesicles (organelles), has been a valuable tool to study specific subcellular localization and dynamic trafficking of proteins (89). Organelle enrichment by density gradient fractionation is one of the major techniques used to reduce the complexity of cellular proteome (85). While sucrose has been the main component of density gradients, synthetic OptiPrep™ (iodixanol) began being used a few years ago for separation of organelles due to its iso-osmotic property (90). We have demonstrated that separation of various subcellular vesicles by OptiPrep density gradient is highly reproducible (87,90). This reproducibility is critical for down-stream analysis, particularly when multiple samples are to be compared. So far, no proteomic study has been reported using subcellular fractionation techniques. However, it is worth noting that O'Morain et al (91) subjected rectal biopsy specimens from CD and UC patients to analytical subcellular fractionation by sucrose density gradient centrifugation, and assayed for various organellar marker enzymes, including plasma membrane, mitochondria, peroxisomes, lysosomes, endoplasmic reticulum, and cytosol. While CD patients had increased plasma membrane activity, UC patients had decreased cytosolic and lysosome activity. CD patients also had selective reduction in particulate activity in non-rectal Crohn's disease, demonstrating lysosomal alterations in both UC and CD (91). This study suggests that the organellar proteomes are different in patients with CD vs UC. Since the differences in healthy controls vs IBD are expected to be even more pronounced, organellar proteomics of IBD will likely identify differentially expressed proteins that may be either involved in IBD pathogenesis or used for IBD biomarkers.

IV.2. Detergent Solubility-Based Fractionation

One of the most frequently used solubility-based fractionation approaches is the separation of detergent-soluble membrane fractions from detergent-insoluble membranes, which include lipid rafts (87,90,92). A large number of recent proteomic studies have generated a stunning list of lipid raft constituents, leading to both enhanced understanding of this unique subcellular structure and controversy (92). Since lipid rafts are lipid microdomains where signaling molecules (particularly in T- or B-cells) are dynamically associated (92), profiling the dynamic changes of signaling proteins in lipid rafts will be an important step to understand molecular mechanism of IBD. However, proteomic study of lipid raft proteins in IBD has not been reported. *Sequential extraction* procedure is another approach that has been described to separate proteins of different solubility (93). Felley-Bosco and Andre used the sequential extraction application in IBD proteomics with the ReadyPrep™ sequential extraction kit (Bio-Rad Inc, Hercules, CA), which is based on four consecutive extractions with a chaotropic agent (15). Application of this procedure to colonic epithelial cells led to 64%, 22%, 10% and 4% of total protein in extracts I-IV, of which, more than 94% of extract IV corresponded to cytoskeleton proteins (including keratin 8, 18, and 19). The authors speculate that phosphorylation of keratins (extract IV) dictated the reorganization of cytoskeletal elements, and hence cellular shape, and is important in the pathogenesis of IBD, particularly since keratin 8-deficient FVB/N mice develop IBD (15,94).

IV.3. Immunoisolation

Immunoisolation of organelles is achieved by using magnetic bead-coupled antibodies against specific protein (antigens) that are exposed at the surface of organelles (or right-side-out membrane vesicles). Compared to density gradient fractionation, immunoisolation yields cell organelles with more purity. A typical example of immunoisolation coupled with proteomics is a recent report by Zhang et al (95), who purified liver plasma membrane (PM) using magnetic beads-coated antibodies against PM proteins flotillin and Na/K-ATPase. Analysis by MALDI-TOF-TOF and ESI-Q-TOF of the purified PM fraction, in which an enrichment of 3-fold in PM and 2-fold less contamination of mitochondria were achieved compared to density gradient fractionation, identified 248 PM proteins.

V. PROTEIN SEPARATION/FRACTIONATION

Designing strategies to enrich or subtract specific groups of proteins for targeted proteomic analysis are based on the unique physical and biochemical properties of different proteins, such as sizes, charges (see **Section V.1**), post-translational modifications (see **Sections V.1 & V.2**), protein-protein interactions (see **Section V.3**). Other approaches of protein separation are antibody-based specific protein purification (see **Sections V.4**). All these methods are effective in protein separation before MS analysis can be directly applied to IBD study.

V.1. Protein separation based on their physical and chemical properties

V.1.1. 2D-Gel Electrophoresis—2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is an approach in which mixtures of proteins are separated by two properties in two dimensions, isoelectric point and protein mass, and enables the simultaneous visualization of thousands of protein spots (22,66). When combined MS and advanced image analysis software, 2D-PAGE allows robust acquisition of important information on protein expression, post-translational modifications, splice variants, and processed proteins (96,97). 2-dimensional difference gel electrophoresis (2D-DIGE) is an adaptation of the 2D-PAGE, in which protein samples are first labeled using fluorescent dyes (Cy2, Cy3 Cy5 etc), and then subjected to 2D separation and multiple images (from different emissions) corresponding to different samples are generated from a single 2D gel (98,99). Several studies using 2D-PAGE to profile protein expression in biopsies from IBD patients have been recently reported: Liu et al (57) analyzed the protein expression in lymphocytes from rat with TNBS-induced colitis and found 26 altered proteins involved in inflammation, apoptosis, metabolism, cell cycle regulation, cell proliferation, and signal transduction. In intestinal epithelial cells from UC patients, 40 proteins with significantly altered expression levels were identified from inflamed compared to noninflamed tissue regions, of which programmed cell death protein 8 (7.4-fold) and annexin 2A (7.7-fold) were the most increased (100). By 2D-PAGE analysis of proteins from the colonic mucosa of UC vs non-inflammatory controls, Hsieh et al (101) identified 13 down-regulated proteins, and 6 up-regulated proteins (mitochondrial proteins, energy generation, cellular antioxidants, and stress-response proteins) differentially expressed in UC mucosa when compared to controls. Using 2D PAGE coupled with MALDI-TOF and LC/MS/MS, Drew et al. (102) analyzed changes of colon proteins to oxidative stress supplemented with salicylic acid in a rat model. Altered protein profiles and associations that were observed may play an important role in protein folding, redox, and oxidative stress mediated mechanisms of IBD.

Using ‘Zoom’ 2D-DIGE techniques, we analyzed the protein profiles of mucosal biopsies from pediatric CD patients and compared with healthy controls (89). From ~3,000 protein spots resolved on the gels, we identified 40 differentially expressed proteins by LC-MS/MS, including 27 up- and 13 down-regulated (89). 7 of the 40 proteins are known to be involved

in inflammation. These studies demonstrated the utility of 2D-PAGE in the applications of IBD proteomics (89). By 'Zoom' 2D-PAGE, we also demonstrated excellent protein resolution of serum samples in which albumin and immunoglobulins were depleted (Fig 1).

V.1.2. Multi-dimensional chromatography—While 2D-PAGE is highly sensitive (depending on staining method) and provides good resolution of proteins, it is labor intensive, time-consuming, and has limitations in protein solubility after isoelectric focusing for hydrophobic proteins, and with its limited molecular weight range (about 7 to 200 kDa) (16). Other methods employed to separate and quantify proteins that do not have some of these limitations include multidimensional chromatography system such as size exclusion chromatography (SEC)/reversed phase (RP), high-performance liquid chromatography (HPLC), or two-dimensional liquid chromatography (2D-LC) (16). When combined with MS, this approach has higher sensitivity, more sample loading capability and is more conformable to automation when compared to 2D-PAGE (16). Collectively, these approaches are sometime referred to as gel-free “shotgun proteomics”, analogous to the shotgun sequencing of genomic DNA (103). Shotgun proteomics has not been reported in IBD research, but this powerful technology is predicted to be widely used.

V.2. Protein enrichment based on their specific post-translational modifications

Posttranslational modifications of proteins exert key functions in a full spectrum of cellular processes such as enzyme regulation, signal transduction, protein trafficking and localization, protein-protein interactions and stability. Due to their crucial functions in cells, protein modifications have become one of the major focuses in proteomic technologies (collectively called “*modificomics*”) (13). Some of the best-characterized modifications include phosphorylation, glycosylation, and ubiquitination (14).

V.2.1 Phosphorylation—Protein phosphorylation occurs at serine, threonine or tyrosine residues. Phosphorylation-dependent signaling is a key regulator of major cellular processes, and has been an important area for proteomic research: *phosphoproteomics* (41,104–106). Tandem-MS has been the most widely used to annotate the phosphoproteome of both whole cells, and subcellular fractions (105,107,108). Other studies include the novel electron transfer dissociation (ETD) approach (109), and those that used MS analysis in combination with substrate-trapping mutants to identify substrates for phosphotyrosine-phosphatases (110,111). Additional phosphoproteomic strategies include MS analysis of 1) immunisolated phosphorylated proteins/peptides using anti-Ser/Thr or anti-Tyr specific antibodies (for proteome-wide phosphorylation profiling) (112), or antibodies against a particular phosphorylated protein of interest (as we identified the novel phosphorylation sites in NHE3 (113), and 2) phosphopeptides or phosphoproteins enriched with metal oxide affinity chromatography (21,41,114). Given the extensive phosphorylation of large number of signaling molecules/kinases during intestinal inflammation [eg: NFkB pathway (115)], phosphoproteomics holds promise in IBD to investigate changes in phosphorylation by specific kinases of interest or on a global proteome-wide scale.

V.2.2 Glycosylation—Protein glycosylation plays a key role in protein stability, folding, activity, and trafficking, and comprises a sub-proteome whose aberrant modifications have also been correlated with malignancies or congenital disorders of glycosylation (116,117). The identification of glycosylated proteins in an entire proteome, referred to as “glycoproteomics”, has therefore gained considerable interest in recent years (118). The most common approach in glycoproteomics has been the 2D-PAGE-MS/MS approach to investigate intact glycoproteins and their glycoforms (119,120). With the novel ETD approach described earlier, many glycosylation sites can be determined from the ETD MS/MS spectra because the glyco group remains on the peptide and is detected in the

fragmentation pattern of such ETD spectra (109). To study enzymatically cleaved glycopeptides, other approaches using 2D-HPLC-MS/MS have been applied (121,122). These glycoproteomic techniques can be readily applied to the study of IBD. However, the value of its application in IBD research is currently uncertain since the role of glycosylation in IBD is not clear.

V.2.3 Ubiquitination—Protein ubiquitination refers to protein modifications by members of ubiquitin superfamily including ubiquitin and small ubiquitin-like modifier proteins (SUMO) (123). Ubiquitination of NF κ B and HIF-1 (hypoxia inducible factor-1) has been recently suggested to play an important role in IBD pathogenesis (124). It's almost certain that ubiquitination of other signaling proteins is also involved. Proteomics tools have been increasingly employed to identify ubiquitinated proteins (123,125–127). Ubiquitinated proteins can be enriched by affinity chromatography and subsequently analyzed by MS for identification and quantification (123,124). Identification and characterization of additional IBD-specific ubiquitinated proteins, along with those known (NF κ B and HIF-1), may not only enhance our current understanding of IBD pathogenesis, but also lead to the identification of additional therapeutic strategies in IBD.

V. 3. Protein affinity subtraction/Serum Fractionation

Since serological biomarkers hold great promise in diagnosis and prognosis of IBD, our discussion will be focused on the serum proteomics. While serum contains significantly high concentrations of proteins that are important in disease biomarker discovery, >99% of total serum also consists of 22 of the most abundant proteins, such as albumin, immunoglobulin, fibrinogen, transferrin, haptoglobin, etc. (128). The high abundant proteins present in the sera significantly mask the low abundant proteins and decrease the resolution of proteomic analysis. It is therefore important to remove the most abundant proteins in order to detect IBD-specific proteins that may be present in low abundance. The most common approach of removing highly abundant serum proteins is immunodepletion (129,130). As an example, we have demonstrated efficient immunodepletion of two major abundant serum proteins, albumin and immunoglobulin from patients with IBD (Fig 1A–C). This approach allows for significant enhancement of the resolution of the low abundant proteins in serum (unpublished data) (Fig 1).

V.4. Immunoproteomics

Boyle's group recently described a novel and rapid semi-quantitative immunoproteomic assay, termed "immunoproteomics" (131,132). They used a single protein G-based reagent (proACTR) for immobilization of antibodies against antigens that are the subjects of interest. Samples (such as serum/plasma or tissue extracts) containing the subjects of interest (antigens) were incubated with proACTR, so the antigens will be captured. The captured antigens were then transferred to a protein chip and directly analyzed by SELDI-TOF-MS. The difference in protein post-translational modifications can also be detected due to mass changes (131,132). This is an alternative approach to ELISA or Western blotting in analyzing changes of any known proteins of interests, such as cytokines/chemokines. The entire assay procedure, from antigen capture to MS quantification, can be completed in only ~45 min (131,132), a significant advantage over other more conventional assay such as ELISA or Western blotting (which takes hours). Using this approach, Boyle et al identified distinct patterns of changes in the levels of cytokines/chemokines among patients with more than a dozen of various inflammatory diseases, including both acute and chronic conditions (131). Specifically, in IBD patients, they found elevations of IL-2, IFN- γ , MCP1, MIP1 α , eotaxin, IP-10 (131). Therefore, this immunoproteomic technique can be really applied in IBD study, particularly in clinical settings due to its rapid and quantitative nature.

CONCLUSIONS

In this review, we have summarized both commonly used and emerging proteomic technologies, with a specific consideration of their potential application in IBD research. It is necessary to point out that another powerful protein/antibody-based technology, multiplex ELISA, was not included in this review. Recently multiplex ELISA has been proven as a robust approach to profile biomarkers of various diseases, and we have demonstrated its potential in profiling and identifying serum cytokines/chemokines as serological IBD biomarkers (32,33). It's evident that the proteomic study of IBD is in a very early stage, when compared to its application in other diseases (such as cancer). With the versatile, robust, high-throughput nature and unique capability of profiling post-translational modifications, proteomic technologies are anticipated to play a major role in our quests to a) unravel the mystery of immunopathogenic mechanisms of IBD and b) discover novel IBD biomarkers. However, like any other technologies, current proteomic methods also have limitations (for e.g. its limited dynamic range). Finally, evolving proteomics technologies will eventually allow establishment of personalized proteome profiles and/or biomarkers, which will be the foundations for individual-based targeted diagnostics and therapeutics in IBD (ie. personalized medicine).

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Fig. 1. Efficient depletion of serum albumin and immunoglobulin in IBD
A. A 2-step protocol was developed to remove albumin and all immunoglobulins (Igs). Proteins obtained from each stage of the depleting process were resolved by SDS-PAGE and stained with Coomassie Blue. **B.** Depletion of Igs (marked by small arrows) by protein A/G beads with a proper serum:bead ratio. Note the gradual depletion of Igs with increasing amounts of protein A/G beads (lanes 2 through 6). Lanes 1 & 2: 120 and 40 μg of whole serum, respectively; lanes 3–6: serum (from 40 μg whole serum per lane) after depletion of Igs with increasing amounts of protein A/G beads; lanes 7–10: recovered Igs removed from serum (with increasing amounts of protein A/G beads; corresponding to lanes 3–6). **C.** Depletion of albumin (marked by large arrows) with ProteoExtract™ Albumin/IgG Removal Kit (EMD Biosciences, La Jolla, CA). The serum in which Igs has been depleted (as shown in **B**) was loaded on the Albumin/IgG column to removal of albumin. Lanes 1&2: 120 and 40 μg of whole serum, respectively; lanes 3: serum (~from 40 μg whole serum) after depletion of albumin; lanes 4–7, recovered albumin that was removed from the serum was loaded on the gel with increasing amounts. Note that since Igs had been removed using procedure described in **B**, only residual amount of Igs [marked by an asterisk (*)] was further removed by the Albumin/IgG column. The far-right lanes in each panel are MW standard (in kDa). Ig-L and Ig-S: large and small subunits of immunoglobulins, respectively. We found that ProteoExtract™ Albumin/IgG Removal Kit was highly effective in depletion of albumin but much less effective in removal of Igs compared with protein A/G beads we used. **C.** High resolution "Zoom" 2D-PAGE gel separation of post-albumin/Igs serum in CD. A representative figure of a Zoom 2D-PAGE showing proteins in the albumin/Igs-free serum from a CD patient precipitated by TCA-acetone (to remove non-protein substances, which otherwise may interfere the resolution of 2D-PAGE). The 1st dimension (IEF) was done using a 24-cm IPG strip (pH4–7; Invitrogen). The IPG strip was then cut into three equal portions, each of which was loaded on a 7 cm pre-cast gradient SDS-gel for 2nd dimension separation. The albumin/Igs-free serum profile was visualized by silver stain. (MW standards are on the far-left). Note the excellent resolution of the total proteins in whole serum with albumin and Igs depleted.