

Application of proteomics in asthma research

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Bronchial asthma is caused by allergic airway inflammation, resulting in reversible airway obstruction, characterized by airway hyper-responsiveness, bronchoconstriction, increased mucus secretion and an increase in lung vessel permeability. The pathophysiological changes in asthma have been attributed to the altered expression of biologically plausible proteins associated with transcriptional pathways, inflammatory mediators, chemokines, cytokines, apoptosis and cell proliferation. Such multifactorial diseases characteristically involve an interplay of many genetic variations of molecular and biochemical pathways and their interactions with environmental factors. The complex nature of the asthma phenotype, together with genetic heterogeneity and environmental influences, has made it difficult to uncover the aspects that underlie this common disease. Recently, genomic and proteomic technologies have been developed to identify associations between genes, proteins and disease. This approach, called 'omics biology', aims to recognize early onset of disease, institute preventive treatment and identify new molecular targets for novel drugs in multifactorial diseases. This article reviews examples of how proteomic technology can be used to find asthma marker proteins (from the cell model to clinical samples). Identification of protein changes in different stages of asthma could provide further insights into the complex molecular mechanisms involved in this disease. These studies provide new insights for finding novel pathological mediators and biomarkers of asthma.

KEYWORDS: asthma • BAL fluid • plasma • proteomics

Asthma is a common chronic inflammatory disorder of the bronchi, characterized by reversible bronchospasm, resulting from an exaggerated bronchoconstrictor response to various stimuli. The pathological features of airway remodeling include goblet cell hyperplasia, subepithelial fibrosis, collagen deposition, mucosal gland hyperplasia, smooth muscle hypertrophy and changes in the extracellular matrix [1]. Inflammation and remodeling are the main causes of airway hyperresponsiveness and chronic airway obstruction, which are features of the pathophysiology of asthma. The pathophysiological changes in asthma have been attributed to the altered expression of biologically plausible proteins associated with transcriptional pathways, inflammatory mediators, chemokines, cytokines, apoptosis and cell proliferation [2].

However, the complex nature of the asthma phenotype, together with genetic heterogeneity and environmental influences, has made it difficult to uncover the aspects that underlie this common disease [3]. Although therapy is still evolving, with the development of technologies such as molecular biology, there is currently no curative treatment for asthma. Over the last

decade, many researchers have attempted to identify underlying mechanism or molecules that are related to asthma pathogenesis using omics technology. This new research field, which includes transcriptomics and proteomics, has contributed to and enlarged our knowledge of asthma. In this article, we will survey current research results and provide some perspective on asthma proteomics.

General aspects of asthma

Prevalence

Although some researchers have reported that the prevalence of asthma has remained the same or fallen in some countries [4–6], a broad consensus exists that in most Western countries the prevalence of asthma has increased over the last four decades of the 20th Century. Currently, asthma is a worldwide disease, with an estimated 300 million individuals affected; this number is expected reach 400 million by 2025 [7].

Classification

Asthma is not currently accepted as a single disease, but is considered a description of various symptoms. The symptoms of asthma include wheezing, chest tightness, coughing and

breathing difficulties, and it is generally accepted that attacks are induced by inflamed airways. Asthma develops as a consequence of the interaction of host factors (which are primarily genetic) and the latter are usually environmental factors. In order to understand the interaction of these two components, the nature of environmental factors should be defined. Many attempts have been made to classify asthma according to etiology as shown in Box 1, particularly with regard to environment sensitizers/triggers. In addition, subtypes of asthma have different patterns of airway inflammation, as well as different response to treatments and clinical courses [8]. On a molecular basis, pathophysiologic mechanisms are largely classified as IgE-dependent acquired immune responses or IgE-independent innate immune responses. Recent studies have identified different asthma subtypes based on variability in clinical and laboratory features and pathophysiological mechanism [9]. The application of proteomic strategies in asthma studies should be carefully considered on the basis of the diversity of asthma subtypes.

Diagnosis & therapy

A correct diagnosis of asthma is essential for drug therapy and investigational studies such as proteomic approaches. A clinical diagnosis of asthma can be prompted by symptoms such as episodic dyspnea, wheezing and cough, as well as by wheezing on auscultation. However, wheezing may be absent even in the presence of the significant airflow limitation of some asthmatics. However, measurements of lung function, and particularly the demonstration of reversibility of lung function abnormalities, greatly enhance diagnostic confidence. The term reversibility is generally applied to rapid improvements in forced expiratory volume

in 1 s (FEV_1) (or peak expiratory flow), measured within minutes after inhalation of a rapid-acting bronchodilator or more sustained improvement over days or weeks after the introduction of effective controller treatment such as inhaled glucocorticosteroids. For patients with symptoms consistent with asthma who have normal lung function, measurements of airway responsiveness to methacholine, histamine, mannitol or exercise challenge may help to establish a diagnosis of asthma. In our study, all patients had a history of dyspnea and wheezing during the previous 12 months plus one of the following: >15% increase in FEV_1 or >12% increase plus 200 ml following inhalation of a short-acting bronchodilator; <10 mg/ml PC20 methacholine; or >20% increase in FEV_1 following inhalation of inhaled or systemic glucocorticosteroids for a period of 2 weeks or longer [10].

Medications to treat asthma can be classified as controllers or relievers. Controllers are medications taken daily or monthly on a long-term basis to keep asthma under symptomatic and physiologic control. Relievers are medications used on an as-needed basis that act quickly to relieve asthma symptoms through reversing bronchoconstriction. Corticosteroids suppress virtually every step of the inflammatory cascade and constitute the cornerstone of asthma treatment. Among the several kinds of corticosteroids, inhaled glucocorticoids have revolutionized the treatment of asthma and have become the mainstay of therapy for patients with chronic asthma [11]. Early intervention, effective dosage and a sufficient period of inhaled steroid therapy would be the best way to control and maintain asthma symptoms [12]. According to asthma management guidelines [13], patients with moderate persistent asthma are recommended to start inhaled corticosteroid (ICS) therapy with medium- or high-dose ICS or low-dose ICS plus long-acting β agonists (LABAs). In patients with severe persistent asthma, medium- or high-dose ICS plus LABA is recommended as a starting therapy. However, a small percentage of asthmatic patients have symptoms that are severe enough to require high doses of glucocorticosteroids in order to establish maximal lung function [14]. In addition, large interindividual variation exists in the treatment responses to ICS [15,16]. Genomic and proteomic approaches are expected to reveal the causative mediators that determine the different responsiveness to inhaled steroids in asthmatics [17].

Leukotriene modifiers, inhaled LABAs, theophylline and cromones have been used as add-on therapies in asthmatics who are uncontrolled on inhaled glucocorticosteroids alone, or as an alternative treatment for adult patients with mild persistent asthma [18–20], some patients with aspirin-sensitive asthma respond well to leukotriene modifiers [21]. Anti-IgE (omalizumab) is a treatment option limited to patients with elevated serum levels of IgE. Its current indication is for patients with severe allergic asthma [18,19] who are uncontrolled on inhaled glucocorticosteroids, although the dose of concurrent treatment has varied in different studies [13].

Factors involved in asthma development

Asthma is a multifactorial disease involving both genetic (familial history of asthma) and environmental factors. It has long been known that asthma runs in families. Initial attempts to quantify

Box 1. Phenotypes of asthma.

Triggers related to the environment

Aspirin or nonsteroidal anti-inflammatory drugs
Environmental allergens
Occupational allergens or irritants
Menses
Exercise
Viral infection
Obesity

Related to inflammatory patterns

Eosinophilic
Neutrophilic
Pauci-granulocytic: epithelial cell?

Related to clinical manifestations

Clinical or physiological phenotypes
Severity-defined: from mild to severe
Exacerbation-prone: brittle versus nonbrittle
Defined by chronic persistent airflow restriction
Treatment resistant: resistance to steroids
Defined by age at onset

Related to biological functions

IgE dependent
IgE independent

the contribution of the heritable element of asthma or its intermediate phenotypes were performed in extended pedigrees [22].

Various molecular biology techniques have been developed, including DNA sequencing and individual gene-association studies, through the association of single-nucleotide polymorphisms with asthma and asthma-related phenotypes. Over 100 genes have been associated with asthma in at least one genetic association study [23].

The completion of the Human Genome Project in 2003 made genome-wide association (GWA) studies possible, which are applied to identify novel genetic variants associated with disease [24]. The first GWA study on asthma was published in 2007 [25]. Moffatt *et al.* found that genetic variants in 'ORM1-like 3' were significantly associated with childhood asthma, and replicated this finding in two independent cohorts [25]. Gudbjartsson *et al.* and Carriere *et al.* also performed GWA studies on asthma and asthma-related phenotypes [26,27].

Asthma certainly has important genetic predispositions, but other contributors also exist. Asthma may be caused by a combination of factors. Various causative substances may sensitize people with genetic predispositions to asthma. These asthma-causing substances can be indoor allergens (e.g., house dust mites, pets, cockroaches and fungi), outdoor allergens (e.g., pollens, dust and fungi), infectious agents (e.g., bacteria, fungi and parasites), occupational contactants, or drug and food additives. It is believed that in a person with a hereditary predisposition toward allergy produces, when sensitized, antibodies that give little immune protection but cause local tissue damage during the antibody–antigen reaction [28]. Contributing factors such as smoking, air pollution, viral infection, diet and parasite infections can increase airway hypersensitivity to triggering materials (FIGURE 1).

Allergen analysis

As mentioned previously, asthma is not considered a single disease, but rather a description of various symptoms. In some cases, it is not enough to diagnose someone with asthma; we should also know the particular phenotype of their asthma to be able to use effective therapies. By knowing causative factors that exacerbate or evoke asthma attacks, we can prevent symptoms simply by avoidance. In fact, several studies have reported an improvement in asthma with the elimination of allergens [29–31]. In cases of allergic (extrinsic) asthma, causative factors can be checked by skin testing. The skin testing can be used to assess indoor allergens, such as dust mite antigens, animal allergens and molds, and outdoor allergens, such as pollens and insects, and

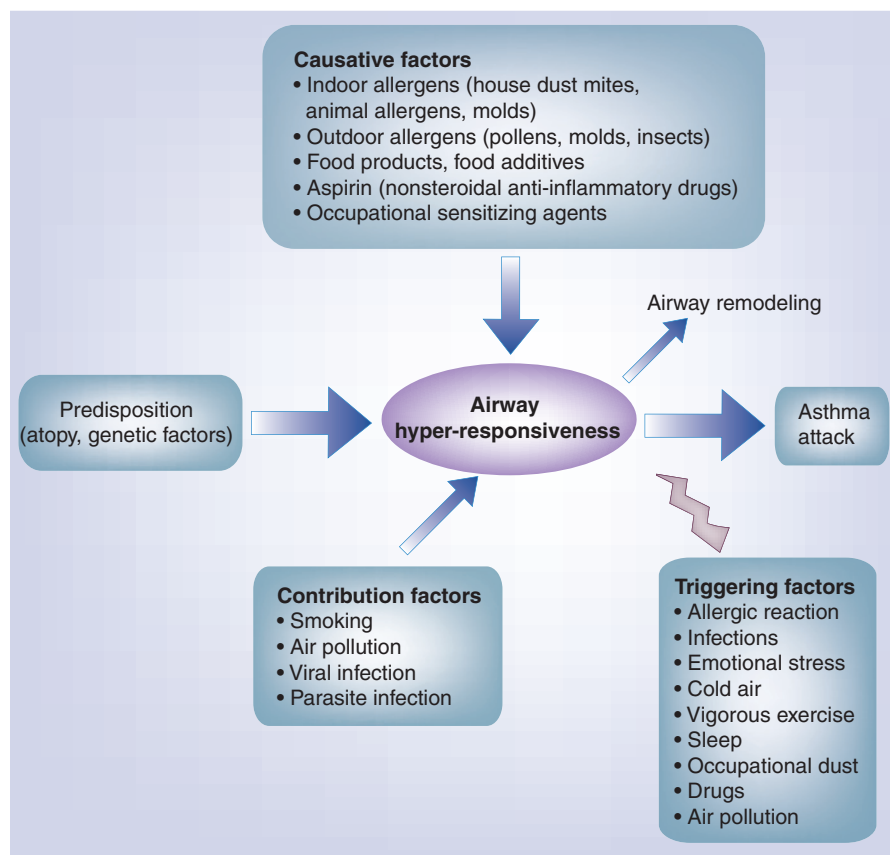


Figure 1. Factors related to asthma development.

Data taken from [8,9,12].

food or food additives. The prick method is the most widely used method in skin testing. In the prick (scratch) test, a few drops of the purified allergen are gently pricked onto the skin surface. This test is usually performed to identify allergies to the most common allergens. Multiple prick tests with various allergens are typically performed.

Pollen & other allergens

Owing to global warming, the amount of airborne pollen has increased [32–34] and the distribution of plants has changed. These changes in plant distribution have resulted in a change in pollen allergens. Allergens that evoke allergic reactions in the body, including pollen, can be analyzed. As shown in FIGURE 2, crude proteins, including target allergens, were separated on 2D electrophoresis (2DE), and then electroblotted onto a polyvinylidene fluoride or nitrocellulose membrane. Blotted proteins were then subjected to Western blot analysis using the patient's serum and a normal healthy control. If the positive spots are only detected with the patient's serum, then the protein spots may contain an allergen. Thus, we can seek to identify the allergen by trypsin digestion and mass spectrometry (MS).

Fujimura *et al.* separated the proteins in *Cryptomeria japonica* (Japanese cedar) pollen on 2DE; the proteins were then immunodetected with IgE in the sera of 40 subjects allergic to *C. japonica* pollen. Numerous *C. japonica* protein spots (allergens) were

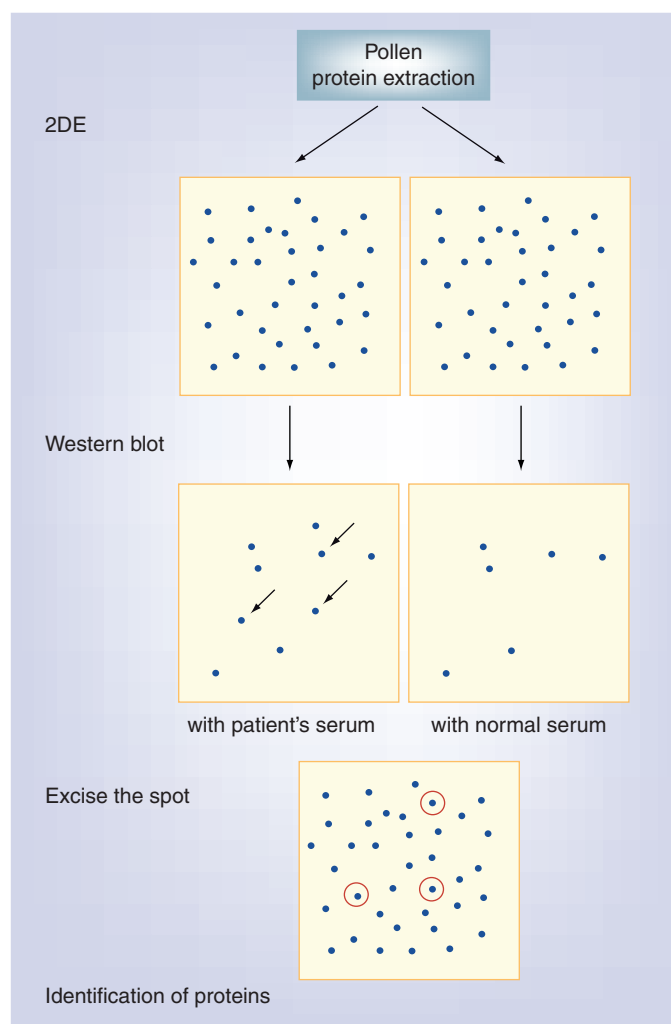


Figure 2. Allergen identification using 2D electrophoresis.

found that showed a higher affinity to IgE than the known *C. japonica* allergens, Cry j 1 and Cry j 2 [35]. Many papers have reported attempts at analyzing pollen allergens by using similar proteomics technology since the year 2000 [36–38].

Rather than detecting antigens by immunoblotting with patient blood, some research groups have used zymography to find proteases, because many major allergens have an associated protease activity closely related to their efficacy in inducing sensitization and inflammation [39–41]. Raftery *et al.* performed a typical experiment using zymography to detect a novel allergen [42]. Pollen diffusates from Kentucky bluegrass (*Poa pratensis*), rye grass (*Lolium perenne*) and Bermuda grass (*Cynodon dactylon*) were assessed for peptidase activity by gelatin zymography. Negatively stained bands on zymography were excised, subjected to tryptic digestion and analyzed by MS [42]. Recently, whole protein spots of pollen that were separated on 2DE were identified [43], as well as all proteins that were differently expressed between high- and low-allergenicity variants [44]. These kinds of high-throughput approaches are expected to be useful with the ever-growing demand for the identification of allergens.

Cell model proteomics

As mentioned previously, many genetic and environmental factors are involved in different asthma outcomes. Therefore, there is no single cell model for asthma research. Thus, proteomics studies using cells in asthma research are primarily performed to analyze the responsible protein expression changes following stimulation of specific cell types.

Cytokine response

T-cell activation or differentiation into Th2 cells is relatively important in the pathogenesis of allergy and asthma. Rosengren *et al.* adopted the 2DE technique to understand the changes induced in human naive CD4⁺ T cells as they were induced by IL-12 to differentiate into Th1 cells [45]. As a result, they found 42 protein spots, including a multifunctional cytokine macrophage migration inhibitory factor, which were differentially expressed following IL-12 stimulation [45]. Cysteinyl leukotrienes (CysLT) and TGF- β 1 are well-known molecules that are increased in asthmatic airways and that influence the pathophysiology of asthma. Altraja *et al.* used 2DE to characterize the inflammatory pathways in bronchial epithelial cells after stimulation with CysLT and TGF- β 1. They found that by downregulating glucocorticoid receptor and heat-shock protein Hsp90a after CysLT and TGF- β 1 treatment, CysLT may interfere with the action of glucocorticoids [46]. Candiano *et al.* demonstrated that IL-4 was a multifunctional cytokine that interacts with many cells, including T cells, B cells, hematopoietic cells and fibroblasts, to stimulate the differentiation of CD4⁺ T cells into Th2 cells and induce B-cell class-switching to IgE. They tried to identify proteins specifically secreted into the airway surface liquid by human bronchial epithelial cells, in both resting conditions and after treatment with IL-4, using 2DE [47]. They found that gelsolin secretion by epithelial cells was increased by IL-4 treatment and suggested that gelsolin might improve the fluidity of airway surface liquid in asthma by breaking down filamentous actin, which may be released in large amounts by dying cells during inflammation [47]. In general, cytokine treatments modify the expression of many proteins. However, the systemic effects of these mediators are not fully understood. In order to analyze the systemic changes following cytokine treatment, proteomics should be useful.

Effects of particles & volatile organic compounds

Associations between asthma exacerbation and both particles and indoor volatile organic compounds (VOCs) are well known [48]. The lung function of patients with chronic obstructive pulmonary disease (COPD) declines more quickly in those living in communities exposed to high levels of air pollution versus those living in areas with less pollution [49]. Some researchers have tried to identify the mediators or elements responsible for particulate matters and VOCs. Nel's group was the first to report that organic diesel exhaust particle (DEP) extracts induced a dose-dependent decrease in the reduced glutathione/oxidized glutathione ratio in macrophage cell cultures, in parallel with a linear increase in the number of newly expressed proteins, identified

by 2DE and MS [50]. They also studied intracellular protein phosphorylation induced or reduced by DEP treatment in macrophage cell lines using the phosphoprotein-specific fluorescent dye Pro-Q® [51]. They showed that DEPs induced the phosphorylation of several phosphoproteins, including the MAPK signaling pathways and other oxidative stress pathways [49]. From these results, they suggested a hierarchical oxidative stress model. With a low amount of oxidative stress induced by particulate matter, phase II antioxidant enzymes are induced via transcriptional activation. With an intermediate amount of oxidative stress, activation of the MAPK and NF- κ B cascades induces proinflammatory responses. With a high amount of oxidative stress, perturbation of the mitochondrial permeability transition pore and disruption of electron transfer results in cellular apoptosis or necrosis [52]. However, particulate matter directly stimulates both macrophages and epithelial cells in airways. Thus, our group has focused on epithelial cells. Treatment of BEAS2B cells with bovine serum albumin-coated titanium dioxide particles altered 20 protein spots on the 2DE gel, and these were then analyzed by nano-liquid chromatography (LC)-MS/MS [51]. Macrophage migration inhibitory factor was one protein that was expressed at a higher level with particle treatment; this was validated by assessing transcription levels by semi-quantitative reverse-transcription PCR and protein levels by Western blot analysis and immunohistochemistry [53].

The effects of VOCs were hardly studied until recently. Mörbt *et al.* exposed human alveolar cell cancer (A549) cell line to styrene and analyzed the effects at the proteome level by 2DE [54]. They found 16 proteins, including superoxide dismutase, to be significantly altered due to exposure to styrene and discussed the relevance of oxidative stress as a primary molecular response mechanism of lung epithelial cells to styrene exposure [54].

Animal model proteomics

Animal models of asthma have been well established and are often used in asthma research. The ovalbumin (OVA)-sensitized and challenged mouse is the most widely used model for allergic asthma, while the toluene-2,4-diisocyanate-induced mouse is generally used for occupational asthma [55].

The simplest experimental design is to compare the protein profiles of asthmatic tissue and healthy controls. Houtman *et al.* were the first to compare the proteome from healthy lung tissue extracts with 2,4-dinitrofluorobenzene-challenged nonallergic asthma mice lung tissues [56]. We chose the OVA-sensitized and challenged mouse model. Using 2DE and MALDI-TOF, we identified 15 protein spots, including YM1 and YM2, that were differentially expressed between normal and allergic asthmatic mouse lung tissues [57]. As an extra validation step, we used reverse-transcription PCR to confirm the increased gene transcription of YM1 and YM2. Our results closely matched those from Zhao *et al.*'s research [58]. They used bronchoalveolar lavage (BAL) fluid instead of tissue extracts, but their results revealed the same proteins as those found in tissue extracts. More recently, Calvo *et al.* analyzed allergen-induced airway inflammation and remodeled tissues using SELDI TOF-MS [59]. In this case, the results

differed from previous studies. The different results from 2DE and SELDI-TOF suggest that different methods may produce different results in protein profiling.

A more aggressive experimental design using animal models is the analysis of protein profile differences before and after various treatments. Kwack *et al.*'s study compared the protein profiles among normal, OVA-challenged asthmatics and asthmatics treated with dexamethasone using 2DE and found 36 differentially expressed protein spots [60]. Most of the identified proteins were related to the cytoskeleton, oxidative stress, apoptosis and metabolism. The authors proposed therapeutic mechanisms of dexamethasone in airway inflammation and goblet hyperplasia in this model. However they did not show the exact mechanism.

Liu *et al.* used a similar research design. Dexamethasone, a corticosteroid commonly used in asthma, may be used to help control asthma (a controller). Inhaled salbutamol was used as the reliever medication. Liu *et al.* chose salbutamol, a short-acting β 2-adrenergic receptor agonist, and identified the differentially expressed proteins between asthmatic and salbutamol-treated asthmatic mouse lung tissues [61].

Animal models may solve ethical problems encountered in human studies for drug-response evaluation or responses to various environmental stimuli, so we expect that much more animal model research will be performed in the future.

Human body fluid proteome analysis

Human body fluid proteomics has been one of the most active areas in recent proteomics-based biomarker research [62]. Many different body fluids have been used for proteomic analysis, including blood (serum or plasma), urine, sweat and saliva. Plasma and serum are the most frequently used and most important body fluids in asthma research. In addition, BAL fluids and sputum are other body fluids that can be analyzed. BAL conducted with fiber-optic bronchoscopy has been widely used to collect cells and other soluble components from the epithelial lining fluids that cover the airway and the alveoli. BAL fluid thus contains proteins secreted from different cell types, including epithelial and inflammatory cells, as well as a wide variety of proteins from the bloodstream [63]. Therefore, analysis of BAL fluid may reveal important pathological mediators in the airway and alveolar wall-lining fluids [64,65]. Sputum is another useful body fluid, especially in respiratory research. Sputum is matter that is expectorated from the respiratory tract, which can then be coughed up and spat out or swallowed. Because sputum originates in the respiratory tract, it reflects the state of lungs, bronchi or respiratory tract.

Plasma or serum proteomics

Plasma or serum is the preferred sample for protein biomarker identification, which reflects physiological, pathological and pharmacological phenotypes. Plasma or serum may reflect systemic changes; however, they are limited in that they only reflect local status with systemic dilution. In addition, individual variation can create further dilution.

Thus, we used plasma in our study; we found a specific cancer marker by simple comparison between healthy normal controls and patients with a specific cancer. However, for diseases that are not so specific, but rather are names for various symptoms, simple comparisons such as asthma versus normal, healthy controls are not expected to yield useful results. Indeed, there has been no previous report involving such a simple comparisons. Rather, there are reports on the identification of proteins responsible for certain subtypes of asthma or finding asthma-related proteins by using challenge strategies.

In 2005, Murphy *et al.* examined the associations between asthma, fetal gender and protein profiles using SELDI-TOF [66]. They compared protein profiles in placental tissue and umbilical vein plasma of ten healthy and 20 asthmatic women. As a result, ten umbilical cord blood peak differences were identified, with four peaks more highly expressed and six peaks less highly expressed in asthmatics. Two placental and five umbilical cord blood peaks were specifically increased in a subgroup of samples collected from asthmatic women who did not use inhaled glucocorticoids and who were pregnant with a female fetus, a group previously found to have altered placental function [66].

Our group also adopted a proteomics approach to understand the protein basis of this pathology and to develop a serologic marker for aspirin hypersensitivity using challenge methods [67]. Aspirin-induced asthma (AIA) is triggered by an inflammatory response of the airways to the ingestion of aspirin (acetylsalicylic acid [ASA]) and other nonsteroidal anti-inflammatory drugs. This syndrome has been characterized by aspirin hypersensitivity, bronchial asthma and chronic rhinosinusitis with nasal polypsis, commonly referred to as the 'aspirin triad' [68]. The exact mechanism of this response is unknown, but may be related to the inhibition of the cyclooxygenase enzyme [69–71]. However, blockage of the cysLTs pathway and its receptor (CysLTR1) by specific antagonists, such as 5-lipoxygenase inhibitors, did not completely protect against AIA [70–73]. In addition, the proposed mechanism fails to explain why ASA does not cause bronchoconstriction in all individuals [74]. These findings suggest the presence of alternative pathways of aspirin hypersensitivity [75,76]. Thus, we adopted a proteomics approach to investigate the protein basis of the pathology and to develop a serologic marker for ASA hypersensitivity [67]. Using 2DE, we found 12 protein spots, including complement C3 and C4, composed of eight proteins that were significantly up- or downregulated after aspirin challenge in patients with AIA. Among them, complement component (C3, C3a, C4 and C4a) levels in plasma were validated using a case-control study. The results of our study showed significantly elevated levels of C3a and C4a in the plasma of patients with AIA. The values of C3a and C4a in patients with AIA were significantly correlated with FEV₁. These results suggest that complement is involved in the pathogenesis of AIA [67].

We also analyzed the proteins that may predispose patients to the appearance of an immediate response to *Dermatophagoides pteronyssinus* allergen challenge and that may provide plasma markers to predict the appearance of an immediate-phase reaction (IPR) with a similar strategy [77]. Allergen inhalation in sensitized asthmatic patients results in an IPR. However, even

subjects strongly sensitized to inhalant allergens showed no IPR in some cases. Although mediators are related to the appearance of an IPR, there has been no reliable indicator to predict conditions for the appearance of an IPR. Before *D. pteronyssinus* antigen challenge, four spots of γ -fibrinogen and its isoforms were significantly decreased and two spots of complement C3 fragments were significantly increased in the positive responders. After *D. pteronyssinus* antigen challenge, complement C3 fragment was persistently higher, while γ -fibrinogen was lower in the positive responders than in the negative responders [77].

Plasma and serum may have limitations in the identification of asthma-specific proteins. However, these human specimens may be good sources for the identification of subtype- or challenge-responsible protein molecules.

BAL fluid proteomics

The BAL test was introduced after the development of bronchoscopy in the 1970s and is now used as an important tool for diagnosing respiratory diseases. A small amount of saline is injected via a fiber-optic bronchoscope into a selective area of lung and immediately aspirated. BAL fluid directly reflects lung physiology and disease status more accurately than plasma does; BAL fluid has been more widely applied in the study of lung diseases [78].

Some time ago, the analysis of proteins in BAL fluids using 2DE was performed. In 1979, Bell and Hook analyzed BAL fluid proteins using 2DE [79]. They compared the 2DE pattern of BAL fluid proteins of healthy volunteers and patients with pulmonary alveolar proteinosis. However, at that time, protein spots were identified by comparing 2DE with serum 2D-polyacrylamide gel electrophoresis (PAGE) protein maps and immunoblot analysis.

Modern proteomics combines a protein separation technique (2DE is most widely used) and protein identification with sensitive instrumentation, in the form of MS. The first proteome analysis of BAL fluid was performed by Wattiez *et al.* [80]. They analyzed BAL fluid proteins by 2DE and identified 182 spots by microsequence analysis and by matching with human blood plasma reference 2DE from the SWISS-2DPAGE database; thus, they created the first 2DE map of BAL fluid proteins [80]. The first report on the BAL fluid proteome profiles of human asthmatics was presented by Lindhal *et al.* [65]. They showed that the concentrations of lipocalin-1, cystatin S, transthyretin and IgBF in BAL fluid and nasal lavage fluid were altered in connection with smoking, upper airway irritation and asthma [65]. However, obtaining a large enough BAL sample for proteomics from asthmatic patients or healthy controls is not easy. Most researchers have used the allergic asthma mouse model to compare BAL protein profiles. Chung *et al.* discovered the downregulated annexin-1, which inhibits the activity of cytosolic phospholipase A2, by allergen challenge [81]. Greenlee *et al.* identified several chemotactic molecules, such as Ym1, S100A8 and S100A9, which possessed putative matrix metalloproteinase (MMP)2 and MMP9 cleavage sites by analyzing the proteome in the BAL fluid of MMP2 and MMP9-knockout mice [82]. Ghosh *et al.* reported the post-translational modification of BAL proteins. They found over 30 different proteins that were targets of nitration following allergen challenge,

including the antioxidant enzyme catalase [83]. This is fairly rare, but there are other reports on the human BAL proteome. Candiano *et al.* found that the amount of gelsolin was increased in the BAL fluid of patients with asthma [47]. More recently, we found 16 proteins that were differently expressed in the BAL fluid of asthma patients and identified them by LC-MS/MS [84]. In the OVA-sensitized/challenged mice model, treatment with antibody against a candidate protein found to be upregulated in asthmatics resulted in a marked decrease in airway hyperresponsiveness, inflammatory cell counts and cytokine production. Moreover, intranasal challenge of BALB/c mice with the candidate protein resulted in the typical infiltration of numerous eosinophils and mucus hyperproduction by bronchial epithelial cells [84].

Identification of proteins in the BAL fluid and their changing expressions in different stages of asthma could provide further insights into the complex molecular mechanisms involved in this disease. A large proportion of these proteins and their expression changes were identified for the first time from BAL fluid, thus providing new insights for finding novel pathological mediators and biomarkers of asthma.

Induced sputum

Induced sputum provides us with a relatively noninvasive method of examining the airway secretions of subjects with lower airway diseases, such as asthma and COPD. These body fluids contain inflammatory mediators or inflammatory cells released from airways. Moreover, Kelly *et al.* suggested that induced sputum directly represents lung oxidative damage [85]. Certainly, induced sputum is a good specimen for analyzing asthma. A standardized protocol of sputum induction applied to all patients, using nebulized saline to loosen phlegm and aid expectoration, is critical for sputum sampling, especially where comparisons in biomarkers of disease are to be made [86]. However, there are technical problems in the analysis of sputum. First, induced sputum samples are often contaminated with saliva. Second, it is difficult to analyze a sticky sputum sample by electrophoresis. To date, few proteomic studies of induced sputum of asthmatics have been reported. Nicholas *et al.* surveyed the proteome of human-induced sputum via a combination of 2DE and gel-based LC-MS/MS. They identified 191 proteins in sputum [87]. Research on diseases associated with sputum proteomics was performed by Gray *et al.* [88]. They compared the proteome of several respiratory diseases, such as asthma, COPD, cystic fibrosis and bronchiectasis, by SELDI-TOF. They suggested that proteomic screening of induced sputum may yield potential biomarkers of respiratory diseases.

Expert commentary

As mentioned previously, asthma is considered to be the name given to various symptoms, not the disease. In addition, bronchial asthma is a multifactorial disease in which environmental, infectious, allergic and psychological elements all play a role. Owing to the complex nature of the asthma phenotype, together with genetic heterogeneity and environmental influences, it has been difficult to identify the aspects that underlie this common disease. Searching for asthma-specific proteins has become an important

issue because such proteins will shed light on early detection, prognosis and treatment of the disease. Thus, large-scale, high-throughput, whole-proteome studies are needed to understand the proteomic contribution to asthma. Many researchers have tried to identify asthma-related proteins using clinical samples or various models of asthma. Some studies have shown promising results. However, we believe that the presently reported studies are just the beginning. Most of the studies adopted 2DE-based proteomics. Therefore, their results were restricted to abundant, soluble and neutral proteins.

Proteomics techniques are currently too limited to identify all the proteins that may play a role in asthma pathophysiology. However, we expect that proteomics approaches, using well-designed experiments, will yield good results.

Five-year view

The application of proteomics to asthma research has progressed relatively slowly. As already described, the use of proteomics in asthma research is quite different from other diseases, such as some cancers. Our aim is not to develop methods to diagnose asthma with one drop of blood or body fluid. Rather, we aim to determine the patient's causal factors so that the patient may avoid them. By finding target molecules that play important roles in asthma pathogenesis, novel therapeutic methods may ultimately be developed.

As we have reviewed, many researchers have adopted proteomics techniques for this purpose. However, most studies have adopted 2DE-based proteomics. Some researchers are currently using a new method. This does not necessitate the removal of abundant proteins or 2D differential gel electrophoresis. Rather, it uses a 1D-LC-MS/MS system, based on capillary LC-ion trap MS/MS or, using a tagging process, relies on chemical labeling reagents, referred to as isotope-coded affinity tags. These non-2DE systems demand more investment and expertise; so many researchers may not have their own equipment. However, these obstacles can be overcome through collaboration, which has already occurred in many cases.

Studying post-translational modification is another direction in which to proceed. By enriching for specific modified proteins, such as phosphorylated proteins, acetylated proteins or nitrated proteins, we can analyze smaller amounts of proteins. After enrichment, the samples can be analyzed by 2DE; therefore, we expect many results will be reported using specific enrichment.

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Key issues

- Asthma is a multifactorial disease with underdetermined genetic, causative, causal factors and triggering factors.
- Asthma is not accepted as a single disease, but is considered a name given to various symptoms. Thus, in some cases, it is not as important as it is for other diseases to determine whether the patient is actually asthmatic. Although it is still important to determine whether or not people have asthma, to identify the subtype by protein identification may be more important than determining whether it is asthma or not.
- Most allergens are proteins. Proteomics is a good choice for identifying causative factors that can exacerbate or evoke asthma attacks.
- The cell model used to analyze responsible protein expression can be changed by many types of stimulation.
- Animal models of asthma have been well established and are often used in asthma research. Tissue, plasma or bronchoalveolar lavage fluid can be used for identifying asthma or its subtype-specific protein markers.
- Plasma and serum are the most frequently used and important body fluids in asthma research. In addition, bronchoalveolar lavage fluid and sputum are other body fluids that can be analyzed.

References

Papers of special note have been highlighted as:

• of interest

•• of considerable interest

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