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Fibrinogen-induced erythrocyte aggregation: erythrocyte-binding site in the fibrinogen molecule

Nobuji Maeda [a], Masahiko Seike [a], Shingo Kume [b], Takeshi Takaku [b] and Takeshi Shiga [a]

[a] *Department of Physiology and* [b] *Central Laboratory, School of Medicine, Ehime University, Ehime (Japan)*

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The effect of fibrinogen and fibrinogen-derived products on the velocity of rouleau formation of human erythrocytes was quantitatively examined with a rheoscope combined with a video-camera, an image analyzer and a computer. (i) The velocity of rouleau formation by naturally occurring low-molecular-weight fibrinogen of 305 kDa and by desialylated fibrinogen was the same as that by native fibrinogen of 340 kDa. (ii) Concerning fibrinogen degradation products by plasmin, the velocity of rouleau formation decreased upon going from fibrinogen > fragment X > fragment Y (the ratio of molar concentration of fibrinogen, fragment X and fragment Y for giving a certain velocity of rouleau formation was approx. 1:2:5). The effect of fragments X and Y on the fibrinogen-induced rouleau formation was additive. (iii) Fragments D and E could not induce rouleau formation and did not affect the fibrinogen-, fragment X- and fragment Y-induced rouleau formation. (iv) Fibrinopeptides A and B and artificial tetrapeptides (Gly-Pro-Arg-Pro and Gly-His-Arg-Pro) did not affect the fibrinogen-induced rouleau formation. (v) The possible erythrocyte-binding site in fibrinogen molecule for leading to rouleaux was proposed to be in Aα-chain (probably, around residues No. 207–303) near the terminal domain of the trinodular structure of fibrinogen.

Introduction

Erythrocytes aggregate in blood flowing at low-shear rates and disintegrate at high-shear rates, reversibly. The erythrocyte aggregation is induced when macromolecules 'interact' with erythrocytes and then 'bridge' between adjacent cells; thus, the binding of macromolecules to erythrocyte surface does not necessarily induce erythrocyte aggregation. The physicochemical properties of bridging macromolecules, such as the molecular weight, the charge, the concentration and so on, are the most important factors for affecting the erythrocyte aggregation [1–3]. Furthermore, the erythrocyte aggregation by macromolecules is sometimes inhibited (or accelerated) by small molecules analogous to the macromolecules, though the small molecules do not induce the erythrocyte aggregation: e.g., IgG-induced erythrocyte aggregation is inhibited by the fragments, Fab and Fc, and so on [3–6]. However, the nature of macromolecular bridging between erythrocytes is still unclear.

Since the mode of macromolecular bridging

Abbreviations: HMW, high-molecular-weight; LMW, low-molecular-weight; GPRP, glycyl-L-prolyl-L-arginyl-L-proline; GHRP, glycyl-L-histidyl-L-arginyl-L-proline.

Correspondence: N. Maeda, Department of Physiology, School of Medicine, Ehime University, Shigenobu, Onsen-gun, Ehime, Japan 791-02.

between erythrocytes cannot be simply understood by the binding isotherm, the kinetics of erythrocyte aggregation (e.g., 'the velocity of rouleau formation') induced by fibrinogen and fibrinogen-derived products is examined in the present study by adopting a rheoscope combined with an image analyzer developed for the velocity measurement of rouleau formation [7].

Fibrinogen in plasma is heterogeneous in molecular weight, due to the partial enzymatic cleavage in plasma [8–10] and in sialic acid content [10–12]. Furthermore, in some diseases many fibrin(ogen)-derived products are present in plasma: fibrinopeptides are released in the process of intravascular coagulation by the action of thrombin, and several fibrin(ogen) degradation products increase in activated states of plasminogen [13–16]. Thus, we are interested in (i) the mode of interaction between fibrinogen and erythrocytes leading to aggregation and (ii) the effect of fibrinogen-derived products (low-molecular-weight fibrinogen, desialylated fibrinogen, fibrinogen degradation products by plasmin, fibrinopeptides, synthetic tetrapeptides inhibiting fibrin formation) on such an interaction with erythrocyte.

Materials and Methods

Erythrocytes and plasma

Fresh blood was obtained from healthy adult males (red cell type, O^+) and treated with 10 units heparin/ml blood. Blood 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/5.6 mM D-glucose (pH 7.4), 285 mosM), and were used within 5 h after collection (during this period, the erythrocyte shape and the ATP content did not change, and the velocity of rouleau formation was not altered significantly).

Measurement of the velocity of rouleau formation

The rheoscope apparatus [17] (composed of a transparent cone-plate viscometer and an inverted microscope equipped with a temperature-controlled stage) combined with a video-camera (Sony, AVC1150, Tokyo, Japan), an image analyzer (Luzex 450, Toyo Ink Co., Tokyo, Japan) and a computer (Hewlett Packard, HP-85, Palo Alto, CA, U.S.A.) was used for the measurement of the velocity of rouleau formation [3–7,18,19] as follows.

The washed erythrocytes were resuspended in an artificial medium composed of fibrinogen (and/or fibrinogen-derived products) of various concentrations and human serum albumin (5.0 g/dl; fatty-acid-free, fraction V; from Miles Laboratories, Naperville, IL, U.S.A.) in isotonic phosphate-buffered saline (pH 7.4). The suspension was immediately applied to the rheoscope, and the erythrocyte aggregation could be observed with a video-camera. The rheoscope was operated in the most suitable conditions, with respect to the sensitivity and the reproducibility (see Ref. 7): i.e., at a final hematocrit of 0.26% (the hematocrit of the original erythrocyte suspension was determined by the microcapillary centrifugation technique), at a shear rate of 7.5 s^{-1} (the shear rate was controlled by adjusting the speed of revolution of the cone) and at 25° C (the temperature was controlled by circulating water of a constant temperature into a microscopic stage, specially made of brass).

The process of erythrocyte aggregation can be distinguished in three stages as follows: In the first stage, some one-dimensional short rouleaux are formed. In the second stage, one-dimensional long rouleaux are produced by growing in the longitudinal direction. In the third stage, three-dimensional (complicatedly branched and piled) aggregates developed. The particle count (i.e., erythrocyte, one-dimensional aggregate ('rouleau') or three-dimensional aggregate) and the total area projected by particles in a frame of the video-image (actual frame size, 190×130 μm^2) were consecutively encoded by the analyzer at an interval of approx. 1.3 s, and transferred to the computer. The velocity of rouleau formation was represented by the increment of area/count per unit time (v, μm^2/min) at the stage of rouleau formation, reflecting the growth of particles [18].

Fibrinogen and fibrinogen-derived products

(1) Fibrinogen (native and partially degraded). Human fibrinogen from AB Kabi (Stockholm, Sweden; grade L, 90% clottable) was used after

treatment with lysine-Sepharose 4B to remove contaminated plasminogen and/or plasmin [19,20]. Commercial human fibrinogen is heterogeneous in molecular weight [9,10,21] due to the enzymatic cleavage in plasma [22], and contains native, high-molecular-weight (HMW)fibrinogen of 340 kDa and partially degradated, low-molecular-weight (LMW)fibrinogen of 305 kDa. The following methods were used for changing the proportion of these fibrinogens.

(i) Gel filtration: 10 ml of fibrinogen solution (0.6–0.8 g/dl) were applied to a column (2.2 × 230 cm) of Ultrogel AcA 34 (from LKB, Bromma, Sweden) equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 10 mM ε-aminocaproic acid, and the effluent, at a flow rate of 30 ml/h, was monitored at 280 nm. The fast-eluting minor peak (content less than 10% of the total) and the slow-eluting major peak were separated. In the major peak, the former (mainly HMW fibrinogen) and the latter (mainly LMW fibrinogen) halves were separately collected, and fibrinogens were precipitated with 2.2 M glycine [23].

(ii) Precipitation with ammonium sulfate: Fibrinogen dissolved in 50 mM potassium phosphate buffer (pH 7.4) was subjected to a step-wise precipitation with ammonium sulfate [24]. The precipitate in 17.5% ammonium sulfate saturation consisted mainly of HMW fibrinogen, and those between 20.5–26.0% ammonium sulfate saturation, mainly of LMW fibrinogen.

(2) Desialylated fibrinogen. 6 mg/ml fibrinogen were incubated with 120 mU/ml neuraminidase (sialidase from *Clostridium perfringens*; the activity, 0.86 unit/mg protein; from Sigma Chemicals, St. Louis, MO, U.S.A.) in 50 mM potassium phosphate buffer (pH 7.4) at 37 °C for 3 h. Desialylated fibrinogen was collected by repeating the precipitation with 2.2 M glycine [23] and dissolution in the buffer, twice. For control fibrinogen, the same procedure was carried out without neuraminidase. The content of sialic acid in fibrinogen molecule was determined by the thiobarbituric acid method [25].

The concentration of HMW, LMW and desialylated fibrinogens was determined spectrophotometrically by using an absorption coefficient of 15.1 [26] for 1 g/dl solution at 280 nm (1 cm in light path).

(3) Fibrinogen degradation products by plasmin. Fibrinogen (10 mg/ml) in 50 mM potassium phosphate buffer (pH 7.4) was digested at 37 °C for various periods by 1 U/ml plasmin (from human plasma; activity, 5.2 units/mg protein; from Sigma Chemicals). After digestion, plasmin was removed by passing the digests through a lysine-Sepharose 4B column (1 × 5 cm). Each degradation product was separated at 4 °C by gel filtration on an Ultrogel AcA-34 column (2.2 × 190 cm) in 50 mM potassium phosphate buffer containing 10 mM ε-aminocaproic acid. The representative fractionations of fibrinogen degradation products are shown in Fig. 1. Each fraction was concentrated by membrane filter (Collodion-bags, SM 13200 from Sartorius, Göttingen, F.R.G.), and was rechromatographed for further purification. Each degradation product could be efficiently prepared by changing the digestion time (e.g., to obtain a lot of fragment X, the digestion was stopped by 60 min).

The concentration of fibrinogen degradation products was determined spectrophotometrically using the following absorption coefficients for 1 g/dl at 280 nm (1 cm lightpath) [26,27]; 14.2 for

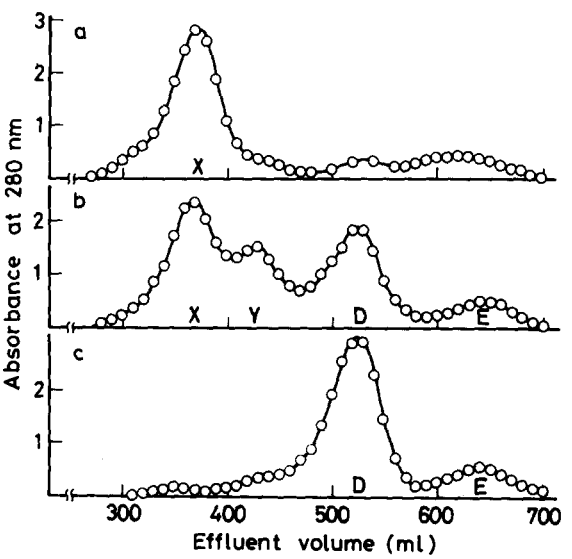

Fig. 1. Gel filtration of fibrinogen degradation products by plasmin. 200 mg fibrinogen digested by 4 mg plasmin for (a) 60 min, (b) 120 min and (c) 180 min at 37 °C were applied to an Ultrogel AcA 34 column (2.2×190 cm), and each fragment (X, Y, D, E) was monitored at 280 nm (details in the text).

fragment X, 17.6 for fragment Y, 20.8 for fragment D, and 10.2 for fragment E.

(4) Fibrinopeptides. Fibrinopeptides A and B were prepared essentially according to the method of Blombäck and Vestermark [28]: 10 mg/ml fibrinogen in 0.15 M ammonium acetate buffer (pH 7.0) was incubated with 50 U/ml thrombin (from bovine plasma; the activity, 10 units/mg protein; from Mochida Pharmaceuticals, Tokyo, Japan) at 25 °C, overnight. After the resulting clot was solubilized with formic acid, the mixture adjusted to pH 4.5 with ammonia was stood 4 h. Insoluble aggregates were removed by centrifugation at $15000 \times g$ for 15 min at 4 °C, and the supernatant was lyophilized. According to the method of Martinelli and Scheraga [29] essentially, fibrinopeptides A and B were separated on a C_{18} reverse-phase column (PepRPC column of 0.5×5 cm, from Pharmacia Fine Chemicals, Uppsala, Sweden) by an isocratic elution with 0.03 M sodium phosphate (pH 4.5)/acetonitrile (85 : 15, by volume) at a flow rate of 0.8 ml/min and were monitored at 214 nm. The amino-acid composition (mol of amino acid/mol of peptide) of isolated fibrinopeptides was as follows: fibrinopeptide A: Asp(1.93), Ser(1.09), Glu(2.20), Gly(4.89), Ala(1.87), Val(0.90), Leu(1.04), Phe(1.05), Arg (1.03); fibrinopeptide B: Asp(2.75), Ser(1.19), Glu (3.23), Gly(2.39), Ala(1.02), Val(0.98), Phe(1.89) and Arg(0.72). The results well agreed with the reported value [30].

(5) Synthetic tetrapeptides. Glycyl-L-prolyl-L-arginyl-L-proline (GPRP) and glycyl-L-histidyl-L-arginyl-L-proline (GHRP) were purchased from Sigma Chemicals.

Electrophoretic analysis of fibrinogen and the degradation products

The purity of fibrinogen and fibrinogen-derived products (5–10 mg/ml) was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Fairbanks et al. [31]. The electrophoresis in 0.1 M Tris/0.2 M sodium acetate/0.02 M EDTA-acetate buffer (pH 7.4) was performed on a slab gel (2 mm in thickness) containing 4.0 or 5.0 g/dl polyacrylamide, 0.21 g/dl *N,N'*-methylene bisacrylamide and 1 g/dl sodium dodecyl sulfate with or without 40 mM β-mercaptoethanol. The densitometry of Coomassie brilliant blue R-250-stained gel was performed by a densitometer (Dual-Wavelength TLC Scanner, model CS-900, Shimadzu, Kyoto, Japan).

Results

(1) Rouleau formation induced by HMW and LMW fibrinogens

The molecular weight heterogeneity of Aα-chain in commercial human fibrinogen [9,10,21,24] (see Fig. 5) was confirmed by polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol. The velocity of rouleau formation induced by the preparations with different proportions of HMW (340 kDa) and LMW (305 kDa) fibrinogens is shown in Fig. 2. As the fibrinogen concentration increased, the velocity of rouleau formation increased, and began to saturate above 15 μM fibrinogen. However, no difference of the velocity among these preparations, i.e., between HMW

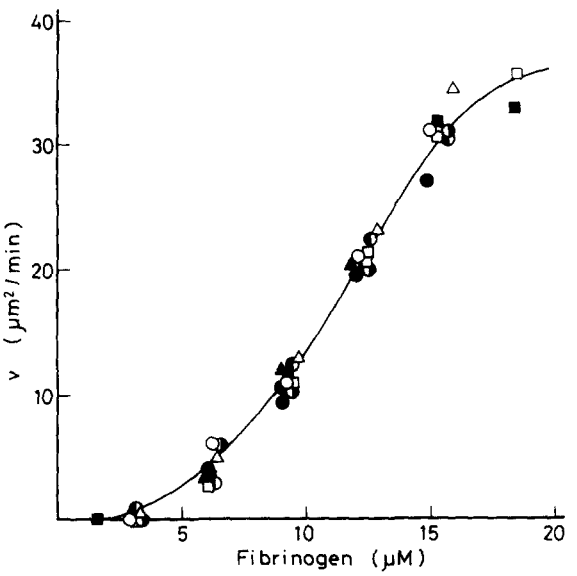

Fig. 2. Effect of fibrinogen(-derivatives) on the velocity of rouleau formation. (i) Fibrinogen fractionated by gel filtration, ○ (HMW fibrinogen/LMW fibrinogen = 89.6/10.4), ◑ (52.2/47.8), ◐ (41.7/58.3), ● (87.8/12.2); (ii) fibrinogen fractionated by ammonium sulfate precipitation, △ (23.1/76.9), ▲ (87.5/12.5) (the ratio of both fibrinogens, determined by 4% polyacrylamide gel electrophoresis); and (iii) desialylated fibrinogen (■, containing 1.32 mol sialic acid/mol) compared with normal fibrinogen (□, containing 7.35 mol sialic acid/mol) (the content of sialic acid, determined by the thiobarbituric acid method).

and LMW fibrinogens, was observed (thus, in the later experiments, fibrinogen was used without purification).

(2) Effect of sialic acid in fibrinogen molecule on the rouleau formation

We have already observed that the fibrinogen-induced rouleau formation is accelerated by desialylation of erythrocyte membrane glycoproteins [19]. Therefore, the removal of sialic acid from Bβ- and γ-chains of fibrinogen molecule (see Fig. 5) may accelerate the rouleau formation due to the strong binding of fibrinogen to negatively charged erythrocytes.

However, the desialylation of fibrinogen molecule did not affect the velocity of rouleau formation (Fig. 2), in spite of the fact that about 80% of sialic acid were removed by neuraminidase.

(3) Effect of fibrinogen degradation products by plasmin on the rouleau formation

Various fibrinogen degradation products by plasmin (fragments X, Y, D and E; see Fig. 5) are useful for deducing the interaction of fibrinogen with erythrocytes which leads to the rouleau formation. The effect of each fragment on the velocity of rouleau formation is shown in Fig. 3 by both (A) normal plot and (B) logarithmic plot.

The velocity of rouleau formation induced by fragment X was much less than that induced by fibrinogen. The rouleau formation induced by fragment Y was further suppressed. Fragments D and E, up to the concentrations $8 \cdot 10^{-4}$ M and $7 \cdot 10^{-4}$ M, respectively, could not induce the rouleaux.

The logarithmic plots between the velocity of rouleau formation (v, $\mu m^2/min$) and the concentration of fibrinogen and the degradation products (C, mol/l) gave straight lines: the slopes were the same among these proteins, but the intercepts were different. Thus, the relation between v and C could be represented by the following equations:

$$\log v = \alpha \log C + \beta \quad \text{or} \quad v = \beta' C^{\alpha}$$

where α, β, β' were constants. α was 2.3, and β and β' were 12.6 and 39.8 ($\times 10^{11}$) for fibrinogen, 11.8 and 6.3 ($\times 10^{11}$) for fragment X, and 11.0 and 1.0 ($\times 10^{11}$) for fragment Y, respectively. Quantitatively, the molar ratio of fibrinogen, fragment X and fragment Y for giving a certain velocity of

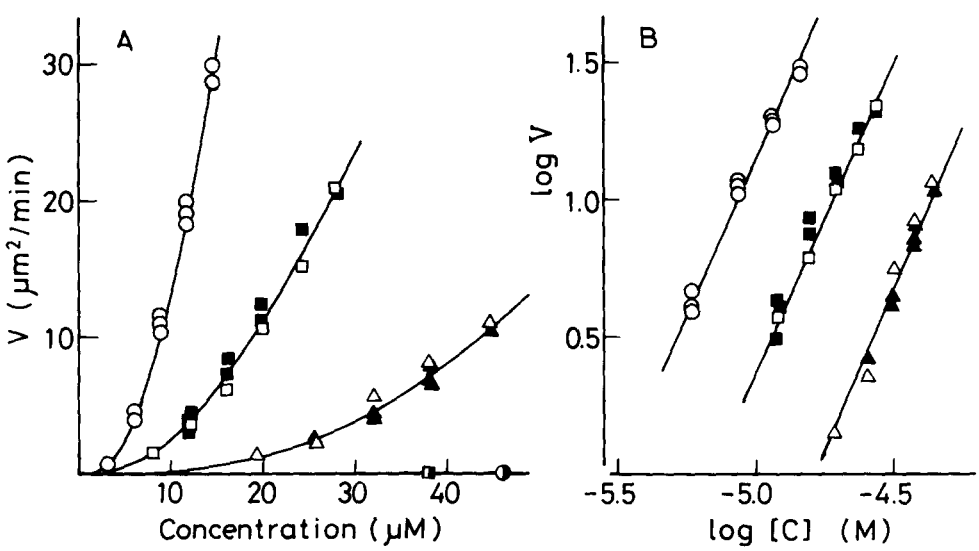

Fig. 3. Effect of fibrinogen and fibrinogen degradation products on the velocity of rouleau formation. (A) Normal plot and (B) logarithmic plot. Fibrinogen (○); fragment X with a purity of 91.4% (□) and 88.4% (■); fragment Y with a purity of 89.7% (△) and 92.6% (▲); fragment D with a purity of 95.6% (◑); and fragment E with a purity of 99.0% (◨) (the purity was determined by 5% polyacrylamide gel electrophoresis).

rouleau formation was approx. 1 : 2 : 5 in the concentration range examined here (e.g., 8.5 μM fibrinogen, 19 μM fragment X and 44 μM fragment Y were equivalent to each other in giving a velocity of 10 $\mu m^2/min$).

The effect of fragments X and Y on the velocity of fibrinogen-induced rouleau formation is shown in Table I. It is clear that with regard to the velocity of rouleau formation, fragment X (or fragment Y) in the mixture can be equivalently substituted by fibrinogen of one-half the concentration of fragment X (or of one-fifth the concentration of fragment Y). Thus, the velocity of rouleau formation induced by the mixture of fibrinogen and the degradation products (v_M) is represented by

$$\log v_M = \alpha \log([F]+[X]/2+[Y]/5)+\beta$$

where [F], [X] and [Y] are the concentrations of fibrinogen, fragment X and fragment Y, respectively. Here, the constants for fibrinogen can be used for α and β. Conclusively, the influence of fibrinogen, fragment X and fragment Y on the velocity of rouleau formation is additive.

The effect of fragments D and E on the velocity of fibrinogen-, fragment X- and fragment Y-induced rouleau formation was also examined. The fibrinogen(up to 12 μM)-induced rouleau formation was not affected by fragment D (up to 6.0 molar excess of fibrinogen) or fragment E (up to 6.8 molar excess), the fragment X(up to 20 μM)-induced rouleau formation not by fragment D (up to 4.4 molar excess of fragment X) or fragment E (up to 5.0 molar excess), and the fragment Y(up to 45 μM)-induced rouleau formation not by fragment D (up to 1.4 molar excess of fragment Y) or fragment E (up to 1.1 molar excess). Conclusively, fragments D and E do not affect the velocity of fibrinogen-, fragment X- and fragment Y-induced rouleau formation.

(4) Effect of fibrinogen-derived small peptides on the fibrinogen-induced rouleau formation

Since small molecules sometimes affect the rouleau formation induced by macromolecules [3–6], the effect of fibrinogen-derived small peptides on the velocity of fibrinogen-induced rouleau formation was examined.

(i) Fibrinopeptides are released from the central domain in the process of fibrin formation [32] (see Fig. 5) and may be present in plasma of hypercoagulability [16]. However, the velocity of fibrinogen(8.8 μM)-induced rouleau formation was not affected by 18 molar excess of fibrinopeptide A and by 14 molar excess of fibrinopeptide B.

(ii) Artificial tetrapeptides, GPRP and GHRP, are known to bind γ- and β-lobes of the terminal domain of fibrinogen, respectively (see Fig. 5) and inhibit the polymerization of fibrin monomer

TABLE I

ADDITIVE EFFECT OF FIBRINOGEN, FRAGMENT X AND FRAGMENT Y ON THE VELOCITY OF ROULEAU FORMATION OF ERYTHROCYTES

Concentration of macromolecules (μM)			Velocity of rouleau formation ($\mu m^2/min$)	
fibrinogen	fragment X	fragment Y	observed ± s.d. (n)	estimated [a]
2.9	–	–	0.6 ± 0.3 (3)	–
2.9	4.0	–	3.6 (1)	2.8
2.9	–	6.4	2.2 (1)	1.8
5.9	–	–	4.2 ± 0.5 (3)	–
5.9	8.0	–	14.3 (1)	14.1
5.9	–	12.9	10.6 (2)	10.1
8.8	–	–	10.9 ± 0.5 (5)	–
8.8	12.0	–	30.3 (1)	29.4
8.8	–	19.4	21.9 (1)	22.3

[a] Calculated by the equation, $v = 39.8 \cdot 10^{11}$ ([fibrinogen]+[fragment]/z)$^{2.3}$, where the concentration is in mol/l, z is 2 for fragment X and 5 for fragment Y (see text).

[30,33–36]. The effect of a 86 molar excess of GPRP and a 200 molar excess of GHRP on the velocity of fibrinogen(8.8 μM)-induced rouleau formation was not observed.

Discussion

In the present study, the characteristic interaction of fibrinogen and fibrinogen-derived products with erythrocyte leading to rouleau formation was examined by a kinetic method. Based on these results are discussed: (1) the characteristics of rouleau formation induced by fibrinogen and fibrinogen-derived products; (2) the effect of fibrinogen-derived products on the velocity of fibrinogen-induced rouleau formation; (3) the binding site of fibrinogen on the erythrocyte surface leading to the rouleau formation; and (4) the rheological consideration of fibrinogen and fibrinogen-derived products.

(1) Characteristics of rouleau formation by fibrinogen and fibrinogen-derived products

Generally, the effectiveness of macromolecules on the erythrocyte aggregation is strongly dependent on the molecular size [1,2]: when a large macromolecule bridges between erythrocytes, the intercellular distance increases and the electrostatic repulsive force between cells weakens, and thus, the erythrocyte aggregation increases [1,2,37]. However, various physicochemical properties of macromolecules, such as the molecular conformation, the charge, and so on, should be taken into consideration for understanding the nature of macromolecular bridging leading to erythrocyte aggregation.

The relation between the molecular size of fibrinogen and fibrinogen-derived products (in molecular weight) and the effectiveness of rouleau formation (in concentration of macromolecule for giving $v = 15\ \mu m^2/min$) is shown in Fig. 4, as are our previous results of rouleau formation by polyglutamic acids [3]. (i) The velocity of rouleau formation decreases on going from HMW fibrinogen (340 kDa) = LMW fibrinogen (305 kDa) > fragment X (250 kDa) > fragment Y (155 kDa), depending on molecular size. (ii) Fragment D (85 kDa) and fragment E (45 kDa) cannot induce rouleaux in the present experimental range.

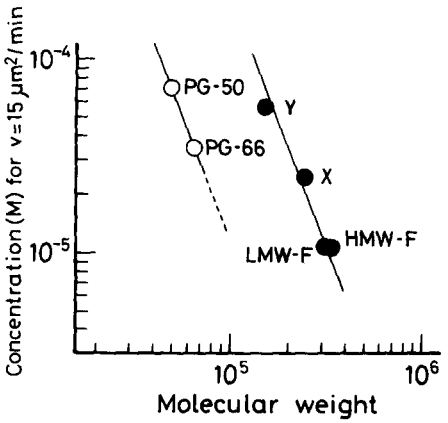

Fig. 4. Relation between the effectiveness of rouleau formation and the molecular weight of bridging macromolecules. The effectiveness of rouleau formation is represented by the concentration (M) of macromolecule for giving $v = 15\ \mu m^2/min$ in the presence of 5 g/dl albumin. HMW fibrinogen (HMW-F), LMW fibrinogen (LMW-F), fragment X (X) and fragment Y (Y) from Figs. 2 and 3. Polyglutamic acids of 66 kDa (PG-66) and 50 kDa (PG-50) from Ref. 3.

The order of the ability of rouleau formation by fibrinogen and the fragments (X, Y, D and E) agreed with the order of the acceleration of erythrocyte sedimentation by these macromolecules [38].

However, the dependency of the velocity of rouleau formation on the molecular weight among fibrinogen, fragment X and fragment Y was clearly different from those of polyglutamic acids (Fig. 4). The results suggest that the interaction between macromolecule and erythrocyte leading to rouleau formation must be explained not only by the difference in molecular weight, but also by the specific 'site' on the macromolecule.

(2) Effect of fibrinogen-derived products on fibrinogen-induced rouleau formation

Rouleau formation induced by macromolecules is sometimes affected by small (macro)molecules with similar molecular characteristics, probably due to the competition at their binding site on the erythrocyte surface, e.g.: (a) IgG-induced rouleau formation is inhibited by the fragments Fab and Fc [4] and by polyglutamic acids of 20 kDa and 8 kDa [5]; (b) one of the peptic fragments of IgG, the $F(ab')_2$-induced one is inhibited by Fab and Fc [4], but accelerated by Fc-derived small fragment (C_H3) [6]; (c) the high-molecular-weight (of

70 kDa) dextran-induced one is inhibited by low-molecular-weight dextrans (of 40 kDa and 10 kDa) [3]; (d) the high-molecular-weight (50 and 66 kDa) polyglutamic acid-induced one is inhibited by polyglutamic acid of 8 kDa, but accelerated by that of 20 kDa [3].

The effect of various fibrinogen-derived products on fibrinogen-induced rouleau formation in the present experiment was essentially different from the above observations. (i) Fragments X and Y show the additive effect for fibrinogen-induced rouleau formation, probably due to the difference of the erythrocyte-binding sites in these fragments from those in fibrinogen. (ii) Fragments D and E can bind to erythrocyte surface [39], but do not affect the fibrinogen-induced rouleau formation. Probably, the erythrocyte-binding sites in these fragments are considerably different from those in fibrinogen, fragment X and fragment Y. (iii) Fibrinopeptides A and B (composed of 16 and 14 amino-acid residues, respectively) also do not affect the fibrinogen-induced rouleau formation. Probably, these acidic peptides do not bind the negatively charged erythrocyte surface, and thus do not affect the interaction between fibrinogen and erythrocyte. (iv) The synthetic tetrapeptides, GPRP and GHRP, do not affect the fibrinogen-induced rouleau formation, in spite of the fact that more than 90% of the tetrapeptide-binding sites (in the γ- and β-lobes, respectively, of the terminal domain of fibrinogen) are saturated in the present conditions [34]. Therefore, these tetrapeptide-binding sites are not related to the interaction of fibrinogen with erythrocytes to leading rouleau formation.

Conclusively, the fibrinogen and fibrinogen-derived products examined here may interact with erythrocytes in their different sites. Thus, these (macro)molecules do not compete each other on the erythrocyte surface for leading to rouleau formation, and small molecules also do not interfere the bridging of macromolecule between erythrocytes.

(3) Site of fibrinogen bridging between erythrocytes

The trinodular structure of fibrinogen is composed of (i) the central domain including fibrinopeptides; (ii) the 'coiled coils' interdomainal con-

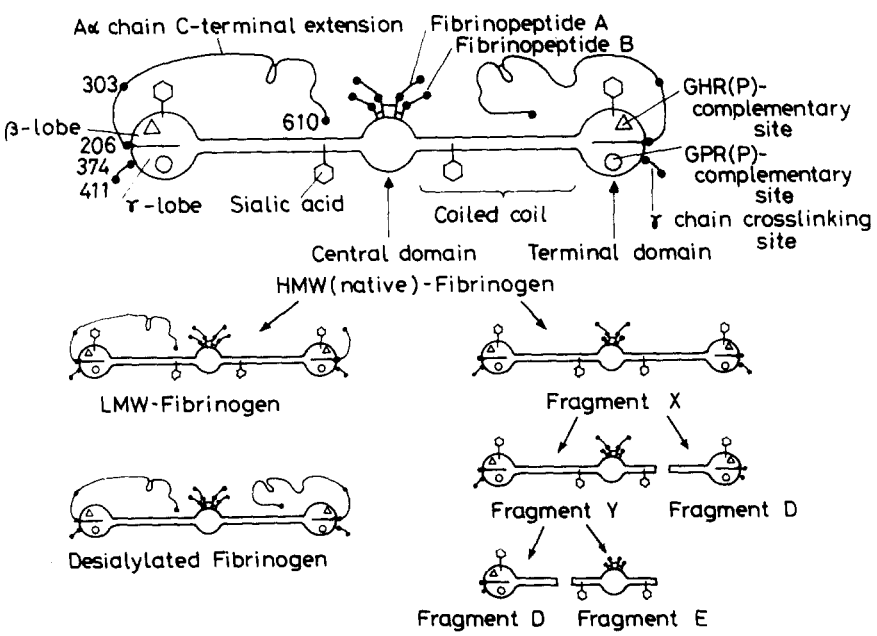

Fig. 5. Schematic model of fibrinogen and fibrinogen-derived products. Modified from Doolittle [35,40] with reference to Pizzo et al. [41], Budzynski et al. [42] and Marder and Budzynski [27]. The locations of sialic acid (○), GPR-complementary site (○) and GHR-complementary site (△) are shown in the plausible region. Numbers show the number of the amino-acid residue.

nector; (iii) the terminal domain consisting of homologous Bβ- and γ-chain segments, (iv) the Aα-chain carboxy-terminal extension and (v) the γ-chain crosslinking site [35], as is shown in Fig. 5 (including schematic models of several fibrinogen-derived products). Rampling [39] has studied the erythrocyte-binding isotherm of fibrinogen and fibrinogen degradation products by plasmin. However, it should be noted that the binding of macromolecules to erythrocyte surface for bridging between adjacent cells and leading to rouleau formation is essentially different from the adsorption of macromolecules on erytrhocyte surface. In the present experiment, the possible erythrocyte-binding site in fibrinogen leading to rouleau formation could be postulated by adopting a kinetic method for the quantitative measurement of rouleau formation, as follows.

(a) Interaction of Aα-chain. (i) Aα-(1–206): by the cleavage of the near terminal domain of the Aα-chain (at Aα-206 [43]) to convert fragment X, most of the carboxy-terminal extensions (corresponding to about 70% of native chain in molecular weight) are removed. The suppressed rouleau formation by fragment X shows that the erythrocyte-binding site in fibrinogen is lost. (ii) Aα-(207–303): this region is preserved in naturally occurring LMW fibrinogen. Since the velocity of rouleau formation induced by LMW fibrinogen is the same as that induced by native HMW fibrinogen, this region is one of the possible erythrocyte-binding sites in fibrinogen molecules. (iii) Aα-(304–610): this region may not contribute to the interaction between fibrinogen and erythrocytes leading to rouleau formation, since the LMW fibrinogen lacking in this region was equivalent to the HMW fibrinogen in the rouleau formation. Lorand [44] has proposed an interaction between fibrinopeptides (in the central domain) and the carboxy-terminal halves of Aα-chain in the compact structure of fibrinogen. Weisel et al. [45] have shown by electron microscopy of rotary-shadowed fibrinogen that the carboxy-terminal regions of Aα-chains are folded back to form an additional domain in the central region of fibrinogen molecule.

(b) Interaction of Bβ- and γ-chains. The fibrinopeptide-containing region (including Aα- and Bβ-chains) in the central domain may be repulsive to negatively charged erythrocyte surface due to the presence of many acidic amino acids.

Unlike the remarkable contribution of sialic acid on the erythrocyte surface for the erythrocyte aggregation [1,19], sialic acids in the terminal domain of Bβ-chain and in the coiled coil of γ-chain near the central domain [46,47] have no effect on the fibrinogen-induced erythrocyte aggregation. This suggests that a local structure of fibrinogen around sialic acid does not contribute to the interaction with erythrocytes leading to rouleau formation.

The release of fibrinopeptides from fibrinogen exposes the polymerization sites of fibrin which interacts with complementary sites on the terminal domain of another fibrinogen molecule [35]. This interaction is inhibited by the specific binding of the artificial tetrapeptides, GPRP and GHRP, to the complementary sites [33–36]. However, the binding of these peptides to such sites did not affect the rouleau formation. This suggests that a possible conformational change around the complementary sites induced by the binding of these tetrapeptides does not influence the erythrocyte-binding site in fibrinogen. Niewiarowski et al. [48,49] have postulated that in platelet aggregation, the platelet-binding sites for fibrinogen are located on the carboxy-terminal portion of the Aα- (Aα-(250–610)) and the γ-chain (γ-(374–411)). We have no information about the γ-chain crosslinking site (γ-(374–411)) at the moment, but, because this region is also preserved in fragments X and Y [42] and these fragments can induce the rouleaux, any contribution of the extended portion of the carboxy-terminus of γ-chain for erythrocyte binding must still be taken into consideration.

Conclusively, one of the most plausible erythrocyte-binding sites in fibrinogen is Aα-(207–303), as far as deduced from the velocity measurement of rouleau formation.

(4) Hemorheological consideration on fibrinogen and fibrinogen derived products

In various diseases [50–53], the increase of plasma fibrinogen accelerates the erythrocyte sedimentation due to the increased erythrocyte aggregation, and decreases the filterability of blood [1,50–53]. The increased fibrin(ogen)olytic activity

by plasmin in various diseases (especially in disseminated intravascular coagulation, hyperfibrinolysis and so on) increases the fibrin(ogen) degradation products in circulating blood [15,16]. Therefore, the rheological consideration for these products in blood is important for modifying the status of microcirculation and the blood viscosity (especially in low-shear region) in these diseases.

The present experiment performed with purified fibrinogen degradation products shows that the ability of rouleau formation is preserved in fragments X and Y, but not in fragments D and E. Although the plasma level of fibrin(ogen) degradation products (even in pathological states) [13,14] is less than those in the present experiments, the local concentration of fibrin(ogen) degradation products may increase at the fibrin(ogen)olytic site, thus the hemorheological consideration on some products (especially, fragments X and Y) should be taken into account.

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