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ORGANIZATIONAL DIFFERENCES IN THE MEMBRANE PROTEINS OF NORMAL AND IRREVERSIBLY SICKLED ERYTHROCYTES

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Summary

Using two-dimensional gels, no unique membrane proteins were detected in irreversibly sickled cells. Membranes from irreversibly sickled cells were shown to cross-link much more readily with dithiobis(succinimidyl propionate) than normal erythrocyte membranes. Increased binding of band 4.5 protein and increased intra-chain disulfides were also demonstrated. These changes may correlate to enhanced cellular rigidity.

Introduction

Recently, there has been much speculation but little direct data concerning the possible role of the plasma membrane to sickled cells in producing the symptoms of sickle cell anemia. Membrane alterations could result in reduced deformability or enhanced adhesivity to endothelia and/or between adjacent erythrocyte thereby producing a blockage of vessels [1—5]. We report here direct evidence for an alteration in the organization and structure of some of the membrane proteins in irreversibly sickled cells.

Materials and Methods

Preparation of blood. Blood from patients homozygous for sickle cell anemia was obtained at the University of Miami Comprehensive Sickle Cell Center and normal bood was obtained from the investigators. The blood was drawn into heparinized centrifuge tubes, cooled to 4°C and spun at 2000 rev./min for

Abbreviations: DTSP, dithiobis(succinimidyl propionate); NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate.

4 min on an I.E.C. HN-5 centrifuge. The serum and buffy coat layer were aspirated away. The cells were then washed three times in phosphate-buffered saline (5 mM sodium phosphate, 0.15 M NaCl), pH 7.6, spun at 2000 rev./min for 4 min and a portion of the uppermost layer of cells was removed with each supernatant aspiration.

Preparation of irreversibly sickled cells. Irreversibly sickled cells were isolated from the sickle cell patient red cells by the method of Zucker and Cameron [6]. The phosphate-buffered saline washed cells were diluted 1 : 4 in a 33% bovine serum albumin solution (1.1 $g \cdot cm^{-3}$) and the suspension was spun at 7710 $\times g$ for 15 min in an SS-34 rotor in a Sorval RC-2B refrigerated centrifuge. The irreversibly sickled cells were removed from the bottom of the tube. Normal blood was treated in an analagous manner; however, all the cells migrated to the upper portion of the bovine serum albumin gradient.

Cross-linking with DTSP. The isolated irreversibly sickled cells and normal cells were washed three times in phosphate-buffered saline (pH 7.6) to remove the bovine serum albumin. The cells were then lysed in a 1 : 10 suspension of 5 mM sodium phosphate (pH 7.0) and spun at 12 000 $\times g$ for 10 min at 4°C in an SS-34 rotor in a Sorval RC-2B refrigerated centrifuge. The supernatant and fibrous button were aspirated away. This was repeated 4 times with a final spin at 50 000 $\times g$. Cells were then assayed for their protein content by the method of Bradford [7]. Protein concentration was adjusted to 4 mg/ml with 5 mM sodium phosphate, pH 7.0. Dithiobis(succinimidyl propionate) (DTSP) was solubilized in Me_2SO at 40 mg/ml and diluted to 100 ml with 5 mM sodium phosphate, pH 7.0. Equal portions of ghosts and DTSP were allowed to react for 0, 5, 10, 20 and 30 min at room temperature. Non-specific disulfide formation was prevented by alkylating all SH groups with an NEM/SDS solution (30 mM *N*-ethylmaleimide, 3% SDS in 0.05 M sodium phosphate), pH 8.0, and allowed to react for 10 min at room temperature at half the total volume of solution already present [8]. After 10 min 0.35 M lysine is added in an amount equal to 1/6 the volume of the total ghost suspension present to quench the reaction.

SDS isoelectric focusing two-dimensional gels were run as previously described [9].

NEM treatment on ghosts. Ghosts were prepared as for DTSP cross-linking and brought to 4 mg/ml with 0.05 mM sodium phosphate, pH 8.0. This was reacted with 30 mM NEM in 0.05 M sodium phosphate for 10 min at room temperature and then washed once in 5 mM sodium phosphate, pH 7.0, and spun at 50 000 $\times g$ for 10 min. The pellet was solubilized in both sample buffer with and without mercaptoethanol and run on one- and two-dimensional gels [10].

Freeze-etch microscopy. To examine the protoplasmic surface of the membranes, ghosts were induced to endocytosis using the method of Steck et al. [11]. The resultant ghosts filled with endocytic vesicles were then prepared for freeze-etching.

Results and Discussion

Initially we compared the two-dimensional gel pattern of normal and irreversibly sickled erythrocytes from twenty different patients using a gel

system which we have previously shown resolves over 200 polypeptides for the human erythrocyte membrane [9]. We compared the charge and molecular weight characteristics of all of these polypeptides from these two preparations and were unable to detect any difference in type or amount of protein present.

We instituted a series of cross-linking experiments using DTSP cross-linking of ghosts isolated in such a way as to retain their sickled morphology [4]. The results using one-dimensional gels quickly revealed a dramatic difference in the cross-linking patterns when comparing normal to irreversibly sickled cell fractions. After cross-linking, specific bands sometimes were present in the sickle cells and absent in the normal and vice versa, but no consistent pattern of inter-chain protein cross-linking could be detected. However, in all cases (ten different donors were used) the degree of cross-linking of all of the polypeptides in the sickle preparation was much greater than that of the normal. This is indicated by the reduction in amount of material entering our 12% slab gels (see Fig. 1). The aggregates thus formed from the cross-linked proteins were greater in molecular weight than $1 \cdot 10^6$ since they also did not enter 2.5% acrylamide agarose gels. Reducing the concentration of cross-linking agent or the time of cross-linking (minimum of 1 min) did not reveal specific lower molecular weight cross-linked products. This cross-linking procedure did not cause any discernible change in intramembranous particle distributions as revealed by freeze-etch electron microscopy. Moreover, the distribution of the globular material on the cytoplasmic surface of the membrane was not altered.

In the control preparations that were treated identically to the cross-link experiments except that the DTSP was left out, a consistent band at a molecular weight of 60 000 appeared in greater amount in the sickled membrane preparations. After investigation it was discovered that this band (band 4.5) was reduced in amount when mercaptoethanol was deleted from the preparation prior to running the gels. Concurrently, an increase in a lower molecular weight component was observed (Fig. 2). This suggested that these two polypeptides were being interconverted depending on the conditions of oxidation and reduction in the buffer. To test this, a two-dimensional gel was run in which the first dimension was a regular SDS gel slab run in the absence of reducing agent. The second dimension was a slab gel run in the perpendicular direction after equilibration for 30 min in sample buffer containing 5% mercaptoethanol with mercaptoethanol placed in the interface between the 2 gels. The results of these gel runs revealed a band running in the upper quadrant of the two-dimensional gels above the diagonal of the rest of the polypeptides (see Fig. 3). This indicated that this protein ran with a higher apparent molecular weight when reduced than when not reduced. This result has held true for all of seven different donors examined to date.

This typical two-dimensional gel pattern was qualitatively identical when comparing sickle and normal membrane preparations. However, we attempted to test the possibility that we were observing an artifact produced during the preparation of the ghosts. To do this the live cells after brief washing were alkylated in NEM prior to formation of ghosts and subsequent solubilization for electrophoresis. When this is done, the shift in molecular weight of band 4.5 is seen in the sickle preparation but is never seen in the normal cell ghosts which always possess only the 60 000 M_w band in the absence of reducing agent.

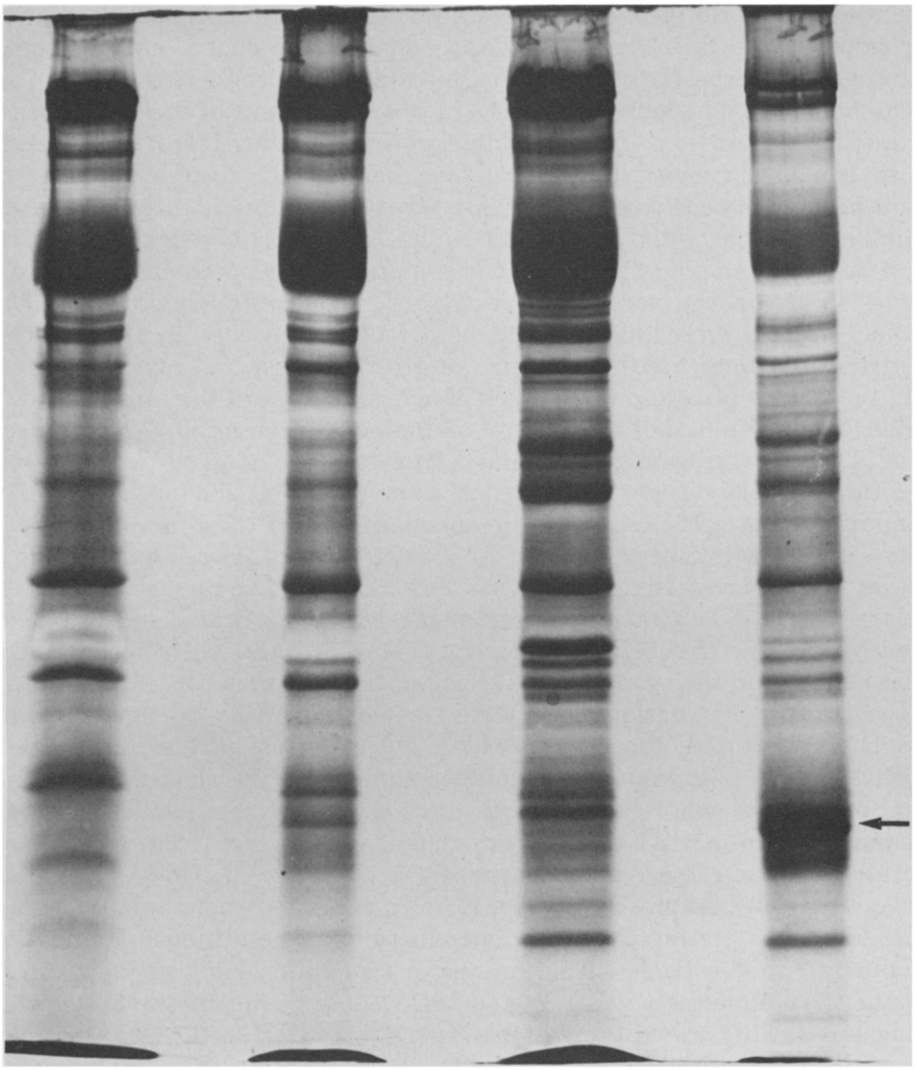

Fig. 1. SDS-polyacrylamide gel showing reduced staining profile of irreversibly sickled cells cross-linked sample. From left to right: normal control, normal cross-linked, irreversibly sickled cell control, and irreversibly sickled cell cross-linked. Note arrow at cross-linked hemoglobin dimer.

We tested the possibility that this phenomenon appeared only in the irreversibly sickled cell fraction by repeating these experiments after alkylation on the non-irreversibly sickled cell supernatant fraction taken from the bovine serum albumin gradients of blood form sickle cell patients. The results of these experiments show that this non-irreversibly sickled cell fraction of cells behaved identically to the normal control cells; namely, when the cells were pretreated with NEM no shift in apparent molecular weight was seen.

In our hands the use of DTSP did not reveal specific alterations in polypeptide interactions as a result of sickling. We have, however, demonstrated that the membrane proteins of irreversibly sickled cell ghosts are much more readily

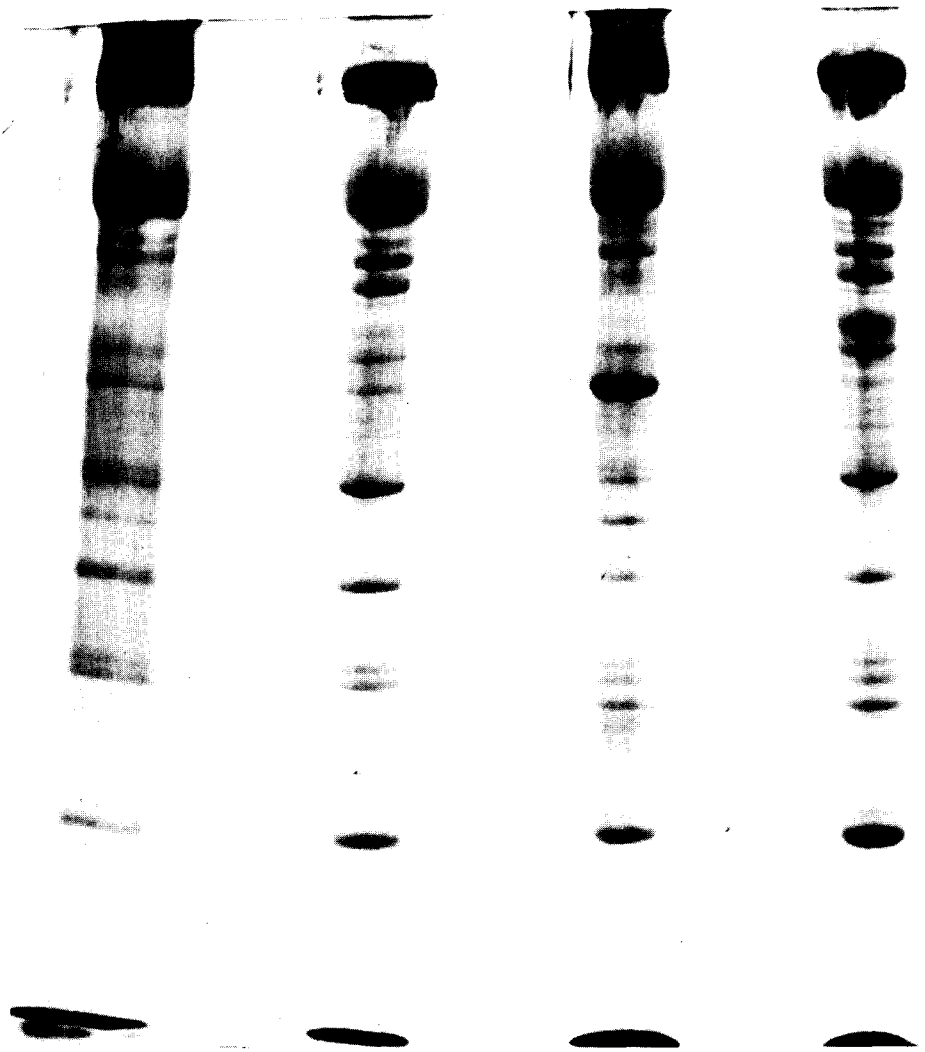

Fig. 2. SDS-polyacrylamide gel showing the effects of pretreatment in NEM and differences in gel pattern due to addition or deletion of β-mercaptoethanol in the solubilization buffer. From left to right: normal control, normal with mercaptoethanol, irreversibly sickled cell control, irreversibly sickled cell with mercaptoethanol.

cross-linked. This may indicate a greater lateral mobility or a more tightly interlocked meshwork of proteins. It is possible that hemoglobin itself is serving as a major cross-linking bridge between the polypeptides in the membrane. Also the actin-spectrin meshwork may be altered in such a way as to allow the easy cross-linking of the other membrane protein with actin and spectrin. However, freeze-etch studies have shown no difference in the distribution of material on the cytoplasmic surface of normal versus irreversibly sickled cells [12]. These biochemical results are identical to those recently reported for cross-linking of Sarcoma 180 cell membranes [13], which like erythrocyte ghosts have an actin meshwork attached to the undersurface.

Fig. 3. Two-dimensional SDS-polyacrylamide gel of a sample run under oxidizing conditions in the first dimension and reducing conditions in the second dimension. Note arrow at presumptive catalase band. In the first dimension, the high molecular weight is on the left and in the second dimension the high molecular weight is on the upper left.

The freeze-etch results indicate that the cross-linking procedure is not disrupting the normal architecture of the membrane in terms of the distribution of the intramembranous particles within the membrane and the distribution of the globular material (spectrin?) on the protoplasmic surface. Moreover, since the intramembranous particles do not become clustered after X-linking, it is probable that the increased cross-linking seen in irreversibly sickled cell membranes is the result of proteins, other than those in intramembranous particles, being either more mobile or already having an altered distribution such that they are more susceptible to cross-linking.

The interconversion of a low molecular weight polypeptide to a higher

60 000 band after reduction suggests that this protein normally possesses one or more intra-chain disulfide linkages which give it a smaller conformation and allow it to sieve more rapidly through the polyacrylamide matrix. When it unfolds after reduction, it runs with a higher apparent molecular weight. Lysing normal cells can induce this disulfide formation causing it to run at a higher apparent molecular weight; whereas if the sickle cells are alkylated prior to the lysing and solubilization procedure the intra-chain disulfide is retained. This is not the case for the normal cells and we conclude, therefore, that sickle cells possess and altered protein structure. The nature of this protein is not yet known although we suggest here that it is catalase. Catalase has a molecular weight identical to this band under conditions of reduction and catalase is known to contain 16 cysteine residues [14]. Catalase is also known to undergo various intra-chain disulfide formations during the isolation of the enzyme from erythrocytes [14,15].

Enhanced catalase binding to irreversibly sickled cell ghosts has been previously reported [3]. There also appears to be more catalase bound to erythrocyte ghosts after ATP depletion and in hereditary spherocytosis [15,16] than in normal cell ghosts. We may be observing a similar phenomenon since irreversibly sickled cells have been reported to contain reduced amounts of ATP [17]. Increased band 4.5 binding, however, was not detected in our original two-dimensional gel study. However, small changes in the amount of a given protein are difficult to detect in small spots on a two-dimensional gel. Sickel cells as well as erythrocyte in hereditary spherocytosis show reduced deformability [18] and thus catalase binding or activity may be directly linked with cellular rigidity. It will be of interest to examine catalase activity in whole blood, erythrocytes only, and erythrocyte ghosts from normal and sickle cell patients.

Another consideration is the age distribution of the irreversibly sickled cell fraction that we use. It might be argued that the results we have obtained are due to the reduced average age of the irreversibly sickled cells as compared to control cells. However, age alone cannot explain our results since the non-irreversibly sickled cell supernatant fractions from sickle cell patients do not show the effects we have described here. They appear to possess a protein organization equivalent in cross-linking potential and degree of catalase oxidation to that of normal cells. This supernatant fraction contains virtually all of the reticulocytes which we find present in much greater amounts in sickle cell patients (up to 30%). Thus, by definition, the supernatant fraction must on the average be younger than the irreversibly sickled cell fraction. We, therefore, can discount cell age as a causative factor in our results.

Since the non-sickle fraction of the red blood cells from sickle cell patients do not show the altered catalase, we suggest that there is an alteration in the organization of the proteins in the membrane during the sicking of the cell. This is indicated by the enhanced cross-linking we observed. This change in protein organization may put stress on certain peptides to bring about an altered protein structure as indicated by the catalase intra-chain disulfide formation. Whether or not these types of protein alterations can result in the functional changes that could produce the symptoms of sickle cell anemia remain to be discovered.

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