Relationship between Contents of Neuraminic Acid and ATP and Adhesiveness of the Platelet

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The relationships among platelet adhesiveness, platelet N-acetylneuraminic acid (NANA) content, platelet ATP content and lactic acid production rate were studied in human and rabbit. The NANA content of non-adhesive platelets was lower than that of platelets adhered on a glass bead surface. The treatment with neuramidase in vitro resulted in a marked decrease of NANA content and adhesiveness index in rabbit platelets. The long-term Warfarin administration reduced both NANA content and adhesiveness index in man and rabbit. NANA contents of platelets obtained from patients with ITP, von Willebrand's disease, SLE, uremia or liver cirrhosis, and patients under long-term Warfarin therapy were markedly reduced, accompanying with a decrease in adhesiveness index. A significant correlation was demonstrated between NANA content and adhesiveness index in platelets. ATP content and lactic acid production rate of non-adhesive platelets were markedly reduced. These results suggested that surface negative charge due to NANA plays a significant role in the mechanism of adhesion of the platelet on a glass bead surface and a metabolic energy supply is required for the following platelet aggregation.

The exact mechanisms of the platelet adhesion still remain to be clarified. Scanning electron microscopic examinations have demonstrated that platelets adhered on a glass surface are aggregated (Hattori et al. 1973). Thus the adhesiveness test in common use seems actually measuring two steps reactions; adhesion of platelets on a glass surface and their following aggregation.

Platelets suspended in plasma or in physiologic saline show a high negative surface charge (Born 1968). This negative surface charge is supposed to attribute in maintaining platelets non-aggregated. However, it seems likely that a reduction of negative surface charge of platelets may decrease their adhesiveness on a surface with positive charge. N-acetyleneuraminic acid (NANA) chiefly attributes to keep the surface negative charge of the cell (Madoff et al. 1964).

The platelet aggregation following its adhesion on a glass surface is supposed to be induced by the release of ADP from the platelet. It has been well established that a supply of metabolic ATP is required for ADP-induced platelet aggregation (Hirsh and Doery 1971).

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In this paper, the relationships among NANA content, ATP content and platelet adhesiveness are studied.

**MATERIALS AND METHODS**

Blood was collected by venous puncture into a siliconized glass tube containing 0.1 volume of 3.8% citrate solution as anticoagulant. The plasma was separated by centrifugation at 290 g at 4°C for 10 min. This platelet rich plasma was again centrifuged in the same manner to reduce further the contamination of red cells and white cells. The platelets were obtained by centrifugation of the above supernatant at 1300 g at 4°C for 30 min. The platelets were washed 3 times by resuspending them in approximately 50 volumes of cold physiologic saline and centrifuging at 1300 g at 4°C for 30 min. They were finally resuspended in plasma or pH 7.4 Tris buffer saline. Platelet counts were determined by the method of Brecker and Cronkite (Brecker et al. 1953). The contamination of red cells and white cells was determined on undiluted final platelet suspension. This did not exceed 200 leukocytes per mm³ and 1500 erythrocytes per mm³.

Adhesiveness of the platelet in vitro was measured by the glass beads column method (Hellem 1960). Citrated platelet rich plasma was filtered with constant speed through a plastic tube containing exactly 5 g of glass beads with a diameter of 0.5 mm. The contact time was 30 sec. The difference in the number of platelets before and after filtration was designated as a number of adhered platelets. Adhesiveness index was expressed as percent of adhered platelet counts to total platelet counts.

NANA was measured by the thiobarbituric acid method of Warren (1959). Pure NANA was used as a reference standard for each assay. Two tenths ml of 0.2N H₂SO₄ was added to the platelet button. Then the mixture was incubated for 1 hr at 80°C. After the incubation it was cooled in an ice bath. One tenth ml of 10% trichloracetic acid (TCA) was added to the ice cooled solution and then the solution was centrifuged at 4000 rpm for 30 min. The total NANA of supernatant was measured by the thiobarbituric acid method. Optical density readings in a Beckmann spectrophotometer were carried out at 549 nm. Platelet protein was measured on sediments of the 10% TCA treated solution by the micro-Kjeldahl method for nitrogen.

The platelet ATP content was measured by the phosphoglycerate kinase (Baehringer) method (Adams 1963).

The lactic acid production rate of platelet was determined by Barker’s method (Barker and Summerson 1941).

**RESULTS**

*NANA content of the rabbit platelet*

NANA content of platelets of 15 normal rabbits averaged 23.72±5.14 nmoles/mg platelet protein as shown in Fig. 1. NANA contents of non-adhesive platelets separated by Hellem’s glass beads method and of platelets which adhered on glass beads were determined on 15 normal rabbits. The NANA content of non-adhesive platelets averaged 11.35±4.32 nmoles/mg platelet protein. The NANA content of adhered platelets averaged 27.12±7.31 nmoles/mg platelet protein. The difference between them was statistically significant (p<0.01).

*Effects of neuramidase on the platelet NANA content and platelet adhesiveness*

One tenth ml of purified neuramidase solution (600 units in 1.0 ml of pH 7.4 Tris buffer saline) was added to 1.0 ml of the citrated rabbit platelet rich plasma and the mixture was incubated at 37°C for 15, 30, and 60 min. The platelet NANA
Adhesiveness of Platelets

*Fig. 1. NANA content of non-adhesive platelet of rabbits. Averages of 15 rabbits.*

*Fig. 2. Effect of neuramidase on platelet NANA content and adhesiveness index.*

content and platelet adhesiveness index were determined after the incubation.

The platelet NANA content before the incubation averaged $23.72 \pm 4.2$ nmoles/mg platelet protein. The platelet NANA contents 15, 30 and 60 min after the incubation averaged $18.8 \pm 3.1$, $14.1 \pm 3.8$, and $10.9 \pm 3.6$ nmoles/mg platelet protein, respectively. Platelet adhesiveness indices of 15 normal rabbits averaged
342  T. Kuroyanagi and M. Saito

Fig. 3. Decrease of platelet NANA content and adhesiveness index due to Warfarin administration in rabbits.

67.4±3.4%. Neuramidase treatment of platelets in vitro reduced the adhesiveness indices. Adhesiveness indices of platelet 15, 30 and 60 min after the incubation were 53.6±5.7, 34.8±5.6, and 16.2±6.5%, respectively.

Effects of long-term Warfarin administration on the NANA content and adhesiveness of platelet in rabbits

The adhesiveness indices and NANA contents of platelet were determined on 12 rabbits which received daily administration of Warfarin (6 mg/day) for 3 weeks. Adhesiveness indices before administration of Warfarin averaged 65.6±4.1%. The long-term administration of Warfarin reduced the platelet adhesiveness indices, averaging 52.4±6.4%. The NANA contents of platelets in rabbits which received daily administration of Warfarin for 3 weeks decreased markedly, averaging 17.2±3.7 nmoles/mg platelet protein. Platelet adhesiveness indices and NANA contents in these rabbits demonstrated a close correlation (r=0.742), as shown in Fig. 4.

NANA content and adhesiveness index of the human platelets in various hemorrhagic disorders

As shown in Fig. 5, NANA content of the normal human platelets averaged 47±11.02 nmoles/mg platelet protein. Those of the human non-adhesive and adhered platelets averaged 34.68±5.01 and 52.34±7.23 nmoles/mg platelet protein, respectively. The difference between non-adhesive and adhered platelets was statistically significant (p<0.05).
Platelet NANA contents and adhesiveness indices were determined on patients with various hemorrhagic disorders. As shown in Fig. 6, NANA contents were decreased in ITP, von Willebrand's disease, SLE, uremia, liver cirrhosis and patients under long-term Warfarin administration, averaging 30.0, 34.5, 23.2, 16.1, 21.1 and 23.0 nmoles/mg platelet protein, respectively. Adhesiveness indices
Fig. 6. Platelet NANA content in patients with various hemorrhagic disorders.

Fig. 7. Correlation between platelet NANA content and adhesiveness index of platelet from patients with various hemorrhagic disorders.
decreased in these patients. Platelet NANA content (x) and platelet adhesiveness index (y) of these patients demonstrated a significant correlation within a 99% confidence limit, as shown in Fig. 7, presenting the following formula: 

\[ x = 3.64 + 0.47y \quad (r=0.88) \]

**ATP content and lactic acid production rate of the human non-adhesive platelet**

As shown in Table 1, ATP contents of the normal human platelets measured by the phosphoglycerate kinase method averaged 107±17.8 nmoles/mg platelet protein. Those of non-adhesive platelets averaged 49.4±10.7 nmoles/mg platelet protein. The difference between these two values was statistically significant \( (p<0.01) \).

Lactic acid production rates of the normal human platelets averaged 94.6±10.5 nmoles/mg platelet protein/hr. Those of the human non-adhesive platelets were significantly lower \( (p<0.01) \), averaging 53.4±14.6 nmoles/mg platelet protein/hr.

**Table 1. ATP content and lactic acid production rate of human non-adhesive platelet**

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<thead>
<tr>
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<th>Normal platelet</th>
<th>Non-adhesive platelet</th>
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<tr>
<td>ATP (nmoles/mg platelet protein)</td>
<td>107±17.8</td>
<td>49.4±10.7</td>
</tr>
<tr>
<td>Lactic acid production rate (nmoles/mg platelet protein/hr)</td>
<td>94.6±10.5</td>
<td>53.4±14.6</td>
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**COMMENTS**

Sialic acids have been demonstrated in most blood cells and other tissues and fluids (Klenk 1958; Gottschalk 1960). NANA is the only type of sialic acids so far been identified in human tissues. It has been well established that NANA content of the human platelet is larger than that of the human erythrocyte (Born 1968). Assuming that the platelet surface area is 28.3 \( \mu \text{m}^2 \) and that the platelet content of \( 10^{10} \) platelets is 18 mg, there are \( 19.1 \times 10^5 \) molecules of NANA per \( \mu \text{m}^2 \) of platelet surface. This is approximately eleven times the concentration in the human erythrocyte.

The precise function of cell NANA is not known. It has been reported that removal of NANA out of the cell constituents resulted in changes in antigenic specificities of the erythrocyte (Speinger 1963), life span of the erythrocyte (Brading et al. 1959) and leukocyte metabolism (Fisher and Ginsberg 1956). When cells were treated with neuramidase, the surface negative charge and electrophoretic mobility were decreased (Jerushalmy et al. 1961; Eyler et al. 1962; Ruhenstroth-Bauer et al. 1962). The adhesiveness of malignant tumor cells to the vascular endothelium was reduced by removal of their NANA by neuramidase treatment (Gasic and Gasic 1962).

Platelets suspended in plasma or physiologic saline have a high negative
charge (Hirsh and Doery 1971). The net electrokinetic charge density of the human platelet in physiologic saline ranges from 2920 esu/cm² to 5800 esu/cm². The intact human platelet has the following electrokinetic constitutions: a) \(3.5 \times 10^5\) katiogenic (positive) groups of \(pK > 9\), b) \(20.5 \times 10^5\) aniogenic (negative) groups \(8.9 \times 10^5\) of which represent NANA ions, c) the majority of the remaining aniogenic groups appear to have a \(pK\) of 4 and are probably carboxyl groups of acidic amino acids in protein; phosphate and sulphate ions probably constitute less than 10% of the remaining aniogenic groups.

The high surface negative charge of the platelet probably attributes in maintaining platelet non-aggregated by electrokinetic repulsion. The treatment of platelets with neuraminidase resulted in a decrease of their electrophoretic mobility and electrokinetic surface charge density. The surface charge density that can be removed by treatment with neuraminidase is associated with NANA. Sixty one % of the total NANA of platelets is supposed to be liberated by neuraminidase treatment. Thus it is evident that a reduction of NANA content of the platelet results in a decrease of surface negative charge.

The high surface negative charge of platelets is related to their electrophoretic mobility (Madoff et al. 1964; Hampton and Mitchell 1966; Born 1968) and is involved in maintaining the platelets in physiologic repulsion. It seems likely that a decrease in surface negative charge of the platelet might result in a decrease in its adhesiveness on a glass surface having positive surface charge. The relationship between platelet NANA content and platelet adhesiveness on a glass surface was studied in this paper. Most sialic acids are presented in a bound form linked to protein through intermediary carbohydrates (Gottschalk 1962). The presence of free sialic acids has been reported in thyroid glands and cerebrospinal fluids (Wollman and Warren 1961; Boroch and Evans 1962). Madoff et al. (1964) reported the presence of free NANA in the platelet. However, since the source of free NANA was obscure, only the total NANA content of the platelet was measured in this study.

Our present studies demonstrate that NANA content of non-adhesive platelets is lower than that of adhered platelets. The treatment of the rabbit platelets with neuraminidase in vitro resulted in a marked decrease in both platelet NANA content and platelet adhesiveness index. The long-term administration of Warfarin reduced platelet NANA content and adhesiveness index both in rabbits and in patients. A close correlation was demonstrated between NANA content of platelets and adhesiveness index.

The adhesiveness test in common use reflects the sensitivity of platelets to a low concentration of ADP. Evidence for this is provided by the observations that the adhesiveness is inhibited by EDTA (Hellem 1960, 1968; Hirsh et al. 1966) and adenosine (Hirsh et al. 1966) in the same way so that ADP-aggregation of the platelet is inhibited by them. The platelet adhesiveness is markedly reduced when platelet rich plasma is used instead of whole blood (Hellem 1960, 1968; Hirsh et al. 1966). The reason is as follows: When the adhesiveness test is performed
in whole blood, ADP is provided by traumatized red cells, but when the adhesiveness test is carried out in platelet rich plasma, little ADP is provided. ADP-induced platelet aggregation is inhibited by blocking both glycolysis and oxidative phosphorylation, but not by blocking either one of these two metabolic pathways (Müller et al. 1967). The platelets also lose their responsiveness to ADP when incubated in a glucose-free medium (Kinlough-Rathbone et al. 1970). Thus it seems likely that a supply of metabolic ATP is required for platelet aggregation following its adhesion on a glass surface in the adhesiveness test.

Our present studies demonstrate that the ATP content of non-adhesive platelets is lower than that of adhered platelets and the lactic acid production rate of non-adhesive platelets is markedly reduced.

From these results it may be suggested that the negative surface charge due to NANA plays a significant role in the mechanism of platelet adhesion on a glass surface and that metabolic energy supply is required in the mechanism of platelet aggregation following adhesion.

Decreased platelet adhesiveness occurs in thrombosthenia and in the majority of patients with von Willebrand’s disease (Salzman 1963; Hellem 1968; Bowie et al. 1969). The decreased platelet adhesiveness has also been reported to occur in macroglobulinemia, scurvy, chronic myeloid leukemia and uremia (Hellem 1968). It has been reported that long-term anti-coagulant therapy reduces platelet adhesiveness (Evans and Irvine 1966).

Our present studies demonstrate that platelet adhesiveness index is reduced in patients with von Willebrand’s disease, ITP, SLE, uremia, liver cirrhosis and long-term Warfarin therapy. Platelet NANA content in these patients is markedly reduced, showing a significant correlation with platelet adhesiveness index.

References


