Analysis of coagulation of blood in different animal species with special reference to procoagulant activity of red blood cell

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We reported previously that human coagulation factor IX (F-IX), when activated by normal human red blood cells (RBCs), causes coagulation. We also identified and characterized the F-IX-activating enzyme in the normal RBC membrane. In the present study, the coagulation of blood in experimental animals, including swine, dogs, rabbits, cattle and sheep, was compared to that in humans, with special reference to the procoagulant activity of RBCs. Rheological measurement showed that coagulation of platelet-free plasma (PFP) in a polypropylene tube did not occur in any of the species. In swine as in humans, coagulation of PFP supplemented with RBCs (RBCs/PFP) occurred. However, in dogs, rabbits, sheep or cattle coagulation of RBCs/PFP did not occur. Fluorescence assays of RBC membranes using a synthetic fluorogenic substrate suggested that F-IX-activating enzyme may be present in swine, dog and rabbit as well as human RBC membranes, but its level may be very low in sheep and bovine membranes. Our data suggest that there is a significant difference in procoagulant activity of RBCs among animal species. In addition, they suggest that appropriate selection of animal species would be important for studying venous thrombus formation, including the evaluation of anticoagulability of materials under stagnant flow conditions.

Key words: red blood cell (RBC), coagulation of blood, factor IX, procoagulant activity, animal species differences.

1. Introduction

The intrinsic coagulation pathway is triggered by the activation of factor XII (F-XII) or F-XI in the presence of contact surfaces, leading to the activation of F-IX and F-X via a series of steps and, ultimately, to the generation of the protease thrombin. It is generally thought that this pathway is not critical in the initiation of thrombin generation, when compared with the extrinsic coagulation pathway that is associated with vascular wall injury. The clot formed by the intrinsic and extrinsic pathways is basically composed
of fibrin and red blood cells (RBCs) with variable amounts of platelets and leukocytes, and is mainly formed under stagnant flow conditions. There are a number of studies on blood coagulation in different animal species. It is considered that the intrinsic coagulation pathway may be essentially the same among mammals, although many differences of coagulation factors, including fibrinogen, prothrombin, F-IX, F-X and F-VII, among species have been reported.

It is generally thought that normal human RBC membranes have no procoagulant activity and that RBCs must lyse to exhibit clot-promoting activity, like damaged platelets and leukocytes. However, in our previous studies, it was shown that normal human RBCs were capable of initiating the coagulation pathway by activating F-IX at stasis. Other factors such as F-X, XI, XII and VII were not activated by RBCs. Recently, we tried to extract, purify and identify the F-IX-activating enzyme from human RBC membranes. We showed that the amino acid sequence of the F-IX-activating enzyme was almost identical with that of human neutrophil elastase except for some small differences. We designated the enzyme as erythroelastase-IX (EE-IX).

In in vitro experiments, it was shown that the F-IX activation by human RBCs was enhanced by risk factors for venous thrombus formation, for example, an elevation of hematocrit, a decrease in flow shear rate, an increase in age and hypercoagulable states such as diabetes and pregnancy. The slow initiation of coagulation triggered through the activation of F-IX by RBCs (approximately 30 minutes in humans) may have little physiologic significance under normal physiological in vivo flow conditions. However, it is reported that the growth of venous thrombi occurs very slowly, over a period of hours or at most a few days. Therefore, it is assumed that this triggering reaction may be sufficiently large to cause clot formation under stagnant flow conditions, that is, to cause venous thrombosis, although at present there is no direct in vivo evidence to support a role for EE-IX in the coagulation process.

In this study, using rheological and biochemical techniques, the coagulation of blood in swine, dogs, rabbits, cattle and sheep was compared to that in humans with special reference to the procoagulant activity of RBCs. These mammalian species were selected for study because they are common laboratory animals.

2. Materials and Methods

2.1 Blood samples

Bovine and swine blood was obtained from a slaughterhouse. Sheep blood was obtained from the Animal Resource Science Center of The University of Tokyo. Dog and rabbit blood was obtained from animals anesthetized with an intravenous injection of pentobarbital sodium. Human blood was obtained from healthy adult volunteers by venepuncture. Care was taken to avoid contamination with tissue factor during blood sample collection. Nine volumes of the sampled blood were immediately poured into a polypropylene test tube containing 1 volume of 3.8 % sodium citrate solution as an anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifuging blood at 100 x g for 15 min at 20°C, and platelet-poor plasma (PPP, platelet count <1 x 10^4/µl) was prepared by centrifuging PRP at 150 x g for 15 min at 20°C. The platelet count of PRP was adjusted to approximately 10 x 10^4/µl by mixing PRP and PPP. Platelet-free plasma (PFP, platelet count<100/µl) was prepared by centrifuging PPP at 16,000 x g for 15 min at 4°C. The platelet count was determined using a Model ACM-403 Platelet Counter (Erma Optical Works, Tokyo, Japan).

To prepare PFP supplemented with RBCs (RBCs/PFP), RBCs were washed three times with Heps-buffered saline (HBS, 50 mM Heps, 115 mM NaCl, 5 mM KCl, 0.1 % (w/v) glucose; pH 7.4) and then resuspended in PFP.
2.2 Coagulation factors
Purified human F-IX (0.2 U/μg), bovine F-IX (100 U/vial), activated human F-IX (F-IXaβ, 0.2 U/mg) and bovine F-IXaβ (100 U/vial) were obtained from the Enzyme Research Laboratory (South Bend, Indiana, USA). Each factor was dissolved in HBS and was stored in a frozen state.

2.3 Rheological measurement of blood coagulation process
The progress of coagulation of a blood sample in a polypropylene tube (length, 3 cm; inner diameter, 0.7 cm) was monitored using a damped oscillation rheometer. The principle of the rheometer has been described in detail elsewhere. In brief, the tube is connected coaxially to a hollow-cylinder aluminum tube, to the bottom of which a coil is attached. The measuring system, consisting of the tube and coil, is suspended from a torsion wire passing through the aluminum tube. Direct current is introduced into the coil between magnetic poles, which generates an initial rotational displacement in the measuring system. When the current is turned off, the system starts a rotational damped oscillation, which produces an induced electromotive force in the coil. A damped oscillation curve of the measuring system is detected as an output voltage every 20 sec. From the curve, a logarithmic damping factor (LDF) is obtained. The LDF is closely related to the fluidity of the blood sample. The change of LDF during the coagulation of a blood sample was monitored at 37°C.

The maximum value of the wall shear rate induced by blood flow in the present tube was estimated to be less than 1 sec⁻¹. Therefore, we assumed that the present measuring system could be used to simulate the condition of stagnant flow.

2.4 Coagulation factor activation assay
One hundred microliters of purified F-IX dissolved in HBS at a concentration of 50 U/ml was mixed with 100 μl of washed packed RBCs (hematocrit, approximately 40%). After 10 μl of a 26 mM CaCl₂ solution was added, the sample was incubated for a predetermined time at 37°C. After the addition of 30 μl of 0.1 M ethylenediamine tetraacetic acid, the RBCs were removed by centrifugation. The supernatant was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5-15 % gel) in the presence of 0.1 % SDS. The proteins were transferred to a PVDF membrane. The membrane was probed with rabbit anti-factor IX immunoglobulin (IgG) and peroxide-conjugated goat anti-rabbit IgG as a secondary antibody, and then stained with 3,3'-diaminobenzidine solution containing 6 ppm H₂O₂.

2.5 Fluorescence intensity of RBCs
A synthetic fluorogenic substrate [Suc(OMe)-Ala-Ala-Pro-Val-MCA (MCA : 4-methyl-cumaryl-7-amide), Calbiochem-Novabiochem, San Diego, Ca, USA], specific for elastase, was dissolved in HBS. On a polypropylene plate, 10 μl of 1.5 μM fluorogenic synthetic substrate was added to 40 μl of packed RBCs suspended in HBS. To avoid diffusion of the MCA product from RBC membranes, the sample was covered with a glass plate. The coverslipped sample was incubated for 30 min at room temperature and the fluorescence intensity was observed with a photo microscope with epi-fluorescent optics (Model AX70, Olympus, Tokyo, Japan) at λ ex = 380 nm and λ em = 460 nm.

To measure the fluorescence intensity of a supernatant of the mixture of RBCs and the synthetic fluorogenic substrate, 3.8 μl of packed RBCs and 3 μl of the synthetic substrate was added to 196 μl of HBS and incubated for 30 min at 37°C. After centrifuging the mixture at 700 x g for 3 min, 100 μl of the supernatant was added to 2ml of HBS. The fluorescence intensity was measured using a fluorescence spectrophotometer (F-1200, Hitachi, Tokyo, Japan) at λ ex = 380 nm and λ em = 460 nm.
3. Results

3.1 Coagulation analysis

An example of the change in LDF during the coagulation of swine whole blood, RBCs/PFP, PRP and PFP is shown in Fig. 1. No change in LDF of PFP occurred within the experimental time period (in excess of 2 h). In contrast, the LDF of whole blood, RBCs/PFP and PRP began to change when the coagulation began to start. The results were essentially the same as those for human blood samples.

The time course of LDF during the coagulation of bovine whole blood, RBCs/PFP, PRP and PFP is shown in Fig. 2. Coagulation of whole blood and PRP occurred, but coagulation of RBCs/PFP and PFP did not. In dogs, rabbits and sheep, coagulation of RBCs/PFP also did not occur.

The times of onset of coagulation of whole blood, RBCs/PFP, PRP and PFP for all of the tested animal species are summarized in Table 1.

Table 1 Time of onset of coagulation of blood samples obtained from different species

<table>
<thead>
<tr>
<th>Blood sample</th>
<th>swine (min)</th>
<th>dog (min)</th>
<th>rabbit (min)</th>
<th>sheep (min)</th>
<th>cattle (min)</th>
<th>human (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>whole blood</td>
<td>6.3±2.1 (8)</td>
<td>38.2±11.7 (6)</td>
<td>27.3±14.7 (9)</td>
<td>28.5±14.3 (6)</td>
<td>10.8±4.0 (8)</td>
<td>30.1±3.8 (50)</td>
</tr>
<tr>
<td>RBCs/PFP</td>
<td>18.0±11.1</td>
<td>∞</td>
<td>∞</td>
<td>∞</td>
<td>∞</td>
<td>30.5±6.4</td>
</tr>
<tr>
<td>PRP</td>
<td>10.8±4.3</td>
<td>41.1±17.5</td>
<td>19.6±10.7</td>
<td>27.4±17.5</td>
<td>15.4±2.7</td>
<td>47.3±6.5</td>
</tr>
<tr>
<td>PFP</td>
<td>∞</td>
<td>∞</td>
<td>∞</td>
<td>∞</td>
<td>∞</td>
<td>∞</td>
</tr>
</tbody>
</table>

∞ means that coagulation did not occur within the experimental time period (in excess of 2 h).
Figures in parentheses indicate the number of times of independent experimental runs. Data is expressed as means ± SD.
3.2 F-IX activation assay

To examine whether F-IX is activated by RBCs, Western blot analysis of bovine F-IX incubated with washed bovine RBCs was performed. A band corresponding to activated F-IX (F-IXa) was not observed (Fig. 3a). For comparison, the result for humans is shown in Fig. 3b. The amount of cleaved F-IX increased with the incubation time. Western blot analysis for swine, dogs, rabbits, and sheep was not performed since the purified F-IX for these animals was not available.

3.3 Enzymatic activity assay of RBCs by fluorescence observation

The issue of whether EE-IX is located in RBC membranes of various animal species was examined using a fluorogenic substrate specific for elastase. Fig. 4 shows an example of fluorescence observation of an incubation mixture of the fluorogenic substrate and human RBCs. The picture clearly shows that EE-IX is present in the human RBC membranes. In contrast, fluorescence was not observed with bovine RBCs (data not shown).

To compare the enzymatic activity among animal species quantitatively, the fluorescence intensity (I) of the supernatant of a mixture of synthetic fluorogenic substrate and RBCs obtained from different animal species was examined. The results are summarized in Table 2. The value of fluorescence intensity for swine, dogs, rabbits, and humans ranged from 7 to 30, and was dependent on the individuals as well as species, but the value for sheep and cattle was almost zero.

3.4 Coagulation-inducing ability of F-IX cleaved by EE-IX

The issue of whether F-IX is capable of initiating coagulation when incubated with RBCs was examined. The addition of F-IX incubated with rabbit and dog RBCs to PFP failed to cause coagulation, whereas coagulation of PFP occurred upon the addition of F-IX cleaved by human RBCs (Fig. 5).

4. Discussion

Using the rheometer employed here, the slight initial change in fluidity during the coagulation of blood, i.e., fibrin polymerization and subsequent network formation, can be sensitively detected because the lowest detectable viscosity is approximately 1 mPa·s\(^{0.20}\). The initial increase
in LDF for a PRP sample (Figs. 1 and 2) reflects an increase in viscosity from about 1 mPa·s to about 8 mPa·s during the fibrin polymerization and subsequent network formation \(^{20,23}\). The subsequent rapid decrease in the LDF is due to the further increase in viscosity and viscoelasticity during clotting. For whole blood and RBCs/PFP, however, an increase in the LDF is not observed (Figs. 1 and 2), as the viscosity of uncoagulated blood is higher than 8 mPa·s \(^{24}\). Therefore, the time of onset of coagulation can be determined from the time at which the LDF value began to change.

Using the PFP sample for all animal species examined, no change in LDF occurred. This indicates that the polypropylene tube used has an anticoagulant and antithrombogenic surface \(^{25}\). The values of time of onset of coagulation may be influenced by many factors such as animal conditions, including mental stress, and variety of coagulation activities in a particular animal species on a particular day fluctuates among individuals. Therefore, the comparison of absolute values of Ti among animal species may not be very meaningful. In this study, we focused only on whether coagulation of RBCs/PFP occurred or not, that is, on the procoagulant activity of RBCs among different animal species.

In our earlier studies using human blood \(^{15}\), it was shown that even if contamination such as activated coagulation factors, including F-VIIa, F-Ixa, F-Xa and F-XIa and tissue factor, as well as leukocytes and platelets are present, they do not play a role in the activation of F-IX. We previously verified that only F-IX among coagulation factors is activated by EE-IX in RBC membranes and that the activated F-IX has the ability to cause coagulation \(^{16}\).

The cleavage of Val-MCA in the synthetic fluororogenic substrate produces a free MCA \(^{30}\). Therefore, the fluorescence intensity may be related to the amount of Val active sites in F-IX cleaved by RBCs, that is, the fluorescence intensity may give information on the presence of EE-IX in RBC membranes. The results shown in Table 2 suggest that EE-IX may be present in swine, rabbit and dog RBC membranes as well as in those of humans.

In addition to the results of fluorogenic analysis, the results of rheological analysis for swine blood were similar to those for human blood. The results suggest that F-IX activated by swine has the ability to cause coagulation. In contrast, coagulation of bovine RBCs/PFP did not occur. Together with the results of Western blot analysis and the fluorogenic assay (Table 2), this suggests that EE-IX may not be present in bovine RBC membranes. The procoagulant activity of sheep RBCs appears to be similar to that of bovine RBCs.

There are at least two possible explanations for the absence of coagulation of rabbit and dog RBCs/PFP. One is a difference in cleavage sites of F-IX by RBCs. The cleavage sites of F-IX by EE-IX extracted from human RBC membranes...
were Thr₁₁₀-Ser₁₁¹, Val₁₁⁰-Val₁₁² and Val₁₁²-Gly₁₁³, which were somewhat different from the Arg₁₄⁵-Ala₁₄⁶ and Arg₁₈⁰-Val₁₈¹ cleaved by F-XIa and from the Thr₁₄⁰-Ser₁₄¹, Thr₁₄⁴-Arg₁₄⁵ and Val₁₈¹-Val₁₈² cleaved by neutrophil elastase. The ability of F-IX cleaved by RBCs to cause coagulation was weaker than that of F-IIXa. The fact that coagulation of dog and rabbit RBCs/PFP did not occur, together with the results of fluorogenic assays of the RBCs and the data shown in Fig. 5, suggests that the enzymatic ability of F-IX cleaved by these RBCs to cause coagulation may be weaker than that of F-IX cleaved by swine and human RBCs.

In our previous report, it was shown that the propagation reaction on RBC membranes (i.e., coagulation reaction leading to clot formation after F-IX activation) was significantly dependent on the individual. It was assumed that the significant delay or absence of coagulation of RBCs/PFP was due to a difference in local structure or properties of the RBC surface that supports the propagation reaction. Therefore, another possible reason for the absence of coagulation of dog and rabbit RBCs/PFP may be the lack of the local surface structure or properties required for the propagation reaction to proceed. To clarify the reason for the lack of coagulation of rabbit and dog RBCs/PFP, further studies using blood samples and purified coagulation factors obtained from individual animal species will be required.

Interestingly, human, swine, dog and rabbit RBCs, which may possess EE-IX in RBC membranes, form rouleaux although the aggregability of RBCs is different in the various animal species. In contrast, sheep and bovine RBCs, which may not possess EE-IX, are not capable of forming rouleaux. Sheep and cattle belong to the Bovidae family. At present, the relationship between the procoagulant activity of the RBCs of various animal families and the ability to form rouleaux is not clear. We reported that the gene for EE-IX in the human RBC membrane might be expressed in bone marrow precursors prior to RBC maturation. A systematic study of F-IX activation by RBCs of different species may provide insight into the relationship between the procoagulant activity of RBCs and the process of evolution of these animal species.

When antithrombogenicity of artificial organs such as vascular grafts, artificial valves and artificial hearts is evaluated by implantation into animal bodies, the amount of white thrombi consisting of aggregated platelets adherent to material surfaces generally tends to be focused upon. Our previous study demonstrated that coagulation of human blood occurred under stagnant flow conditions even when the endothelial cells or material surface offered an inert surface with respect to blood coagulation and thrombus formation. Therefore, in vitro evaluation of antithrombogenicity of artificial materials, it is necessary to pay attention to the formation of

| Table 2 Fluorescence intensity (I) for different species. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | swine           | dog             | rabbit          | sheep           | cattle          | human           |
| Mean±s.d.       | 13.7±3.5(7)     | 17.6±5.8(6)     | 8.5±0.6(8)      | 2.5±0.2(5)      | 1.9±1.4(5)      | 14.0±5.9(30)    |
| Imax/ Imin      | 19.0/9.5        | 24.8/8.1        | 9.6/7.9         | —               | —               | 29.2/9.0        |

Imax and Imin: the maximum and minimum values of the fluorescence intensity for individual animal species. Figures in parentheses indicate the number of times of independent experimental runs. Data is expressed as means ± SD.
red thrombi consisting of fibrin and RBCs on foreign surfaces at stagnant flow regions. Moreover, in studies of blood coagulation using animal species, as well as in the evaluation of antithrombogenicity, appropriate choice of the animal species may be important.

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Analysis of coagulation of blood in different animal species with special reference to procoagulant activity of red blood cell (43)


