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Simultaneous influence of erythrocyte deformability and macromolecules in the medium on erythrocyte aggregation: a kinetic study by a laser scattering technique

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Abstract

The aggregation and sedimentation kinetics of human erythrocytes was studied by modifying the cellular properties and medium compositions simultaneously. Dextrans of average molecular weight 70400 and 494000 were used to provide suspending medium modifications, while diamide (diazene dicarboxylic acid bis(*N,N*-dimethylamide)) was used to alter the membrane structural properties. Laser scattering method was employed for this study, and it was compared with a kinetic method combined with a low-shear rheoscope and an image analyzer. From scattered light intensity profiles continuously obtained during aggregation of erythrocytes and sedimentation of the aggregates, characteristic kinetic parameters were computed. Kinetic parameters obtained from a phase of the one-dimensional aggregate formation and sedimentation corresponded well to the velocity of rouleaux formation obtained by the low-shear rheoscope technique. Dextrans accelerated the erythrocyte aggregation and the sedimentation, and diamide treatment suppressed the process by decreasing the erythrocyte deformability. The aggregating force by dextrans overcame the disaggregating force by the decreased deformability. However, the arrangement of erythrocytes as expressed in specific units for aggregates (i.e., rouleaux) became irregular by decreasing the erythrocyte deformability. In conclusion, the progression of erythrocyte aggregation and the structure of the aggregates were dependent on both erythrocyte properties and macromolecules in the medium.

Key words: Erythrocyte aggregation; Erythrocyte sedimentation; Light scattering; Dextran; Diamide; Spectrin

1. Introduction

Erythrocyte aggregation leading to rheological alterations is a function of many factors and they are grouped as chemical and physical factors [1,2]. Chemical factors are concerned with modifications of either erythrocytes or suspending medium. Plasma proteins are the major factor which induces erythrocyte aggregation to a greater extent [2–4]. Pathophysiologically, fibrinogen and immunoglobulins are the main contributors. Plasma additives such as dextrans and hydroxyethyl starch also influence the erythrocyte aggregation [5,6]. Physical factors such as shear stress, temperature, gravitational and magnetic fields are found to affect erythrocyte aggregation [1,2,7–10].

As compared to the physical factors, the influences of chemical factors are many folds. Although a large number of studies has been reported mainly on the alterations of suspending medium, some investigations have pointed out the importance of cellular modifications to the erythrocyte aggregation, especially in relation to erythrocyte deformability [11–13]. The contributions of the cellular alterations to the erythrocyte aggregation are smaller in magnitude, but the influence is significant. For example, natural process such as aging of cells [12,14] and the disease such as sickle cell anemia [15,16] alter the erythrocyte membrane characteristics leading to modification of their aggregation properties. In most studies, either the suspending medium is altered keeping the cellular factors unaltered or vice versa. Whereas, in reality both cell and

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medium may simultaneously undergo modifications, but this situation remains unanswered.

Dextrans (Dx) are the most commonly employed polysaccharide in both in vivo and in vitro to study the cell-medium interaction in the development of erythrocyte aggregates [1,2,5,17–20]. Diamide, diazene dicarboxylic acid bis(*N,N*-dimethylamide), is preferably used for the quantitative modification of cytoskeletal proteins, spectrin, in erythrocyte membrane and thus for the quantitative decrease of erythrocyte deformability [11,21,22].

In the present study, we used two kinds of dextrans, Dx-70 and Dx-500 (the average molecular weight, 70 400 and 494 000, respectively), to provide suspending medium alterations, and diamide to provide erythrocyte membrane alterations (i.e., to decrease the membrane deformability). Methodologically, a laser light scattering method was employed [23]. This method is based on continuously recording the forward scattered light from settling erythrocytes and/or their aggregates under gravitational field entering and leaving an observation window. The purpose of this study is to analyze the variations in kinetic parameters in the process of erythrocyte aggregation and sedimentation due to the simultaneous alterations of suspending medium and erythrocyte properties. Furthermore, the method was compared with a kinetic method using a low-shear rheoscope combined with an image analyzer and a computer [24].

2. Materials and methods

2.1. Apparatus for measuring the erythrocyte aggregation and sedimentation

The system for the measurement is shown in Fig. 1. A linearly polarized He-Ne laser (05LHP121, Melles Griot, USA: generation power 2.0 mW, wavelength 632.8 nm, beam diameter 1.0 mm) was passed through erythrocyte suspension in a chamber (50 × 10 × 1 mm in height × breadth × width; made of microscopic glass plates). Erythrocyte suspension was filled up to 40 mm in height. The forward scattered light intensity through the sample column was detected with a high speed, low noise pin silicon photodiode-amplifier (S3887, Hamamatsu Photonics, Japan). The scattered light signal was digitized using a 12 bit analog to digital converter (AD12–8T 98, Contec, Japan) and continuously recorded with a computer (PC9801m, NEC, Japan). The graphical events of the scattered light were displayed in real time on the monitor. The digitized data was stored in the computer for further analysis. The experiments were carried out at 25°C.

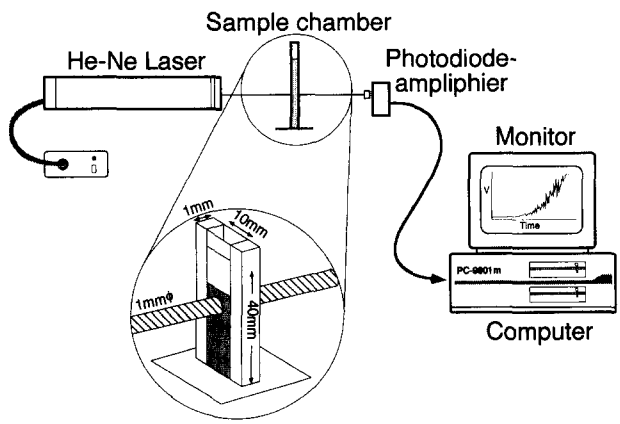

Fig. 1. Schematic diagram of an arrangement of light scattering experimental system.

A well-mixed erythrocyte suspension (hematocrit, 5%) was gently introduced into the chamber using a syringe with long needle, and laser beam was exactly passed through the center of the sample column (at a height of 20 mm). Immediately after the sample was introduced, the forward scattered light signal at zero angle was continuously digitized at a rate of 100 ms and stored in a computer. Data acquisition was continued until the signal reached a saturation (this maximum light intensity showed the completion of the erythrocyte aggregation and sedimentation at the monitoring point). After the signal was normalized with the maximum light intensity, transmittance was displayed on-line on the monitor.

2.2. Kinetic method for measuring the velocity of rouleaux formation

The apparatus combined a rheoscope (composed of a transparent cone and plate viscometer and an inverted microscope) with an image analyzer and a computer was used for the measurement of the velocity of rouleaux formation, as reported previously [24]. In the process of erythrocyte aggregation under a constant shear rate (usually, 7.5 s^{-1}), the count of particles (i.e., single erythrocytes, one-dimensional aggregates (rouleaux) or three-dimensional aggregates) and the total area projected by particles in a frame of the video image were consecutively encoded by the analyzer at a constant interval (usually, 1 s), and transferred to the computer. The velocity of erythrocyte aggregation was expressed by the increment of area/count per unit time (μm^2/min).

2.3. Preparation of control and hardened erythrocytes

Fresh blood samples were obtained from the cubital vein of healthy human subjects with heparin (10 IU/ml of blood). Samples were centrifuged at 3000 rpm for 5 min at 4°C, plasma was separated and buffy coat discarded. Control erythrocytes were obtained by washing

three times with isotonic phosphate buffered saline solution (PBS, composed of 50 mM sodium phosphate, 3 mM KCl, 90 mM NaCl, 0.1 g/dl D-glucose, pH 7.4).

Hardened erythrocytes (with decreased deformability in different levels) were obtained by treating with diamide (purchased from Sigma, USA), according to the method of Maeda et al. [11]. Washed (control) erythrocytes (hematocrit, 20%) were incubated with 1.5 mM and 4.0 mM diamide solutions at 37°C for 60 min. Then the cells were washed three times with PBS. The oxidative crosslinking of spectrin was examined by SDS-PAGE, according to Maeda et al. [11]. The degree of crosslinking ('% crosslinking of spectrin') was expressed by $100 \times (1 - Sd/Sc)$, in where Sd and Sc were (non-crosslinked spectrin β)/(band 3) in diamide-treated cells and in control cells, respectively (the content of band 3 was used as an internal marker, because of no alteration by the diamide treatment).

Erythrocyte deformability was determined using a high shear rheoscope [2,21], and the deformation index was expressed by $(L - S)/(L + S)$, where L and S were the long and short radii of ellipsoidal erythrocytes deformed under the uniform shear stress, respectively [21,25].

2.4. Suspending medium for erythrocytes

Plasma as a medium was obtained by re-centrifugation of separated plasma at 15000 rpm for 15 min at 4°C to remove platelets.

Suspending medium alterations were made by adding two kinds of dextran, Dx-70 and Dx-500 (purchased from Pharmacia Fine Chem., Sweden: at a concentration of 2.0 g/dl and 1.5 g/dl of Dx-70 and Dx-500 in isotonic PBS, respectively). To all samples 0.5 g/dl of bovine serum albumin was added to prevent erythrocyte adhesion to glass chamber. The final hematocrit was adjusted to 5% in all samples, because of the linear relation between the transmittance and the hematocrit.

2.5. Microphotographic examination of erythrocyte aggregation

In order to get information on the structure of erythrocyte aggregates, microphotographic technique was employed during the process of erythrocyte aggregation and sedimentation of the aggregates, using a microscope (BH, Olympus Optics, Japan). The sample chamber was gently placed on the microscopic stage during different phases as shown by the light scatering profile in Fig. 2. Microphotographs were taken at the center of the sample column where the laser light was passed through. Furthermore, fine structure of erythrocytes and the aggregates were obtained with a scanning electron microscope (S-500, Hitachi, Japan).

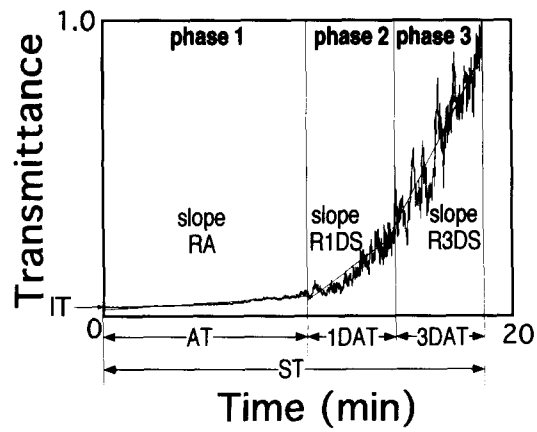

Fig. 2. A typical pattern of the changes of light scattered transmittance in the process of aggregation and sedimentation of control erythrocytes in autologous plasma. From the signal pattern and the morphological examination, three phases were differentiated, and the kinetic parameters (time and rate parameters) were defined (details in the text).

2.6. Statistical analysis

Kinetic parameters for erythrocyte aggregation and sedimentation were expressed as mean ± standard deviation (S.D.). The statistical evaluation of the results was performed by Student's *t*-test.

3. Results

3.1. Erythrocyte aggregation-sedimentation process monitored by light scattering

A typical transmittance variation for a control sample in autologous plasma is shown in Fig. 2. On the basis of signal pattern and microphotographic observation, three phases were differentiated, and the kinetic parameters (times and rates) were defined.

Phase 1: The initial phase was mainly due to the orientation of single erythrocytes in the process of tiny aggregate formation. In this smooth phase, the signal intensity slightly increased. The time at which the clear fluctuation began to appear was termed as aggregation time (AT). The rate of aggregation (RA) was obtained from the slope of this phase.

This fluctuating signal continued to increase exponentially and reached a maximum transmittance level, and this time was termed as sedimentation time (ST).

Phase 2: The second phase was mainly due to the formation of small and one-dimensional aggregates and their sedimentation. In this phase, the fluctuation of signal intensity was clear and regular, the amplitude was relatively small, and the mean signal intensity gradually increased. The interval of second phase was termed as one-dimensional aggregation time (1DAT).

Table 1
Kinetics of erythrocyte aggregation and the sedimentation of the aggregates in autologous plasma (autologous plasma), Dextran T-70 medium (Dx-70 in PBS) and Dextran T-500 medium (Dx-500 in PBS): effect of erythrocyte deformability on the kinetic parameters

Parameter	Autologous plasma			2 g/dl Dx-70 in PBS			1.5 g/dl Dx-500 in PBS		
	control ($n = 9$)	1.5 mM diamide ($n = 7$)	4 mM diamide ($n = 7$)	Control ($n = 7$)	1.5 mM diamide ($n = 7$)	4 mM diamide ($n = 7$)	Control ($n = 7$)	1.5 mM diamide ($n = 7$)	4 mM diamide ($n = 7$)
Duration of phase (min)									
AT	7.72 ±0.84	8.59 ±1.10 (NS)	8.75 ±0.98 ($P < 0.05$)	6.79 ±0.09	6.90 ±0.63 (NS)	6.49 ±0.59 (NS)	6.39 ±0.51	6.95 ±0.78 (NS)	5.83 ±1.27 (NS)
1DAT	6.96 ±1.06	8.48 ±1.42 ($P < 0.05$)	11.26 ±1.76 ($P < 0.01$)	3.63 ±0.51	4.27 ±0.56 ($P < 0.05$)	5.17 ±0.81 ($P < 0.05$)	3.38 ±0.55	3.06 ±0.54 (NS)	2.29 ±0.56 ($P < 0.01$)
3DAT	3.47 ±0.84	3.56 ±1.29 (NS)	4.25 ±1.54 (NS)	3.14 ±1.00	2.38 ±0.49 (NS)	2.96 ±0.90 (NS)	3.00 ±0.70	2.51 ±1.21 (NS)	2.19 ±0.50 ($P < 0.05$)
ST	18.16 ±1.98	20.11 ±1.28 ($P < 0.05$)	23.21 ±1.63 ($P < 0.01$)	13.89 ±0.67	13.54 ±1.02 (NS)	14.62 ±1.07 (NS)	12.65 ±0.56	13.18 ±1.93 (NS)	10.20 ±2.24 ($P < 0.05$)
Rate in phase (/ min)									
RA	0.0037±0.0004	0.0034±0.0005 (NS)	0.0027±0.0003 ($P < 0.01$)	0.0035±0.0002	0.0035±0.0001 (NS)	0.0032±0.0003 ($P < 0.05$)	0.0035±0.0002	0.0034±0.0008 (NS)	0.0034±0.0003 (NS)
R1DS	0.047 ±0.010	0.038 ±0.010 (NS)	0.025 ±0.006 ($P < 0.01$)	0.104 ±0.010	0.072 ±0.009 ($P < 0.01$)	0.051 ±0.007 ($P < 0.01$)	0.130 ±0.012	0.125 ±0.027 (NS)	0.150 ±0.008 ($P < 0.01$)
R3DS	0.169 ±0.028	0.158 ±0.027 (NS)	0.131 ±0.022 ($P < 0.05$)	0.243 ±0.037	0.234 ±0.029 (NS)	0.228 ±0.047 (NS)	0.285 ±0.005	0.201 ±0.075 ($P < 0.05$)	0.303 ±0.114 (NS)

Data are presented as means±S.D. Statistical comparison with control sample was made using the Student's t-test. (NS), statistically not significant. In diamide samples,% crosslinking of spectrin in 1.5 mM and 4 mM diamide-treated cells were 50% and 79%, respectively. The deformation index ('deformability' determined with a high shear rheoscope) at 200 and 290 dyn/cm^2 was 0.493±0.038 and 0.508±0.062 for control cells, 0.343±0.052 and 0.381±0.064 for 1.5 mM diamide-treated cells, and 0.223±0.056 and 0.259±0.062 for 4 mM diamide-treated cells, respectively.

Slope of this phase was termed as rate of sedimentation of one-dimensional aggregates (R1DS).

Phase 3: The third phase was mainly due to the formation of large and three-dimensional aggregates and their sedimentation. In this phase, the fluctuation was rapid and irregular, and the amplitude was fairly large. The interval of this phase was termed as three-dimensional aggregation time (3DAT), and the rate of sedimentation of three-dimensional aggregates (R3DS) was obtained from slope of this phase.

The demarcation of each phases was essentially performed by the above characteristic fluctuation of signal intensity. In addition, the demarcation was automatically determined with a computer on the basis of the amplitude of signal fluctuation: the difference of transmittance between the raw signal intensity digitized at a rate of 100 ms and the moving average of 6 s duration was tentatively set to 0.007 for the onset of phase 2 and to 0.12 for the onset of phase 3. Both methods for the onset of each phases agreed well.

3.2. Comparison of kinetic parameters between the laser scattering technique and the low-shear rheoscope technique

Various kinetic parameters obtained by light scattering technique was compared with the velocity of rouleaux formation by low-shear rheoscope technique. In the concentration dependency of Dx-70, the velocity of rouleaux formation (v, μm^2/min) determined by the low-shear rheoscope technique corresponded to R1DS (/min) and 1/1DAT (/min) obtained by the laser scattering technique, as shown in Fig. 3. The concentration of Dx-70 giving maximum velocity and/or rate

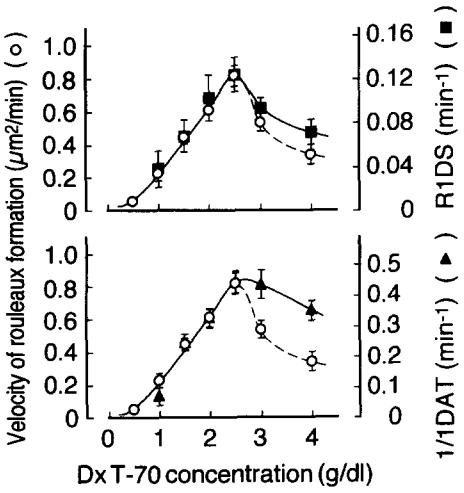

Fig. 3. Comparison of kinetic parameters obtained by different techniques for the measurement of erythrocyte aggregation. As a function of Dextran T-70 concentration, 1DAT and 1/R1DS obtained by the light scattering technique were compared with the velocity of rouleaux formation measured at a shear rate of 7.5 s^{-1} with the low-shear rheoscope.

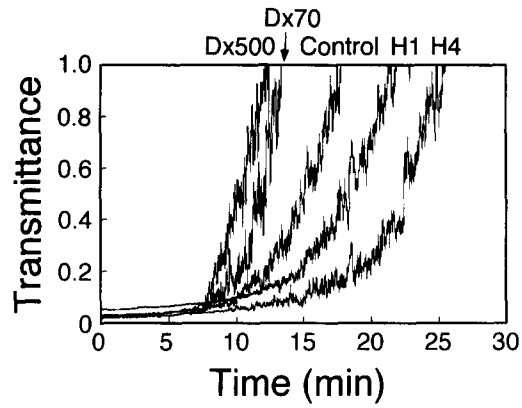

Fig. 4. Changes of light scattered transmittance in the process of aggregation and sedimentation of erythrocytes. Control: normal erythrocytes in autologous plasma; H1: 1.5 mM diamide-treated erythrocytes in autologous plasma; H4: 4 mM diamide-treated erythrocytes in autologous plasma; Dx70: control erythrocytes in PBS containing 2 g/dl dextran 70; Dx500: control erythrocytes in PBS containing 1.5 g/dl dextran 500. Hematocrit was 5% in all samples.

of erythrocyte aggregation agreed well between both techniques. The different behavior in the high concentration of Dx-70 was clearly due to the methodological difference (see the discussion of Section 4).

3.3. Effects of diamide-treatment and dextrans on erythrocyte aggregation

The effect of erythrocyte properties (i.e., decreased deformability) and macromolecule (i.e., dextran) on the process of erythrocyte aggregation and sedimentation is shown in Fig. 4. The signal variation showed three distinct phases in all samples, namely a smooth initial phase, a gentle fluctuation phase in the middle and a rapid fluctuating phase toward the end. Furthermore, the good reproducibility of signal pattern was obtained by repeated measurements, as ascertained by good superposition of the signal patterns.

Diamide-treated cells suspended in autologous plasma (diamide sample) clearly showed a smooth increase in the transmittance in phases 1 and 2, compared with control cells. However, the increase of transmittance in phase 3 was similar to that in control cells. Therefore, the decrease of erythrocyte deformability inhibits the aggregate formation of erythrocytes.

On the other hand, the cells suspended in PBS containing Dx-70 or Dx-500 (dextran sample) showed a steep transmittance variation after a short smooth phase in the beginning. This characteristics was prominent in phase 2, compared with that of control cells. It should be noted that the cells suspended in only PBS did not show any observable variation in light transmittance within 30 min duration.

The kinetic parameters obtained for control, dextran and diamide samples are summarized in Table 1.

Comparison of these kinetic parameters provided the following characteristics, despite of the difference of medium between diamide samples and dextran samples.

On the time parameter, the aggregation time (AT) was significantly decreased for dextran samples along with sedimentation time (ST) and one-dimensional aggregation phase (1DAT) ($P < 0.01$). Whereas, the corresponding parameters for diamide samples showed a significant increase. However, the three dimensional aggregation phase (3DAT) did not show any significant deviation.

On the rate parameter, the rate of aggregation (RA) showed no significant difference for dextran and 1.5 mM diamide samples, but 4 mM diamide sample showed a highly significant decrease. Rate of one-dimensional aggregate sedimentation (R1DS) showed significant increase for dextran samples ($P < 0.01$) and significant decrease for 4 mM diamide sample (not significant for 1.5 mM diamide sample). The rate of three-dimensional aggregate sedimentation (R3DS) was significantly high for dextran samples ($P < 0.01$) but slightly low for 4 mM diamide sample.

In short, the aggregating macromolecules, dextrans, decrease the time parameters and increase the rate parameters, while the decrease of erythrocyte deformability increases the time parameters and decreases the rate parameters.

3.4. Effects of simultaneous alterations of cellular deformability and aggregating macromolecules on erythrocyte aggregation

Typical light scattering profiles for erythrocytes with decreased deformability suspended in dextran medium are shown in Fig. 5. The diamide-treated cells in Dx-500 medium showed a very steep transmittance variation after a short flat phase in the beginning. Other diamide-treated cells in Dx-70 medium were in between control and Dx-500 samples. It must be noted particularly that 4 mM diamide-treated cells in Dx-500 medium showed a faster aggregation and sedimentation as compared to 1.5 mM diamide-treated cells in the same medium along with quicker saturation.

The kinetic parameters are summarized in Table 1: AT was rather reduced in 4 mM diamide-treated cells, though not significant. 1DAT significantly increased in Dx-70 medium, but decreased in Dx-500 medium. 3DAT did not show any significant difference except a less significant decrease for 4 mM diamide-treated cells in Dx-500 medium. ST (= AT + 1DAT + 3DAT) in 4 mM diamide-treated cells was slightly prolonged in Dx-70 medium (though not significant), but it was significantly shortened in Dx-500 medium.

The rate of aggregation, RA did not show any difference except for a less significant decrease in 4 mM diamide-treated cells in Dx-70 medium. RlDS significantly decreased in Dx-70 medium with decreasing the erythrocyte deformability, but increased in Dx-500 medium. R3DS did not show such a characteristic variation.

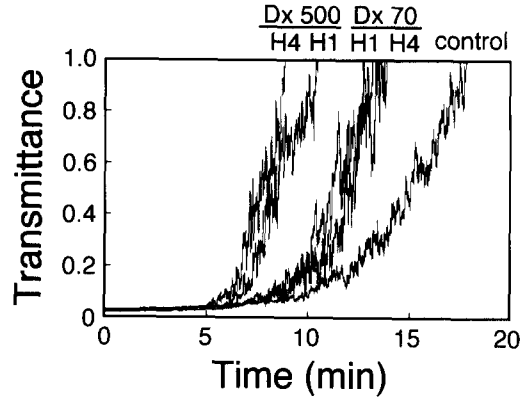

Fig. 5. Changes of light scattered transmittance in the process of aggregation and sedimentation of erythrocytes: aggregation of erythrocytes with decreased deformability in the presence of macromolecular dextrans (Dx70 or Dx500) in PBS. Erythrocytes with decreased deformability were obtained by treating with 1.5 mM diamide (H1) or 4 mM diamide (H4). Control cells were suspended in autologous plasma. Hematocrit was 5% in all samples.

The characteristic changes of kinetic parameters in various media by decreasing erythrocyte deformability are summarized in Fig. 6. In short, the characteristic changes mainly appeared in phase 2: with decreasing erythrocyte deformability with diamide, the duration of phase 2 (1DAT and thus ST) prolonged in autologous plasma and in Dx-70 medium, and the rate in phase 2 (R1DS) decreased. On the other hand, the opposite relations were observed in Dx-500 medium.

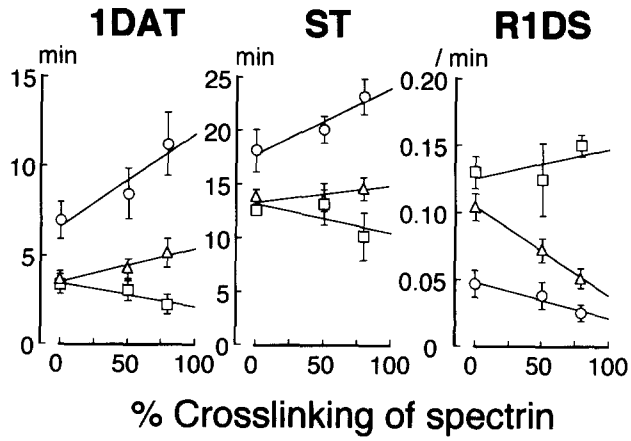

Fig. 6. Changes of characteristic kinetic parameters in the process of erythrocyte aggregation and sedimentation in various media: effect of erythrocyte deformability (i.e., crosslinking of spectrin). Experiments were performed in autologous plasma (○), in PBS containing 2 g/dl Dx70 (△) and in PBS containing 1.5 g/dl Dx500 (□). Data from Table 1.

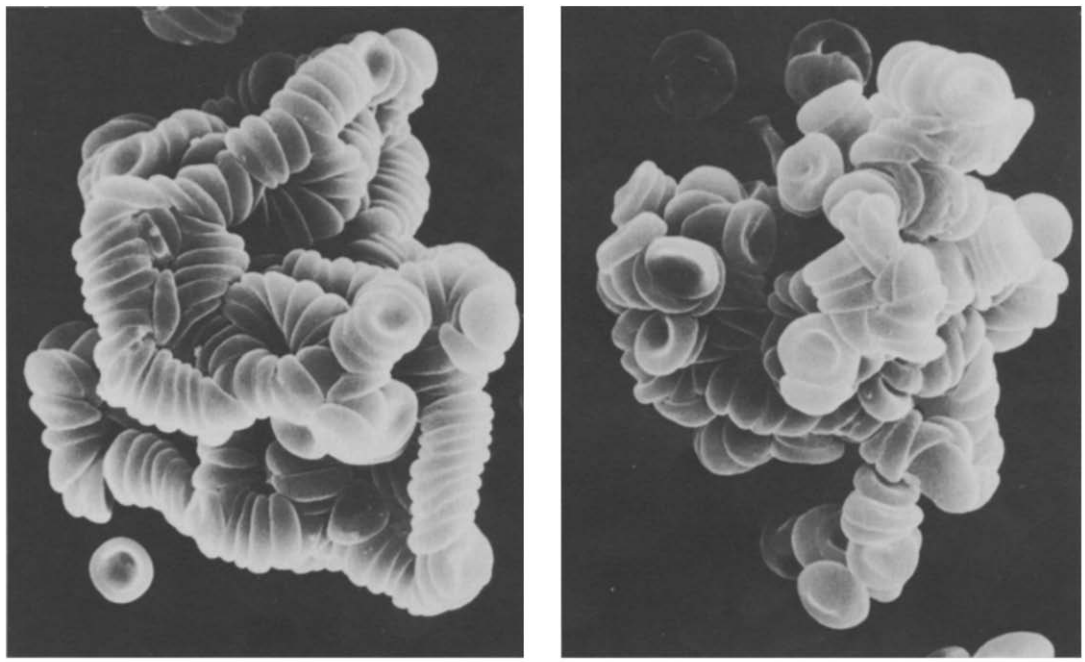

Fig. 7. Comparison of shape of three-dimensional aggregates observed with the scanning electron microscope. Three-dimensional aggregates of erythrocytes formed in the presence of 1.8 g/dl Dx-70: normal cells (left) and 4 mM diamide-treated cells (right).

Scanning electron microscopic observation of erythrocyte aggregates formed by Dx-70 showed distinct difference between normal and hardened erythrocytes. As shown in Fig. 7, rouleaux assembled into three-dimensional aggregates were homogeneous and linearly arranged in normal erythrocytes, while those were inhomogeneous and irregular in hardened cells. Moreover, the erythrocyte aggregates formed by normal cells were dense, while those formed by hardened cells were rough.

4. Discussion

Erythrocyte aggregation is an important rheological phenomenon responsible for the low-shear non-Newtonian behavior of blood. (a) Macromolecular substances in suspending medium of erythrocytes greatly contribute to this behavior. The interaction of erythrocytes with plasma proteins (fibrinogen and immunoglobulins) or plasma substitutes (such as dextrans) leads to erythrocyte aggregation and their strength depends on the availability of these macromolecules [1,2]. The dependence of erythrocyte aggregation on these macromolecules has been extensively studied [1–5,17–19,26,27]. (b) Properties of erythrocytes, such as viscoelastic properties of erythrocyte membrane, internal viscosity, cellular concentration (or hematocrit), cell size and cell shape, also contribute to erythrocyte aggregation [1,2]. (c) In an in vivo situation, vascular factors, such as vessel size, pressure-flow relation, capillary distribution and cell-vessel wall interaction, also contribute to erythrocyte aggregation. Alteration of one of the above factors affects other factors in vicious cycle [28]. In the process, the aggregation characteristics of erythrocytes changes momentarily and influences the rheological properties of blood, i.e., the blood flow.

In most of the pathophysiological conditions, both cells and suspending medium simultaneously undergo modifications. Therefore, erythrocyte aggregation represents a net contribution from both factors. Many cardiovascular diseases exhibit both cells and medium alterations. In diabetes mellitus, the erythrocyte aggregation is increased and the erythrocyte deformability is decreased [29,30]. Increase of plasma proteins (fibrinogen and α_2-globulin) in myocardial infarction presents the similar effect [31]. In sickle cell anemia, the altered cell membrane combined with increase in plasma fibrinogen increases the severity of the disease [32]. These alterations lead to the decreased deformability and increased adhesiveness of sickle cells to vascular endothelium [33]. In a two-component system of plasma and erythrocytes, it is quite obvious that the alteration of either one will effectively alter the overall characteristics. Therefore, similar studies on the simultaneous influence of suspending medium and erythrocyte membrane alterations on erythrocyte aggregation

are important in understanding the pathophysiology of diseases.

The present study has been aimed to simulate a severe pathological situation where both cell and medium simultaneously undergo alterations. We approached the individual modifications of cell and medium on erythrocyte aggregation and both alterations by using dextran and diamide. For this purpose, we used the change of forward scattered laser light intensity during the entire process of erythrocyte aggregation and sedimentation, and we obtained the kinetic parameters during different stages of the process by automatic computation.

4.1. Comparison of light scattering technique with a low-shear rheoscope technique with an image analyzer

Light scattering technique has the advantages of quickly providing distinct profiles for different samples with a significantly varied kinetic parameters of different phases. Moreover, these profiles and the kinetic parameters can provide a better picture of the interactions of macromolecules in forming aggregates of erythrocytes and in their sedimentation. Therefore, these kinetic parameters can be effectively employed to quantify the rheological behavior erythrocytes in pathological conditions. Furthermore, the simple technique based on light scattering can be useful for the clinical medicine for the rapid scanning of blood sample.

The present study has proved that 1DAT and 1/R1DS obtained by the light scattering technique correspond well with the velocity of rouleaux formation obtained with a low-shear rheoscope-image analyzer [24,26]. The different dose-dependent changes of kinetic parameters in high concentration of Dx-70 may be due to the methodological difference (Fig. 3): the rheoscope technique measures purely the process of rouleaux formation at a constant shear rate, while the light scattering technique monitors both processes of erythrocyte aggregation and sedimentation of erythrocytes and/or their aggregates under a gravitational field. The erythrocyte aggregation process in higher concentration of dextran (namely, in more viscous media) will be more suppressed under a constant shear rate (i.e., 7.5 s^{-1}) than under the gravitational field (i.e., nearly 0 s^{-1}), because of the increase of disaggregating force due to the increased shear stress (= medium viscosity × shear rate).

4.2. Effect of decreased cellular deformability and dextrans on the erythrocyte aggregation

Maeda et al. [11] have reported the inhibition of erythrocyte aggregation by the crosslinking of membrane cytoskeleton with diamide. Furthermore, the effect of cell modifications on erythrocyte aggregation has been reported in detail by Nash et al. [12] and Sowemimo-Coker et al. [13].

In the present study, we have employed diamide to alter the membrane elastic properties, as diamide crosslinks only spectrin quantitatively, cytoskeletal protein of erythrocyte membrane [11] and leads to the decrease of erythrocyte deformability by increasing the membrane stiffness [11,21,34]. Because the diamide-treated cells behave like solid particles, the cell-to-cell contact leading to aggregation is suppressed in autologous plasma (Fig. 4). Thus, we could observe that the aggregates formed by diamide-treated cells are small, and the erythrocytes in the three-dimensional aggregates are loosely packed.

Dextrans induce erythrocyte aggregation by macromolecular adsorption on the membrane and connecting adjacent erythrocytes by bridging [1,2,18,26]. The rate and degree of dextran-induced erythrocyte aggregation depend both on the molecular weight and the concentration of dextran with an optimum concentration leading to a maximum aggregation [18,26]. Morphologically, with increase in molecular weight of dextran, the size of erythrocyte aggregates increased and their structure became tightly packed.

4.3. Effect of simultaneous alterations of erythrocyte deformability and macromolecules on erythrocyte aggregation

Macromolecules in plasma play a major role in bringing the cells together, while cellular constituents probably determines the structural characteristics of the aggregates. Cytoskeletal membrane proteins in erythrocytes impart the mechanical strength and stability and the maintenance of their shape [35]. Therefore, alterations of these proteins affect their rheological properties. Some of the cellular factors can contribute to their deformability, which is a function of surface area to volume ratio, membrane viscoelasticity and internal viscosity [1,2].

Decrease of erythrocyte deformability alone can produce inhibitory effect on the erythrocyte aggregation. However, the presence of some aggregating macromolecules accelerates the aggregation leading to a complicated situation. The reduction of erythrocyte deformability in dextran media (as in Dx-500 medium) significantly decreases the time parameter (especially, 1DAT) and increases the rate parameter (especially, R1DS) (Table 1 and Fig. 6): consequently the erythrocyte aggregation and sedimentation processes are accelerated. It has been shown that cell shrinkage increases the degree and strength of erythrocyte aggregation [12]. Our morphological study shows that 4 mM diamide-treated cells in Dx-70 medium forms big mass of aggregates, but the aggregates are rough and the typical rouleaux in the aggregates are hardly observed.

Clearly, macromolecules in medium bridges among erythrocytes to produce aggregates, whereas the shape, strength and stability of the aggregates depend on the alterations of membrane structure. Such morphological difference between erythrocyte aggregates formed by normal and hardened cells should be taken into consideration for the interpretation of kinetic parameters.

According to Chien and Jan [18], macromolecular bridging over a limited area of two adjacent cells brings the neighboring areas into sufficiently close range for further bridging to proceed. The progression of bridge formation is facilatated by the deformation of cells, and the maximum contact area of cell surface bridged by the macromolecules is resulted. However, when the deformation of cells to get such a wide contact area is considerably impaired and the bridging (aggregating) force of macromolecules is extremely strong, cells may makes sparse and thus large aggregates quickly without getting maximum contact area between the cells. This may be a case of 4 mM diamide-treated erythrocytes in Dx-500 medium.

In conclusion, both erythrocytes and macromolecules in suspending medium effectively contribute to many folds increase in the aggregation of erythrocytes. Dextrans and diamide provide an excellent experimental models for this study in giving insight of cell-medium interactions of erythrocyte aggregation. Quantifying the erythrocyte aggregation in pathological conditions, both the cell and medium contributions should be taken into consideration especially in cases where cell factors are likely to undergo alterations. The laser scattering method could conveniently and precisely quantify these alterations during the different phases of erythrocyte aggregation and sedimentation.

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