Analysis of erythrocyte membrane proteins from dystrophic hamsters

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To search for potentially mutant proteins, we have investigated erythrocyte ghost proteins from normal and dystrophic hamster by two-dimensional gel electrophoresis. No significant differences are observed between dystrophic and normal erythrocytes in their peptide patterns on SDS–polyacrylamide gel electrophoresis while on two-dimensional gels a protein spot of approximate M_r 20000 with an approximate isoelectric point of 4.5 is found in erythrocytes from dystrophic animals and is consistently absent in normal erythrocytes. A large population of erythrocyte (60%) from dystrophic hamsters shows distorted shape as visualized by scanning electron microscopy. The nature of this protein and its relevance in hamster muscular dystrophy are at present not known.

Muscular dystrophy *Hamster* *Erythrocyte* *Membrane protein* *2-D gel electrophoresis* *Scanning electron microscopy*

1. INTRODUCTION

Although muscular dystrophy (MD) is characterized by major pathological changes in voluntary muscle [1], abnormalities have been reported in the respiratory system [2], nervous system [3], heart [4], skin fibroblast [5], lymphocytes [6], and erythrocytes [5]. Due to their simplicity, erythrocytes have been extensively investigated and several abnormalities, often conflicting, have been reported from dystrophic patients and animals [7–16].

Since MD is an inheritable disease, attempts have been made to search for altered protein(s) which reflect mutation(s) in the genetic material in MD. However, the available data are inconclusive and conflicting [17,18]. This might be due to low resolving power of the techniques employed in the earlier studies [19]. With the development of two-dimensional polyacrylamide gel electrophoresis [20] and silver staining procedure [21], the resolution for analysing peptide composition from complex systems has greatly improved. We therefore used these two modifications to study peptide composition of erythrocyte ghosts from normal (F_1B) and dystrophic (BIO 14.6) hamsters.

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Abbreviations: IEF, isoelectric focusing; MD, muscular dystrophy; M_r, relative molecular mass; SEM, scanning electron microscopy; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Animals*

Dystrophic hamsters of strain BIO 14.6 were used in these experiments. This homozygous line of BIO 14.6 was originally derived from BIO 1.50. Animals of strain BIO 14.6 show various degrees of cardiomyopathy. The cardiomyopathic animals were recognised after 55 days of age by the presence of whitish dots of 1–5 mm diam. located at the lower and lateral surfaces of tongue. Histologically, these lesions were shown to be

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calcified focal areas of myolysis [22]. Animals of strain F_1 B served as controls.

2.2. *Erythrocyte ghost preparation*

Blood was collected by direct cardiac puncture from anaesthetized animals and hemoglobin-free ghosts were prepared as in [23]. Care was taken to minimize endogenous proteolysis by removing leukocytes early during isolation procedure and by keeping the isolation time to minimum.

2.3. *Gel electrophoresis*

Samples were prepared for two-dimensional electrophoresis as in [20] omitting the use of nucleases. For SDS–PAGE, samples were prepared by solubilizing erythrocyte membranes in a buffer containing 2% SDS and 5% 2-mercaptoethanol. Two-dimensional electrophoresis was performed as in [20] with minor modifications. Isoelectric focusing (IEF) gels were prepared using 2% ampholines, pH 3–10 (Bio-Rad) and polymerized in glass rods 125 mm long and 3 mm diam. IEF was carried out for 16–20 h at 400 V followed by 60 min at 500 V. Electrophoresis in the second dimension was done in slab gels 1.5 mm thick with 10% acrylamide, using the discontinuous buffer system of Laemmli [24]. The IEF gel was applied to the second dimension without prior equilibration and was pre-run in 'high SDS electrode buffer' at 20 mA for 20 min [20]. SDS–PAGE in the second dimension was carried out at 20 mA for 1 h followed by 4–6 h at 30 mA. After completion of electrophoresis, gels were fixed and silver stained as in [21]. The pH gradient in the IEF gels was determined using 5 mm sections equilibrated overnight in capped tubes containing 0.5 ml water/tube. M_r-Values were determined by the use of appropriate markers. Protein determinations were as in [25] using bovine serum albumin as protein standard.

2.4. *Scanning electron microscopy*

The samples for SEM were prepared as in [8] with minor modifications. Freshly drawn blood was immediately dispersed in large volume of 1% glutaraldehyde in 0.1 M phosphate buffer (pH

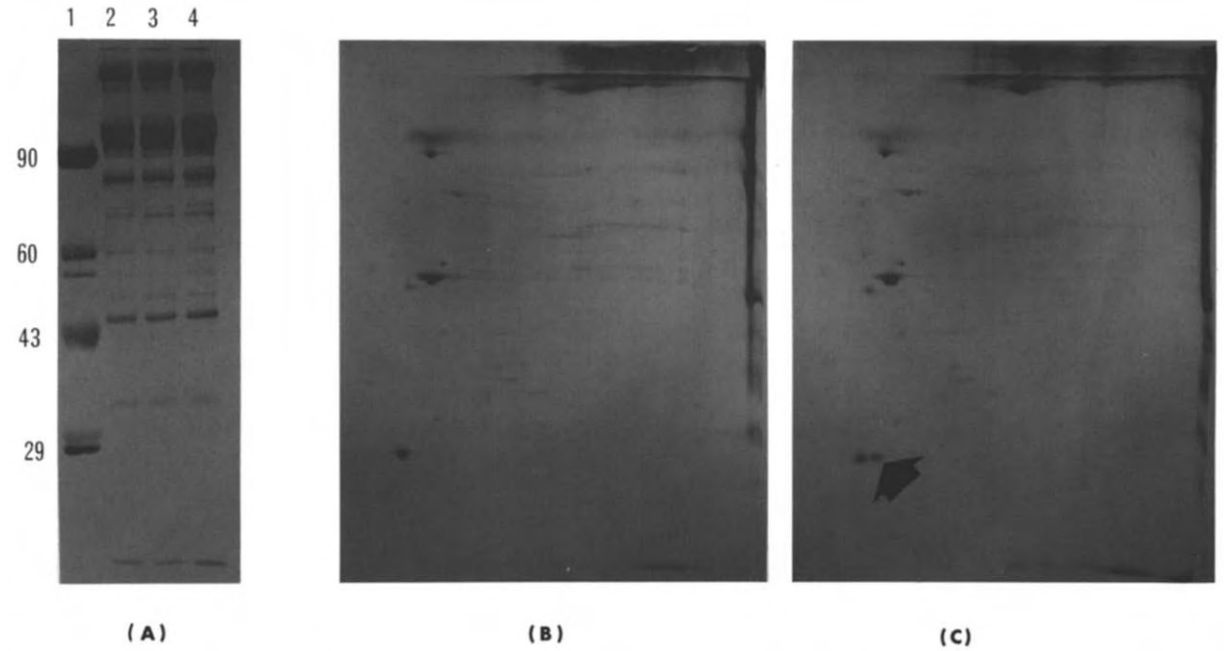
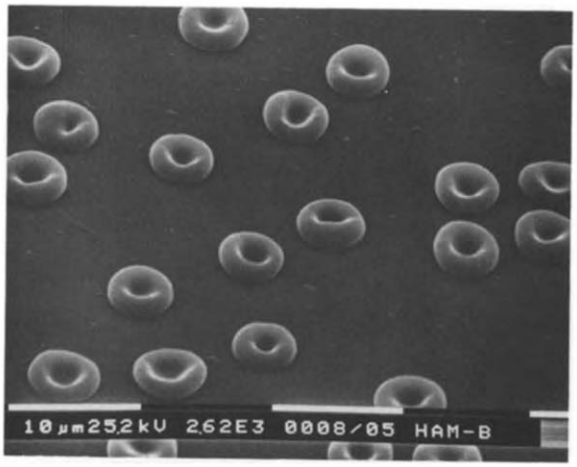
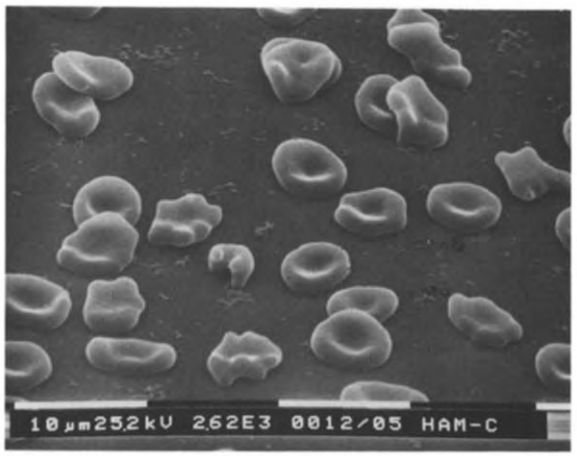


Fig.1. Electrophoretic analysis of erythrocyte ghost proteins from normal and dystrophic hamsters: (A) SDS–PAGE electrophoretogram of normal (track 3) and dystrophic (tracks 2,4) erythrocytes (track 1 shows M_r standards); (B,C) two-dimensional electrophoretograms showing protein constellation of normal (B) and dystrophic (C) erythrocytes. Arrow in C indicates a protein spot at M_r ~20000 and pI ~4.5. Erythrocytes were obtained from anaesthetized animals and hemoglobin-free ghosts were prepared as in [22].

7.4). After 1 h at 4°C, erythrocytes were placed on collagen-coated coverslips, dehydrated through graded series of ethanol and air-dried. The coverslips were coated with ~150 Å of gold target and examined in a Philips SEM 505 at 25 kV at 55° tilt. In the coded samples, areas were selected at random and photographed at 2600 × and cell counts were made directly from such photographs.



(A)



(B)

Fig.2. Scanning electron micrographs of erythrocytes: (A) normal hamster; (B) dystrophic hamster. The samples were prepared according to [8] with minor modifications. The bar in photographs represents 10 µm.

Table 1

Percentage of distorted erythrocytes in normal and dystrophic hamsters

Group	Total no. of cells counted	Bicon-cave cells	Distorted cells	% Distorted cells
Control (n = 4)	503	454	49	10
Dystrophic (n = 4)	517	212	305	59

3. RESULTS

3.1. *Gel electrophoresis*

Analysis of erythrocyte ghost proteins on one-dimensional SDS–PAGE (fig.1A) did not reveal any consistent significant differences in peptide profile of dystrophic and normal erythrocyte ghosts. However, two-dimensional electrophoretic analysis of dystrophic ghosts revealed two protein spots (fig.1C) in the M_r 20000 and pI 4.5 area while normal ghosts showed only one protein spot (fig.1B). This extra protein spot was observed in all the 5 dystrophic animals studied. Other minor differences in protein profiles were occasionally detected and were probably due to individual variations.

3.2. *Scanning electron microscopy*

A large proportion of the cells in the dystrophic hamsters (fig.2) showed surface irregularities involving variable protrusions from the cell surface. They resemble echinocyte and have been observed in dystrophic mice [26]. Very few erythrocytes with irregular shapes were observed in normal hamsters. Table 1 is a summary of these observations and shows a high incidence of distorted cells in dystrophic hamsters.

4. DISCUSSION

SDS–PAGE analysis of dystrophic and normal erythrocyte ghosts does not reveal any differences in their peptide profiles. Earlier reports on the polypeptide composition of erythrocyte in MD have yielded conflicting results. Observations reporting differences in the peptide profile [17] and

no differences in the peptide profile [11] from dystrophic erythrocyte ghosts have been made. These differences could be due to the differences in the techniques employed.

Two-dimensional electrophoresis resolved erythrocyte membrane proteins into a much larger number of components. In case of dystrophic animals an additional protein spot was consistently observed in the region of M_r 20000 and pI 4.5. This difference was not observed on one-dimensional SDS–PAGE since a protein with same M_r is present in normal animals. Obviously these two peptides differ in the amount charge present on the molecule. Since a uniform series of spots which is a characteristic of deamination artifacts is largely absent from the electrophoretograms we suspect that this additional protein could be a result of point mutation involving a charged amino acid.

Our observation of altered erythrocyte shape in dystrophic hamsters is also supported by earlier similar observations made in patients with Duchenne muscular dystrophy [7] and dystrophic mice [26]. The molecular mechanism of the shape alteration is, however, not known. It has been suggested [28] that cytoskeletal proteins and membrane proteins play a vital role in maintenance of morphology and deformability of erythrocyte membrane. In this context, presence of the additional protein seems to be important. Though, at present, there is no evidence that the additional protein is involved in the shape alteration, such a possibility does exist and experiments to explore such a possibility are in progress. In any case these observations together with a recent report on skin fibroblasts [29] from dystrophic patients further strengthens the hypothesis that in MD, an altered gene produce is expressed in non-muscle cells.

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