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ALTERATION OF RHEOLOGICAL PROPERTIES OF HUMAN ERYTHROCYTES BY CROSSLINKING OF MEMBRANE PROTEINS

NOBUJI MAEDA, KAZUNORI KON, KAZUHIKO IMAIZUMI, MISUZU SEKIYA and TAKESHI SHIGA *

Department of Physiology, School of Medicine, Ehime University, Shigenobu, Onsen-gun, Ehime 791-02 (Japan)

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The crosslinking of membrane proteins of human erythrocytes by diamide (diazene dicarboxylic acid bis(*N*,*N*-dimethylamide)) was quantified by 4% polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate. The relation between the crosslinking of membrane proteins and erythrocyte functions (rheological and oxygen transporting) was quantitatively examined. (i) The crosslinking of membrane protein was induced by diamide, without changing the shape and the contents of intracellular organic phosphates (adenylates and 2,3-diphosphoglycerate). The intensity of spectrin 2 in SDS-polyacrylamide gel electrophoresis decreased proportionally to diamide concentration. The percentage decrease in spectrin 2 (using band 3 as an internal standard) was the most appropriate indicator for crosslinking ('% crosslinking'). (ii) The suspension viscosity of erythrocytes increased in proportion to the percentage of crosslinking, in the range of applied shear rates of 3.76–752 s^{-1}. (iii) Erythrocyte deformability (measured by a high-shear rheoscope) was reduced by the crosslinking. The change was detectable even at 5% crosslinking. (iv) Rouleaux formation (measured by a television image analyzer combined with a low-shear rheoscope) was inhibited by the crosslinking. The inhibition was also sensitively detected at more than 5% crosslinking. (v) Hemoglobin in erythrocytes was chemically modified by higher dose of diamide (probably by the binding of diamide with sulfhydryl groups). Also the oxygen affinity of hemoglobin increased and the heme-heme interaction decreased. (vi) The reduction of the crosslinking of membrane proteins by dithiothreitol apparently reversed the intensity of spectrin bands in SDS-polyacrylamide gel electrophoresis and the erythrocyte functions (the suspension viscosity and the deformability), though not completely.

Introduction

Erythrocyte membrane proteins have very important roles in maintaining the deformability of erythrocytes, which is controlled by the viscoelastic properties of membrane, the internal viscosity and the shape [1,2]. The status of cytoskeletal proteins especially regulates the membrane organization and maintains the normal biconcave disc shape of erythrocytes [2–4]. The erythrocytes in the circulation are always exposed to some (natural and artificial) oxidizing agents. Malondialdehyde, a peroxidative product of phospholipids during aging, has been shown to induce the crosslinking of spectrin [5] and to reduce the deformability of erythrocytes [6]. Recently, a role of peroxidation in red cell aging has also been proposed for rat erythrocytes, in which higher molecular weight proteins are formed [7]. Furthermore, the crosslinking of membrane proteins has been observed in erythrocytes from patients with glucose-6-phosphate dehydrogenase deficiency [8], and in erythrocytes exposed to some oxidizing agents [9].

Diamide, diazene dicarboxylic acid bis(*N*,*N*-dimethylamide), which was introduced by Kosower et al. [10] as a specific oxidant for the sulfhydryl

* To whom correspondence should be addressed.

group, crosslinks the erythrocyte membrane proteins into higher molecular weight complexes, without changing the biconcave disc shape of normal erythrocytes [11]. When the erythrocytes were treated with diamide, the deformability strongly increased [12–15], and the suspension viscosity reduced [16]. The hardened erythrocytes resisted flow in capillaries and stagnated [17,18]. It seems, therefore, that the diamide-treated erythrocytes are a good model for studying rheological impairments due to the crosslinking of membrane proteins. However, as a quantitative description on the relationship between the degree of crosslinking of membrane proteins and the rheological parameters is lacking in these studies, one cannot estimate the pathophysiological significance of crosslinking of membrane proteins. Thus, we deal with the quantitative correlation between the degree of spectrin crosslinking (not the concentration of diamide) and the changes in rheological parameters.

The present paper concerns the quantitative study of the crosslinking of membrane proteins by diamide and on the relation between the crosslinking and various erythrocyte functions, such as (i) the suspension viscosity of human erythrocytes (measured by a cone-plate viscometer), (ii) the deformability (measured by a high-shear rheoscope), (iii) the rouleaux formation (measured by the quantitative kinetic image analysis of the progressive rouleaux formation in a low-shear rheoscope), and (iv) the hemoglobin function (estimated by the oxygen equilibrium curve). Furthermore, the functional reversibility upon reducing the diamide-induced crosslinking is described.

Materials and Methods

Materials

An erythrocyte suspension was prepared from heparinized fresh human blood collected from cubital vein. After removal of the plasma and buffy coat by centrifugation at 3000 rpm, the erythrocytes were washed three times with isotonic solution at 4°C.

Two kinds of isotonic buffer were used: isotonic phosphate-buffered saline (50 mM sodium phosphate, 3 mM KCl/90 mM NaCl/0.1% D-glucose (pH 7.4)) and isotonic Hepes-buffered saline (50 mM Hepes-NaOH/3 mM KCl/126 mM NaCl and 0.1% D-glucose (pH 7.4)). Diamide (diazene dicarboxylic acid bis(*N*,*N*-dimethylamide)) was purchased from Sigma and dithiothreitol from Wako. Reagents for polyacrylamide gel electrophoresis were obtained from Nakarai and other chemical reagents were of analytical grade.

Crosslinking of human erythrocyte membrane proteins

The washed erythrocytes in isotonic Hepes-buffered saline (hematocrit, 10%) were incubated with various concentrations of diamide for 30 min at 37°C. After incubation, the cells were washed with isotonic phosphate-buffered saline three times.

The shape of erythrocytes was not altered by diamide treatment, as far as was judged by scanning electron microscopy. The contents of intracellular organic phosphates (adenylates and 2,3-diphosphoglycerate) were not significantly altered under our experimental conditions (see Table II).

Reduction of diamide-induced crosslinking

With the intention of reducing the crosslinking of membrane proteins, the diamide-treated erythrocytes were further incubated with 5 mM dithiothreitol in isotonic Hepes-buffered saline for 30 min at 37°C (hematocrit, 10%). After incubation, the cells were washed with the isotonic phosphate-buffered saline three times.

Electrophoretic analysis of membrane proteins

The membrane ghosts were prepared by hypotonic hemolysis, according to the method of Dodge et al. [21]. The solubilized membrane proteins from a known number of ghosts were used for the electrophoresis. The polyacrylamide gel electrophoresis was performed on a slab gel (2 mm in thickness) containing 4.0% polyacrylamide, 0.21% *N*,*N*′-methylene bisacrylamide and 1.0% sodium dodecyl sulfate (SDS) without disulfide reducing agent, essentially according to the method of Fairbanks et al. [22]. The densitometric scanning of Coomassie brilliant blue R-250-stained gel was performed by a densitometer (Dual-Wavelength TLC Scanner, Shimadzu CS-900). The degree of crosslinking of membrane proteins was estimated from the intensity of band 2 (spectrin 2) to band 3 (as an internal standard) (see Results).

Rheological measurements

(i) Viscometry. A cone-plate viscometer (Tokyo Keiki Co., model E, mounted 0.8° cone) was used at 37°C. The erythrocyte concentrations of samples were adjusted on the basis of the hematocrit. The hematocrit was determined by microcapillary centrifugation technique, without correction the buffer volume trapped among cells.

(ii) Rheoscopy. A rheoscope [23] was constructed by attaching a cone-plate viscometer (Tokyo Keiki Co., model B, with 0.8° transparent cone) to an inverted microscope (Olympus, model IMT) [24]. The flash photographs of deformed cells under high shear were taken on Kodak Tri-X film. The degree of deformation (i.e., 'deformability index') was quantitatively expressed by the ratio of the short radius to the long radius.

(iii) Measurement of rouleaux formation. The apparatus for estimating the velocity of rouleaux formation was constructed by combining a rheoscope (as described above) with a particle analyzer (Luzex 450, Toyo Ink Co.) and a computer (Hewlett Packerd, HP-85), as described elsewhere [25,26]. The washed erythrocytes were resuspended in 70% diluted or non-diluted autologous plasma as to give a final hematocrit of 0.26%. The mixture was immediately applied to the rheoscope. Rouleaux formation was observed at a shear rate of 7.5 s^{-1} at 25°C. The number of particles (i.e., erythrocytes, rouleaux and aggregates) and the total area occupied by particles in a television image were consecutively encoded by the particle analyzer, and transfered to the computer. The time-course of averaged area per particle (of 23 televion images over a period of 30 s) was computed and displayed. The increment of the area per particle gave an estimate of the velocity (μm^2/min) of rouleaux formation.

Oxygen equilibrium curve

The oxygen equilibrium curve of erythrocyte suspension was measured in isotonic phosphate-buffered saline at 37°C, according to the method of Kon et al. [27].

Results

Crosslinking of membrane proteins with diamide

The decrease in bands 1 and 2 (spectrins) and the appearance of new bands between origin and band 1 (crosslinked proteins) were observed in SDS-gel electrophoresis by diamide treatment, as already reported [11–13,28]. No significant alterations in other proteins were observed within diamide concentrations less than 5 mM. Since crosslinking of spectrin produces various kinds of molecular species, it is difficult to represent the degree of crosslinking by the amount of crosslinked proteins. Band 1 (spectrin 1) was not a good indicator for crosslinking, because of incomplete separation between band 1 and a minor crosslinked protein band. Instead of band 1, the percentage decrease of band 2 (spectrin 2, with lower molecular weight than spectrin 1) may be used for the quantitative representation of crosslinking. The integrated values of band 2 and band 3 densitometric peaks were proportional to the amount of ghosts applied to slab gel for electrophoresis, up to $3 \cdot 10^7$ cells per gel groove (with a dimension of 2×10 mm). Although the membrane proteins corresponding to the same amount of erythrocytes were applied to electrophoresis, the minor deviation by the staining procedure (within 5% for band 2) was inevitable. The amount of band 3 determined on the densitogram was scarcely altered by diamide treatment, thus it was used as

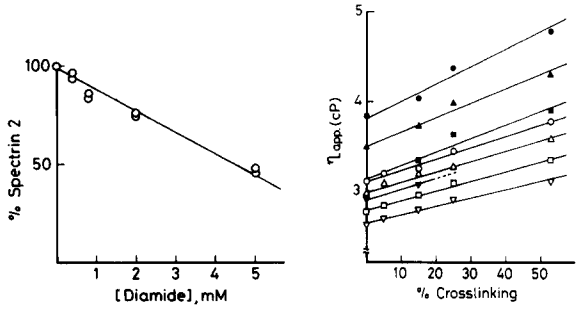

Fig. 1. Crosslinking of membrane proteins by diamide. Erythrocytes were treated with various concentration of diamide in isotonic Hepes-buffered saline (pH 7.4) for 30 min at 37°C. Percentage of spectrin 2 (using the intensity of band 3 as an internal standard) was estimated from the densitogram of SDS-polyacrylamide gel, as described in the text.

Fig. 2. Suspension viscosity of diamide-treated erythrocytes. The suspension viscosity was measured at 37°C in the absence (open symbols; hematocrit, 40%) and presence (closed symbols; hematocrit, 38%) of 80% autologous plasma by a cone-plate viscometer. Shear rates (s^{-1}): ○, ●, 75; △, ▲, 150; □, ■, 375; ▽, ▼, 752.

an internal standard for the amount of membrane proteins applied for electrophoresis, in order to correct the minor deviation of the staining.

The intensity of band 2 to band 3 decreased proportionally to the diamide concentration with good reproducibility (Fig. 1). In the erythrocytes treated with 5 mM diamide, about 50% of spectrin 2 was crosslinked. The crosslinking was completely annulled upon adding dithiothreitol to the ghost membrane: the intensity of spectrin recovered and the bands of crosslinked proteins disappeared. Therefore, the crosslinking should be mainly the reversible disulfide linkage.

Suspension viscosity of erythrocytes

The apparent viscosity (η_{app}) of diamide-treated erythrocytes in suspension is shown in Fig. 2. At all shear rates measured, the apparent viscosity increased proportionally to the degree of crosslinking. The addition of autologous plasma (in 80% of total extracellular fluid) further increased the suspension viscosity: the response of percentage crosslinking to increasing the viscosity became slightly larger, probably due to the interactions of plasma proteins with erythrocytes.

At higher shear rates ($38 < \dot{\gamma} < 752\ s^{-1}$), the Casson plot [29], $\sqrt{\tau} = \sqrt{\eta_c} \cdot \sqrt{\dot{\gamma}} + \sqrt{f_c}$ ($\tau = \dot{\gamma} \cdot \eta_{app}$; η_c, Casson viscosity; f_c, Casson yield value), gave straight lines in all diamide concentrations. As the crosslinking of membrane proteins progressed, the Casson viscosity increased in proportion to percentage crosslinking, and was further augmented upon addition of autologous plasma. The yield value was not affected by the degree of crosslinking, but increased in the presence of plasma.

Rheoscopic observation of erythrocytes

In order to analyze the cause of the increase in suspension viscosity of diamide-treated erythrocytes, the deformability of erythrocytes was mea-

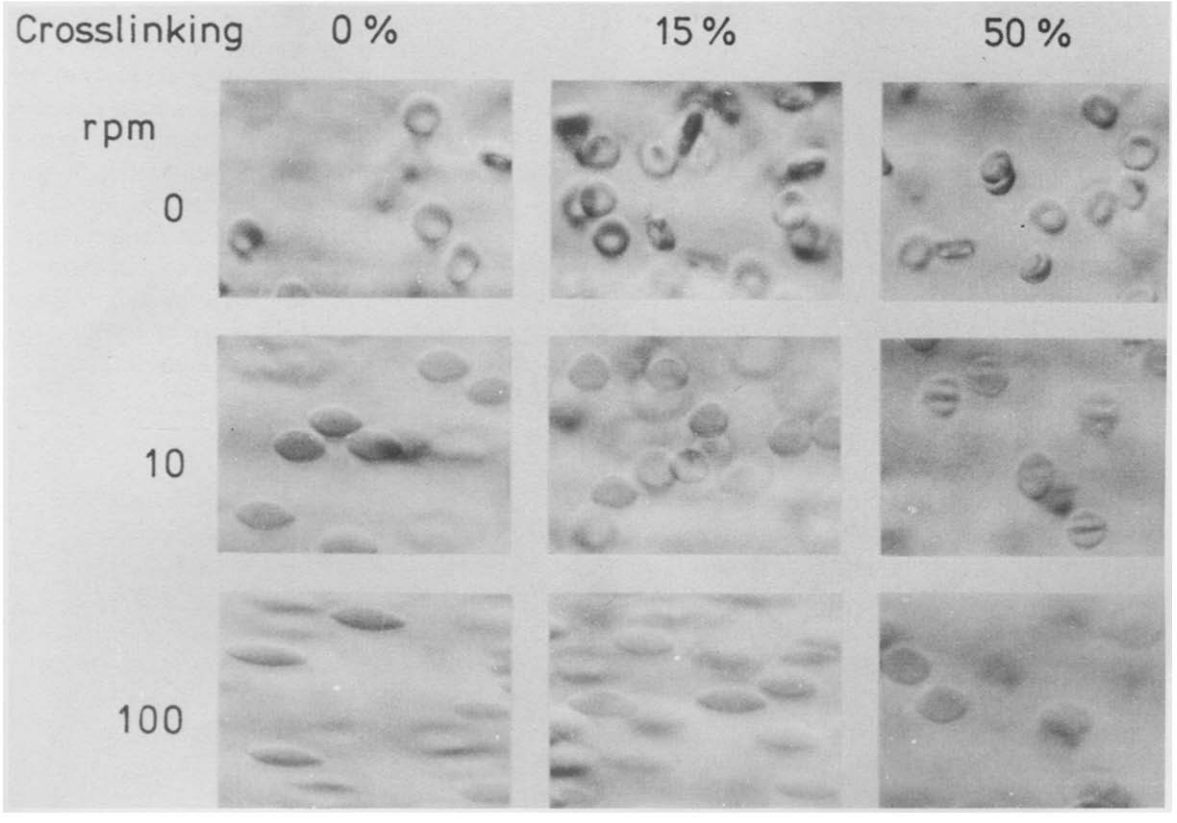

Fig. 3. Representative high-shear deformation of diamide-treated erythrocytes observed by the rheoscope. Measurement was performed at 25°C.

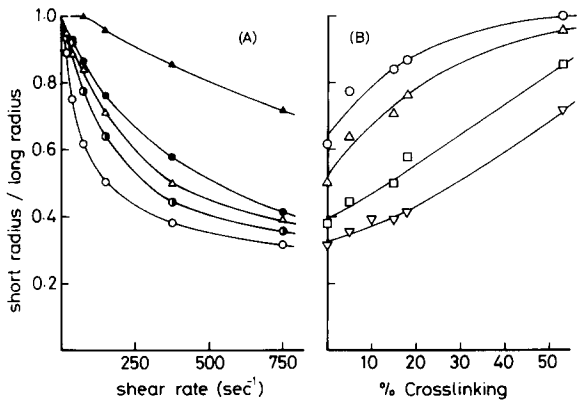

Fig. 4. Quantitative representation of high-shear deformation of diamide-treated erythrocytes. (A) Effect of diamide on the high-shear deformation. The degree of deformation (represented by ellipticity, short radius/long radius, of deformed cells; $n = 50$–110 cells; S.D. of the value was about 10%) is plotted against shear rate. Diamide concentrations treated (mM); 0 (○), 0.4 (◑), 0.8 (△), 1.2 (●) and 5.0 (▲). (B) Plot of the degree of deformation against percentage crosslinking at various shear rates. Shear rate (s^{-1}): ○, 75; △, 150; □, 375; and ▽, 752.

sured by the rheoscope under high shear stress (i.e., in 20% Dextran T-40 dissolved in isotonic phosphate-buffered saline; 20 cP) [23,24]. The representative patterns are shown in Fig. 3. The normal discocytes easily deformed to an ellipsoidal disc form, but the deformation became difficult as the crosslinking proceeded. These phenomena have been shown in the cases of human [12,13], dog [14] and rat [15] erythrocytes. Furthermore, a buckling pattern, having furrows parallel to the flow direction as found by Fischer et al. [30], was recognized clearly at 50% crosslinking (very slightly at 15% crosslinking).

The quantitative expression of the deformability of diamide-treated erythrocytes is shown in Fig. 4. With increasing shear rate, the erythrocytes deformed progressively, but the deformation was inhibited as the degree of crosslinking increased (Fig. 4A). The deformability index approached a value for undeformed cells in all shear rates, as the crosslinking proceeded (Fig. 4B).

Rouleaux formation of diamide-treated erythrocytes

With an increase in the degree of crosslinking of membrane proteins, the velocity of rouleaux formation under a low shear rate (7.5 s^{-1}) was impeded remarkably, even at 5% crosslinking (Fig. 5). The dilution of plasma in the medium reduced the velocity of rouleaux formation, due to the decrease of protein factors for accelerating the rouleaux formation (i.e., fibrinogen, γ-globulin, α_2-globulin and so forth) [1].

Effect of diamide on the hemoglobin function

The oxygen equilibrium curve of erythrocyte suspension was measured to examine the effect of diamide on an intracellular major protein, hemoglobin. The results were expressed as the oxygen affinity (P_{50}, the oxygen tension at half oxygenation of hemoglobin) and the heme-heme interaction (the Hill coefficient, n), as shown in Table I.

No significant alteration in hemoglobin function (both P_{50} and n) was detected in erythrocytes treated with less than 0.8 mM diamide. In 0.8–2 mM diamide, the decrease in the n value was

TABLE I

EFFECT OF DIAMIDE ON THE HEMOGLOBIN FUNCTION AND INTRACELLULAR ORGANIC PHOSPHATES

For hemoglobin function, the oxygen equilibrium curve was measured in isotonic phosphate-buffered saline (pH 7.4) at 37°C. P_{50}, the oxygen tension at half oxygenation of hemoglobin. n, Hill's coefficient. Expressed as mean ± S.D. of three runs. 2,3-Diphosphoglycerate (2,3-DPG) was determined by the enzymatic method of Maeda et al. [19]. Adenylates (ATP, ADP and AMP) were determined for acid-extract by high-performance liquid chromatography using an anion-exchange resin (Permaphase AAX, purchased from Shimadzu Manuf. Co.) [20].

Treatment	Hemoglobin function		Organic phosphates (mol/l of packed cells)			
	P_{50} (mmHg)	n	2,3-DPG	ATP	ADP	AMP
Control	24.4 ± 0.4	2.49 ± 0.06	4.87	1.11	0.22	0.02
0.8 mM diamide	24.3 ± 0.9	2.42 ± 0.03	4.92	1.00	0.23	0.03
2.0 mM diamide	24.4 ± 1.1	2.34 ± 0.03	5.00	0.96	0.23	0.03
5.0 mM diamide	21.5 ± 0.1	2.02 ± 0.01	4.98	0.98	0.23	0.02

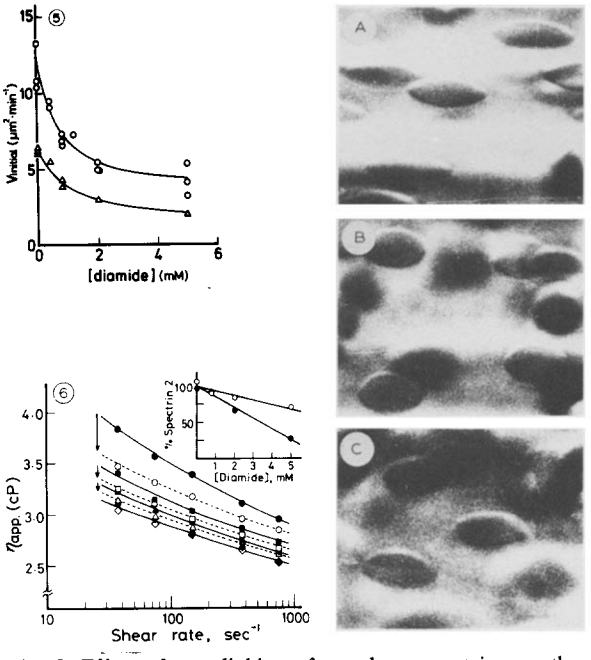

Fig. 5. Effect of crosslinking of membrane proteins on the velocity of the rouleaux formation. The velocity of rouleaux formation was measured at 25°C in the presence of 70% (△, pH 7.99) and 100% (○, pH 8.64) autologous plasma, and was represented by the increasing velocity of area per particle, as described in the text.

Fig. 6. Reversibility of the suspension viscosity of diamide-treated erythrocytes by dithiothreitol. The suspension viscosity of erythrocytes treated with 0 (◆), 0.8 (▲), 2.0 (■) and 5.0 (●) mM diamide was reversed in isotonic Hepes-buffered saline at 37°C for 30 min by 5 mM dithiothreitol, as shown by the corresponding open symbols. The percentages of remaining spectrin 2 before (●) and after (○) reduction of diamide-induced crosslinking by dithiothreitol is shown in the inserted figure.

Fig. 7. Reversibility of high shear deformation of erythrocytes by dithiothreitol. The rheoscopy was performed at 25°C. The representative deformation patterns obtained at the shear rate of 752 s^{-1} in the 20% Dextran T-40 are shown in the figure. (A) Control; (B) treated with 2 mM diamide; (C) reduced by 5 mM dithiothreitol.

evidently detected without significant alteration of P_{50}. However, a slight decrease in P_{50} was observed in erythrocytes treated with 5 mM diamide. In this connection, 2,3-diphosphoglycerate concentration, which is a major factor affecting the oxygen affinity, was not altered by diamide treatment (Table I).

No polymerized hemoglobin was detected in erythrocytes treated with 5 mM diamide, as examined by the gel filtration. However, starch gel electrophoresis in a Tris-EDTA-borate buffer system at pH 8.6 [31] clearly showed the presence of a new hemoglobin band moving slightly faster to the anode than the normal major band. These results may suggest the chemical modification of sulfhydryl groups of the hemoglobin molecule by diamide without the polymerization of hemoglobin.

Functional reversion of diamide-treated erythrocytes by dithiothreitol

In order to examine the reversibility of the rheological functions, the crosslinking of the diamide-treated erythrocytes was reduced by 5 mM dithiothreitol in isotonic Hepes-buffered saline.

The increased suspension viscosity of diamide-treated erythrocytes was clearly, but not completely, reduced by 5 mM dithiothreitol, as shown in Fig. 6. A further increase in the concentration of dithiothreitol did not improve the viscosity any further. The electrophoretic analysis of membrane proteins also showed that 50–60% of crosslinked spectrin 2 was recovered by dithiothreitol, as shown in the insert in Fig. 6.

The deformability of erythrocytes in 20% Dextran T-40 at high shear rates (observed by the rheoscope) was also recovered (but not completely), as clearly shown in Fig. 7. The erythrocytes treated with 2 mM diamide were clearly elongated much more by the treatment with 5 mM dithiothreitol.

Discussion

Membrane proteins of the erythrocytes are related not only to the transport phenomena of various substances, but also to the rheological functions. In order to discuss the pathophysiological significance of naturally occurring oxidative crosslinking of membrane proteins [5–9], we have dealt quantitatively with the effect of diamide, a preferential crosslinking agent of spectrin, on the rheological properties of erythrocytes and on the hemoglobin function.

Crosslinking of membrane proteins with diamide

Several oxidizing and alkylating agents have

been used for the crosslinking of membrane proteins [32,33]. Diamide, a membrane-permeable sulfhydryl reagent developed by Kosower et al. [10,37], does not change the absorption spectra of hemoglobin and does not form the Heinz body at a concentration up to 5 mM. Fischer et al. [12] have shown that diamide crosslinks only membrane proteins and, preferentially, spectrin at low concentration (less than 0.05 mM). Haest et al. [11,13] have shown that the dimerization of band 3 protein by diamide occurs in glutathione-reduced erythrocytes, but not in intact cells.

In the present experiment, the intensity of band 2 (spectrin 2) in SDS-polyacrylamide gel electrophoretic pattern decreased in proportion to the diamide concentration (Fig. 1), and some new bands due to crosslinking of spectrin appeared between the origin and band 1. The intensities of band 3 and the other bands were scarcely altered by diamide treatment. Therefore, the percentage decrease in band 2 (using the intensity of band 3 as an internal standard) could be reasonably adopted as a measure of spectrin crosslinking.

Dithiothreitol (a specific agent for disulfide linkage) reduced (though not completely) the diamide-induced crosslinking in erythrocytes (as shown in Fig. 6). However, when the ghosts prepared by hemolyzing the erythrocytes were treated with dithiothreitol in the presence of SDS, the crosslinking was completely reduced. It seems that the conformation of spectrin network in the diamide-treated erythrocytes is fairly altered, or it may be that dithiothreitol cannot attack some disulfide linkages in the crosslinked spectrin.

In addition, the chemical modification of hemoglobin (possibly the binding of diamide with sulfhydryl groups) was suggested in the present experiment: (i) the change in the oxygen equilibrium curve of erythrocytes treated with more than 0.8 mM diamide, because it is generally recognized that the binding of thiol reagents to hemoglobin increases the oxygen affinity of hemoglobin and decreases the heme-heme interaction [34,35]; (ii) the appearance of a new hemoglobin band in starch gel electrophoresis; and (iii) the absence of polymerized hemoglobin in the gel-filtration pattern.

Reduced deformability of diamide-treated erythrocytes

The deformability of erythrocytes measured by the high-shear rheoscope decreased remarkably as the crosslinking of membrane proteins proceeded (Fig. 3 and 4). The change in deformability is detectable for erythrocytes at 5% crosslinking of spectrin. The main cause of the decreased deformability originates from the alteration in viscoelastic properties due to crosslinking of spectrin, because the shape is maintained in biconcave disc and the internal viscosity may not be affected so much by the minor modification of hemoglobin, as described above. The present results were qualitatively consistent with those reported by many authors [12–15], though the degree of decreased deformability against diamide concentration was different, due to the difference in experimental conditions (i.e., pH, composition of buffer, and so forth). Therefore, we tried to quantitate the degree of spectrin polymerization and to relate it to the rheological alteration.

The viscoelastic behavior of the erythrocyte membrane is attributed to membrane organization [36], i.e., (i) protein organization, (ii) lipid organization, (iii) protein-lipid interaction and (iv) membrane-cytoplasmic constituent interaction.

(i) Protein organization. The crosslinking of spectrin with diamide fixes the cytoskeleton of erythrocyte membrane through the formation of disulfide bonds [28,37], and will restrict the translational mobility of various membrane proteins [38]. Fischer et al. [12] have suggested from the rheoscopic observation of erythrocytes treated with impermeable and permeable SH reagents that proteins responsible for the shear elasticity of erythrocyte membrane are located on the internal surface of membrane. The increase in membrane shear modulus has been theoretically and experimentally ascertained by the buckling phenomenon [30], which is interpreted as the compression of membrane tending to develop at right angles to the direction of stretch, due to the requirement of constant surface area as the length of the cell increases. In our experiment, the similar pattern was observed at more than 15% crosslinking of spectrin. Thus, the spectrin organization in the cytoskeleton must be very important for the deformability of erythrocytes. The larger contribution

of spectrin organization for the decreased deformability of diamide-treated erythrocytes was justified from the reversion of the deformability by dithiothreitol (i.e., by the reduction of disulfide bonds formed among spectrin molecules).

(ii) Lipid organization and (iii) protein-lipid interaction. The fluidity of the lipid bilayer influences various erythrocyte functions [36], for example, the cholesterol loading to human erythrocytes decreased the deformability [39,40] and led to retarded deoxygenation [27,39] as a consequence of the reduced acyl chain motion. It has been suggested that the crosslinking of spectrin by diamide enhances the rate of transbilayer movement of phospholipids [41,42]. Until now, however, no distinct alteration in acyl chain motion, due to diamide-treatment, has been shown.

(iv) Membrane-cytoplasmic constituent interaction. The intracellular ATP level is important for the maintenance of the biconcave disc shape and the deformability [43,44]. The results reported for the ATP level in the diamide-treated cells are ambiguous as concerns diamide concentration and/or the composition of incubation medium; for example (a) Kurantsin-Mills and Lessin [28] observed 25% decrease of ATP in 10 mM diamide (for 30 min in isotonic 5 mM phosphate-buffered saline (pH 7.4) at 37°C) and (b) Johnson et al. [14] showed no change of ATP level in dog erythrocytes in 0.4 mM diamide (for 90 min in isotonic Tris-buffered saline (pH 7.4) at 37°C). The present experiment showed no decrease in ATP level (also, no increase in ADP and AMP) even in 5 mM diamide, which induced 50% crosslinking of membrane proteins.

Conclusively, the main cause for the decreased deformability of diamide-treated erythrocytes will be the increase in membrane shear modulus caused by the crosslinking of cytoskeletal proteins.

Reduced aggregation of diamide-treated erythrocytes

The most sensitive change in the rheological properties of diamide-treated erythrocytes was the inhibition of the rouleaux formation, which could be observed even at 5% crosslinking. Fischer et al. [16] have also described the reduced aggregation of diamide-treated cells. The phenomenon may be explained as follows.

(i) Physical difficulty of adhesion between the hardened erythrocytes. Fischer et al. [30] have pointed out the contribution of the spectrin network to the bending stiffness of the membrane. The crosslinking of cytoskeletal proteins by diamide increases the membrane stiffness; thus the flexible contacts among erythrocytes will become difficult. Furthermore, an erythrocyte in contact with another cell may detach even in a low shear rate.

(ii) Structural alteration of membrane organization. The reaction of diamide with sulfhydryl groups in proteins leads to the formation of intermolecular and/or intramolecular disulfide bonds in proteins [37], which induces the remarkable conformational changes of proteins and results inevitably in the alteration of membrane organization. As the alterations in the cell surface will affect the interaction with plasma protein which causes the aggregation [1], it is reasonably expected that the chemical modification alters the interaction of erythrocyte membrane with various plasma proteins and impedes the cell-cell adhesion to form the rouleaux.

Increased suspension viscosity of diamide-treated erythrocytes

The suspension viscosity of diamide-treated erythrocytes increased remarkably (Fig. 2). The increased viscosity originates mainly from the decreased deformability, as pointed out by many investigators [12–15]. The presence of plasma in the erythrocyte suspension increased the viscosity further due to the additional plasma viscosity and the interaction of plasma proteins with erythrocytes. Especially, the difference in the Casson viscosity of diamide-treated erythrocytes in the presence and absence of plasma suggests the marked alteration of interaction of plasma proteins with erythrocytes by diamide treatment, as discussed before.

At the low shear rate of 3.76 s^{-1}, the suspension viscosity of diamide-treated erythrocytes in plasma was elevated (data not shown), in spite of the fact that the rouleaux formation (and/or aggregate formation) was remarkably inhibited. This suggests that the effective volume of diamide-treated erythrocytes in shear flow is increased as in the case of glutaraldehyde-treated erythrocytes [1]. Furthermore, the difference in the mechanical

flexibility of rouleaux made of normal and hardened erythrocytes should be taken into account, as has been observed for glutaraldehyde-fixed rouleaux [45]. The rouleaux made of hardened erythrocytes may not be flexible in the shear flow, compared with those made of normal cells, though the velocity of rouleaux formation of diamide-treated erythrocytes is indeed impeded. These findings may explain the increased suspension viscosity of diamide-treated erythrocytes at low shear rates.

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