Nuclear Proteins in the Heart of the Cardiomyopathic Syrian Hamster

Fractionation of Phenol-Soluble Nonhistone Proteins by Two-Dimensional Polyacrylamide Gel Electrophoresis

CHOONG-CHIN LIEW AND MICHAEL J. SOLE

SUMMARY Nonhistone nuclear proteins (NHNP) were isolated from the hearts of hamsters in the myolytic, hypertrophic, and failing phases of cardiomyopathy and from paired controls. These proteins were solubilized in phenol and fractionated by polyacrylamide gel electrophoresis. One-dimensional gel electrophoresis, using either isoelectric focusing or sodium dodecyl sulfate, showed quantitative differences between the dystrophic hearts and the controls. A high resolution of NHNP was achieved by two-dimensional gel electrophoresis, revealing both quantitative and limited qualitative differences between the two groups. Proteins focusing from pH 5.0 to 5.6 with molecular weights of 55,000 (P5) and 100,000 (P6) were strikingly increased in the cardiomyopathy. A protein (P1) with an isoelectric point of 5.1 and some NHNP from dystrophic hearts in the entire region from pH 7.0 to 9.0 with molecular weights ranging from 35,000 (P1) to 68,000 (P4) were marked reduced or absent. Differences in NHNP could be detected during the myolytic phase of cardiomyopathy but were most striking during the terminal phase of the disease. There were no detectable differences between the profiles of proteins derived from whole heart homogenates of dystrophic hamsters and controls. There were no significant differences at the failing phase between NHNP isolated from a purified preparation of myocardial cells and those isolated from whole heart. Therefore, the differences in NHNP appear to be reflections of alterations in nuclear composition of the dystrophic myocardial cell. Some of these observations may represent changes secondary to heart disease. However, if NHNP interacting with DNA play a major role in genetic expression, some of the manifestations of hamster cardiomyopathy could be due to a different constitution of NHNP in the dystrophic heart.

THE CARDIOMYOPATHIES may be defined as disorders of heart muscle in which the pathological process originates in the myocardium itself rather than in associated structures such as the coronary arteries, heart valves, lungs, or peripheral vessels. In many varieties of cardiomyopathy, genetic factors play a significant role. The group of cardiomyopathies associated with the degenerative heredofamilial neuromyopathic diseases, notably Duchenne's progressive muscular dystrophy, and Friedrich's ataxia, have a definite genetic basis.1 In a very high percentage of the cases of idiopathic hypertrophic cardiomyopathy, a heart muscle disorder characterized by an excessive growth of ventricular myocardium, the trait is transmitted as an autosomal dominant.2,3 The cardiomyopathic hamster is a reproducible, spontaneous model of cardiac hypertrophy, dilation, and congestive failure and is thus an important paradigm of myocardial disease.4 Homburger et al.5 have shown this defect to be transmitted by an autosomal recessive gene. Many investigators have described changes in catecholamine metabolism, oxidative phosphorylation, enzyme activity, and membrane composition.6-9 As it is known that genetic expression in eukaryotic cells may be regulated primarily by DNA-associated proteins,6-9 we decided to isolate and characterize the nuclear proteins of the hearts...
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of both cardiomyopathic and normal hamsters. This report deals with the changes in phenol-soluble nonhistone nuclear proteins (NHNP) of myopathic hamsters during various stages in the development of the cardiomyopathy.

Methods

Chemicals

All chemicals used in these studies were of reagent grade. Acrylamide, N,N'-methylene-bis (acrylamide), N,N',N'-tetramethylethylenediamine, ammonium persulfate, and sodium dodecyl sulfate (SDS) were purchased from Eastman Organic Chemicals; ampholine (40% pH range 3.5 to 10) was from LKB Produkter; and Coomassie blue was from Schwarz/Mann Co.

Animals

Cardiomyopathic (Bio 53.58) and control (RB) Syrian hamsters of both sexes, 35-270 days of age, were not allowed access to food overnight before use. Hamsters were age and sex matched and bred by Telaco, Bar Harbor, Maine. The course of hamster cardiomyopathy can be divided into several stages as described by Gertz. Hamsters used for studies on the "myolytic" and "hypertrophic" phases were from 35 to 70 days and 140 to 180 days of age, respectively. "Failing" phase animals were older than 240 days. Hamsters in heart failure had ascites, subcutaneous edema, hepatic engorgement, and, occasionally, pulmonary edema.

Isolation of Nuclei

The hamsters were killed by decapitation and their hearts were removed and rinsed. The ventricles were dissected free of the atria and great vessels and placed in 15 volumes of ice-cold medium A containing 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 3 mM MgCl₂, and 0.1 mM phenylmethylsulfonylfluoride (PMSF), a protease inhibitor. The tissue was minced with scissors and homogenized with a Polytron homogenizer (Brinkman Instruments) for 12 seconds at position 4. The homogenate was centrifuged at 2000 rpm for 10 minutes in a swinging bucket IEC-J-6 refrigerated centrifuge. The pellet was suspended in 20 volumes of medium B containing 0.1% Triton X-100 in medium A. The nuclear suspension was centrifuged at low speed once prior to the isolation of myocardial nuclei. Recently, we have followed the method of Cutilleta et al. for the isolation of myocardial cells, and similar results were obtained.

As a control, cardiomyopathic hearts were perfused with hyaluronidase and collagenase. After the enzyme digestion, the whole minced tissue was pelleted at 1000 g to remove the enzyme solution. Isolation of heart nuclei then proceeded.

Polyacrylamide Gel Electrophoresis

NHNP were fractionated by the three following methods:

SDS Polyacrylamide Gel Electrophoresis

Fractionation of the proteins was carried out by SDS-polyacrylamide gel electrophoresis as described by Laemmli. NHNP were dialyzed gradually from urea to 0.1% SDS phosphate buffer as described by Teng et al. About 80 µg of these proteins were applied to the gels. Gel electrophoresis was carried out, using a current of 2 mA/tube for about 5-6 hours until the tracing dye, bromophenol blue, had reached the bottom of the gel. The gels were stained with 0.1% Coomassie blue, destained in 7% acetic acid, and then were scanned at A570 nm with a UNICAM 1800 spectrophotometer as described previously.

Isoelectrofocusing Gel Electrophoresis

NHNP were dialyzed in urea buffers containing 0.02 M glycine to prevent possible carbamylation during dialysis, as described previously. These protein samples were mixed with 38.4% acrylamide, 1.6% N,N'-methylene-bis(acrylamide) 40% ampholine (pH 3.5–10) and 0.125% ammonium persulfate (in 10 M urea, freshly prepared) in proportions of 1.25:0.5:0.25:3.0 (by volume). The mixture was immediately pipetted into an acid-washed glass tube (2.5 × 90 mm) to a height of 68 mm. After overlayering with water, the gel was allowed to polymerize.
Two-Dimensional Polyacrylamide Gel Electrophoresis

NHNP were also subjected to isoelectrofocusing in the first dimension and SDS-polyacrylamide slab gel electrophoresis in the second dimension. The slab gel was prepared by the method of Laemmli. The following stock solutions were used to prepare the gel: (i) 38.9% acrylamide-1.06% N,N'-methylene-bis(acrylamide) wt/vol; (ii) 1.5 M Tris-HCl, pH 8.8; (iii) 0.5 M Tris-HCl, pH 6.8, (iv) 1.0% SDS, (v) 0.08% ammonium persulfate; and (vi) 2.0% TEMED (wt/vol). The lower small-pore gel was prepared according to the proportions of volumes: 1(i):1(ii):0.4(iv):1.5(v):0.1(vi) and large-pore gel (i.e., cap gel) required 0.45(i):1.0(iii):0.4(iv):1.5(v):0.2(vi) and 0.45 parts of distilled water, as described previously. This method is similar to that described by O’Farrell.

Following electrophoresis, the gels were fixed with 10% trichloroacetic acid and stained with 0.1% Coomassie blue-10% acetic acid for 4 hours. Destaining of the gels was carried out in 25% ethanol-10% acetic acid. The gel was finally kept in 7% acetic acid. In some experiments, the dye from the stained spot was extracted with 0.5 ml dimethylsulfoxide at 40°C overnight. The absorbance of the protein dye was measured at 615 nm as described previously.

Determination of Protein, DNA, and Molecular Weight

Protein determination was carried out by the method of Lowry et al. using bovine serum albumin (BSA) as the standard. DNA was estimated by the method of Burton. Molecular weights of NHNP were estimated by using known protein markers, e.g., human carbonic anhydrase (29,000), ovalbumin (45,000), BSA (67,000), and phosphorylase A (91,000).

Results

Isolation of Nuclei from Ventricles

The nuclear fractions obtained from the ventricles of control and cardiomyopathic hamsters (failing phase) contained minimal cytoplasmic contamination. Most of the outer nuclear membranes were removed by treatment with 0.1% Triton X-100. The nuclei appeared to be intact, as shown by the electronmicrograph in Figure 1, A and B. Nuclear enlargement and vacuolization are characteristics of the cardiomyopathy.

The recovery of nuclei from the homogenate, as estimated by the quantity of DNA, was about 25% in the control and 30% in the cardiomyopathic hamsters, as shown in Table 1. The nuclear pellet obtained from cardiomyopathic hamster hearts was consistently larger in volume than that from the controls. The nuclear protein-

Table 1 Recovery of Nuclei from the Homogenate

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cardiomyopathy</th>
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<tbody>
<tr>
<td>Homogenate</td>
<td>1693 ± 127</td>
<td>1273 ± 142</td>
</tr>
<tr>
<td>Filtrate</td>
<td>883 ± 79</td>
<td>965 ± 232</td>
</tr>
<tr>
<td>Nuclei</td>
<td>423 ± 35</td>
<td>383 ± 38</td>
</tr>
</tbody>
</table>

Results are expressed as μg of DNA per g of ventricle. These results represent the mean ± SD of three separate experiments.

Table 2 Fractionation of Nuclear Proteins

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cardiomyopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear suspension</td>
<td>581</td>
<td>552</td>
</tr>
<tr>
<td>0.14 M NaCl</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>0.25 M HCl</td>
<td>277</td>
<td>256</td>
</tr>
<tr>
<td>Phenol-soluble NHNP</td>
<td>55</td>
<td>110</td>
</tr>
</tbody>
</table>

Results are expressed as μg protein per g of ventricle. The cardiomyopathic hamsters were in the failing phase.
Fractionation of Nuclear Proteins

The nuclei were fractionated into three major fractions (Table 2). Approximately 2-5% of the nuclear proteins were extracted into 0.14 M NaCl and 50% of the nuclear proteins were acid-soluble proteins. It was found that over 90% of the acid-soluble fraction was histone. The recovery of acid-insoluble proteins which were solubilized in the phenol fraction was about 10-20% of nuclear proteins.

to-DNA ratio was approximately 3.1 in the control and 4.5 in the cardiomyopathic hamsters.

Fractionation of NHNP from Three Different Stages of Cardiomyopathy

NHNP isolated from hearts in the failing phase of the cardiomyopathy and their controls were fractionated by SDS-polyacrylamide gel electrophoresis. As shown in Figure 2, at least 15 major fractions of NHNP could be separated in both cardiomyopathic and control hamsters. Fractions of NHNP that had approximate molecular weights of 100,000 and 80,000 were increased markedly in the diseased heart as compared to the control. Two other fractions with molecular weights of 60,000 and 45,000 were also increased significantly in the cardiomyo-
Isoelectrofocusing gel electrophoresis. Seventy-five micrograms of protein from the hearts of cardiomyopathic ( ) and control (—) hamsters 240-270 days old were fractionated by gel electrophoresis.

These findings clearly demonstrated that some NHNP were altered quantitatively in the cardiomyopathic hamster. These proteins also could be separated along a pH gradient ranging from 3.5 to 10.0, as shown in Figure 3. Several fractions in the pH gradient from 7.2 to 8.3 were decreased in the cardiomyopathic hamster. One fraction at pH 5.2 was significantly increased in the dystrophic heart as compared to the control. Actin obtained from cardiac muscle migrated to this region.

Two-dimensional gel electrophoresis was used to fractionate the proteins according to differences in their isoelectric points in the first dimension and their molecular weights in the second dimension. As shown in Figure 4, more than 150 NHNP were detected by this system. NHNP isolated from cardiomyopathic heart nuclei were remarkably different from the control. In the pH region 5.0 to 5.6, protein(s) with a molecular weight of 100,000 (P1) from the failing phase of dystrophic myocardium (Fig. 4F) showed a striking increase. At pH 5.1, a protein of molecular weight 55,000 (P5) was markedly increased. Protein(s) of molecular weights 60,000 (P2, P3) and 50,000 (P4) in the pH region 5.2 to 6.2 were decreased quantitatively in the cardiomyopathic hamster. NHNP in the entire region from pH 7.0 to 9.0, with molecular weights ranging from 35,000 (P2) to 68,000 (P4), were markedly reduced or absent.

Differences in NHNP between dystrophic and control hearts were also found in the early stages of heart disease. As shown in Figure 4M, significant differences of NHNP began to appear in the myolytic phase. These differences became very noticeable in the hypertrophic phase (Fig. 4H). The P5 fraction was significantly increased, whereas the P2 to P4 and P6 to P7 fractions were relatively decreased as compared to the controls (Fig. 4N). The P1 fraction showed a significant increase during heart failure (Fig. 4F). Control hamsters 35-270 days old showed no changes in their NHNP patterns.

Nonhistone Nuclear Proteins Isolated from Cardiomyopathic Myocardial Cells

The differences in NHNP that we observed could have been due to proteins contributed by nonmuscle cells in the myopathic hearts. We therefore carried out the following experiments: (1) Whole heart homogenates from three different stages of hamster cardiomyopathy and their controls were analyzed in a fashion similar to the nuclear fraction. The phenol-soluble proteins of the whole heart homogenates were fractionated by two-dimensional polyacrylamide gel electrophoresis. As shown in Figure 5, more than 150 proteins could be separated on the gel system. In contrast to the striking alterations in NHNP composition (Fig. 4), the differences in these proteins between the myopathic hamsters and their respective controls were minimal. (2) Myocardial cells were isolated as described in Methods. NHNP isolated from the purified myocardial cells of the cardiomyopathic hamster were then fractionated by two-dimensional polyacrylamide gel electrophoresis. The electrophoretic patterns of these myocardial nuclear proteins were relatively similar to those of proteins derived from whole heart nuclei (Fig. 6). These findings provide clear evidence that the differences in NHNP found between the myopathic and control hearts were a reflection of differences in NHNP of myocardial cell nuclei.

Discussion

We have shown that there are striking differences in cardiac NHNP between myopathic hamsters and their controls. These differences in NHNP appeared early in the course of the cardiomyopathy and intensified as the disease progressed. At the terminal stages of the cardiomyopathy, differences between the diseased and control groups were most striking. One-dimensional polyacrylamide gel electrophoresis such as isoelectrofocusing, which separates polypeptides based on their isoelectric points, or SDS-polyacrylamide gel electrophoresis, which fractionates the polypeptides according to their molecular weight, was able to detect quantitative differences in NHNP between the two groups. However, qualitative differences could not be analyzed satisfactorily by one-di-
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**FIGURE 4** Two-dimensional gel electrophoresis. Three hundred micrograms of NHNP from the hearts of control hamsters (N), and cardiomyopathic hamsters in the failing (F), myolytic (M) and hypertrophic (H) phases were analyzed by two-dimensional polyacrylamide gel electrophoresis. The dotted lines delineate regions of particular interest, referred to in the text.

M.W.  
91K — 67K — 45K — 29K —  
P1 — P2 — P3 — P4 — P5 — P6 — P7 —  

N  

M  

F  

H  

pH  
3 4 5 6 7 8 9 10

Mentional gel electrophoresis because several proteins may have had the same isoelectric point or molecular weight. Therefore, we employed high-resolution two-dimensional gel electrophoresis in an attempt to "screen" the NHNP. Both qualitative and quantitative differences in NHNP could be demonstrated by two-dimensional gel electrophoresis.

It is unlikely that our observations were artifacts due to traces of cytoplasmic proteins contaminating our nuclear preparations. The observed differences in NHNP profiles were exactly reproducible from experiment to experiment. Furthermore, the acid-insoluble, phenol-soluble proteins of whole heart homogenate (nuclei and cytoplasm) from the various stages of the cardiomyopathy and their controls were found to be identical by two-dimensional polyacrylamide gel electrophoresis. It is believed that some NHNP are not extractable in phenol-buffer. Thus, variation in extractability could possibly give rise to some of the quantitative differences observed. However, if this is true, then variation in extractability should also reflect actual differences in the composition of NHNP between the two groups.

The contribution of nonmuscle cell nuclei is an important consideration in any comparative investigation of cardiac nuclear biochemistry. In the very early stages of hamster cardiomyopathy, there should be similar amounts of nonmuscle cell nuclei in both control and myopathic hearts, yet distinct differences in NHNP could be recognized. The utility of the cardiomyopathic hamster as a paradigm for the study of nuclear events in myocardial disease is enhanced by a relatively limited proliferation of connective tissue during the later stages of the cardiomyopathy. Histological studies have demonstrated few new degenerative lesions beyond the myolytic stage of the disease; healing, fibrosis, and calcification are virtually completed during the early stages of hypertrophy. Biochemical studies support these pathological observations. With repair of the myolytic lesions, the total amount of collagen in cardiomyopathic hearts was found to increase only from 0.44% to 0.73% of cardiac wet weight. Cardiac connective tissue, as reflected by both collagen content and concentration, did not show a further increase during the subsequent stages of the heart disease. Our DNA data (Table 1) support these findings. Thus, beyond
the early stages of the cardiomyopathy, the relative number of nuclei contributed by nonmuscle cells appears to be approximately constant. Despite this, we found that the differences in NHNP constitution progressively increased. In addition, NHNP isolated from purified cardiac muscle cells in the failing phase of the cardiomyopathy showed electrophoretic patterns similar to those derived from the whole heart nuclei. It appears that the differences in NHNP composition between myopathic and control hearts are a reflection of differences in myocardial cell nuclear constitution.

NHNP are believed to be important both in the maintenance of chromatin structure and function and in the regulation of genetic expression in eukaryotic cells. These proteins are highly heterogeneous, exhibiting some quantitative and qualitative variations in different tissues. We found, on the other hand, that NHNP of normal hamster heart, when analyzed by either one- or two-dimensional gel electrophoresis, appeared to be similar to those obtained from normal human hearts. It is of interest that the NHNP electrophoretic pattern of the failing myopathic hamster heart bore a remarkable resemblance to that found in the human heart with idiopathic hypertrophic cardiomyopathy.

The particular function of any given NHNP identified by our analysis is, as yet, unknown. However, Suria and Liew have recently shown that in liver cells, the group of NHNP found in the region pH 7.0–9.0 and mol wt 35,000–40,000, plays an important role in the transport of messenger RNA from the nucleus to the cytoplasm (ribonucleoprotein particles, see review Georgiev). There was an almost total loss of cardiac NHNP in this region during the terminal stages of hamster cardiomyopathy. If the functions of these NHNP in the heart are similar to those found in the liver, this nuclear defect could be expected to lead to a severe impairment of protein synthesis.

We conclude that the NHNP isolated from the hearts of cardiomyopathic hamsters are quantitatively and qualitatively different from controls. These differences become

![Figure 5](http://circres.ahajournals.org/)

**Figure 5** Phenol-soluble heart homogenate proteins from three different stages of cardiomyopathy. Whole heart homogenates from myolytic (M), hypertrophic (H), and failing (F) phases were extracted with 0.25 M HCl. The acid-insoluble proteins were then solubilized in phenol. These proteins from both the cardiomyopathic hamsters and their paired controls (N) were fractionated by two-dimensional polyacrylamide gel electrophoresis.
more marked with the progression of the cardiomyopathy. Some of our observations may represent changes secondary to the heart disease and perhaps reflect a basic biochemical process associated with the deterioration of cell function characteristic of myocardial failure. Since NHNP interacting with DNA appear to play a major role in genetic expression, it is also possible that some of the manifestations of this cardiomyopathy are due to a different constitution of NHNP in the dystrophic heart.

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References
Nuclear proteins in the heart of the cardiomyopathic Syrian hamster. Fractionation of phenol-soluble nonhistone proteins by two-dimensional polyacrylamide gel electrophoresis.

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