ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 815–824

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Proteomics — the protein expression technology to study connective tissue biology

G. Westergren-Thorsson [a], J. Malmström [a], G. Marko-Varga [b,*]

[a] *Cell and Molecular Biology, University of Lund, Box 124, 221 00 Lund, Sweden*
[b] *Astra Zeneca R&D Lund, Molecular Sciences, Scheelevägen 8, SE-22187 Lund, Sweden*

Received 16 May 2000; received in revised form 22 November 2000; accepted 23 November 2000

Abstract

During the formation of peribronchial fibrosis in asthma, remodeling of connective tissue is due to an increase in deposition of extracellular matrix components like that of specific types of collagens and proteoglycans. By taking bronchial biopsies, we were able to isolate cell cultures derived from asthmatic patients and healthy volunteers, which provides a good model system to study differences regarding cell morphology and key connective tissue proteins in the remodeling process. Proteomics, utilizing two-dimensional electrophoresis and modern image analysis systems have made it possible to study protein expression and regulation of proteins in biological systems. By using this powerful tool, it is possible to quantitatively study protein regulation and to obtain increased knowledge about the mechanism behind the inflammatory process and formation of peribronchial fibrosis. We have optimized a proteomic protocol enabling detailed investigation of the protein expression pattern in human lung cells. An increased expression pattern was obtained, whereby 20 protein spots could be detected by image analysis in the <45 kDa region. Out of these, specific regulations of four spots were found by quantitative image analysis and spots of interest were identified by MALDI TOF-MS. This protocol enables us to study 1000–2000 proteins simultaneously and the possibility to correlate protein expression to the physiological status of the cell culture investigated. We have found that two proteins, actin and tropomyosin, are increased in expression due to transforming growth factor-β stimulation. These proteins are correlated to the transformation of normal fibroblasts to myofibroblasts which are involved in the remodeling processes observed in asthma.

Keywords: Proteomics; Asthma; Proteoglycans

1. Introduction

Analytical technology development directed towards mapping protein expression is currently in the focus of biological understanding. Differential display expression analysis is directed towards events taking place in cells, tissues, and organs as

* Corresponding author. Tel.: +46-46-336887; fax: +46-46-337383.
E-mail address: gyorgy.marko-varga@astrazeneca.com (G. Marko-Varga).

0731-7085/01/$ - see front matter
PII: S0731-7085(00)00548-3

well as in a number of biofluids [1,2]. The current activity of mapping the human genome certainly has a strong link to the resulting protein expression why proteomics has entered into the highlights of life science [1]. The key to facilitate protein identifications and their structure that is to be linked to the protein function all comes back to applying high quality analytical techniques and methods. Proteomics is a research area with fast improvements in methodology as well as new technology introductions [1]. The most efficient high resolution separation technique available today for protein separations is still two-dimensional gel electrophoresis [3,4]. The proteins of interest are then identified by mass spectrometry. Accuracy from modern mass spectrometry instruments are typically in the 5–10 ppm range which is in line with the search routines used in data base mining and data base identifications.

By using this powerful tool it is possible to get increased knowledge about the mechanism behind the inflammatory process and formation of peribronchial fibrosis seen in asthma. In asthma, a profound change of the organization of the extracellular matrix have been recorded, where peribronchial fibrosis is a distinct feature as well as smooth muscle hyperplasia and hyper secretion of mucus [5]. Among the proteins that are elevated in the diseased extracellular matrix are some of the proteins in a group of proteins called proteoglycans which are substituted with carbohydrates [6–8]. Bensadoun et al., 1997 showed that one of the proteoglycans, versican, is one of the first molecules seen in the formation of new extracellular matrix [9]. It is believed that versican has a regulatory function in the formation of both normal and diseased extracellular matrix. The mechanism behind these profound changes around the bronchia in asthmatics may be related to induced changes of proteoglycan synthesis. The effect of this altered proteoglycan synthesis may also be direct cell surface receptor activation, effect on cytokine receptors, storage/inactivation or activation of cytokines and control of protease activity. The cell responsible for the elevated proteoglycan production is proposed to be the bronchial myofibroblasts [10,11]. The pro-fibrotic cytokine TGF-β is important for the transformation of fibroblasts to myofibroblasts, which have increased amounts of α-smooth muscle actin [12,13]. TGF-β have also been shown to alter the proteoglycan synthesis in normal proteoglycan synthesis [6,7,14] and is over-expressed in an asthmatic lung and, thus, regarded as important for the formation of fibrosis. [15–18]. In this study, we have compared the expression patterns of TGF-β stimulated and non-stimulated fibroblasts in the low mass range of proteins. We also have an expression profile of the proteoglycans in primary human lung cells derived from human asthmatics. Proteomics is applied to study a fibroblasts cell line to gain understanding about the alterations in connective tissues biology from asthmatic patients.

2. Experimental

2.1. Cell culture

Fibroblast cultures were established from central bronchial lung biopsies. Control cells were normal human lung fibroblasts (HFL-1) purchased from American Type Culture Collection (ATCC). Cells were grown in 25 cm^2 dishes in Eagel's minimum essential medium (EMEM) supplemented with 10% new-born calf serum and 1% glutamine, at 37°C in a humidified 5%/CO_2/95% air atmosphere. Confluent cultures were trypsinized and split 1:2 before replating. They usually became confluent after 3–4 days. The cells were regularly checked for mycoplasma using 4,6-diamidine-2-phenyl-dihydrochloride. Experiments were performed on cells between passage five and nine for fibroblasts from central lung biopsies and between passage 15 and 21 for the HFL-1 cells. For morphological identification, fibroblasts were studied by light microscopy after staining with crystal violet. The HFL-1 cells were grown in T-75 flasks with MEM medium (Sigma, M-4655) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in 5% CO_2 until confluence. The cells were stimulated with TGF-β 10 ng/ml (R&D Systems, Minneapolis, MN) or vehicle for 24 h in same medium as above, but

with 0.4% FBS. The adherent cells were scraped from the tissue culture flasks, and then harvested by centrifugation ($1000 \times g$). The cell pellets were stored at -80°C until further use. The amount of cells per T-75 flask was estimated to 10×10^6 cells by cell counting of trypsinated cells in both control and TGF-β treated cultures.

2.2. Preparation and identification of proteoglycans

At confluence, fibroblast cultures were labeled with 200 μCi/ml [^{35}S]-sulphate from ARC (St. Louis, MO, USA) for 24 h in sulfate-poor EMEM supplemented with 10% FBS. The culture medium was harvested an analyzed regarding amount and type of secreted proteoglycans.

The total amount of proteoglycans were separated by ion exchange chromatography as previously described [19]. Briefly, the cell medium was passaged over columns of (0.7×4 cm) DEAE-cellulose DE-52 from Whatman (Madistone, UK), which previously had been equilibrated with 6 M urea, 50 mM acetic acid, pH 5.8, 5 mM *N*-ethylmaleimide and 5 μg/ml of ovalbumin. To remove unincorporated radioactive precursors, the columns were washed with 60 bed volumes of the same solvent. Hyaluronan was eluted with six bed volumes of 6 M urea, 500 mM acetic acid, and 5 μg/ml of ovalbumin. Finally, total proteoglycans were eluted twice with three bed volumes of 4 M guanidium chloride, 50 mM sodium acetate, pH 5.8, and 5 μg/ml of ovalbumin.

Proteoglycans were then identified and separated into different types of proteoglycans by sodium doedecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After ethanol precipitation, electrophoresis was performed on 3–12% polyacrylamide gradient gel ($T/C = 30/0.8$) from Scot Lab. (Luton, UK), and by further using a 3% stacking gel and the buffer system of Laemmli [20], as described by Lindblom et al. [21]. The intensity of the different bands containing versican, perlecan, biglycan or decorin were further analyzed and visualized on an image plate which was scanned by a Fuji BAS 2000 image analyzer (Seikagaku Kogyo, Tokyo, Japan). These proteoglycans have earlier been identified in earlier work using Western blot, with specific antibodies against the various proteoglycans [22].

2.3. Sample preparations and gel electrophoresis

2.3.1. Sample preparation

Human fetal lung cells in various passages 15–21 were growth arrested by incubation in MEM with 0.4% FCS for 24 h. Following stimulation was achieved by incubation with TGF-β 10 ng/ml (R&D Systems, Minneapolis, MN) or vehicle for 24–48 h in MEM-medium (Sigma, M-3911) supplemented with 0.4% dialyzed FCS, 1 mM L-glutamine and 0.5 μg/ml cold methionine (Sigma, M-7520 St Louis, IL). The cells were washed with Hanks buffer and then lyzed in a mixture of 8 M urea and 2% CHAPS $40 + 80$ μl.

2.3.2. Two-dimensional gel electrophoresis

Immobiline dry strips (180 mm, pH 3–10 NL, Amersham Pharmacia Biotech, Uppsala Sweden) were rehydrated in 350 μl of the solubilization solution containing 7 M thiourea, 2 M urea, 4% CHAPS, 10 mM DTT, and 0.5% IPG 3–10 buffer, together with the fractionated samples.

The iso electro focusing (IEF) step was performed at 20°C in a IPGphor™ (Amersham Pharmacia Biotech, Uppsala, Sweden) and run according to the following schedule, (1) 30 V for 10 h; (2) 500 V for 1 h; (3) 1000 V for 1 h and (4) 4000 V until approximately 45 000 V h were reached. The strips were equilibrated for 10 min in a solution containing 65 mM DTT, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS and 50 mM Tris–HCl pH 8.8. A second equilibration step was also carried out for 10 min in the same solution except for DTT, which was replaced by 259 mM iodoacetamide. The strips were soaked in electrophoresis buffer (24 mM Tris base, 0.2 M Glycine and 0.1% SDS) just before the second-dimensional gel electrophoresis. The strips were applied on 14% homogeneous Duracryl (Genomic solutions, Chelmsford, MA, USA) slabgel. The strips were then overlaid with a solution of 1% agarose in electrophoresis buffer (kept at 60°C). Electrophoresis was carried out in an Hoefer™ DALT gel apparatus (Amersham Pharmacia Biotech, San Francisco, CA, USA) at 20°C and constant 100 V for 18 h.

2.3.3. Gel staining

Gels were stained with silver according to [23] Briefly, the gels were fixed in 50% ethanol and 5% acetic acid and then sensitized in 0.02% sodium thiosulfate. The staining was performed in 0.1% silver nitrate.

2.3.4. Spot analysis

Gels were scanned using a Fluor-S™ MultiImager (Bio-Rad Laboratories, Sundbyberg, Sweden) and Quantity One (version 4.0.3, Bio-Rad Laboratories, Sundbyberg, Sweden). Spot analysis was performed using the PDQUEST (version 6.1.0) two-dimensional gel analysis system (Bio-Rad discovery series, Bio-Rad Laboratories, Sundbyberg, Sweden).

2.4. Mass tryptic digest and extraction of peptides

After the image analysis Gels spots were excised from the gel and transferred to separate Eppendorf vials. Next the gel pieces were washed with ammonium bicarbonate buffer (50 mM) followed by acetonitrile extraction and reduction and alkylation. The in gel digestion of protein spots were performed overnight with Trypsin from porcine (Boehringer Mannheim Germany). For protein spots not identified in this first mode, further extraction of the gel pieces were performed using acetonitrile and 5% formic acid.

2.5. Mass spectrometry identification

The MALDI-TOF instrument was a Voyager DE-PRO (Perseptive Biosystems Inc., Framingham, MA, USA) mass spectrometer. The instrument, equipped with a delayed extraction ion source, utilize a nitrogen laser at 337 nm and was operated in reflectron mode at an accelerating voltages of 20 kV. The sample probes were made of polished stainless steel. Sample deposition of nanoliter fractions was made on stainless steel MALDI-target plates and on a 100 position stainless steel target plate (Perseptive Biosystems). The nanoliter fractions were gained by manually eluting small droplets from microextraction columns onto the MALDI target plate according to [24]. Gel extraction prior to MALDI identifications were made according to Shevchenko et al [23].

3. Results

Central bronchial biopsies were obtained from asthmatic patients and healthy volunteers. From these biopsies, cell cultures were established from central biopsies. The cell cultures derived from the biopsies were incubated in growth conditions favoring fibroblasts. The morphology of the cells obtained from central biopsies were typical for a fibroblast (see Fig. 1A and B). The shapes of the cells as well as the production of connective tissue are typical of human lung fibroblasts. Fig. 1C show cells from a well characterized cell line, HFL-1, which are similar to the cells derived from the biopsies regarding cell shape and connective tissue production. However, it could be noticed that the fibroblasts from the asthmatic were surrounded by more connective tissue than the HFL-1 cells (see Fig. 1). To be able to obtain sufficient amounts of cells, the cells were grown on plastic plates and split every week. This was repeated until the cells had been splitted five times and, thus, were in passage five.

The proteoglycan composition secreted from fibroblast cultures established from central lung biopsies, were further characterized after ion exchange chromatography with support material of DEAE-cellulose. The ion exchange chromatography was followed by SDS-PAGE where the different type of proteoglycans are lined up according to size (Fig. 2A). The major constituent produced by the biopsy derived fibroblasts were perlecan, versican, decorin and biglycan, which have been identified in earlier work by Western blot [22].

The ratio between the connective tissue components were approximately 6:6:4:3, respectively (see Fig. 2B). These quantities were calculated from the image scanned calculations. The proteoglycan pattern was found to be stable after passage five. Furthermore the variance between cell cultures derived from different biopsies in proteoglycan production is shown in Fig. 2. The interplay between the proteoglycans and the lung fibroblasts is regarded important in the formation of

peribronchial fibrosis. Therefore, we used a proteomics approach to search for proteins important for matrix formation in a diseased state. The overall work-scheme is presented in Fig. 3 where the main objective was to investigate the correlation between the intracellular proteins and the proteoglycan synthesis seen in the asthmatic cells.

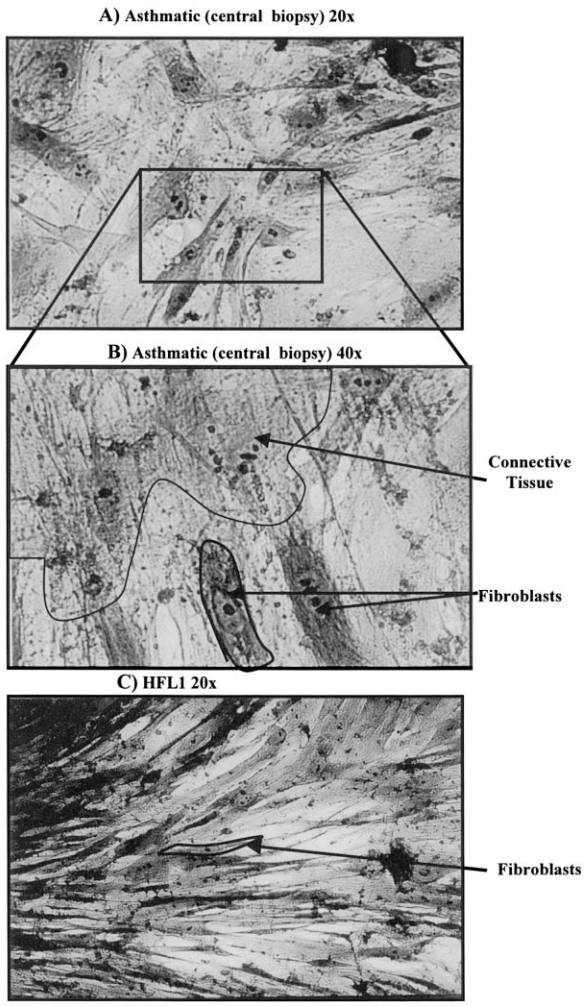

Fig. 1. Light microscopy pictures on, (a) human lung primary lung cells derived from central biopsies; (b) the same lung fibroblast from a central lung biopsy at a magnification of $\times 40$; (c) human lung fibroblasts (magnification $\times 20$). To visualize the cells easier, cells were stained with crystal violet. The HFL-1 cells were in passage 18 and the asthmatic cultures in passage five.

3.1. Two-dimensional gel electrophoresis analysis

In order to make extensive protein analysis by proteomics, a human fetal lung cell line, HFL-1 was used. By studying this human lung fibroblast cell line HFL-1, it was concluded that a significant number of proteins in the fibrosis process also showed altered expression. Prior to two-dimensional gel electrophoresis, the cells were treated with TGF-β which is regarded to be important in the development of peribronchial fibrosis [18]. The samples were run in triplicates and visualization of the separated proteins was achieved by silver staining according to methodology by Shevchenko [23]. The gels were analyzed by the PDQuest software, which assign each spot on the gel a given integrated optical density (IOD) value. This value was compared with the total amounts of valid spots, which was made in order to be able to reduce gel variations, like unequal staining or small differences throughout the gel work. Thus, each spot is expressed as a PPM of the total IOD of all valid spots. Every spot on the gel from TGF-β treated samples were compared with the corresponding spot on the gel with the control treated samples. The spots that had a statistically significant difference ($P < 0.05$) were regarded as regulated. After image analysis, gels spots were excised from the gel and transferred to separate Eppendorf vials and digested overnight.

The resulting peptide mixture extracted from the gel spots were identified by MALDI-MS. The dry droplet MALDI crystallization technique was applied using 1 μl of the sample. Some proteins were identified directly after submission of the peptide masses to the prowl search engine (www.prowl.com). In most cases, additional clean up and enrichment steps had to be performed using small reversed phase microcolumns [24] and direct elution with matrix. The amount of Trypsin used in the digestion step was found to be of importance in order to generate the autodigest fragments. If lower amounts are being used, the autodigest peaks will not appear in the spectrum, which makes the internal calibration impossible. External calibration in these situations will yield less good mass accuracy. A too high amount of Trypsin will result in strong autodigest signals

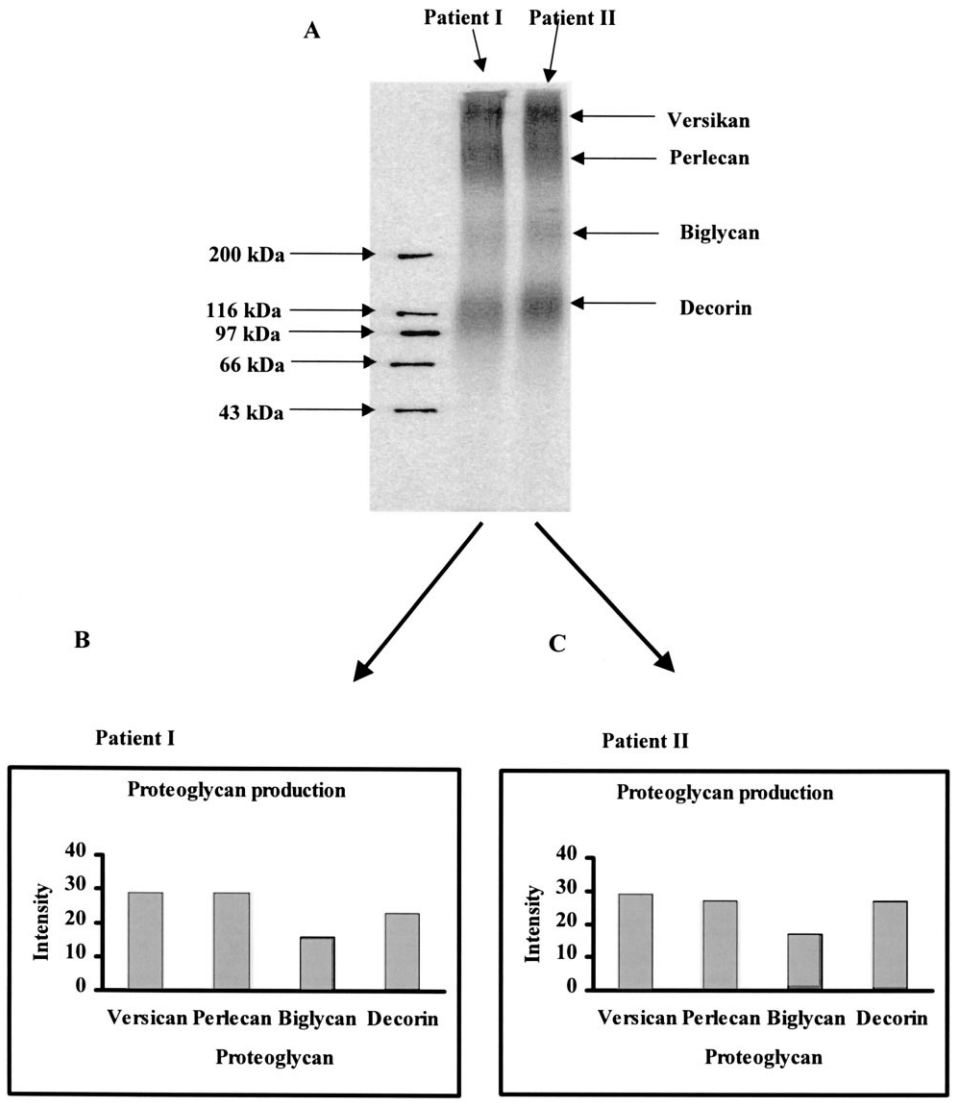

Fig. 2. Fibroblasts from patients with asthma were labeled for 24 h with [^{35}S]. Total secreted amount of proteoglycans was isolated by ion exchange chromatography. The various types of proteoglycans were further separated into different types of proteoglycans by SDS-PAGE, shown in (A). The radioactivity in the different bands was visualized and intensity was measured by Fuji BAS 2000 image analyzer, shown in (B). For further details see Section 2.

that will suppress the ionization of the peptides from the protein spot.

Fig. 4A shows a protein identification map of proteins identified on a human fibroblasts cell line. Some of the identified proteins were also significantly regulated. By using IPG strips with a more narrow pH 4.5–5.5 areas of interest can be studied in detail (see Fig. 4B). By using these zoom gels, proteins that are building blocks of the contractile machinery in fibroblasts could be studied in detail, regarding different isoforms and phosphorylation forms. Different isoforms of tropomyosin was increased by a factor of six to ten and actin was increased by a factor of three to five.

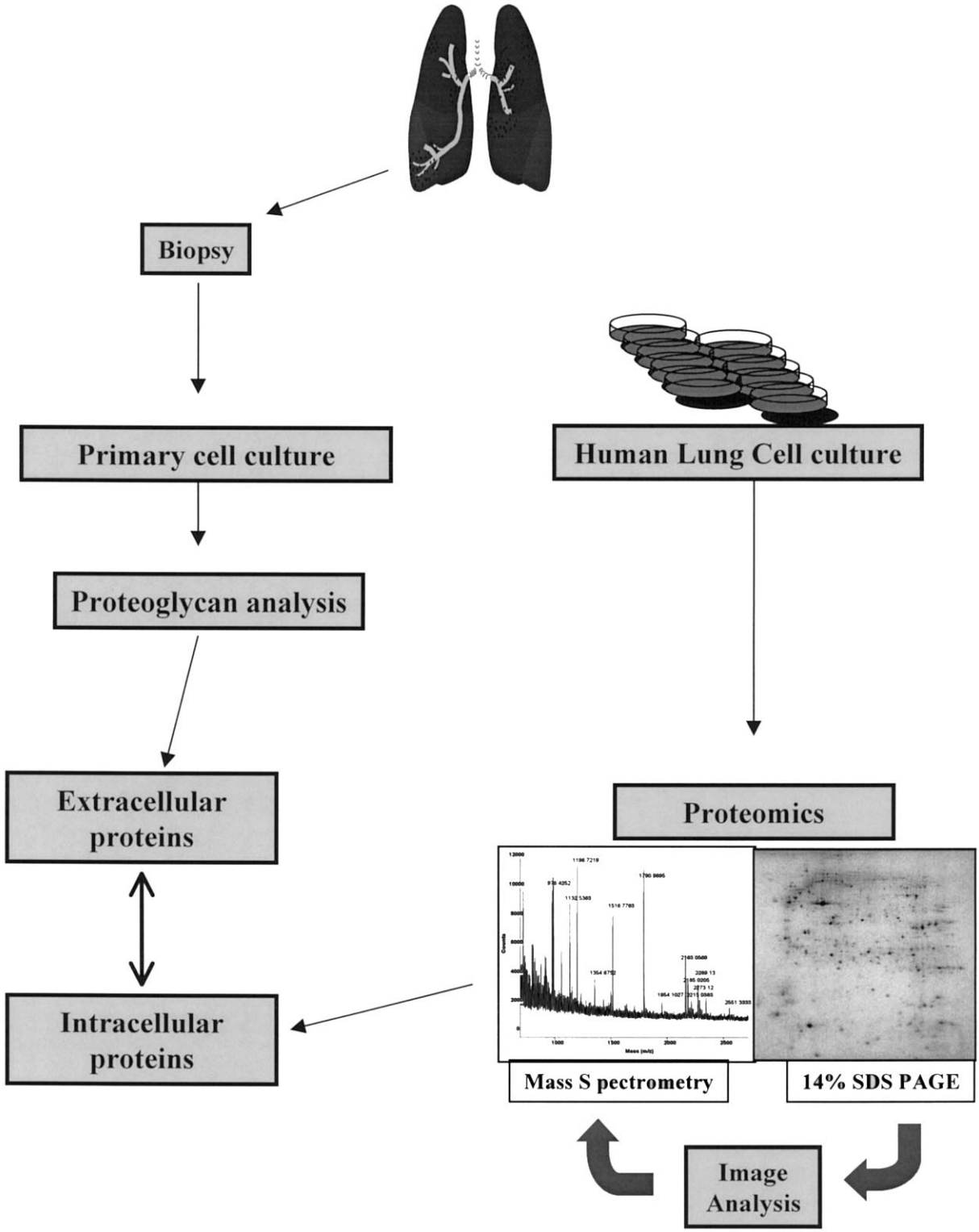
Biopsy
Primary cell culture
Proteoglycan analysis
Extracellular proteins
Intracellular proteins
Human Lung Cell culture
Proteomics
Mass S pectrometry
14% SDS PAGE
Image Analysis

Fig. 3.

4. Discussion

During formation of peribronchial fibrosis in asthma, remodeling of connective tissue is due to an increased deposition of extracellular matrix components like that of specific types of collagens and proteoglycans [25]. The increased deposition of these components causes the subepithelial layer to be more than double in diameter [26,27] and this thickening correlates to the asthma severity score [28–31]. The increased rigidity of the thickened lamina reticularis reduces the basement membrane capacity to fold during smooth muscle contraction. This in turn results in increased airway responsiveness [13,32,33]. By taking central bronchial biopsies, we were able to isolate cell cultures derived from asthmatic patients and healthy volunteers. These cell cultures were cultured in growth medium favoring fibroblast outgrowth and, thereby, obtaining a pure population of fibroblasts cells. These cells provide a good model system to study differences in asthmatics and healthy volunteers regarding cell morphology. By the use of ion-exchange chromatography, we were able to determine the stoichiometric relation between the four major proteoglycans. The proteoglycans are key molecules in the remodeling process in human tissue, and are important in peribronchial fibrosis and inflammation. The proteoglycan ratio shown in Fig. 2 show a normal proteoglycan pattern. However, in fibrotic extracellular matrix perlecan which is a proteoglycan rich in heparan sulfate side chains are increased [34]. The heparan sulfate side chains have several different biologically important features. Functions like binding cytokines [35], proteases [36], as well as having direct effect on the fibroblasts [37] are known effects. An increase in perlecan is seen in the fibrotic tissue, which mediates a different protease and cytokine pattern within the tissue [34]. Early in a fibrotic process or in vitro stimulation with TGF-β, biglycan and collagen are increased whereas decorin is decreased. This is of special interest since decorin is considered as one of the organizers of the collagen fibrils [38]. An increase in collagen with a decrease in decorin results in a large amount of unorganized collagen. Then, when decorin is activated this usually results in a fibrotic extracellular matrix [38]. Therefore, the ration between the component is of importance in the formation of normal extracellular matrix.

We have optimized a proteomic protocol enabling detailed investigation of the protein expression pattern in human lung fibroblasts, which are the main extracellular matrix producing cells. This protocol enables us to study 1000–2000 proteins simultaneously and a possibility to correlate the protein expression to the physiological status of the cell culture investigated. We could MS-identify three isoforms of tropomyosins and actin and, thereby, identify the regulation when using TGF-β as stimulus. These two proteins are important in the formation of contractile fibers. It has also been found that contractile proteins correlated to the transformation of normal fibroblasts to myofibroblasts [13]. The myofibroblasts are responsible to the remodeling in both normal wounds as well as in peribronchial fibrosis. At least two actin filament pools exist in the vertebrate cells, one which is stable and associated with tropomyosin. Tropomyosin isoforms organization and gene expression is responsive to alterations in actin gene expression [39]. It has previously been documented that TGF-β upregulated both actin and tropomyosins [40,41], however, how the different isoforms are affected is unclear. The resolution achieved by 2-D electrophoresis offers a possibility to view these factors at the same time. At least three different isoforms of tropomyosin are found to be expressed in our gels and all three of these isoforms are statistically significant upregulated. Current work is ongoing to identify in detail which isoforms respond to TGF-β and which do

Fig. 3. Flowchart of the work flow. The cells are extracted from the tissue according to the experimental section and HFL-1 cells were solubilized in a CHAPS and urea solution. The proteins are separated in the first dimension by isoelectric focusing and in the second dimension with a SDS-PAGE gel electrophoresis. The visualization was performed with Silver and the images were analyzed with the PDQuest software. Protein identification is determined with MALDI-TOF.

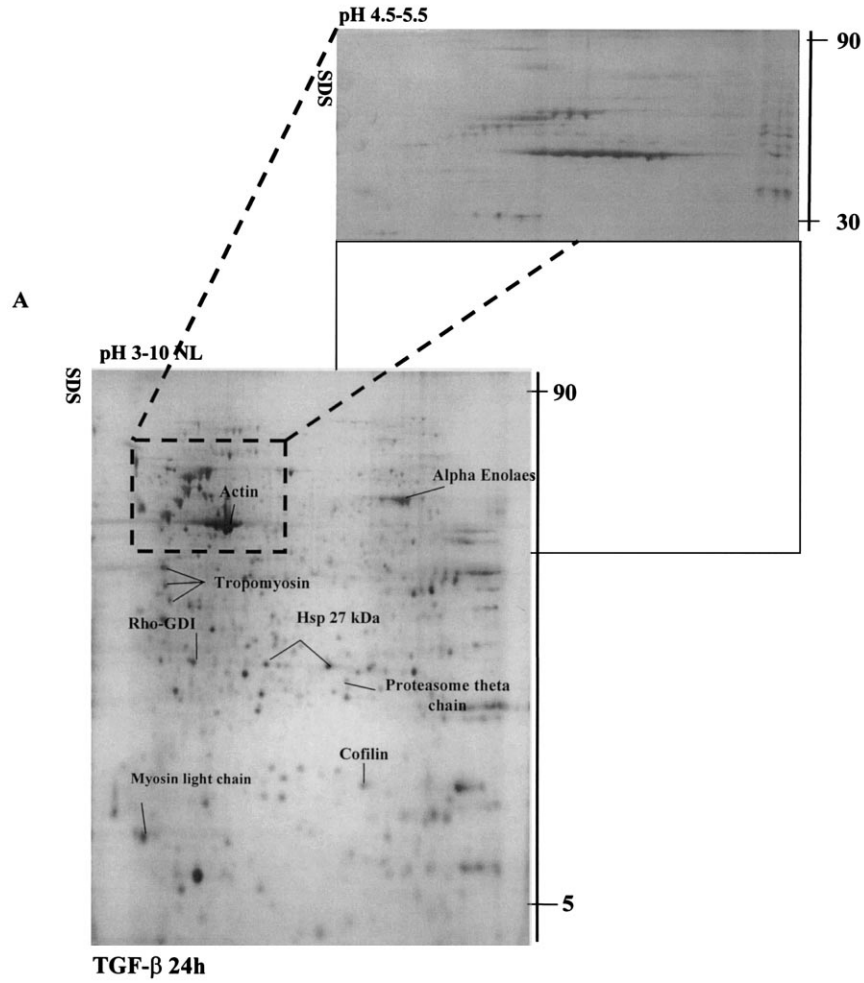

Fig. 4. Gels were run according to the experimental section. The load was 100 μg of total cellular protein. For the second dimension, 14% duracryl gels were used. Protein identification was accomplished with mass spectrometry. Area of key interest is enlarged with zoomgels with a narrow pH interval of 4.5–5.5.

not. The protocol that we have developed enable us to visualize both intracellular and extracellular proteins which all have been linked to the remodeling in peribronchial fibrosis. In the current study the co-expression of these factors allows us to search for the role of the organization of the cytoskeleton and the remodeling of the extracellular matrix, focusing on the proteoglycans. In a future perspective, the exact link between these proteins can be of major importance in determining the mechanisms behind the physiological and morphological changes induced by different exogenous stimulation.

References

[1] A. Abbott, Nature 402 (1999) 715–720.
[2] M.R. Wilkins, K.L. Williams, R.D. Appel, D.F. Hochstrasser, Proteome Research: New Frontiers in Functional Genomics, Heidelberg, 1996.
[3] P.H. O'Farrell, J. Biol. Chem. 250 (1975) 4007–4021.
[4] F. Lottspeich, Angew. Chem. Int. Ed. 38 (1999) 2477–2492.

[5] A.E. Redington, Clin. Exp. Allergy 30 Suppl. 1, 42–45.
[6] G. Westergren Thorsson, L. Coster, A. Akesson, F.A. Wollheim, J. Rheumatol. 23 (1996) 1398–1406.
[7] G. Westergren Thorsson, J. Hernnas, B. Sarnstrand, A. Oldberg, D. Heinegard, A. Malmstrom, J. Clin. Invest. 92 (1993) 632–637.
[8] G. Westergren Thorsson, M. Norman, S. Bjornsson, U. Endresen, Y. Stjernholm, G. Ekman, A. Malmstrom, Biochim. Biophys. Acta 1406 (1998) 203–213.
[9] E.S. Bensadoun, A.K. Burke, J.C. Hogg, C.R. Roberts, Eur. Respir. J. 10 (1997) 2731–2737.
[10] J.V. Jester, P.A. Barry Lane, H.D. Cavanagh, W.M. Petroll, Cornea 15 (1996) 505–516.
[11] J.V. Jester, J. Huang, P.A. Barry Lane, W.W. Kao, W.M. Petroll, H.D. Cavanagh, Invest. Ophthalmol. Vis. Sci. 40 (1999) 1959–1967.
[12] M.B. Vaughan, E.W. Howard, J.J. Tomasek, Exp. Cell. Res. 257, 180–189.
[13] C.E. Brewster, P.H. Howarth, R. Djukanovic, J. Wilson, S.T. Holgate, W.R. Roche, Am. J. Respir. Cell Mol. Biol. 3 (1990) 507–511.
[14] G. Westergren Thorsson, P. Antonsson, A. Malmstrom, D. Heinegard, A. Oldberg, Matrix 11 (1991) 177–183.
[15] J. Varga, J. Rosenbloom, S.A. Jimenez, Biochem. J. 247 (1987) 597–604.
[16] H.Y. Zhang, M. Gharaee Kermani, K. Zhang, S. Karmiol, S.H. Phan, Am. J. Pathol. 148 (1996) 527–537.
[17] M.L. Kruse, P.B. Hildebrand, C. Timke, U.R. Folsch, W.E. Schmidt, Regul. Pept. 90 (2000) 47–52.
[18] J. Gauldie, P.J. Sime, Z. Xing, B. Marr, G.M. Tremblay, Curr. Top. Pathol. 93 (1999) 35–45.
[19] K. Tiedemann, A. Malmstrom, G. Westergren Thorsson, Matrix Biol. 15 (1997) 469–478.
[20] U.K. Laemmli, Nature 227 (1970) 680–685.
[21] A. Lindblom, I. Carlstedt, L.A. Fransson, Biochem. J. 261 (1989) 145–153.
[22] E. Tufvesson, G. Westergren Thorsson, J. Cell Biochem. 77 (2000) 298–309.
[23] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Anal. Chem. 68 (1996) 850–858.
[24] J. Gobom, E. Nordhoff, E. Mirgorodskaya, R. Ekman, P. Roepstorff, J. Mass Spectrom. 34 (1999) 105–116.
[25] W.R. Roche, R. Beasley, J.H. Williams, S.T. Holgate, Lancet 1 (1989) 520–524.
[26] A. Chetta, A. Foresi, M. Del Donno, G. Bertorelli, A. Pesci, D. Olivieri, Chest 111 (1997) 852–857.
[27] L. Boulet, M. Belanger, G. Carrier, Am. J. Respir. Crit. Care Med. 152 (1995) 865–871.
[28] L.P. Boulet, M. Laviolette, H. Turcotte, A. Cartier, M. Dugas, J.L. Malo, M. Boutet, Chest 112 (1997) 45–52.
[29] A. Chetta, A. Foresi, M. Del Donno, G.F. Consigli, G. Bertorelli, A. Pesci, R.A. Barbee, D. Olivieri, Am. J. Respir. Crit. Care Med. 153 (1996) 910–917.
[30] B.R. Wiggs, C.A. Hrousis, J.M. Drazen, R.D. Kamm, J. Appl. Physiol. 83 (1997) 1814–1821.
[31] M. Okazawa, L. Wang, R.K. Lambert, L. Verburgt, S. Vedal, J.L. Bert, K.L. Pinder, P.D. Pare, Chest 107 (1995) 88s.
[32] R.K. Lambert, S.L. Codd, M.R. Alley, R.J. Pack, J. Appl. Physiol. 77 (1994) 1206–1216.
[33] A. Desmouliere, L. Rubbia Brandt, G. Grau, G. Gabbiani, Lab. Invest. 67 (1992) 716–726.
[34] G.M. Tremblay, M.J. Lafreniére-Allard, G. Westergren Thorsson, A. Malmström, J. Chakir, J. Dubé, L.P. Boulet, Am. J. Respir. Crit. Care Med. 157, (1998).
[35] E. Feyzi, F. Lustig, G. Fager, D. Spillmann, U. Lindahl, M. Salmivirta, J. Biol. Chem. 272 (1997) 5518–5524.
[36] V. Kainulainen, H. Wang, C. Schick, M. Bernfield, J. Biol. Chem. 273 (1998) 11563–11569.
[37] J. Malmstrom, G. Westergren Thorsson, Glycobiology 8 (1998) 1149–1155.
[38] P.J. Neame, C.J. Kay, D.J. McQuillan, M.P. Beales, J.R. Hassell, Cell Mol. Life Sci. 57 (2000) 859–863.
[39] F. Canonne Hergaux, J. Zwiller, D. Aunis, Neurochem. Int. 25 (1994) 545–553.
[40] P.D. Arora, C.A. McCulloch, Am. J. Pathol. 155 (1999) 2087–2099.
[41] A. Tada, H. Kato, K. Takenaga, S. Hasegawa, Cancer Lett. 121 (1997) 31–37.