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Review

Database of bronchoalveolar lavage fluid proteins

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Abstract

Bronchoalveolar lavage during fiberoptic bronchoscopy is extensively used for investigating cellular and biochemical alterations of the epithelial lining fluid in various lung disorders. Two-dimensional electrophoresis (2-DE) offers the possibility to simultaneously display and analyze proteins contained in bronchoalveolar lavage fluid (BALF). We present the current status of 2-DE of BALF samples with an updated listing of the proteins already identified and of their level and/or posttranslational alterations in lung disorders. Alternatives to 2-DE of BALF samples and future prospects of proteomics to unravel lung functions and pathologies are discussed.

Keywords: Reviews; Bronchoalveloar lavage; Proteomics; Proteins

Contents

1. Introduction

Bronchoalveolar lavage (BAL) performed during fiberoptic bronchoscopy is a relatively safe technique which has proven useful to collect cells and a wide variety of soluble components from the human lung: phospholipids, nucleic acids and proteins, originating from the thin layer of epithelial lining fluid (ELF) that covers the airways [1]. Centrifugation of BAL allows the separation of cells from the supernatant BAL fluid (BALF) that contains the soluble components of the ELF.

The cellular content of BAL mainly consists of

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alveolar macrophages (80–95% of the cells), lymphocytes (<10%), neutrophils (<5%), eosinophils (<5%) and sometimes plasma cells. Squamous epithelial cells, bronchial epithelial cells, type II pneumocytes, basophils and mast cells are also found in BAL [2–5].

Phospholipids, responsible for the decrease of alveolar surface tension, are synthesised by the lung epithelial cells and represent the main components of surfactant (90% [6]). Nucleic acids are found occasionally in BAL during infections by pathogens [7]. Soluble proteins in BALF are very diverse and may originate from a broad range of sources: diffusion from serum across the air–blood barrier, production by pulmonary T cells, synthesis by alveolar macrophages, bronchial epithelial cells, alveolar TI and TII cells, Clara cells, etc. For example, BALF interleukin-10 has four possible origins: production by pulmonary T cells [8], alveolar macrophages [9], bronchial epithelial cells [10], and diffusion from serum across the air–blood barrier.

Careful analysis of each of these BAL components enables accurate diagnosis and follow-up of a number of lung diseases. For example, acute respiratory distress syndrome (ARDS) is characterized by a significant decrease in the percentage of phosphatidylcholine and phosphatidylglycerol and an increase of phosphatidylinositol in total phospholipids (reviewed in Ref. [6]), granulomatous and allergic lung diseases are characterized by an increase in the lymphocyte count [5], *Pneumocystis carinii* pneumonia can be diagnosed by nested polymerase chain reaction on BALF samples [7].

Due to the variety of origins for proteins present in BAL, differences in the amounts of lung-specific proteins in BAL may result from many different kinds of phenomena. Indeed, reduction of BALF surfactant proteins (SPs) may be caused by reduction in the amount of secreting cells, or by decreased synthesis and/or release by these cells. On the contrary, increase of BALF SPs may result from an increase in the synthesis, from augmented release by secreting cells or from impaired clearance by alveolar macrophages, mucociliary transport, degradation, and absorption into the bloodstream. For instance, increased synthesis and/or release are the most plausible mechanisms explaining BALF surfactant protein A (SP-A, the major protein component of the surfactant) increase in patients with sarcoidosis and hypersensitivity pneumonitis (HP) [11]. BALF SP-A increase in pulmonary alveolar proteinosis (PAP) is also due to impaired removal/degradation of surfactant [12].

Due to the huge diversity of proteins present in BALF as well as the wide variety of origins of each protein considered, analysis of the protein content of BALF is of outstanding interest to diagnose most lung diseases. However, whereas quantification of the expression level of a single protein represents the integration of a multitude of different mechanisms involved in its synthesis, release and/or clearance, measuring changes in the levels of only one particular protein species gives insights only into one particular piece affected in the puzzle of a defined lung disease. Establishing an unambiguous diagnosis of one particular disease, allowing proper treatment and follow-up, requires the combined analysis of a repertoire of protein markers in BALF samples.

Proteomics, a technology-based science which studies levels and post-translational modifications of a large number of proteins simultaneously, their differences between healthy and diseased states and under the influence of environmental factors, fits these requirements [13]. Current interest in the application of proteomics to study human disease is huge and covers a wide variety of biomedical areas including cardiovascular diseases [14], cancer [15] and neurological disorder research [16]. Moreover, proteomics is the only global expression profiling technique applicable to body fluids, which cannot be analyzed via nucleic acid-based approaches [17]. Great developments have thus been encountered in the field of body fluid proteomics, exemplified by the identification of bladder squamous cell carcinoma biomarkers in urine [18] or of the 14-3-3 brain protein in cerebrospinal fluid as a marker for spongiform encephalopathies [19].

In this context, the use of two-dimensional electrophoresis (2-DE) and mapping of BALF as the first step of a proteomic approach is of high interest. The hunt for new lung disease markers in BALF via the 2-DE display and subsequent interpretation of levels and post-translational modifications of the total protein content of BALF, together with the search for differences between healthy versus diseased states is described in this review.

2. History of proteomics with BALF samples

The origins of differential display proteomics with BALF samples go back to the late 1970s. Searches for disease-associated protein markers were undertaken within lavage effluent proteins displayed using gradient gel electrophoresis or isoelectric focusing in a one-dimensional fashion [20,21].

The first two-dimensional map displaying the major soluble proteins present in lung lavage was published in 1979 [22]. The identification of 23 serum-derived proteins, accounting for 97% of the total BALF protein content, was performed by immunostaining, comparisons with purified reference standards and pattern matching with 2-DE maps of serum samples [23]. Hypothesis concerning the origins of these serum-specific proteins in BALF were given, including transudation through the air–blood barrier, local synthesis by lung cells and/or selective transport [22,23]. A few lung-specific proteins, termed apoproteins because of their presumed interactions with phospholipids to form lipid–protein complexes, were also reported on the 2-DE map, but no identification of these proteins could be provided at that time [24].

From these first steps into proteomic analysis of BALF samples, it was demonstrated that the approach could provide a reference 2-DE map of proteins contained in BALF, lead to the identification of putative disease markers by comparing the maps between healthy individuals and diseased patients and, finally, propose mechanistic hypotheses to help understand disease processes. This knowledge, gained in the beginning of the 1980s on 2-DE of BALF samples, remained almost unchanged for about 10 years.

3. 2-DE of BALF: state of the art

2-DE of BALF samples encountered impressive modernization due to many different technical improvements introduced in the beginning of the 1990s. However, current work still aims at the construction of an exhaustive 2-DE reference database of bronchoalveolar lavage fluid proteins, the search for new disease protein markers, their identification and the understanding of the molecular processes involved in lung diseases.

The most important technical progress brought in in BALF proteomics resides in the method used for isoelectric focusing (IEF). Indeed, a major contributor to the variability observed in 2-DE patterns was the carrier ampholyte determining the first-dimensional separation based on the charge of the proteins. Limitations included discontinuities in the gel gradient, drifts in the focusing pattern and difficulty to produce precast gels, lowering intra- and inter-laboratory reproducibility. The use of immobilized pH gradients (IPGs), introduced in 1983 [25], overcomes these restrictions: the pH gradient, created by covalently incorporating acidic and basic buffering groups into the polyacrylamide gel, is an integral part of the gel matrix. In 1990, Lenz et al. used this improved method for isoelectric focusing for the first-dimensional separation of proteins from dog bronchoalveolar lavage fluid [26].

Two other major problems were also associated with 2-DE of BALF proteins: the low protein content of BALF and the high salt concentration in BALF, that derives from the phosphate-buffered saline used for the lavage procedure. Of the multiple procedures used to circumvent these two problems, Lenz et al. retained a 24-h dialysis against water to efficiently remove salts and evaporation in a vacuum centrifuge to concentrate the samples [26]. Using this procedure, the authors obtained satisfactory and reproducible separation of dog BALF proteins with isoelectric point (pI) values ranging from 4 to 9 and could identify dog SP-A by pattern matching with purified SP-A [26]. A similar procedure applied on human BALF samples also allowed them to separate 400 polypeptides within a pI range of 4 to 7 [27].

Depending on the laboratory considered, other techniques may be preferred that also enable salt removal (gel filtration [28], trichloroacetic acid (TCA) precipitation [29], ion-exchange chromatography [30]) and sample concentration (vacuum filtration [26], centrifuge ultrafiltration [26], lyophilisation [27]).

Another question that was tackled by Lenz et al. is the impaired detection and identification of scarce proteins in BALF hindered by over-represented proteins like albumin (50%), transferrin (5 to 6%), α1-antitrypsin (3 to 5%) and immunoglobulins A

Table 1
List of proteins of the 2-DE map of human bronchoalveolar lavage fluid identified by pattern matching with published 2-DE maps (PMs), comigration with purified reference proteins (RPs), Western blotting (WB), N-terminal Edman degradation (E), internal Edman degradation (EI) and mass spectrometry (MS)

	Protein name	Genbank Accession number	M_r (kDa)	p*I*	Number of spots	Method of identification	Refs.
	Actin β chain	GI481515	42	5.1	1	MS	[30]
	Actin γ chain	GI7441428	42	5.3	3	MS	[30]
1	Acyl coA binding protein like	GI118275	10.1	5.8	1	EI	[31]
2	Albumin	GI113576	69 to 66	5.6 to 5.8	7	PMs, RPs, WB, E	[28]; [29]
3	α-1 Antichymotrypsin	GI112874	64 to 59.6	4.6 to 4.8	5	PMs, E	[28]; [31]
	α-1 Antiproteinase	GI1703025	46.9	5.4	1	MS	[30]
4	α-1 Antitrypsin	GI1703025	58 to 56	4.9 to 5.1	6	PMs, RPs, E, MS	[28]; [29]; [30]
	α-1 β Glycoprotein	GI112892	72	5.2	4	PMs, MS	[28]; [30]
	α-1 Microglobulin	GI825614	35 to 32	5.2 to 5.5	3	PMs	[43]
5	α-2 Antiplasmin	GI112907				E, PMs	[29]
6	α-2 HS Glycoprotein	GI112910	58 to 50	4.6 to 4.8	5	PMs, EI	[28]; [31]
	α-2 Macroglobulin	GI112911	164	5.6 to 5.7	11	PMs	[43]
	Amyloid P	GI14720863	28	5.6	1	PMs	[43]
7	Antioxidant enzyme (AOEB166)	GI6912238	15 and 12.3	6.9 and 6.5	2	E, MS	[29]; [31]
	Antithrombin III chain A	GI113936	53.1 and 49.4	6.3 and 6	2	PMs	[28]; [30]
	Apolipoprotein D	GI4502163	32.3 to 30.3	4.4 to 4.8	4	PMs, MS	[28]
8	Apolipoprotein A I	GI113992	25	4.9 to 5.2	3	PMs, E	[43]; [29]; [31]
9	Apolipoprotein A II	GI114000	12.6	5.2	1	E, MS	[29]
	Apolipoprotein A IV	GI178779	43.4	5.2	1	MS	[30]
10	β 2 Microglobulin	GI114773	10.9	6.2	1	E, MS	[29]
11	Calcyclin (S100A6 protein)	GI116509	9.1	5.2	1	EI	[31]
12	Calgizzarin (S100c protein)	GI1710818	10.4	6	1	EI, MS	[31]
13	Calgranulin A	GI115442	10.2 to 9.9	6.5 to 6.7	3	E	[29]
14	Calgranulin C	GI2507565	9.8	5.9	1	E	[29]
15	Calreticulin	GI117505	63	4.5 and 4.6	2	E, MS	[31]
16	Calvasculin (S100A4 protein)	GI115601	10.1	5	1	EI	[31]
17	Cathepsin D (heavy chain)	GI115717	31	4.3 to 5.7	3	E, MS	[29]; [31]
18	Cathepsin D (light chain)	GI115717	13.8 to 10.6	4.9 to 5.7	5	E, MS	[29]; [31]
19	Ceruloplasmin	GI116117	140 to 112	5.4 to 5.7	12	PMs	[43]; [31]
20	Clara cell protein 16	GI256397	5.9 to 6.5	4.6 to 5.2	4	RPs, WB, E, MS	[43]; [29]; [31]; [30]
21	Clusterin	GI116533	39 to 35	4.7 to 5	9	PMs	[31]
22	Complement C3	GI116594	70	6.5 to 6.8	5	PMs, E	[28]; [29]
23	Complement factor 4 gamma	GI116602	33	6.1 and 6.3	2	PMs, E	[28]; [29]
24	Complement factor B	GI584908	98	5.9 to 6.3	5	PMs	[29]
	Cystatin S	GI399336	4.9 and 5.1	13 and 13.5	2	E, MS	[45]

25	Epididymal secretory protein	GI3182993	24 to 19.5	7	6	E	[31]
26	Fatty acid binding protein, adipocyte	GI119781	12.5	5.9	1	EI, MS	[31]
27	Fatty acid binding protein, epidermal	GI232081	13.3	6.2	1	EI, MS	[31]
28	Fibrinogen beta chain	GI399492	58	6.1 to 6.3	3	PMs	[28]; [31]
29	Fibrinogen gamma chain	GI120142	54 to 52	5.2 to 5.6	8	PMs, E	[28]; [29]
	Glutathione S transferase, chain A	GI494066	23.4	5.4	1	MS	[30]
30	GTP cyclohydrolase I feedback regulatory protein	GI2506906	10	6.4	1	E	[29]
31	Haptoglobin-1 (α 1 chain)	GI123507	12.9 to 10.6	5.6 to 5.7	3	PMs, E, MS	[43]; [31]
32	Haptoglobin-2 (α 2 chain)	GI123508	17	5.3 to 5.7	3	PMs, E, MS	[28]; [29]
33	Haptoglobin-2 (β 2 chain)	GI123508	44 to 40	4.8 to 5.6	11–18	PMs, E, MS	[28]; [29]
	Heat shock cognate 70 kDa	GI5729877	71	5.4	1	MS	[30]
34	Hemoglobin α chain	GI122412	12	7.5 to 8.3	4	E	[29]
35	Hemoglobin β chain	GI122615	13 to 12	6.9 to 7	4	E	[29]
36	Hemopexin	GI1708182	76 to 71	5.3 to 5.6	5	E, PMs	[28]; [29]
37	Immunoglobulin binding factor	GI131436	13.6	5.6	1	E	[29]; [45]
38	Immunoglobulin A, S chain		98 to 88	4.7 to 5.8	11–25	PMs	[28]; [43]; [29]
	Immunoglobulin A, α chain		64	5.2 to 6.7	20–25	PMs	[43]
39	Immunoglobulin G, heavy chain γ	GI123845	59 to 54	5.9 to 9.0	30–40	PMs, RPs, E	[28]; [29]
40	Immunoglobulin G, γs intermediate chain		38.1 to 37.6	6.8 to 7.3	5–12	PMs	[28]; [31]
41	Immunoglobulin light chains	GI125780	30 to 26	5.4 to 8.9	90–120	PMs, RPs, E	[28]; [29]
	κ, λ	GI125788					
		GI125811					
		GI126565					
		GI126568					
	Immunoglobulin M, μs intermediate chain		50	5.9 to 6.1	3	PMs	[28]
	Immunoglobulin M, μ chain		80	5.9 to 6.1	3	PMs	[43]
42	Instestinal trefoil factor	GI585328	14	4.8	1	E, MS	[29]
43	Lactoferrin	GI6175096	85	7.1 to 7.6	1	RPs, WB, MS	[43]; [31]
44	Leucine-rich α-2 glycoprotein	GI112908	57.3 to 43.2	4.7 to 5.6	4	PMs	[31]
45	Lipocalin 1	GI401346	18 to 17	5.2 to 5.5	8	E	[31]; [45]
	Lipocortin 1	GI113944	38 to 34	5.9 to 7.4	7	RPs, WB	[43]
46	Lysozyme C	GI126615	13.9	9 to 10	1	RPs, WB, E	[43]; [29]
	Macrophage colony stimulating factor 1	GI2118667	26.3	5.1	1	MS	[30]
	MHC class II antigen DRB3	GI5834330	11.1	5.4	1	MS	[30]
	Nuclear chloride ion channel 27	GI4337097	27	5.3	1	MS	[30]
	Orosomucoid	GI3868933	51 to 49	3.6 to 4.0	4	PMs, RPs	[28]
47	Peptidyl-prolyl *cis–trans* isomerase A	GI14781854	17.3	7.2 and 7.5	2	E, MS	[29]
48	Peptidyl-prolyl *cis–trans* isomerase B	GI14755223	22.8	9.6 and 9.7	2	E	[29]
	Peripheral blood leukocytes peptide 474aa	GI455970	54.5	5.3	1	MS	[30]
49	Phosphatidylethanolamine binding protein	GI1352726	23.3	7.4	1	E	[29]
50	Plasma retinol binding protein	GI132404	20.2 to 19.8	5.1 to 5.2	3	E	[31]

Table 1. Continued

	Protein name	Genbank Accession number	M_r (kDa)	p*I*	Number of spots	Method of identification	Refs.
51	Plasminogen	GI130316	105 to 101	6.4 to 7.9	8	PMs, E	[28]; [29]
	L-Plastin	GI4504965	70.8	5.2	1	MS	[30]
	Prothrombin	GI135807	80.2 to 79.9	4.9 to 5.1	4	PMs, MS	[28]
	Retinol binding protein	GI4558179	20.7	4.9	1	MS	[30]
	Rho GDP dissociation inhibitor α	GI4757768	23.3	5	1	MS	[30]
52	Saposin D chain	GI134218	9.9	4.7	1	E	[31]
53	SH3 domain binding glutamic acid-rich protein like	GI4506925	13.5 and 12.8	5.2 and 5.3	2	E, MS	[29]; [30]
54	Superoxide dismutase (Cu/Zn)	GI134618	25	5.2 to 5.7	6	EI	[31]
55	Superoxide dismutase (Mn)	GI134665	22	6.7 and 6.9	2	E, MS	[29]
56	Surfactant protein A	P07714	38 to 31	4.3 to 5.1	20	E, MS	[29]; [31]; [30]
57	Thioredoxin	P10599	12	4.9	1	E, MS	[29]; [30]
58	Transferrin	GI136191	83 to 77	5.9 to 6.4	24	PMs, RPs, E	[28]; [29]
59	Transthyretin	GI136464	13.8	5.4 and 5.6	2	PMs, E, MS	[28]; [29]; [45]; [30]
60	Triosephosphate isomerase	GI136060	27.6 and 26.9	6.6 and 6.8	2	E	[29]
61	Tropomyosin α-chain skeletal muscle type	GI136092	27	4.7	3	EI	[31]
	Tubulin β2	GI5174735	50.3	4.8	1	MS	[30]
62	Ubiquitin	GI136670	9.2 and 9.1	5.1 and 6.8	2	E	[29]
	Unknown protein (homology to coactosin)	GI1196417	16	5.5	1	MS	[30]
63	Vimentin (fragment)	GI418249	22	4.7	1	E	[29]
64	Vitamin D binding protein	GI139641	58 to 54	5.1 to 5.4	4	E, MS	[29]; [30]
65	Zinc α-2 glycoprotein	GI4502337	41	4.9	2	EI, MS	[31]; [30]

and G (together about 30%). Whereas removing the albumin fraction by binding to Affigel-Blue is giving quite unsatisfactory results because other proteins are lost, visualization of low-abundance proteins below a molecular mass of 40 kDa can be obtained by massive overloading since the upper-mentioned overlapping proteins are all above 40 kDa [27].

Additional improvements of the 2-DE map of human BALF samples could be obtained with the arrival on the market of new pH gradients for the first dimensional separation. Indeed, since most of the proteins in BALF have isoelectric points comprised in the 4 to 7 range, the use of nonlinear gradients (3–10 NLs) instead of linear gradients (3–10 Ls) could partly solve the problem because the p*I* gradient in nonlinear IPG gels provides a higher resolution in the 4–7 p*I* range [28]. Moreover, the use of larger IPG gels allows a better overall resolution of every proteins spots by increasing the distance between them. Improvements also arose from the second dimensional separation: the use of ultra-thin polyacrylamide gradient gels further increases resolution of the 2-DE map. To illustrate this, in 1999, using 11 cm 3-10 linear gels for the first dimension and linear sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for the second dimension, we could detect 211 silver stained spots whereas 1 year after, using 18 cm 3-10 nonlinear gels for the first dimension and gradient SDS–PAGE for the second dimension, 600 to 1000 spots were detected [31].

Very recently, the use of a series of narrow-range IPG strips covering the pH interval 4.5–6.7 enabled the detection of more low abundant proteins in BALF [30]: 678 spots were detected using a 3–7 pH gradient, 409 were detected with a 4.5–5.5 strip and 425 spots were visualized using a 5.5–6.7 strip. Splitting a p*I* range into multiple narrower p*I* ranges of equal length produces a proportionate broader reconstituted 2-DE map. Accordingly, the use of these narrow range gradient IPG gels for IEF improves the overall quality of BALF 2-DE maps in that the resolution power is enhanced, since proteins differing from each other by few p*I* subunits are much far away from each other on a narrow-range gradient than on a wide-range gradient, thus much more resolved. Moreover, using narrow-range gradient gels is a little bit like doing prefractionation: more protein extract can be loaded on a narrow-range gradient and, as a consequence, the probability to detect scarce proteins will be higher.

To conclude with this chapter, the establishment of the 2-DE map is improving on an every-day basis, relying on many different technical improvements, at every step of the two-dimensional electrophoresis process as well as the protein identification methods used. The BALF sample preparation (salt removal, sample concentration and fractionation), the technique used for sample loading (cup-loading, paper bridge application), the choice of the p*I* range and the second dimensional gel to be used are critical to obtain optimal 2-DE resolution of the widest range of proteins present in BALF. Finally, it goes without saying that the ultimate quality of the BALF 2-DE map also strongly depends on the highly sensible detection and identification methods, of which silver staining and peptide fingerprinting using mass spectrometry are without any doubt.

4. Updated database of proteins present in BALF 2-DE maps

Mainly three research groups have published identification of proteins present in the BALF 2-DE map as it stands nowadays (see proteins listed in Table 1 and displayed in Fig. 1).

In 1995, Lindahl et al. published a map comprising about 1000 protein spots with p*I* values ranging from 4 to 7 [28]. Using a combination of Western blotting, pattern matching with a reference plasma 2-DE map and co-migration with reference standards, they identified 25 different proteins, all originating from plasma. Later, the same group enriched this map with seven other plasma proteins, again by pattern matching with the human plasma 2-DE map, and 8 clinically interesting proteins (lysozyme, lactoferrin, lipocortin-1, Clara cell protein 16, lipocalin-1, cystatin S, transthyretin and immunoglobulin binding factor) by Western blotting, co-migration with reference standards and N-terminal microsequence analysis by Edman degradation [28].

In 1999, wishing to systematically characterize the nature of protein BALF components, we undertook an exhaustive identification of all proteins present in human BAL fluid [29]. Our strategy to construct a

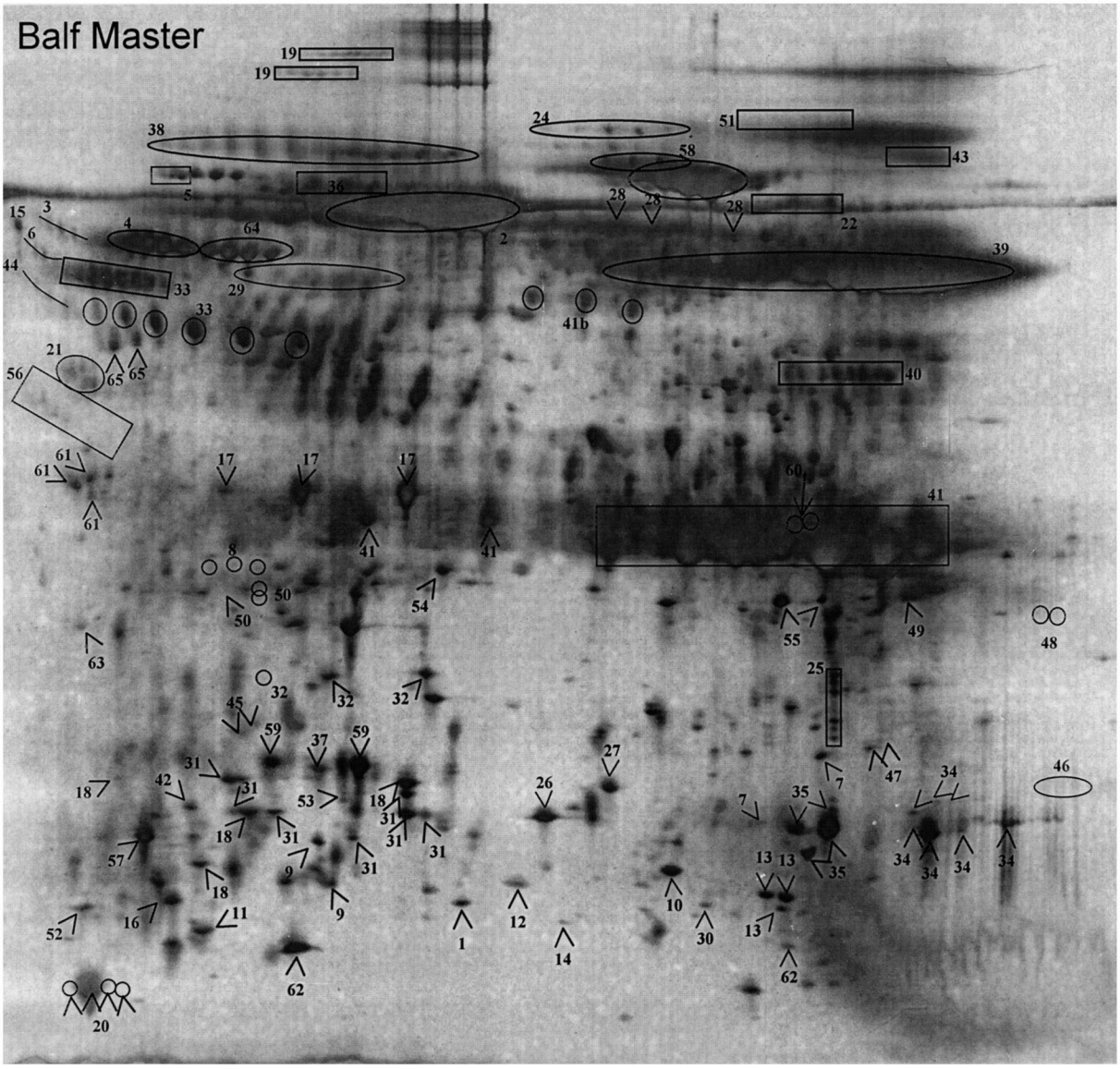

Fig. 1. Analytical silver-stained 2-DE gel from a patient with IPF and superimposed spot identities referring to Table 1 (proteins identified by our working group). A 25-μg amount of proteins dissolved in 9 *M* urea, 0.5% (v/v) Triton X-100, 2% (v/v) Pharmalyte 3-10, 65 m*M* DTE and 8 m*M* phenylmethylsulfonyl fluoride (PMSF) were loaded on pH 3–10 non-linear IPG strips for isoelectric focusing. Second-dimensional separation of the proteins was done on ExcelGel XL 12–14% and detected by silver staining.

protein map that contains the widest range of proteins was based on the analysis of individual and pooled BAL fluid samples from patients suffering from various lung disorders. Our present BALF 2-DE map comprises more than 1200 silver stained spots. Over 900 protein spots (intact proteins, their isoforms, protein subunits and fragments), corresponding to about 78 different protein species, were identified by matching with the human plasma reference 2-DE map or with other miscellaneous cell line maps available from SWISS-2D PAGE (macrophage-like, epidermal keratinocyte, liver), by N-

terminal or internal amino acid microsequencing and mass spectrometry (peptide mass fingerprinting). A dynamic 2-DE database for human BALF was made available on the Worldwide Web in 2000 (http://w3.umh.ac.be/~biochim/proteomic.htm [31]).

Very recently, 12 previously unidentified proteins were described in BALF samples displayed on narrow-range gradient IPG gels and identified by peptide mass fingerprinting [30].

Insights into the function of a total of 93 identified proteins can be inferred from the groupings that have been made with these proteins.

First, classification of the proteins can be performed according to their origins: many proteins identified in BALF are found in plasma too, probably originating from it by passive diffusion due to the permeability of the air–blood barrier. However, among these proteins, 13 are synthesized in the lung too (α-1 antitrypsin [32], α-2 macroglobulin [33], apolipoprotein A1 [34], β-2 microglobulin [35], ceruloplasmin [36], complement factor 3 and 4 [37], fibrinogen γ chain [38], immunoglobulins [39] and transferrin [36]), probably to provide local extra-protection against invading microorganisms, oxidative damages and proteases released during inflammatory processes taking place in the lung. More interestingly, a certain number of proteins are present in BALF and not in plasma, suggesting that they are specifically produced in the airways. These proteins are therefore good candidates for becoming lung-specific biomarkers.

Several functional classes of proteins can be distinguished among the 93 proteins identified in the 2-DE map: proteins involved in lipid metabolism (acyl CoA binding protein like, FABP-E, FABP-A and saposin D chain), in immunological response and inflammation (HSP70, lipocortin-1, IgBF, CC-16, lipocalin-1, complement C3 and C4, MCSF-1, MHC class II antigen DRB3, peripheral blood leukocytes peptide, vitamin D binding protein), in protecting the lung from microorganisms (cystatin S, secretory IgA, SP-A, transferrin), oxidative damage (AOEB166, ceruloplasmin, GST, superoxide dismutase, thioredoxin) or proteases (α-1 antitrypsin) and proteins involved in tissue repair and cell proliferation (calcyclin, calgizzarin, calvasculin, cathepsin D, zinc α-2 glycoprotein, clusterin, fibrinogen γ chain, intestinal trefoil factor).

In contrast to the major surfactant protein SP-A, the three other SPs are not displayed on the 2-DE reference map. SP-B and SP-C, tightly associated with lipids of the surfactant [40], are probably not recovered due to the incompatibility between their hydrophobicity and the techniques used to prepare BALF samples prior to 2-DE. Moreover, the low molecular mass of these proteins after their maturation renders them impossible to detect in standard 2-DE gels. The absence of detection of SP-D, mainly existing as a soluble form not associated with surfactant lipids, is probably caused by its low levels of expression compared to SP-A [40].

Several intracellular proteins that are specifically found in BALF and not in plasma probably originate from cellular lysis consecutive to cellular turnover and death, which may be more pronounced in the BALF compartment than in peripheral blood. This is the case for L-plastin, Rho-GDP dissociation inhibitor, β and γ actin, tubulin β 2, and nuclear chloride ion channel 27.

2-DE studies also allowed the identification of new proteins of unknown function in BALF. Human AOEB166 was for instance identified in our BALF 2-DE gels and microsequenced to enable reverse cloning of the complete gene [41]. The protein, a peroxiredoxin widely expressed in human tissue, is believed to have a protective role in oxidation and inflammatory processes since LPS (lipopolysaccharide)-induced inflammation in rat lung is accompanied by an increase of lung AOEB166 mRNA levels [41]. Other unknown proteins detected in BALF, their tissue-specificity, their involvement in various lung disorders and their validations as lung disease markers are currently under investigation.

5. 2-DE mapping of BAL protein alterations in lung disease

The present chapter is dedicated to the description of up- or down-regulated proteins that have been detected on the BALF 2-DE map so far (Table 2).

In 1993, Lenz et al. identified the major surfactant protein, SP-A, as a series of spots located at the acidic side of the map by comparison with the 2-DE pattern of purified SP-A [27]. They showed that BALF SP-A had reduced in expression levels in

Table 2
List of proteins differentially expressed in BALF 2-DE patterns of healthy individuals and patients with various lung disorders

Protein name	Sarcoidosis	Idiopathic pulmonary fibrosis	Hypersensitivity pneumonitis	Cigarette smoking	Refs.
α-1 Antitrypsin	↑	↑	↑		[31]
Transferrin	↑	↑	↑		[87]; [31]
Transthyretin	↑	↑	↑		[31]
IgG	↑	↑	↑		[27]; [31]
IgA	↑	↑	↑	↓	[27]; [43]; [31]
Albumin	↑	↑	↑	↓	[87]
Ceruloplasmin				↓	[43]
Pro-apolipoprotein A1				↓	[43]
Cystatin S				↓	[45]
CC-16				↑	[44]
Lipocalin-1				↑	[45]
Immunoglobulin binding factor				↑	[45]
SP-A		↓	↓		[27]; [31]
Saposin D		↑	↑		[31]
Cathepsin D		↑	↑		[31]
Ubiquitin-like protein		↑	↑		[31]
A-FABP		↑	↑		[31]
E-FABP		↑	↑		[31]
Calvasculin		↑	↑		[31]
Intestinal trefoil factor		↑	↑		[31]
Tropomyosin		↑	↑		[31]
Calreticulin		↑	↑		[31]
Calcyclin		↑	↑		[31]
Calgranulin A	↑	↑	↑		[31]

patient with idiopathic pulmonary fibrosis, which is consistent with previously observed changes in alveolar composition during IPF. These results have been confirmed by our 2-DE results [31] and also by previous reports demonstration that enzyme-linked immunosorbent assay (ELISA)-detected SP-A was significantly decreased in IPF [42], possibly reflecting changes in alveolar type II cell function. IgG, IgA were increased in sarcoidosis [27], which is correlated with increased alveolar–capillary permeability and enhanced IgG production during the active state of this disease.

Lindahl et al. showed that IgA, ceruloplasmin and the pro- form of apolipoprotein A1 were down-regulated in smokers [43]. Moreover, lipocortin-1 and CC-16 could turn out to be useful markers of lung inflammation. Indeed, isoform distribution of CC-16 and lipocortin-1 was shown to be altered in BALF from smokers [44]. These two proteins have been proposed to function as modulators of inflammatory reactions in vivo.

In 1999, the same authors described the identification, by N-terminal sequencing, of several new and clinically interesting proteins [45]. Lipocalin-1 may function as a scavenger against toxic and proinflammatory lipids and has cysteine proteinase inhibitor function. Bronchial secretions from patients with cystic fibrosis were shown to contain higher levels of lipocalin-1, due to an up-regulated expression of the LCN1 gene [46] and BALF from smokers contain more lipocalin-1 than BALF from nonsmokers [45]. Cystatin S is thought to protect the lung surface against cysteine proteinases inhibitors originating from invading microorganisms or lysosomes. The two identified forms of cystatin S, probably differing from each other by their phosphorylation status, were decreased in BALF from smokers versus nonsmokers [45]. Immunoglobulin binding factor (IgBF), which is abundant in human seminal plasma but has also been found in BALF [29,47], may act as a modulator of local immune responses by binding to immunoglobulins, and is higher in smokers than in nonsmokers.

In 2000, our group published a comparison of 2-DE of BALF from IPF, sarcoidosis patients and healthy controls, shown in Fig. 2 [31]. The study was

done on 15 healthy subjects, 10 patients with IPF and 15 with sarcoidosis, all being nonsmokers.

Dramatic increases of some plasma proteins (transferrin, haptoglobin, α-1 antitrypsin and immunoglobulins) are observed in patients with sarcoidosis. This result is a confirmation of previous observations reporting increases in serum proteins in BALF of sarcoidosis patients. The most likely mech-

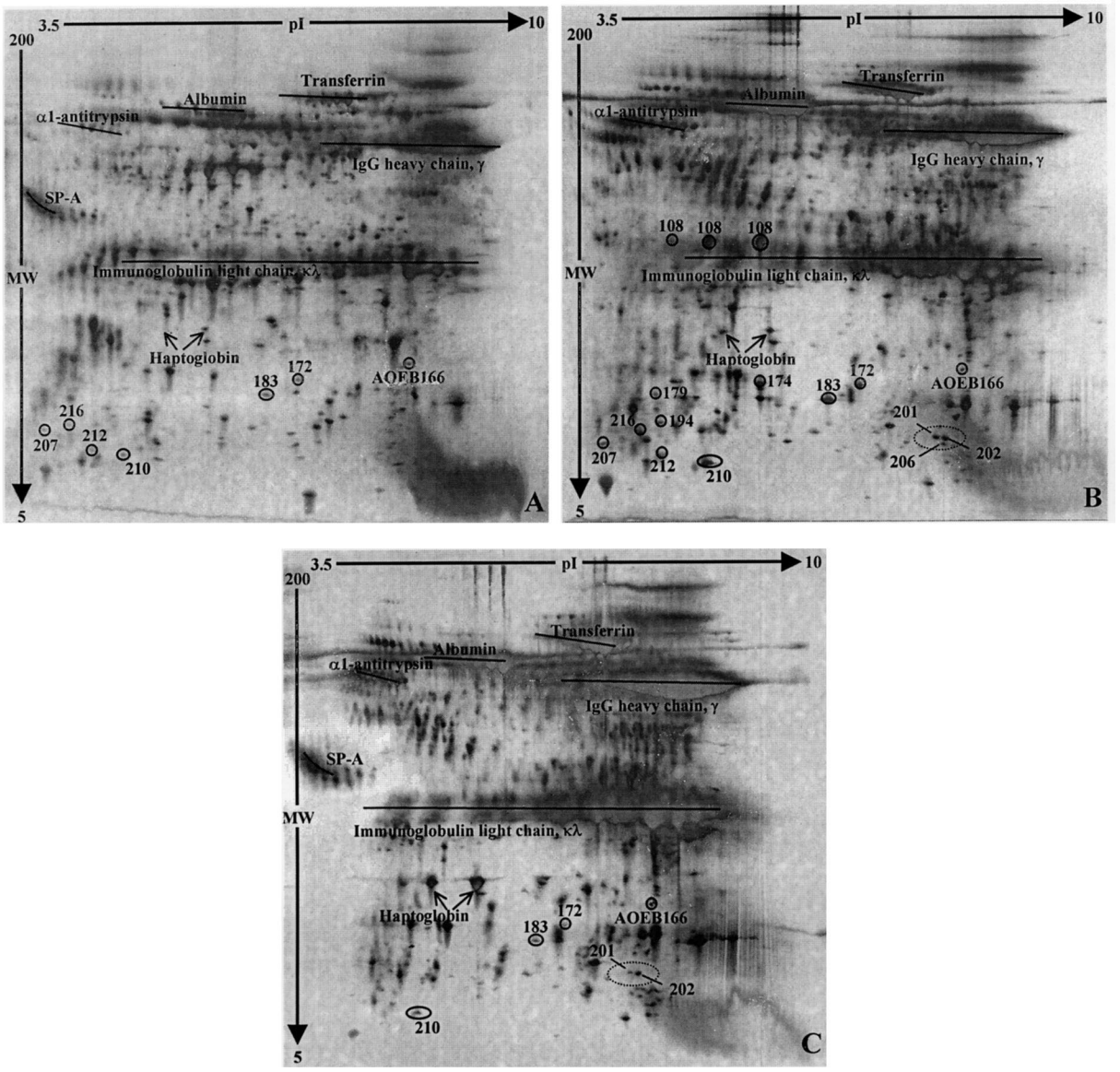

Fig. 2. Analytical silver-stained 2-DE gels of human BALF from a healthy subject (A) a patient with IPF (B) and a patient with sarcoidosis (C). A 25-μg amount of proteins dissolved in 9 *M* urea, 0.5% (v/v) Triton X-100, 2% (v/v) Pharmalyte 3-10, 65 m*M* DTE and 8 m*M* PMSF were loaded on pH 3–10 non-linear IPG strips for isoelectric focusing. Second-dimensional separation of the proteins was done on ExcelGel XL 12–14% and detected by silver staining. Bars and arrows indicate plasma proteins that are increased in BALF of patients with IPF and sarcoidosis. SP-A is absent in BALF of patients with IPF. Circles indicated small acidic proteins up regulated in IPF (108: cathepsin D, heavy chains; 172: FABP-E; 174: cathepsin D, light chain; 179: intestinal trefoil factor; 183: FABP-E; 194: cathepsin D, light chain; 201, 202 and 206: calgranulin A; 207: saposin, D chain; 210: ubiquitin-like protein; 212: calcyclin; 216: calvasculin) and the matching spots in healthy control.

anism for this elevation is an increased protein leakage from the bloodstream to the lung tissues caused by inflammatory damage to the alveolar–capillary barrier [48].

Concentration of SP-A, the most abundant pulmonary surfactant protein, is down regulated in BALF of patients with IPF, which has been observed previously in 2-DE gel experiments [27] and ELISAs [42] and is probably the consequence of an alteration of the synthesis and/or release of this lung-specific protein, caused by alveolar type II cells damage. SP-A being involved in various host defence mechanisms through its interaction with alveolar macrophages [49], a decrease in SP-A concentration is responsible for the ineffectiveness of the inflammatory response in IPF patients.

High levels of three forms of calgranulin A are also observed in BALF of IPF and sarcoidosis patients compared to healthy individuals [31]. However, the three detected forms of this protein (spots 201, 202 and 206) were differently distributed according to the type of pathology: spot 206 was only found in IPF whereas spot 202 had approximately the same level in both IPF and sarcoidosis. Spot 201 was significantly reduced in IPF compared to sarcoidosis patients.

IPF patients were also characterized by an increase in number and intensity of acidic low-M_r proteins: calcium binding proteins (calcyclin, spot 212 and calvasculin, spot 216), lipid binding proteins (epidermal fatty acid-binding protein, spot 174 and adipocyte fatty acid-binding protein, spot 183), enzymes (cathepsin D, spots 108, 174 and 207; saposin D chain, spot 210) and miscellaneous proteins (intestinal trefoil factor, spot 179 and ubiquitin-like protein, spot 212). Some of these proteins have been involved in a variety of processes related to cell proliferation. However, the exact role of these proteins in IPF pathogenesis remains to be clearly understood.

Recent data suggest that increased calcyclin gene expression plays a role in the response of pulmonary fibroblasts to increased mechanical tension and, by altering the regulation of the fibroblast cell cycle, promotes cell proliferation [50]. Calvasculin is capable of regulating cell cycle progression, modulating intercellular adhesion and invasive and metastatic properties of cancer cells, notably by sequestration and disabling the p53 suppressor protein [51]. The presence of cathepsin D in BALF correlates with reports of its expression by alveolar macrophages, bronchial epithelial cells and type I pneumocytes [52]. Cathepsin D increase in BALF of IPF patients is consistent with its potential role in remodelling processes occurring during fibrogenesis [52]. The trefoil factor family (TFF) domain peptides, which are involved in mucosal wound healing of the gastrointestinal tract, are hypothesized to perform the same roles on mucosal surfaces of the human respiratory tract [53].

In 2001, Griese et al. analyzed proteolysis of SP-A and total BALF proteins of patients suffering from cystic fibrosis displayed on 2-DE maps, before and after treatment with α1-protease inhibitor, which is believed to restore the protease–antiprotease imbalance [54]. Reduction in the number and in the intensity of protein spots with a molecular mass under 20 kDa and disappearance of lower-molecular-mass degradation products of SP-A were observed upon treatment, consistent with the presumed antiproteolytic action of α1-antiprotease. Thus, proteomics analysis of BAL fluid also enables monitoring of the actual impact of therapeutic interventions on BALF proteins.

6. 2-DE mapping of protein alterations in other body fluids in lung disease

Since bronchoalveolar lavage is not the only way of collecting ELF, other techniques have been designed that allow sampling of the distal airways in a less invasive fashion.

Sputum induction using hypertonic saline (HS) has been developed over the last decade, allowing minimally invasive assessment of airway inflammation without subjects having to undergo bronchoscopy [55]. It involves the inhalation of hypertonic saline aerosol, a stimulus known to cause bronchoconstriction in asthmatic subjects. This method, mainly restricted to the assessment of airway inflammation in asthmatic patients [56], is currently extended to the sampling of airways of subjects with cystic fibrosis [57], tuberculosis [58] and various interstitial lung diseases [59]. Two-dimensional electrophoresis of sputum induction samples has not

been developed yet, though specific alterations in protein composition upon lung disorders have already been described. For example, immunoglobulin binding factor is higher in the respiratory tract of patients with chronic airway diseases than in healthy individuals [60], and plasma protein leakage can be assessed by induced sputum as well as in BALF [61].

Condensation of exhaled breath is a newly described non-invasive way to collect material originating from the lung, including the lower respiratory tract. Exhaled breath condensate is obtained by freezing exhaled air under conditions of spontaneous breathing. It is therefore fully applicable to the follow-up of airway inflammation in very young children and to the analysis of healthy subjects [62,63]. To date, exhaled breath condensates samples have been mainly devote to the analysis of NO metabolites [64,65], 8-isoprostane [66,67], hydrogen peroxide [68] and various inflammatory cytokines [62], but knowledge of the total protein composition of these samples may provide a handy means of assessing airway disorders.

Nasal lavage fluid contains a large number of proteins originating from both proximal and distal airways. In 1995, Lindahl et al. provided a first 2-DE map of about 1000 protein spots, giving the identity of 26 of them [28]. The map was further enriched by the same authors to reach a total of 38 different protein species in 2001 [69], most of them being found in plasma and BALF reference maps too. Several alterations of NLF protein composition upon disease were detected: isoform distribution and protein levels of lipocortin-1 and CC-16 were strongly modified in allergic rhinitis and asthma, lipocalin-1, transthyretin and palate lung nasal epithelial clone protein levels are modified in the NLF 2-DE pattern of individuals after exposure to irritating chemicals.

Finally, search for lung-specific proteins in serum samples has proven useful to diagnose certain lung disorders. Indeed, several lung-specific proteins can be detected in serum, as a result of their spontaneous leakage across the air–blood barrier, and thereby provide another means of assessing the integrity of the alveolar–capillary barrier, less invasively than by measuring the levels of serum antigens in BALF [40,70]. Lung-specific SP-A, SP-B, SP-D, CC-16 and mucin-associated antigens are immunodetected in serum by ELISAs and their respective amounts are modified according to definite lung disorders. Serum SP-A significantly increases in patients with IPF and pulmonary alveolar proteinosis [71]. Cardiac lung oedema and acute respiratory distress syndrome (ARDS) are accompanied by a significant increase in SP-A and SP-B in serum [72]. SP-D is significantly higher in serum of patients with fibrosis, pulmonary alveolar proteinosis, and tuberculosis [73,74]. Serum CC-16 is higher in patients with chronic bronchitis, sarcoidosis and pulmonary fibrosis [75,76]. Mucin KL-6 is elevated in serum of patients with active sarcoidosis [77] and interstitial lung disorders like hypersensitivity pneumonitis [78] and idiopathic pulmonary fibrosis [79]. Mucins 17-B1 and 17-Q2 increase in serum from patients with cystic fibrosis and ARDS, respectively [80,81].

7. Future directions of BAL fluid proteomics

Efficient proteomics-based diagnosis of lung diseases will focus on different topics, in the near future.

First, completion of the human BALF 2-DE protein map will require a significant increase in the number and intensity of displayed protein spots. Fractionation, specific removal of major serum proteins (with albumin antibodies, for example), solubilization of hydrophobic and/or basic proteins, use of BALF samples from the widest range of pulmonary disorders, narrow-range pH gradients for the first dimensional separation, increased staining sensitivity (fluorescent staining), raised detection limits for spot identification are part of the tools that will have to be used to get closer to comprehensiveness of the BALF 2-DE map.

Other analytical methods are available that skip the 2-DE step which is, in the particular case of BALF samples, somehow limiting.

For example, SELDI-TOF (surface-enhanced laser desorption/ionisation-time-of-flight; reviewed by [82]) is able to determine, in total protein extracts, unique protein profiles describing the progression from healthy to diseased states and back. This technique, if implemented to the analysis of BALF samples, will, in a high-throughput fashion, allow BALF-based early diagnosis and therapy follow-up

of lung disorders and enable high-throughput identification of BALF components.

High-performance capillary electrophoresis has recently been successfully implemented to analyze small-scale biological compartments such as tear fluid and lung airway surface fluid [83]. Using a capillary sampling procedure for direct collection in the distal trachea, the authors could identify and quantify the major protein species present in the alveolar surface fluid [84]. Although the analytical resolution achieved with capillary electrophoresis cannot compete with two-dimensional electrophoresis, the analysis of microscale samples renders possible spatial information of different sites in the lung and temporal information by taking multiple samples over time, which is not feasible with macroscale sampling techniques.

Another procedure that could be well adapted to the analysis of BALF samples is based on the peptidomic approach [85]. One has to mention that BALF is already known to contain peptides, that these peptides may carry a functional importance in disease (reviewed in Ref. [86]), but that, unfortunately, classical SDS–PAGE separation methods are not suited to separate proteins of such a low molecular mass. Using the ''peptidomic-based'' technique, low-molecular-mass proteins (peptides) would be separated in the ''first'' dimension according to their hydrophobic properties via a reversed-phase high-performance liquid chromatography (RP-HPLC). Fractions would then be collected, each of them being separately submitted to the ''second'' dimensional separation, according their mass, via a mass spectrometry device (MALDI-TOF). A virtual 2-DE map would then be reconstituted with, as the *x*-axis, the retention time on the RP-HPLC column and, as the *y*-axis, the m/z ratio. The ''intensity'' of the ''spots'' displayed on this 2-DE map would be determined by the amount of consecutive HPLC fractions containing the protein considered, enabling map-to-map comparisons between healthy and diseased states to identify new disease markers of low molecular mass. Such procedure has already been implemented and is particularly well suited for the high-throughput analysis of body fluids.

Molecular biological methods to unravel functions of the disease markers identified by all those upper-described high-throughput approaches are the next step in the comprehension of the mechanisms involved in lung pathogenesis. In vitro generation of recombinant antibodies against putative disease markers will enable sensitive and integrative readouts of what could possibly be wrong at the protein level in a patient exhibiting lung deficiencies, thanks to simultaneous monitoring of proteins in BALF or any other body fluid using immunoblotting, flow cytometry or antibody arrays. Integration of the results gained in each of the obtainable biological samples—condensate, induced sputum, BALF, serum, nasal lavage fluid—about every affordable biomarker: not only proteinic, but also phospholipidic, nucleic acidic and any other assayable metabolite, will in the long term complete the global view of lung function and pathology.

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References

[1] H.Y. Reynolds, Lung 178 (2000) 271.
[2] Z. Chlap, U. Jedynak, K. Sladek, Pneumonol. Alergol. Pol. 66 (1998) 321.
[3] J.A. Jacobs, E. De Brauwer, Hosp. Med. 60 (1999) 550.
[4] A.H. Tiroke, B. Bewig, A. Haverich, Clin. Transplant. 13 (1999) 131.
[5] G. Mikuz, A. Gschwendtner, Verh. Dtsch. Ges. Pathol. 84 (2000) 129.
[6] M. Griese, Eur. Respir. J. 13 (1999) 1455.
[7] F. Moonens, C. Liesnard, F. Brancart, J.P. Van Vooren, E. Serruys, Scand. J. Infect. Dis. 27 (1995) 358.
[8] P. Ralph, I. Nakoinz, A. Sampson-Johannes, S. Fong, D. Lowe, H.Y. Min, L. Lin, J. Immunol. 148 (1992) 808.
[9] L. Armstrong, A.B. Millar, Thorax 52 (1997) 442.
[10] T.L. Bonfield, M.W. Konstan, P. Burfeind, J.R. Panuska, J.B. Hilliard, M. Berger, Am. J. Respir. Cell. Mol. Biol. 13 (1995) 257.
[11] H. Hamm, J. Luhrs, J. Guzman y Rotaeche, U. Costabel, H. Fabel, W. Bartsch, Chest 106 (1994) 1766.
[12] Y. Honda, H. Takahashi, N. Shijubo, Y. Kuroki, T. Akino, Chest 103 (1993) 496.

[13] R.E. Banks, M.J. Dunn, D.F. Hochstrasser, J.C. Sanchez, W. Blackstock, D.J. Pappin, P.J. Selby, Lancet 356 (2000) 1749.
[14] J. Macri, S.T. Rapundalo, Trends Cardiovasc. Med. 11 (2001) 66.
[15] V.E. Bichsel, L.A. Liotta, E.F. Petricoin, 3rd, Cancer J. 7 (2001) 69.
[16] C. Rohlff, Int J Neuropsychopharmacol. 4 (2001) 93.
[17] S. Kennedy, Toxicol. Lett. 120 (2001) 379.
[18] J.E. Celis, H. Wolf, M. Ostergaard, Electrophoresis 21 (2000) 2115.
[19] M.G. Harrington, C.R. Merril, D.M. Asher, D.C. Gajdusek, N. Engl. J. Med. 315 (1986) 279.
[20] G.E. Hook, D.Y. Bell, L.B. Gilmore, D. Nadeau, M.J. Reasor, F.A. Talley, Lab. Invest. 39 (1978) 342.
[21] B. Muller, P. von Wichert, Am. Rev. Respir. Dis. 130 (1984) 674.
[22] D.Y. Bell, G.E. Hook, Am. Rev. Respir. Dis. 119 (1979) 979.
[23] D.Y. Bell, J.A. Haseman, A. Spock, G. McLennan, G.E. Hook, Am. Rev. Respir. Dis. 124 (1981) 72.
[24] B. Muller, P. von Wichert, Klin. Wochenschr. 63 (1985) 781.
[25] R. Westermeier, W. Postel, J. Weser, A. Gorg, J Biochem. Biophys. Methods 8 (1983) 321.
[26] A.G. Lenz, B. Meyer, H. Weber, K. Maier, Electrophoresis 11 (1990) 510.
[27] A.G. Lenz, B. Meyer, U. Costabel, K. Maier, Electrophoresis 14 (1993) 242.
[28] M. Lindahl, B. Stahlbom, C. Tagesson, Electrophoresis 16 (1995) 1199.
[29] R. Wattiez, C. Hermans, A. Bernard, O. Lesur, P. Falmagne, Electrophoresis 20 (1999) 1634.
[30] F. Sabounchi-Schutt, J. Astrom, A. Eklund, J. Grunewald, B. Bjellqvist, Electrophoresis 22 (2001) 1851.
[31] R. Wattiez, C. Hermans, C. Cruyt, A. Bernard, P. Falmagne, Electrophoresis 21 (2000) 2703.
[32] A. Boutten, P. Venembre, N. Seta, J. Hamelin, M. Aubier, G. Durand, M.S. Dehoux, Am. J. Respir. Cell. Mol. Biol. 18 (1998) 511.
[33] R. White, A. Janoff, H.P. Godfrey, Lung 158 (1980) 9.
[34] H. Oku, T. Toda, J. Nagata, M. Ishikawa, K. Neyazaki, C. Shinjyo, I. Chinen, Biosci. Biotechnol. Biochem. 61 (1997) 286.
[35] H.L. Chen, D. Gabrilovich, A. Virmani, I. Ratnani, K.R. Girgis, S. Nadaf-Rahrov, M. Fernandez-Vina, D.P. Carbone, Int. J. Cancer 67 (1996) 756.
[36] S. Szabo, Z. Barbu, L. Lakatos, I. Laszlo, A. Szabo, Respiration 39 (1980) 172.
[37] R.C. Strunk, D.M. Eidlen, R.J. Mason, J. Clin. Invest. 81 (1988) 1419.
[38] P.J. Haidaris, Blood 89 (1997) 873.
[39] A.J. Hance, C. Saltini, R.G. Crystal, Am. Rev. Respir. Dis. 137 (1988) 17.
[40] C. Hermans, A. Bernard, Am. J. Respir. Crit. Care Med. 159 (1999) 646.
[41] B. Knoops, A. Clippe, C. Bogard, K. Arsalane, R. Wattiez, C. Hermans, E. Duconseille, P. Falmagne, A. Bernard, J. Biol. Chem. 274 (1999) 30451.
[42] F.X. McCormack, T.E. King Jr., D.R. Voelker, P.C. Robinson, R.J. Mason, Am. Rev. Respir. Dis. 144 (1991) 160.
[43] M. Lindahl, B. Stahlbom, J. Svartz, C. Tagesson, Electrophoresis 19 (1998) 3222.
[44] M. Lindahl, J. Svartz, C. Tagesson, Electrophoresis 20 (1999) 881.
[45] M. Lindahl, B. Stahlbom, C. Tagesson, Electrophoresis 20 (1999) 3670.
[46] B. Redl, P. Wojnar, H. Ellemunter, H. Feichtinger, Lab. Invest. 78 (1998) 1121.
[47] F. Ogushi et al., Am. J. Respir. Crit. Care Med. 152 (1995) 2133.
[48] M. Jordana, M. Dolovich, M. Newhouse, Sarcoidosis 4 (1987) 116.
[49] M.J. Tino, J.R. Wright, Am. J. Physiol. 270 (1996) L677.
[50] E.C. Breen, Z. Fu, H. Normand, Am. J. Respir. Cell. Mol. Biol. 21 (1999) 746.
[51] F. Cajone, G.V. Sherbet, Clin. Exp. Metastasis 17 (1999) 865.
[52] M. Kasper, P. Lackie, M. Haase, D. Schuh, M. Muller, Virchows Arch. 428 (1996) 207.
[53] E. dos Santos Silva, M. Ulrich, G. Doring, K. Botzenhart, P. Gott, J. Pathol. 190 (2000) 133.
[54] M. Griese, C. von Bredow, P. Birrer, Electrophoresis 22 (2001) 165.
[55] P.D. Jones, R. Hankin, J. Simpson, P.G. Gibson, R.L. Henry, Am. J. Respir. Crit. Care Med. 164 (2001) 1146.
[56] A. Spanevello, G.B. Migliori, A. Satta, M. Neri, P.W. Ind, Monaldi Arch. Chest Dis. 50 (1995) 208.
[57] N.R. Henig, M.R. Tonelli, M.V. Pier, J.L. Burns, M.L. Aitken, Thorax 56 (2001) 306.
[58] C. Anderson, N. Inhaber, D. Menzies, Am. J. Respir. Crit. Care Med. 152 (1995) 1570.
[59] D. Olivieri, R. D'Ippolito, A. Chetta, Curr. Opin. Pulm. Med. 6 (2000) 411.
[60] W. Ichikawa, F. Ogushi, K. Tani, K. Maniwa, M. Kamada, Y. Ohmoto, M. Sakatani, S. Sone, Respirology 4 (1999) 375.
[61] D.F. Schoonbrood, R. Lutter, F.J. Habets, C.M. Roos, H.M. Jansen, T.A. Out, Am. J. Respir. Crit. Care Med. 150 (1994) 1519.
[62] L. Scheideler, H.G. Manke, U. Schwulera, O. Inacker, H. Hammerle, Am. Rev. Respir. Dis. 148 (1993) 778.
[63] P. Montuschi, M. Corradi, G. Ciabattoni, J. Nightingale, S.A. Kharitonov, P.J. Barnes, Am. J. Respir. Crit. Care Med. 160 (1999) 216.
[64] B. Balint, L.E. Donnelly, T. Hanazawa, S.A. Kharitonov, P.J. Barnes, Thorax 56 (2001) 456.
[65] M. Corradi, P. Montuschi, L.E. Donnelly, A. Pesci, S.A. Kharitonov, P.J. Barnes, Am. J. Respir. Crit. Care Med. 163 (2001) 854.
[66] C.T. Carpenter, P.V. Price, B.W. Christman, Chest 114 (1998) 1653.
[67] P. Montuschi, J.V. Collins, G. Ciabattoni, N. Lazzeri, M. Corradi, S.A. Kharitonov, P.J. Barnes, Am. J. Respir. Crit. Care Med. 162 (2000) 1175.
[68] P.N. Dekhuijzen, K.K. Aben, I. Dekker, L.P. Aarts, P.L. Wielders, C.L. van Herwaarden, A. Bast, Am. J. Respir. Crit. Care Med. 154 (1996) 813.
[69] M. Lindahl, B. Stahlbom, C. Tagesson, Electrophoresis 22 (2001) 1795.

[70] C. Hermans, A. Bernard, Eur. Respir. J. 11 (1998) 801.
[71] Y. Kuroki, S. Tsutahara, N. Shijubo, H. Takahashi, M. Shiratori, A. Hattori, Y. Honda, S. Abe, T. Akino, Am. Rev. Respir. Dis. 147 (1993) 723.
[72] I.R. Doyle, A.D. Bersten, T.E. Nicholas, Am. J. Respir. Crit. Care Med. 156 (1997) 1217.
[73] Y. Honda, Y. Kuroki, E. Matsuura, H. Nagae, H. Takahashi, T. Akino, S. Abe, Am. J. Respir. Crit. Care Med. 152 (1995) 1860.
[74] A. Kondo et al., Kekkaku 73 (1998) 585.
[75] C. Hermans, B. Knoops, M. Wiedig, K. Arsalane, G. Toubeau, P. Falmagne, A. Bernard, Eur. Respir. J. 13 (1999) 1014.
[76] F. Broeckaert, A. Clippe, B. Knoops, C. Hermans, A. Bernard, Ann. N. Y. Acad. Sci. 923 (2000) 68.
[77] J. Kobayashi, S. Kitamura, Chest 109 (1996) 1276.
[78] M. Ando, M. Suga, H. Kohrogi, Curr. Opin. Pulm. Med. 5 (1999) 299.
[79] A. Yokoyama, N. Kohno, H. Hamada, M. Sakatani, E. Ueda, K. Kondo, Y. Hirasawa, K. Hiwada, Am. J. Respir. Crit. Care Med. 158 (1998) 1680.
[80] C.B. Robinson, W.R. Martin, J.L. Ratliff, P.V. Holland, R. Wu, C.E. Cross, Am. Rev. Respir. Dis. 148 (1993) 385.
[81] J.Y. Shih, S.C. Yang, C.J. Yu, H.D. Wu, Y.S. Liaw, R. Wu, P.C. Yang, Am. J. Respir. Crit. Care Med. 156 (1997) 1453.
[82] M. Merchant, S.R. Weinberger, Electrophoresis 21 (2000) 1164.
[83] K. Govindaraju, D.K. Lloyd, J. Chromatogr. B Biomed. Sci. Appl. 745 (2000) 127.
[84] K. Govindaraju, E.A. Cowley, D.H. Eidelman, D.K. Lloyd, J. Chromatogr. B Biomed. Sci. Appl. 705 (1998) 223.
[85] P. Schulz-Knappe, H.D. Zucht, G. Heine, M. Jurgens, R. Hess, M. Schrader, Comb. Chem. High Throughput Screen 4 (2001) 207.
[86] S.M. Travis, P.K. Singh, M.J. Welsh, Curr. Opin. Immunol. 13 (2001) 89.
[87] M. Lindahl, T. Ekstrom, J. Sorensen, C. Tagesson, Thorax 51 (1996) 1028.