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Dilated Cardiomyopathy: Computer-Assisted Analysis of Endomyocardial Biopsy Protein Patterns by Two-Dimensional Gel Electrophoresis

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Summary: In order to identify disease-associated alterations in the myocardial protein patterns in dilated cardiomyopathy, we used 2-dimensional gel electrophoresis to analyse the proteins of endomyocardial biopsies from patients and controls. Proteins (150 µg) from biopsies (1–3 mg wet weight) were first separated by isoelectric focusing, then applied to large 2-dimensional gels. A computer-assisted system (PDQUEST®) was used for spot detection, quantification and comparison of 2-dimensional protein patterns. From a single endomyocardial biopsy about 1000 different protein species were resolved. The spot pattern was influenced by the concentration of protein during sample preparation, by the amount of protein loaded onto the gels and by the development time of silver staining. Variances of spot position in the first and second dimension and in the long diagonals were less than 5%. Coefficients of variance for the spot quantities in 8 gels were 16 ± 8%. Contaminating blood proteins could be identified in the biopsy patterns. Computer-assisted comparison between cardiomyopathy (n = 5) and controls (n = 5) over the whole gel revealed that 55 protein spots were increased 100%, 27 protein spots decreased 100%. Four proteins showed significant quantitative differences between the cardiomyopathic hearts and controls.

Fourteen proteins were identified by amino acid analysis or microsequencing. An isoelectric point and molecular mass grid was laid over the whole gel based on these identified protein species, resulting in approximate isoelectric point values and molecular masses for all other protein species.

Thus, myocardial 2-dimensional protein patterns obtained from endomyocardial biopsies can be used for the characterization of cardiac diseases.

Introduction

Qualitative and quantitative alterations of myocardial proteins have been found in different cardiovascular diseases (1, 2), representing primary and secondary changes in myocardial metabolism. Both may contribute to functional impairment (3). Analysis of the myocardial protein patterns in specific cardiovascular diseases associated with heart failure may contribute to the understanding of morphological, biochemical and functional changes in this syndrome. Two-dimensional gel electrophoresis provides a means of separating up to 2000 proteins from small samples like endomyocardial biopsies in a single assay (4, 5). Image processing using pattern recognition techniques enables the qualitative and quantitative evaluation of the resulting protein patterns (6, 7). Single proteins can be identified by peptide mapping, immunoblotting, co-electrophoresis, amino acid analysis or microsequencing procedures and thus be brought into a functional context (5, 8, 9).

We used 2-dimensional electrophoresis followed by silver-staining, computer-assisted evaluation and amino acid analysis to detect changes in the myocardial protein pattern in dilated cardiomyopathy. The small gel technique, for screening purposes, and the large gel technique for final analysis were both optimized to yield highly reproducible patterns with high resolution from endomyocardial biopsies of a few milligrams of tissue.

The identification of single proteins by amino acid analysis was used as a base for the creation of a myocardial protein database. Significant differences in the protein pattern between cardiomyopathy patients and controls were demonstrated.

Materials and Methods

Myocardial samples

The study included endomyocardial biopsies from 5 patients with dilated cardiomyopathy and 5 patients in whom cardiomyopathy or myocarditis was suspected, but in whom the results of a complete left and right heart catheterization with biventricular and coronary angiography and endomyocardial biopsy were normal (tab. 1) (10, 11).

For methodological studies, samples were taken from a donor heart that was not transplanted because of single vessel disease affecting a diagonal branch. Samples were taken from an area of the right ventricular septum, where biopsies were usually sampled, which was not affected by the disease.

The biopsies were frozen less than 15 s after sampling, stored in dry ice and kept at −70 °C until analysis. Samples from the donor heart were also quickly frozen and kept at −70 °C.

Tissue preparation

Biopsies from 1 to 3 mg or samples of corresponding size from the explanted heart were pulverized under liquid nitrogen and solubilized in 20 µl buffer (9 mol/l urea, 25 mmol/l Tris/HCl pH 7.1, 50 mmol/l KCl, 3 mmol/l ethylenediamine tetraacetic acid, 2.9 mmol/l benzamidine, 2.1 µmol/l leupeptin, 70 mmol/l dithiothreitol and 20 ml/l ampholyte (Servalyte, pH 2−4, Serva, Heidelberg)). Finally 0.4 µl of additional protease inhibitors (5 µmol/l pepstatin, 50 mmol/l phenylmethylsulphonyl fluoride) were added. The resulting protein concentration was 15−30 g/l; 5−10 µl, corresponding to 150−200 µg protein, were used for one large gel electrophoresis and 3 µl corresponding to about 30−70 µg protein for a small gel (5, 11).

Protein concentration was determined according to *Lowry* et al., modified according to *Petersen*, using serum albumin as a standard (12, 13).

Two-dimensional gel electrophoresis, small gel technique and large gel technique

The isoelectric focusing for the first dimension and the sodium dodecyl sulphate polyacrylamide gel electrophoresis for the second dimension for both techniques were used as described previously (14, 15).

The isoelectric focusing gels contained 9 mol/l urea, 5% glycerol, 3.5% acrylamide, 0.3% piperazine diacrylamide and 4% of a complex ampholyte mixture pH 2−11 (WITA, Teltow, Germany). The gels were 7.7 cm or 20.2 cm long and 0.15 cm thick. Samples were placed at the anodic site. Focussing was done for 5.5 h with 1842 Vh (small gels) or 21 h with 8867 Vh (large gels). After focussing the gels were equilibrated for 10 min with buffer (125 mmol/l Tris/phosphate pH 6.9, 40% glycerol, 76 mmol/l dithiothritol, 3% sodium dodecyl sulphate) and frozen at −70 °C.

For the second dimension, sodium dodecyl sulphate polyacrylamide gel electrophoresis according to *Laemmli* (16) was used omitting the stacking gel. Gels contained 150 g/l acrylamide and 3 g/l bisacrylamide. The 2-dimensional electrophoresis gels measured 6.5 × 8 × 0.15 cm (small gels) and 23 × 29 × 0.15 cm (large gels). Gels were run for 90 min or 7 to 7.5 hours. After completion of the runs the gels were fixed overnight in fixation solution consisting of methanol/water/acetic acid (50 + 40 + 10, by vol.).

For detection of protein spots in the nanogram range, a silver-staining was used (17). After staining gels were dried at 90 °C under vacuum.

Computer-assisted analysis of 2-dimensional electrophoresis gels

Scanning and spot detection

The PDQUEST® system from pdi (protein and dna imageware system Inc., N. Y., USA) was used for the computer-assisted analysis of the gels. A scanner converted the original silver-stained gel into a digitized image. The gel image was cleaned from horizontal and vertical streaks and background. Spot detection yielded a synthetic image in which each protein spot was fitted by a two-dimensional *Gaussian* curve and described by five parameters (6, 7). Out of these *Gaussian* parameters a synthetic image (gel spot file) was constructed which represented a data-reduced approximation of the original image. Automatic spot detection accurately detected a high percentage of spots, but always some spots were not or incorrectly detected by this procedure. Therefore it was necessary to correct the results of spot detection by reviewing the spots and editing the saturated regions for undetected spots or spots that had not been properly *Gaussian* fitted in 2 dimensions.

Gel matching

After monitoring and correcting the spot-fitting of all images, a single "standard" image was created. After matching, this contained information on every protein spot of all gels. In the matching process all members are compared one with the other, in order to investigate qualitative or quantitative differences in sets of gels. The matching is based on landmark spots, which are manually identified well-resolved spots in all gels. The accuracy of matching depends upon the number of landmarks used and the geometrical distortion of the gels.

Normalization

For quantitative comparison of spot intensities of different silver-stained gels a normalization is required. We normalized the absolute intensities of each spot of each member into relative intensities by dividing each absolute intensity by the sum of the intensities of all valid matched-to-all spots. Since the normalization is done for each gel and the sum is calculated using the same matched spots in each gel, differences caused by staining or exposure processes are reduced.

Quantitative analysis

For quantitative analysis it is important to know how many valid protein spots are resolved in each gel, how many and which spots are saturated and how many protein species can be found in every gel. The number of spots that are increased or decreased in intensity can then be ascertained, and their relative intensities can be determined. Statistical tests are used to reveal significant differences in cardiomyopathy versus control.

Statistics

Data are given as median and range, if not indicated otherwise. For comparison of spot quantities between controls and dilated cardiomyopathy, the U test of *Mann-Whitney* was used.

Tab. 1 Patients included in the study
a) suspected cardiomyopathy or myocarditis, but normal cardiological findings (n = 5)
b) dilated cardiomyopathy (n = 5)

Patient	Age (a)	Sex	Diagnosis	Therapy					Cardiological findings				
				Digitalis glycosides	Diuretics	Angiotensin converting enzyme inhibitors	β-Blockers	Calcium-antagonists	Ejection fraction (%)	Left ventricular enddiastolic volume index (ml/m²)	Left ventricular enddiastolic pressure (mmHg)	Pulmonary artery pressure (mean) (mmHg)	Cardiac index (l/m² min)
Group a													
Di	64	♂	Hypertension	−	+	+	−	+	54	138	10	20	3.1
Kö	50	♂	Aortic stenosis 1, Aortic regurgitation 1	−	+	−	−	−	55	120	12	20	5.2
Kr	67	♂	AV-Tachycardia	−	−	−	+	−	51	124	12	20	2.7
Mi	59	♂	Hyperlipo-proteinaemia	−	−	−	−	−	63	96	10	15	2.8
Se	66	♂	Diabetes mellitus, Hypertension	−	+	−	+	−	66	97	12	18	3.7
Group b													
Ay	42	♂	Dilated cardiomyopathy, Chronic obstructive lung disease	+	+	+	−	−	33	156	10	12	2.8
Dit	51	♂	Dilated cardiomyopathy	+	−	−	−	+	50	120	16	23	3.9
Ka	51	♂	Dilated cardiomyopathy	+	+	−	−	−	25	159	15	23	2.5
Le	25	♂	Dilated cardiomyopathy	+	+	+	−	−	25	226	27	40	2.8
Si	61	♂	Dilated cardiomyopathy, Diabetes mellitus	+	+	+	−	−	28	114	30	48	2.6

Results

Recovery of spots from small gels in comparison with large gels

With the small gel technique it was possible to obtain reproducible protein patterns from small amounts of protein with reasonable resolutions. In the experiment represented in figure 1a and 1b, 239 spots have been resolved from only 30 µg myocardial protein. In contrast, up to 1092 valid spots were detected in the large gels, but about 150 µg protein are needed for such patterns (fig. 2). Thus, the small gel technique is useful for quick screening assays to check sample preparation, amount of protein loaded or buffer systems if a new assay method is being established, but large gels are needed for final analysis with high resolution.

Reproducibility of spot position and of spot quantity

In 8 small gels and 32 large gels the distances representing the 1st dimension, 2nd dimension and diagonals were measured. The coefficient of variation for the first dimension was 4.7% and 3.6% for the small and large gels, respectively, for the second dimension 1.2% and 4.9% respectively, and in the diagonal direction it was 2.5% and 3.4–4.2%.

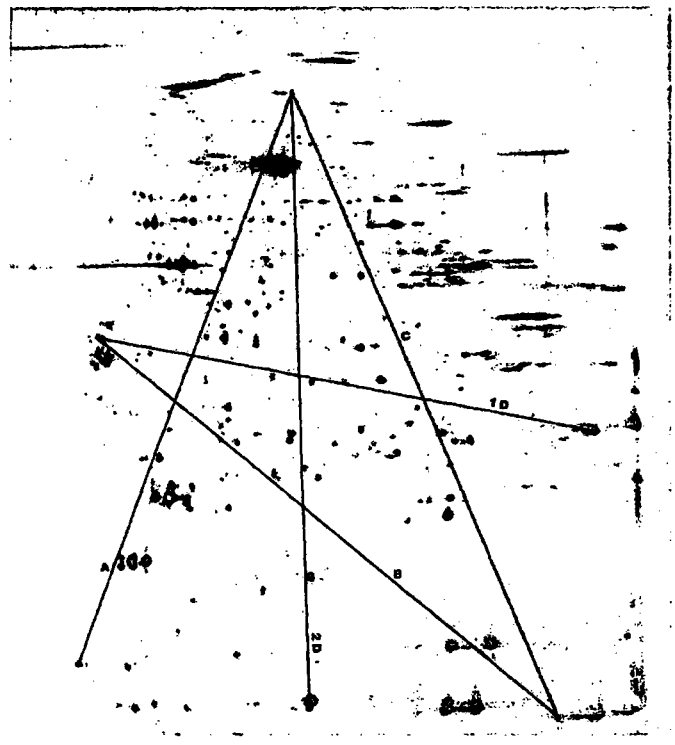

Fig. 2 Original gel scan of a silver-stained large gel from a control patient (endomyocardial biopsy, 170 µg protein/gel). Distances in the first and second dimension (1D, 2D) and in the diagonals A, B, C have been used to assess the reproducibility of spot position.

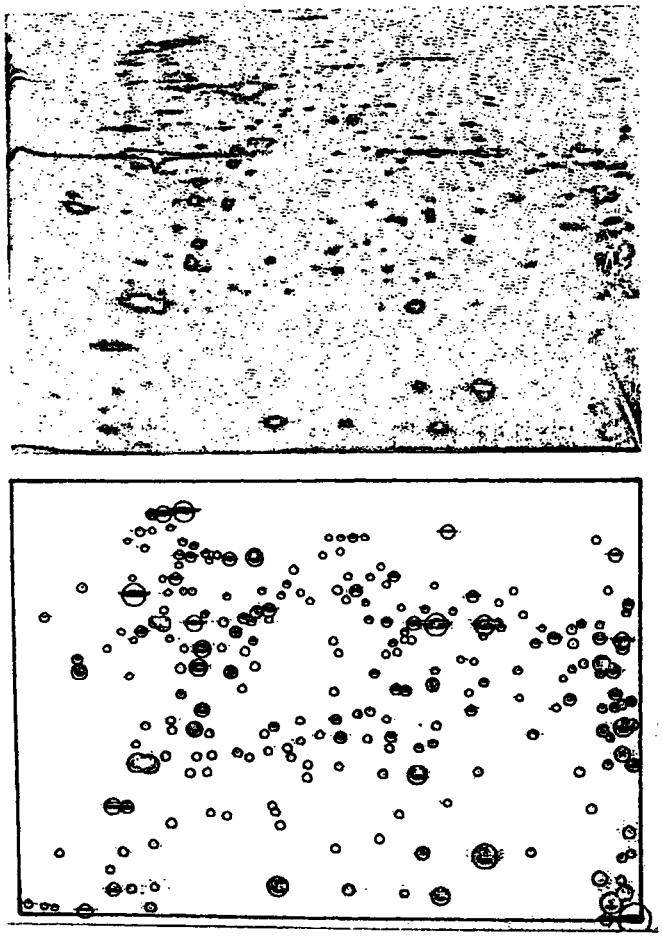

Fig. 1 Gel scan of a silver-stained small gel (above) and the corresponding result of the automated spot detection procedure (below). Proteins (30 µg) from an explanted heart were loaded on the gel. Twohundred thirty nine spots were automatically detected.

To assess the reproducibility of spot quantity, the mean value of spot quantity in protein data units was determined for 16 arbitrarily selected spots in 8 small gels from the same sample (tab. 2). The coefficient of variation ranged from 8% (spot 5) to 35% (spot 13), with a mean of 18.5%.

Effect of the staining procedure and the protein mass on spot quantity

In two series of 8 small gels that differed only in the development time (3.5 or 7 min) of silver staining, 5 spots were compared. If staining was prolonged, the quantities of the 5 different protein spots increased by about the same factor, i.e. 11.4 ± 2.3 fold.

The effect of the amount of protein loaded on a gel was determined in 8 gels that differed only in this variable. For each of 8 spots, measured protein data units increased linearly with increasing amount of proteins on the gel. However, the slopes were different, indicating that increasing the amount of protein does not increase the measured spot quantities of different proteins by the same factor (fig. 3).

Tab. 2 Reproducibility of spot quantity

Spot	Protein data units ($\bar{x} \pm s$)	CV (%)
1	74 ± 8	11
2	241 ± 27	11
3	91 ± 13	14
4	102 ± 11	11
5	165 ± 15	9
6	79 ± 20	25
7	142 ± 24	17
8	171 ± 51	30
9	136 ± 17	12
10	279 ± 34	12
11	36 ± 8	22
12	27 ± 3	11
13	46 ± 16	35
14	207 ± 61	29
15	265 ± 78	29
16	273 ± 49	18
$\bar{x}$		18.5

Mean spot quantity obtained from 8 different gels in protein data units with the corresponding standard deviations for 16 arbitrarily selected spots. The coefficients of variation (CV) range from 8 to 35% for different spots (mean 18.5%).
Protein data units: The sum of data from all spots is set 1000000. One protein data unit corresponds to 10^{-6} of the data sum.

Identification of blood proteins

A two-dimensional gel from whole blood is shown in figure 4. Four groups of protein spots are found in the blood as well as in the endomyocardial biopsy pattern (spot 1, 2, 3, 4). Group No. 4 corresponds to serum albumin. Spots 5 and 6 of the whole blood samples could not be identified in the endomyocardial biopsy pattern.

Comparison of large gel protein patterns in endomyocardial biopsies from cardiomyopathic hearts and controls

Myocardial protein patterns from 10 patients ("matchset-members") were analysed, 5 gels corresponding to dilated cardiomyopathy, 5 gels to controls. After scanning, spot detection, visual control and correction of poorly fitted spots, 887 ± 161 (range 706–1092) protein species were found in the ten gels; 374 ± 30 (range 351–428) protein species were found in at least more than one gel ("matched spots"). Altogether 55 spots were saturated. For these spots no further quantitative analysis was possible.

With regard to all non-saturated spots that were found in more than one gel, 82 spots differed by at least 100%,

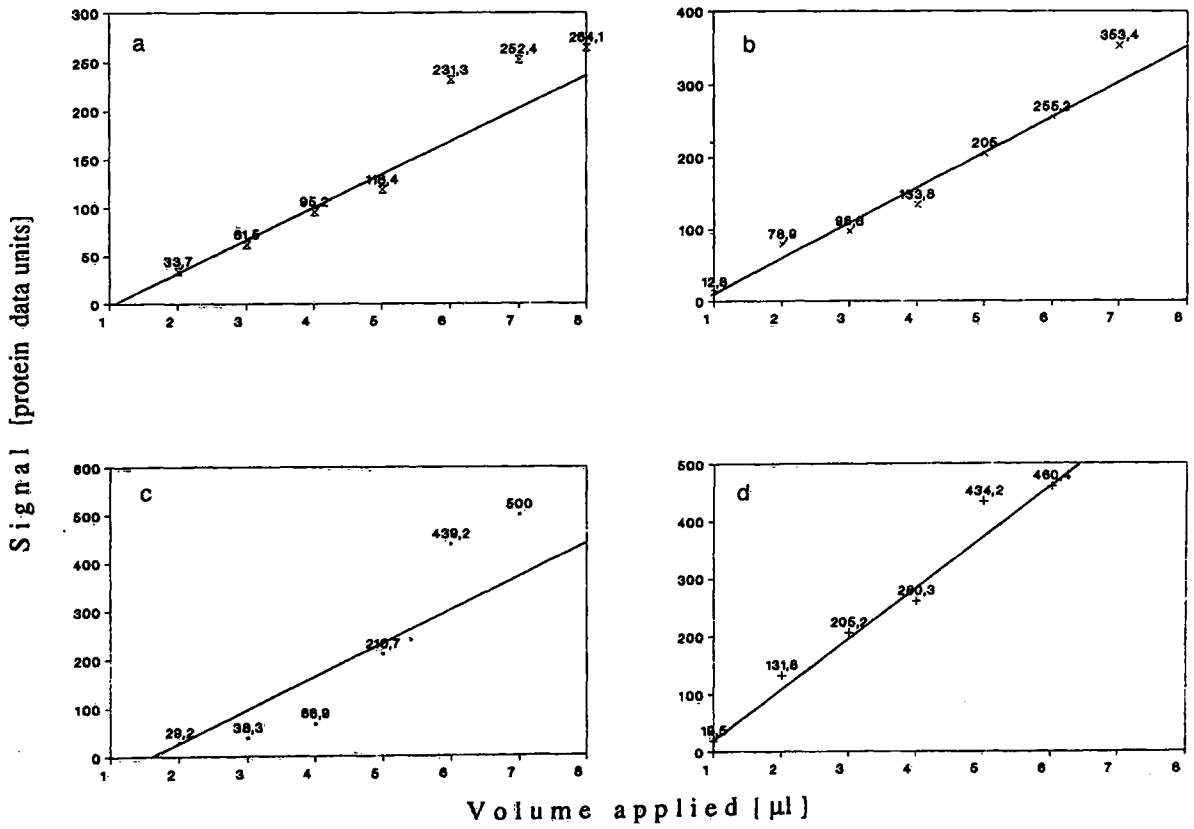

Fig. 3 Effect of the amount of protein on the spot quantification. For 4 different spots, the increasing absorbances in protein data units (PDU) are shown for increasing amounts of sample (1, 2, 3, 4, 5, 6, 7, 8 µl; protein 15 g/l) loaded into the gels. For each spot, the measured protein data units increased with the amount of protein on the gel; however, the slopes for the different spots vary, indicating when the amount of protein is increased, the resulting proportional increase is different for each measured spot:
a) spot 1, slope 35.6 PDU/µl
b) spot 2, slope 49.3 PDU/µl
c) spot 3, slope 69.9 PDU/µl
d) spot 4, slope 90.8 PDU/µl

Fig. 4 Blood protein pattern.
Only 10 µl, a volume that could reasonably contaminate an endomyocardial biopsy were applied to the gel. Spots 2 to 4 could be identified in the biopsy patterns.

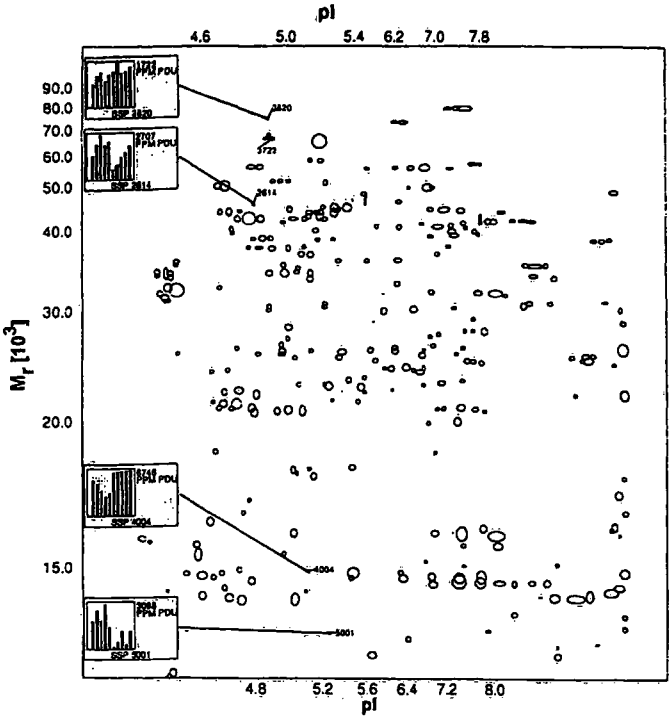

Fig. 5 Standard image with the 4 protein species that were found to differ significantly between cardiomyopathic patients and controls, with their positions and relative intensities in protein data units (PDU). The first 5 lines in the indicated quantitation histograms correspond to the relative protein intensities in the 5 cardiomyopathy patients, the second 5 lines to the 5 control patients. Molecular mass and isoelectric point (pI) scales were obtained as described in figures 6a and 6b.

55 spots were found to be increased 2-fold and 27 spots were found to be increased 2-fold in dilated cardiomyopathy.

We identified 298 spots from their identical position in all gels. As 40 of those 298 spots were saturated, the resulting 258 spots were used for further quantitative comparisons.

With regard to those 258 non-saturated protein spots, which were found in all 10 gels, 7 spots were found to be increased 2-fold and 6 spots were found to be decreased 2-fold in dilated cardiomyopathy. Using the *Mann-Whitney* test, 4 of these 13 protein spots were identified as significantly different ($p < 0.05$) between gels from patients with dilated cardiomyopathy and controls (fig. 5). Table 3 shows these 4 spots with their molecular mass/isoelectric point-values and their spot intensities.

Computer-assisted analysis did not reveal any qualitative differences between the dilatative cardiomyopathy gels and control gels, i. e. no spot was exclusive to the patient or control group.

Tab. 3 Cardiomyopathy-associated protein variants

Spot number	Isoelectric point (pH)	Relative molecular mass (10^3)	Mean protein data units		
			Cardiomyopathy (Group a)	Controls (Group b)	p
2614	4.8	45.8	2107	1270	0.0317
3820	4.9	76.6	1147	1476	0.0158
4004	5.1	14.9	4011	6612	0.0079
5001	5.2	11.7	2194	623	0.0079

Protein species differing between dilated cardiomyopathy and controls. Mean values in protein data units are shown for dilated cardiomyopathy and controls. p values are based on the *Mann-Whitney* test. The positions of the variant proteins are indicated in figure 5.
For characterization of the patient groups see tab. 1.
For definition of protein data units see legend to tab. 2.

Discussion

To detect yet unknown differences between cardiomyopathic and control hearts at the protein level, the so called "substractive approach" was chosen. This approach assumes that disease-associated proteins may be found by substracting a typical control pattern from a pattern characteristic for a specific disease. In contrast to the "candidate-gene" or "candidate-protein" approach, where alterations can only be found in genes or proteins that are suspected to be disease-associated, the subtractive approach has the potential to detect yet unsuspected changes. The disadvantages of this approach on the protein as well as on the cDNA level are the

technical difficulties in creating representative cDNA banks or 2-dimensional protein patterns for subtraction and in distinguishing interindividual variability from disease associated markers.

Two-dimensional gel electrophoresis with consecutive computer-assisted analysis enables the assessment of complex protein patterns from a single endomyocardial biopsy followed by biochemical identification of interesting proteins. In this first study, we investigated basic aspects of the method and the quantitation of differences in the protein patterns from patients with dilated cardiomyopathy and controls.

Technical considerations: Reproducibility and sensitivity of 2-dimensional gel electrophoresis

We demonstrated that the protein concentration during sample preparation and the amount of protein loaded onto the gel influence the resulting protein pattern, probably due to the different solubility and the different staining behaviour of different proteins. If these influences are kept constant, the variance for the spot position in 2-dimensional gel electrophoresis from identical samples can be reduced below 5%. Such a high reproducibility is a prerequisite for the use of computer supported matching programs such as PDQUEST® (19). An even lower variability would be needed to increase the number of "matches-to-all" spots.

With the small gel technique, patterns with about 300 spots can be obtained from as little as 30 µg of protein (fig. 1). Therefore, this technique is suitable for methodological studies and quick screening purposes, for investigating new types of samples, for example different cell cultures, serum or liquor proteins or buffer systems. In contrast, the large gel technique is used for final analysis with high resolution.

Inheritant problems of 2-dimensional gel electrophoretic analysis of endomyocardial biopsies

The use of 2-dimensional gel electrophoresis to detect disease-associated changes in endomyocardial biopsies is complicated by the inhomogenity of the material under investigation. Variable proportions of lymphocytes, fibroblasts, smooth muscle cells or endothelial cells cause quantitative or qualitative changes in myocardial protein patterns, which must be differentiated from disease-associated patterns. In different studies, we have therefore investigated patterns obtained from various human heart cell cultures, in order to identify cell-specific proteins. As the most abundant cells in the myocardium are myocytes, it may be speculated that the most abundant reproducible protein species on the gels represent myocyte proteins, i. e. mainly the "matched-to-all" spots in our analysis. As the resulting number of 258 spots is high enough for quantitative analysis, we did not try to increase the number of "matched-to-all" spots by increasing the amount of protein loaded or the staining time, or by the use of less stringent matching conditions. The variability between gels is probably determined by the different tissue composition of different biopsies (leading to the non-appearance of the low abundance components) and by the remaining 5% positional variance between gels, together with local distortions.

In addition to tissue composition, factors like genetic variability, age, sex, metabolic state and medical therapy may result in the expression of differing protein isoforms, in varying translational activity or in the post-translational modifications of proteins. The genetic variability of proteins has been studied previously in the mouse brain and liver (15, 20). Genetic variability affected less than 5% of all protein species. Liver showed a higher variability than brain; in both organs, quantitative changes (1.6−10.1% of protein species investigated) occurred more frequently than qualitative changes (0−1.5%).

To reduce the effects of age, sex, metabolic state and therapy as much as possible, relatively homogenous groups − Caucasians, males, age 20−60, studied at rest after a 12 h fast − were chosen for our study. However, increasing the numbers of control as well as cardiomyopathic patients will be necessary to reduce the remaining variance and to detect more differences between the groups.

Quantitation for protein species with 2-dimensional gel electrophoresis

In the endomyocardial biopsies, silver staining was used, because it yields a 100fold increased sensitivity in comparison with Coomassie blue and because of the high dynamic range of the silver stain which is linear up to the 40fold protein concentration (21). This high dynamic range is a prerequisite for the quantification of proteins from silver-stained gels. Sophisticated programmes have been developped for this purpose (19, 22). We showed that the measured spot intensity increases linearly with the amount of protein loaded although the slope is not identical for all proteins. However, some variability in spot quantity cannot be attributed to the gel production or to the staining behaviour of single proteins. In our study, this variability was in large part attributable to the spot quantification algorithm (*Pleissner* et al., unpublished). Improvement of

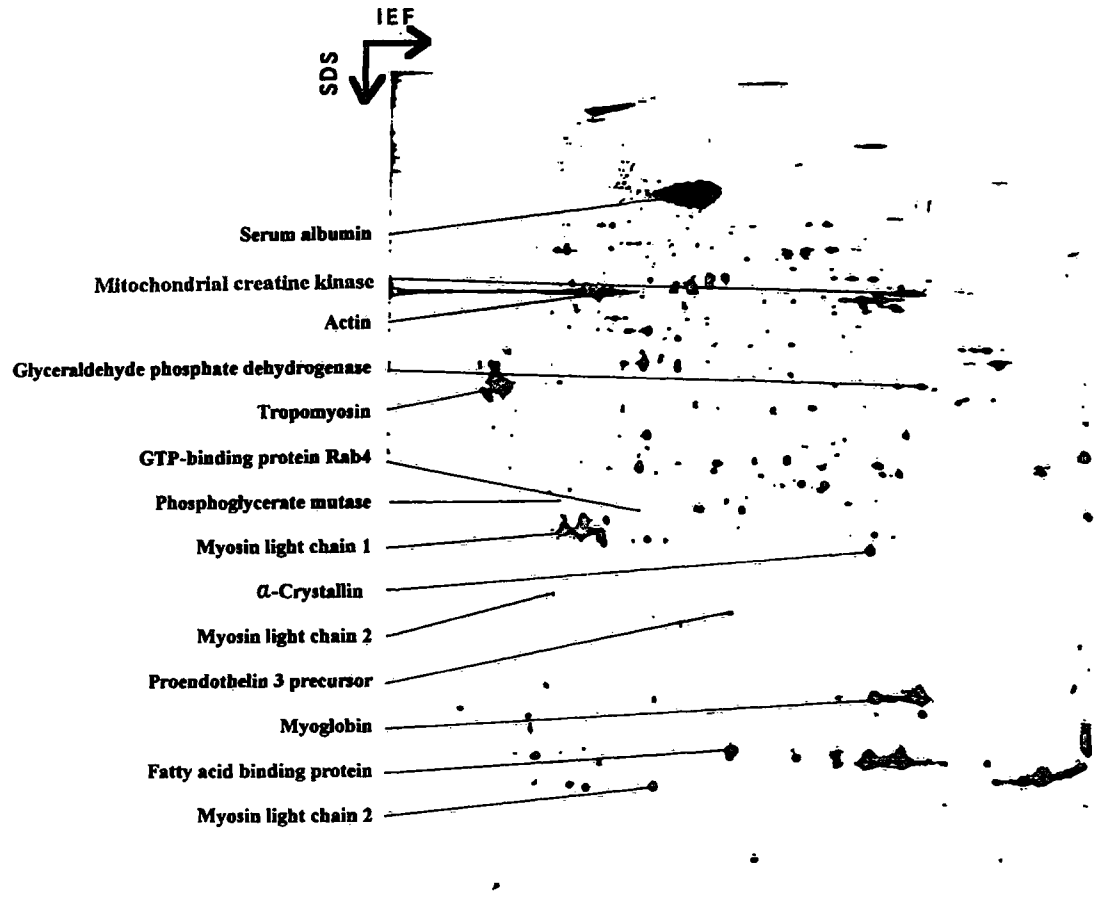

Fig. 6a Silver stained large gel. Fourteen protein species identified by amino acid analysis and microscale sequencing in a parallel investigation (25) are shown.
IEF = Isoelectric focussing; SDS = SDS gel electrophoresis

the PDQUEST® spot detection and quantitation algorithm, which is based on two-dimensional *Gauss*ian fitting, or the introduction of convex area detection (23) or morphological image processing (24) may be the next steps to reduce this variability.

Biochemical identification of proteins

In a parallel investigation, single proteins in the human endomyocardial biopsy pattern were identified by amino acid analysis or microsequencing (25). The first proteins identified with known molecular mass and isoelectric point values were used to construct a grid overlaying the whole gels and to calculate pI values and molecular masses for other protein species (fig. 6a and 6b). Only approximative values are obtained in this first approach based on a restricted number of identified proteins, but amino acid composition as well as sequence information from more proteins will rapidly become available.

Cardiomyopathy-associated protein variants

Thirteen of the 258 protein species that were found in all gels differed by least for 100% in dilated cardiomyopathy, 7 proteins increased 2-fold and 6 proteins de-

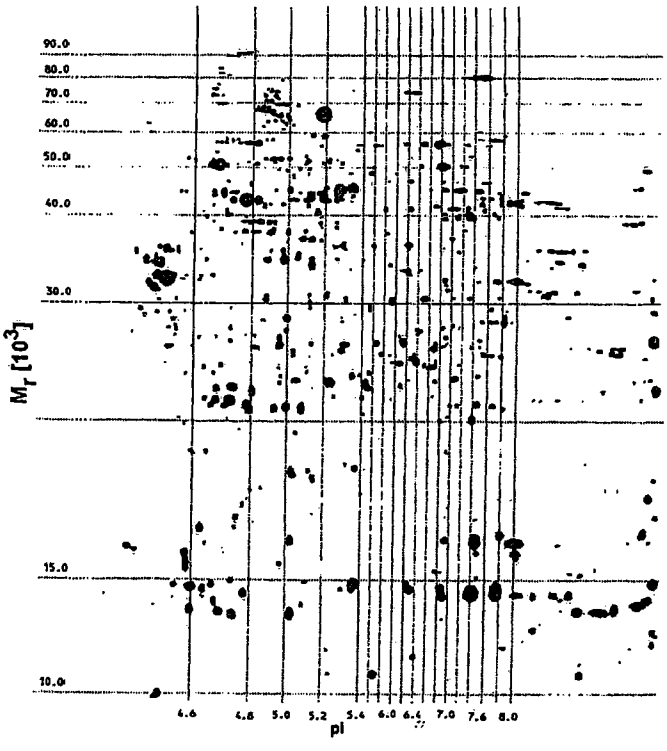

Fig. 6b Synthetic gel spots corresponding to figure 6a. Fourteen protein species previously identified by amino acid analysis or microscale sequencing, with known molecular mass and isoelectric point values (figure 6) were used to construct the grid of isoelectric point (pI) and molecular mass values for overlaying the image.

creased 2-fold. Out of these proteins only 4 proteins differed in quantity significantly between cardiomyopathy and controls (fig. 5). Our study probably underestimates the number of biologically different proteins, because only spots that were found to be statistically different in spite of high variations within the small groups were accepted. So far, direct sequencing of the 4 variant proteins has not been possible, because their intensities were to weak. Preparative gels and possibly the combination of spots from several gels will be needed for this purpose. However, biochemical characterization of the variants by approximate pI and molecular mass is possible (tab. 3). As the variant proteins are represented by spots of low intensity, as the interindividual variabilities are high even within the groups and as corrections for the amounts of protein loaded on the gels must be made before quantitative comparisons are possible, differences in the patterns cannot be detected just by inspecting the gels by eye.

Conclusions

Even with the inherent problems in spot recognition and image processing we found 13 proteins to differ more than 2 fold between controls and cardiomyopathy patients. Four of these proteins differed significantly even in the small groups studied. We expect that this percentage will increase with better reproducibility of gel systems, spot detection and image processing. Identification of more proteins by biochemical procedures and the creation of protein databases for different cardiovascular tissues will be possible in the near future.

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