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Erythrocyte aggregation: bridging by macromolecules and electrostatic repulsion by sialic acid

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Relation between aggregating force (of fibrinogen and IgG) and disaggregating force (due to electrostatic repulsion among erythrocytes) in erythrocyte aggregation was investigated with a rheoscope combining a video camera, an image analyzer and a computer. (i) Erythrocyte aggregation was augmented with the increase of molecular weight of bridging macromolecules as far as examined for fibrinogen and the degradation products and IgG and the related macromolecules, and the augmentation seemed to be dependent on the molecular length of macromolecules. In accelerating the erythrocyte aggregation, fibrinogen was more effective than IgG, and some interaction between fibrinogen and IgG in their coexistence was suggested. (ii) The decrease of sialic acid content on the erythrocyte surface accelerated IgG-induced erythrocyte aggregation much greater than fibrinogen-induced one. (iii) Counteraction between aggregating force and disaggregating force in leading to erythrocyte aggregation was discussed relating to molecular length of bridging macromolecule and electrostatic repulsive force by sialic acid.

Introduction

Erythrocyte aggregation reduces blood flow at low shear regions by increasing blood viscosity and also impedes the capillary flow through sludge formation. Essential factors participating the erythrocyte aggregation are (i) shear stress in flow, (ii) properties of erythrocytes (cell number, deformability, surface charge, shape), (iii) properties of macromolecules bridging between adjacent cells (molecular weight, conformation, charge), and (iv) properties of medium suspending erythrocytes (pH, temperature, osmolarity, ionic strength, and so on) [1,2].

Erythrocyte–erythrocyte interaction is characterized by their aggregation at low shear rates and by the disintegration of the aggregates at high shear rates. Therefore, erythrocyte aggregation is induced, when aggregating force overcomes disaggregating force. However, the detailed relationship between aggregating force and disaggregating force in erythrocyte aggregation is still unknown in both rheological and biochemical standpoint.

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As aggregating force, fibrinogen is the most important bridging macromolecule pathophysiologically, thus extensive studies have been carried out relating fibrinogen to erythrocyte aggregation as well as to the blood viscosity and/or to the erythrocyte sedimentation [1,2]. On the other hand, various immunoglobulins have a great influence on the erythrocyte aggregation in various pathological conditions (infectious diseases, multiple myeloma, macroglobulinemia, and so on), since the hyperviscosity syndrome is induced sometimes [1,3]. Thus, the contribution and/or the interaction of fibrinogen and IgG in the erythrocyte aggregation is of pathophysiological interest to be investigated in their coexistence.

Role of sialic acid on the surface of erythrocyte membrane has been studied as an electrostatic repulsing force in erythrocyte–erythrocyte interactions [6,7]. Negative charge of erythrocyte surface, mainly due to the carboxyl group of sialic acid and partly due to that of acidic amino acid residues in sialoglycoproteins, produces the electrostatic repulsive force between adjacent erythrocytes in leading to inhibition of erythrocyte aggregation. The increased erythrocyte aggregation has been observed in patient with sialic acid deficiency [6].

In the present study, the relation between aggregating force and disaggregating force to erythrocyte aggre-

gation was mainly investigated by using fibrinogen and IgG as bridging macromolecule and by altering sialic acid content on the surface of erythrocytes as electrostatic repulsive force. A close relation between molecular length of bridging macromolecules and repulsion of sialic acid between erythrocytes was clarified by comparing both fibrinogen-induced and IgG-induced erythrocyte aggregation.

Materials and Methods

Erythrocytes. Fresh blood from a healthy adult male (red cell type, O^+) was heparinized (10 units heparin/ml blood), and was centrifuged at $1200 \times g$ for 5 min at 4°C. After removing plasma and buffy coat, erythrocytes were washed twice with isotonic phosphate-buffered saline (42.6 mM Na_2HPO_4, 7.4 mM NaH_2PO_4, 90 mM NaCl, 5 mM KCl, 5 mM glucose (pH 7.4); 285 mosM). Erythrocytes were used within 5 h after collecting blood.

Proteins. Human serum albumin (fatty acid free, fraction V) was purchased from Miles Lab. Inc. (Naperville, IL, U.S.A.). Human fibrinogen (grade L; 90% clottable) was purchased from AB Kabi (Stockholm, Sweden), and was passed through a lysine-Sepharose 4B column to remove contaminated plasminogen and/or plasmin [5,7]. Fragments X and Y of fibrinogen were prepared as described elsewhere [8].

IgG was purchased from Miles Lab. Inc. (Naperville, IL, U.S.A.). IgM, IgA, $F(ab')_2$, Fab and Fc were purchased from the Green Cross Corp. (Osaka, Japan). All proteins were used after dialyzing against isotonic phosphate-buffered saline at 4°C overnight. The final concentration of albumin, fibrinogen, IgG, IgM, IgA, $F(ab')_2$, Fab and Fc was determined spectrophotometrically by using absorption coefficients of 5.5, 15.1, 14.0, 13.3, 13.4, 14.1, 14.2 and 14.1 for 1 g/dl solution at 280 nm, respectively, according to their Chemical Credential.

Measurement of the velocity of erythrocyte aggregation. A rheoscope apparatus [9] (composed of a transparent cone-plate viscometer and an inverted microscope) combined with a video camera (Sony, AVC 1150, Tokyo, Japan), an image analyzer (Toyo Ink Co., Luzex 450, Tokyo, Japan) and a computer (Hewlett Packard, HP-85, Palo Alto, CA) was used for the measurement of the velocity of erythrocyte aggregation [10] under a constant temperature [11].

Erythrocytes were suspended in isotonic phosphate-buffered saline (pH 7.4) containing various macromolecules in the presence of 5 g/dl human serum albumin (albumin was used for preventing the adhesion of erythrocytes and/or aggregates on glass plate of the rheoscope [12]). The suspension was immediately applied to the rheoscope. With respect to the sensitivity, the reproducibility and the kinetic (quantitative) analysis of erythrocyte aggregation, the measurement was carried out after diluting the stock erythrocyte suspension to a hematocrit of 0.3% (measured with a microhematocrit centrifuge, Kubota Manuf. Co., model KH-120, Tokyo, Japan), at constant shear rate and at 25°C. The count of particles (i.e., single erythrocytes, one-dimensional aggregates (rouleaux) or three-dimensional aggregates) and the total area projected by the particles in a frame of the video image (actual frame size, $180\ \mu m \times 130\ \mu m$) were consecutively encoded by the image analyzer at an interval of approx. 1.3 s, and transferred to the computer. The velocity of erythrocyte aggregation was expressed by the increment of area/count per unit time (v, μm^2/min) in the process of rouleaux formation [10,11,13]. Since erythrocyte aggregation is dependent on the shear stress [1,2], the velocity, if necessary, was compared at same shear stress by correcting the velocity on the basis of (i) viscosity of media and (ii) shear rate dependency of the velocity, as described in our previous paper [14]. Furthermore, for the use of dilute erythrocyte suspension, velocity, μm^2/min, can be easily converted to 'number of cells/min' by dividing by 12 μm^2 (projected area of one erythrocyte in aggregates) [13,14].

Alteration of sialic acid content in erythrocytes. Washed erythrocytes (hematocrit, 20%) were incubated with 2–90 mU/ml neuraminidase (sialidase from *Clostridium perfringens*, 0.86 unit/mg protein in activity using *N*-acetylneuramin-lactose; purchased from Sigma Chem. Co., St. Louis, MO, U.S.A.) in isotonic phosphate-buffered saline at 37°C for 80 min with gentle shaking [5]. After incubation, the reaction mixture was cooled and the erythrocytes were washed with cold isotonic phosphate-buffered saline three times to remove neuraminidase.

Determination of sialic acid in erythrocyte membrane. Ghosts were prepared from a known number of erythrocytes by hypotonic hemolysis, according to the method of Dodge et al. [15]. The ghosts were treated with 0.05 M H_2SO_4 at 80°C for 60 min to release sialic acid, and then the thiobarbituric acid method [16] was adopted for the determination of sialic acid. *N*-Acetylneuraminic acid was used as a standard.

Results

Bridging by macromolecules: aggregating force

The erythrocyte aggregation induced by (i) fibrinogen and the degradation products and by (ii) IgG and the related macromolecules in isotonic phosphate-buffered saline containing 5 g/dl albumin is compared in Fig. 1.

Macromolecules with higher molecular weight clearly gave higher velocity of erythrocyte aggregation. Other related macromolecules with molecular weight of less than 100 000 did not induce erythrocyte aggrega-

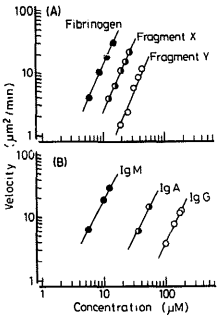

Fig. 1. Erythrocyte aggregation induced by fibrinogen and the degradation products (A) and by IgG and the related macromolecules (B). The velocity of erythrocyte aggregation was measured at a shear rate of 7.5 s^{-1} in isotonic phosphate-buffered saline containing 5 g/dl albumin, at 25°C (velocity, not corrected for the different viscosities of the various media). Data for fibrinogen and the degradation products (A) were cited from our previous results [8]: fibrinogen (●), fragment X (◑) and fragment Y (○). Present results for IgM (●), IgA (◑) and IgG (○) are shown in B. Each point was the mean value of three measurements.

tion: effect of $F(ab')_2$, Fab and Fc were examined up to 300 μM, 600 μM and 600 μM, respectively; fragments D and E have been examined up to 800 μM and 700 μM, respectively [8]. It must be particularly noted that $F(ab')_2$ could not induce erythrocyte aggregation by itself, though $F(ab')_2$ accelerated the fibrinogen-induced erythrocyte aggregation (vide infra). Furthermore, it was ascertained that fibrinogen-induced ery-

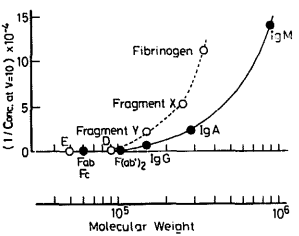

Fig. 2. Comparison of erythrocyte aggregation by macromolecules with different conformation. Fibrinogen and the degradation products are presented by open symbols, and IgG and the related macromolecules by closed symbols. The reciprocal of concentration of macromolecules (in M), giving a velocity of erythrocyte aggregation of 10 μm^2/min, is shown on the ordinate. Data from Fig. 1.

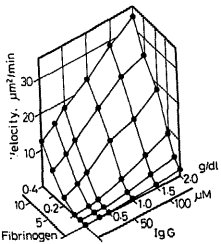

Fig. 3. Erythrocyte aggregation in the presence of fibrinogen and IgG in different proportions. The velocity of erythrocyte aggregation was measured at a shear rate of 7.5 s^{-1} in isotonic phosphate-buffered saline containing 5 g/dl albumin, at 25°C, and then corrected to the velocity at a constant shear stress of 1.3 dyn/cm^2, which is at 7.5 s^{-1} in a medium containing 8.8 μM (0.3 g/dl) fibrinogen, 130 μM (2 g/dl) IgG and 5 g/dl albumin. Each point was the mean value of three measurements.

throcyte aggregation in the present study did not differ from those in our previous results [8].

In order to compare the erythrocyte aggregation induced by different molecular species, the reciprocal of concentration of macromolecule giving a velocity of erythrocyte aggregation, 10 μm^2/min, i.e., the ability of macromolecule in leading to erythrocyte aggregation, was plotted against the molecular weight of macromolecules, as shown in Fig. 2.

Clearly, as far as compared the macromolecules in the similar molecular weight class, fibrinogen and the degradation products aggregated erythrocytes much greater than IgG and the related macromolecules. This qualitative conclusion was not changed, even if the velocity was compared at same shear stress by correcting for the different viscosities of the various media (also see Fig. 3 for the fibrinogen and IgG).

Interaction among macromolecules in inducing erythrocyte aggregation

For understanding the effectiveness of fibrinogen and IgG on the erythrocyte aggregation in plasma, velocity of erythrocyte aggregation was measured in artificial medium containing both fibrinogen and IgG in their different proportion in the presence of 5 g/dl albumin. The detailed experiment is shown in Fig. 3.

At physiological concentration of IgG (2 g/dl = 133 μM), the increase of velocity per μM fibrinogen under a shear stress of 1.3 dyn/cm^2 was approx. 2.8 μm^2/min, while at physiological concentration of fibrinogen (0.3 g/dl = 8.82 μM) the increase of velocity per μM IgG was approx. 0.15 μm^2/min. Therefore, near the physiological condition, the change of fibrinogen concentration was about 20-times as effective in

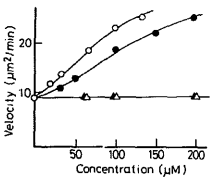

Fig. 4. Acceleration of fibrinogen-induced erythrocyte aggregation by IgG and $F(ab')_2$. Erythrocyte aggregation induced by 0.3 g/dl fibrinogen was accelerated by IgG (○) and $F(ab')_2$ (●), but not by Fab (△) and Fc (▲). The velocity was measured in isotonic phosphate-buffered saline containing 5 g/dl albumin at 25°C at 7.5 s^{-1} (velocity, not corrected for the change of shear stress). Each point was the mean value of three measurements.

the ability of erythrocyte aggregation as that of IgG concentration.

As far as assumed from Fig. 3, the erythrocyte aggregation may be induced by some interactions between fibrinogen and IgG in their bridging between adjacent erythrocytes. For example, 0.1 g/dl fibrinogen or 0.5 g/dl IgG did not induce the erythrocyte aggregation by themselves, but their mixture clearly induced erythrocyte aggregation. The possible evidence for the acceleration of fibrinogen-induced erythrocyte aggregation by IgG was ascertained by $F(ab')_2$, a peptic fragment of IgG, which could not induce erythrocyte aggregation by itself (Fig. 2). As shown in Fig. 4, $F(ab')_2$ clearly accelerated the fibrinogen-induced erythrocyte aggregation. However, the accelerating effect of $F(ab')_2$ was smaller than that of IgG. Other small fragments of IgG, Fab and Fc, did not accelerate the fibrinogen-induced erythrocyte aggregation.

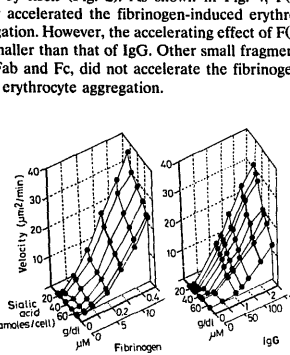

Fig. 5. Effect of sialic acid content on fibrinogen-induced and IgG-induced erythrocyte aggregation. Sialic acid in erythrocytes (expressed by amol (10^{-18} mol)/ cell) was varied by treating with various concentration of neuraminidase (details in the text). The velocity was measured in isotonic phosphate-buffered saline containing fibrinogen or IgG in the presence of 5 g/dl albumin at 25°C and at 7.5 s^{-1} (velocity, not corrected for the change of shear stress). Each point was the mean value of three measurements.

Electrostatic repulsion by sialic acid: disaggregating force

In order to understand the relation between aggregating force by macromolecules and electrostatic repulsive force by sialic acid (as disaggregating force), detailed experiments for both fibrinogen-induced and IgG-induced erythrocyte aggregation were carried out at a constant shear rate (7.5 s^{-1}) in artificial medium using erythrocytes with various contents of sialic acid. Relation among velocity of erythrocyte aggregation, sialic acid content and concentration of fibrinogen or IgG are summarized in Fig. 5.

With decreasing sialic acid content, the erythrocyte aggregation was accelerated in both fibrinogen-containing and IgG-containing media. At the physiological concentration of fibrinogen and IgG, the increase of velocity per amole (10^{-18} mol) sialic acid decreased/ cell was 0.20 μm^2/min and 0.36 μm^2/min, respectively, which correspond to 2.3% and 4.6% increase of aggregation velocity of untreated erythrocytes, respectively. Therefore, the acceleration of IgG-induced erythrocyte aggregation by decreasing sialic acid content was twice as effective as that of fibrinogen-induced one.

Discussion

Blood flow in low shear regions, especially in venous circulation, is greatly reduced by the enhancement of erythrocyte aggregation. Erythrocyte aggregation is induced by the bridging of macromolecules between adjacent erythrocyte surfaces. Therefore, both the properties of bridging macromolecules and the surface structure of erythrocytes are important in erythrocyte aggregation. The contribution of these factors affecting the erythrocyte aggregation is of deep interest under various pathophysiological conditions. In the present study, the relative difference of aggregating force of fibrinogen and IgG (as bridging macromolecules) and the counteraction of the macromolecular bridging to electrostatic repulsive force of sialic acid on the surface of erythrocytes was investigated.

Aggregating force by macromolecules and erythrocyte-binding site in the macromolecules

Since erythrocyte aggregation is greatly influenced by the physical shear stress, kinetic data obtained in different media (with different viscosities) were compared at the same shear stress. As far as examined within physiological concentrations of fibrinogen and IgG and at their lower concentrations, velocity of erythrocyte aggregation induced by fibrinogen was proved to be about 20-times greater than that induced by IgG.

Plausible erythrocyte-binding site in fibrinogen molecule for leading to erythrocyte aggregation has been proposed to be in the terminal domain of the trinodular structure of fibrinogen [8]. In the present

experiment, it was suggested that IgG bridges in the vicinity of the NH_2-terminal end of Fab to an erythrocyte and in the vicinity of the COOH-terminal end of Fc to other erythrocyte, because $F(ab')_2$ does not induce erythrocyte aggregation (see Fig. 2) in spite that the molecular length of $F(ab')_2$ is rather larger than that of IgG due to the increased flexibility at the hinge region [17]. The difference of aggregating force between fibrinogen and IgG greatly depends on the distance between erythrocyte-binding sites (about 48 nm and 16 nm in fibrinogen- and IgG-induced erythrocyte aggregation, respectively (see Ref. 14)), since the electrostatic repulsive force between erythrocytes decreases exponentially with increasing the distance [2]. The difference must be quantitatively evaluated in future by determining the binding strength of these bridging macromolecules to erythrocytes and the number of bridging macromolecules (not the number of binding macromolecules).

Interaction among macromolecules in bridging between erythrocytes

In the present study, it must be noted that IgG and $F(ab')_2$ accelerate the erythrocyte aggregation induced by fibrinogen (Fig. 4), in spite that $F(ab')_2$ cannot especially induce erythrocyte aggregation by itself (Fig. 2). Imaizumi and Shiga [18] have observed the acceleration of erythrocyte aggregation in dilute plasma by IgG and $F(ab')_2$. The phenomena may be explained by some interaction between fibrinogen and IgG or $F(ab')_2$ in bridging of fibrinogen among erythrocytes. Furthermore, we could observe in the present study that such acceleration of fibrinogen-induced erythrocyte aggregation by IgG and $F(ab')_2$ was inhibited by Fab and Fc (data not shown here), as have been observed in dilute plasma by Imaizumi et al. [19]. However, the detailed mechanism is still unknown, though we assume that such inhibition probably occurs at bridging site of macromolecules on the erythrocyte surface and/or at interaction site between fibrinogen and IgG (or $F(ab')_2$).

Relation between aggregating force and repulsive force

For understanding the influence of repulsive force between erythrocytes due to the negative charge of sialic acid on the bridging force of macromolecules, fibrinogen- and IgG-induced erythrocyte aggregations were compared using neuraminidase-treated erythrocytes. However, the erythrocyte aggregation is affected by (i) morphological, (ii) rheological and (iii) biochemical properties of erythrocytes: (i) Shape of erythrocytes is important for making appropriate contact between adjacent cells [1,10]. The shape and the hematological indices of erythrocytes are not altered by neuraminidase-treatment, as previously reported [20,21]. (ii) Erythrocyte deformability, especially tank-treading motion of membrane, is important to get sufficient contact area between adjacent erythrocytes in rouleau formation [22–24]. The treatment with neuraminidase does not alter the erythrocyte deformability [20,21]. (iii) Not only the modification of glycoproteins by proteolytic enzymes [21], but also the removal of sialic acid by neuraminidase [4,5] accelerates the erythrocyte aggregation.

The present results showed that in the physiological concentrations of fibrinogen and IgG, the accelerating effect of erythrocyte aggregation by reducing sialic acid was greater for IgG than for fibrinogen (Fig. 5). The phenomena may be qualitatively explained as follows.

As the molecular length of fibrinogen (approx. 48 nm) is large, the electrostatic repulsive force is not so strong, thus the large aggregating force can be resulted. Even if sialic acid is decreased, the repulsive force is not so much decreased, and the aggregating force does not increase so remarkably. On the other hand, the molecular length of IgG (approx. 16 nm) is shorter than that of fibrinogen. So, the electrostatic repulsive force by sialic acid is fairly large in bridging between erythrocytes by IgG, thus the aggregating force is weak. Under such conditions, when the electrostatic repulsive force is decreased by removing sialic acid, the aggregating force is remarkably strengthened and the velocity of erythrocyte aggregation greatly increases. Another kind of experiment will be necessary in future for quantitative evaluation on the bridging force by fibrinogen and IgG (which may be calculated by number of bridging macromolecules and the bridging force of each macromolecule to erythrocyte surface) and the electrostatic repulsive force by sialic acid.

Acknowledgments

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