Altered Sedimentation Behavior and Ultrastructure of Platelets in Hyperlipidemia

By Jean W. Saleh, M.D. and Sami A. Hashim, M.D.

SUMMARY

The influence of hyperlipidemia on platelet sedimentation behavior and ultrastructure was studied in samples of plasma obtained from 22 normal subjects, 7 patients with Type II and 12 patients with Type IV hyperlipoproteinemia, all fasting: also from 11 normal subjects prior to and at intervals up to 6 hours following ingestion of a meal containing 65 g of fat. Platelet counts were made on mixed platelet rich plasma after centrifugation of whole blood at 1,000 rpm, on top plasma layers after high speed centrifugation (10,000 rpm), and on the sediments resuspended in imidazole buffer. The mean ± SE number of platelets per mm³ in top layer among normal, Type II and Type IV plasmas were 4,090 ± 405; 9,000 ± 70, and 21,500 ± 1,950, respectively. The rise and subsequent fall in plasma triglycerides in response to fat ingestion in normal subjects paralleled those of platelets in the top layers. No such correlation was found with plasma cholesterol. The presence of platelets in the top layers of plasma was demonstrated further by electron microscopy. The buoyant platelets revealed the presence of osmophilic (lipid) particles within their open canalicular system, and pseudopod formation. The sedimented platelets from normal subjects had a smooth contour, where those from patients with hyperlipidemia displayed a striking formation of pseudopods. The data suggest that the sedimentation behavior of platelets and their normal structure are altered significantly as they interact with abnormal concentrations of plasma lipoproteins.

Additional Indexing Words:
Hyperlipoproteinemia
Prandial hyperchylomicronemia
Platelet electron microscopy

Since 1886, blood platelets have been known to be essential for thrombus formation and for their ability to adhere to each other as well as to vessel walls. In 1936, Tocantins and Cantarow observed a moderate "lipemia" in response to administration of anti-platelet serum. It was shown that plasma freed from fat by high speed centrifugation could no longer be clotted by addition of Russel's viper venom, but that added fat restored its coagulability. In studies in man, thrombocytopenia followed repeated infusions of intravenous fat, and in a similar experiment, a significant fall in circulating blood platelets was observed after oral and intravenous administration of fat in emulsion form. Administration of heparin intravenously three hours after the ingestion of the emulsified fat showed a reduction in the turbidity of the plasma together with a prompt but transient rise in the count of circulating platelets. Furthermore, platelet-rich lipemic plasma is cleared less rapidly and completely than lipemic but plateletpoor plasma, obtained respectively after centrifugation at 1500 rpm and 3200 rpm. Even though it seems clear that heparin, platelets, and fat transport and clearing are intimately related, no satisfactory explanation of the mechanism of interaction of platelets and plasma lipids has been forthcoming.

The interaction of platelets with endogenously produced lipoproteins also has been a subject of interest. Increased platelet adhesiveness in hyperlipidemia has been reported, while isolated rabbit and human beta-lipoproteins have been shown to accelerate significantly adenosine diphosphate and thrombin-induced platelet aggregation. Furthermore, ultracentrifugation and electrophoresis are well known techniques for separation of plasma lipoproteins whereas low speed centrifugation, at 1500 rpm or lower, is used to recover the platelet-rich plasma (PRP). At higher speeds of plasma centrifugation, all platelets are believed to sediment. Thus, lipids have been known to interact with platelets and to influence their function. The present study was undertaken to determine the sedimentation behavior of platelets in plasma from normal subjects.

From the Department of Medicine, St. Luke's Hospital Center, and Institute of Human Nutrition, Columbia University College of Physicians and Surgeons, New York, New York.
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Address for reprints: Dr. Sami A. Hashim, Department of Medicine, St. Luke's Hospital Center, Amsterdam Avenue and 114th Street, New York, New York 10025.
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prior to and at intervals following a fat meal, and from patients with Type II and Type IV hyperlipoproteinemia. Electron microscopic studies were made of platelets with altered sedimentation behavior.

Methods

Venous blood samples were drawn in EDTA-containing siliconized glassware during the post-absorptive state from twenty-two normal subjects (8 men and 14 women, aged 19-71, median 35), seven patients with Type II (4 women and 3 men, aged 38-70, median 60), and twelve patients with Type IV (9 men and 3 women, aged 30-75, median 50) hyperlipoproteinemia. Phenotyping of hyperlipoproteinemia was made according to the criteria described by Fredrickson et al. Type II disease was documented by the demonstration of a heavy beta band in the lipoprotein electrophoretogram, a distinct elevation of plasma total cholesterol level, a normal plasma triglyceride concentration, and a clear plasma. Type IV disease was characterized by the demonstration of a heavy pre-beta band by plasma electrophoresis, a striking elevation of plasma triglyceride and to a lesser degree of total cholesterol concentrations in a lactescent plasma. All blood samples were centrifuged at 1000 rpm for 5 min to obtain platelet-rich plasma (PRP), and at 10,000 rpm ("high speed") for 10 min in a Sorvall SS-4 centrifuge. Platelet counts were made according to the method of Miescher and Gerarde on mixed PRP and, after high speed centrifugation on top layers and on the sediment, the latter was suspended to original volume in imidazole buffer (0.05M) containing 1% EDTA. For the purpose of platelet counting and electrophoresis, the top layer of plasma obtained after "high speed" centrifugation was sampled by the capillary action of a standardized twenty microliter disposable pipette brought carefully in contact with the surface of the plasma. For this purpose, the descent of the pipette to the surface of the plasma was controlled by attaching it to a stand equipped with a vertical slide. Lipoprotein electrophoresis, and total cholesterol and triglyceride concentrations were performed on all fasting plasmas. The effect of ultracentrifugation on platelet sedimentation was studied in plasma samples from two normal subjects, two patients with Type II and two patients with Type IV hyperlipoproteinemia. The samples were subjected to centrifugation at 42,000 rpm for 30 minutes in a Beckman preparatory ultracentrifuge (Model L2-65B), using a TI50 rotor. In order to characterize further the top layer of plasma after high speed centrifugation, electrophoresis was done on top layers of plasma from two normal subjects, two patients with Type II and three patients with Type IV hyperlipoproteinemia. In order to study the effect of diet-induced hypercholesterolemia on platelet sedimentation behavior, eleven normal volunteers were fed, after 12-hour fast, a meal containing approximately 65 g of fat (three mediumsized eggs, 7 parts of butter, 25 g of bacon, and 120 ml of whole milk). Plasma samples were obtained prior to breakfast and at hourly intervals up to 6 hours. Platelet counts were made on each mixed PRP and, after centrifugation at 10,000 rpm, on each top layer and on the sediment resuspended in buffer. Also, plasma cholesterol and triglycerides were measured concurrently in all fasting and postprandial plasma samples. Electrophoresis was performed on top layers of the plasma samples following high speed centrifugation.

The ultimate identification of the platelets in the top layers of plasma was made by transmission electron microscopy. Fasting samples of PRP from normal subjects and patients with Types II and IV hyperlipoproteinemia were fixed in 0.1% glutaraldehyde in White's solution and centrifuged at 10,000 rpm. The top layers of plasma had an additional fixation in 3% glutaraldehyde followed by a final fixation in 1% osmic acid. The platelets were trapped on a Millipore filter, washed in buffer, dehydrated in alcohol and embedded in Epon 812. Thin sections were cut with a diamond knife and stained with lead citrate and uranyl acetate. All observations were made in a Zeiss 95 microscope. The sedimented platelets of fasting PRP from normal subjects and patients with Types II and IV hyperlipoproteinemia were fixed in a similar manner and examined by scanning electron microscopy. For this purpose, the fixed platelets were trapped on a Millipore filter and coated with a suspension of colloidal gold. Scanning was performed by use of an AMR 900 microscopy set at 20 K v and 20° tilt.

Results

The total number of platelets in mixed PRP and in top layer of plasma of the normal subjects and the patients with Types II and IV hyperlipoproteinemia, together with values of plasma triglycerides and total cholesterol are shown in Table 1. In the patients with Type IV disease, a significantly higher proportion of platelets was recovered in the top layer of plasma, when compared with the normal subjects (P < .0001). This layer, when subjected to lipoprotein electrophoresis in three subjects displayed a pre-beta lipoprotein pattern of migration. In contrast, in the patients with Type II disease a significantly smaller proportion of platelets was recovered in the top layer of plasma when compared with that from patients with Type IV (P < .001). In two patients with Type II disorder, the electrophoretogram of the top layer disclosed a beta-lipoprotein pattern of migration. However, the proportion of platelets in top layer of plasma obtained from patients with Type II disease was significantly higher (P < .001) than that in the top layer from the normal subjects. It is apparent that a certain proportion of platelets “float” in the top layer of normal plasma, that this number increases significantly in patients with Type II disease and that such an increase is even higher in patients with Type IV disease. In contrast, the number of sedimented platelets derived from patients with Types II and IV was significantly lower (P < .001) than that of the normal controls. Also, significantly more platelets sedimented (P < .001) in the plasmas of patients with Type II than in those with Type IV disease (Table 1).

Ultracentrifugation at 42,000 rpm for 30 minutes done on the plasma of two normal subjects disclosed platelet counts of 410,000 and 380,000 after the sedimented “plugs” were resuspended in buffer to the original volume of plasma, and the counts in the top plasma layers were 2,000 and 3,000. The mixed PRP counts were 412,000 and 415,000, respectively. Elec-
Table 1
Platelet Counts, Plasma Triglyceride, and Total Plasma Cholesterol Concentrations

<table>
<thead>
<tr>
<th>Plasma lipoprotein status (number of subjects)</th>
<th>PRP (mean ± se)</th>
<th>Top layer (mean ± se)</th>
<th>Top layer % of PRP (mean ± se)</th>
<th>Plasma triglycerides (mean ± se)</th>
<th>Plasma total cholesterol (mean ± se)</th>
<th>Platelets in sedimented plug resuspended to original plasma volume (mean ± se)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (22)</td>
<td>332,000 ± 13,000</td>
<td>4,090 ± 405</td>
<td>1.2 ± 0.1</td>
<td>113 ± 12</td>
<td>233 ± 12</td>
<td>328,000 ± 13,985</td>
</tr>
<tr>
<td>Type II (7)</td>
<td>288,000 ± 3,600</td>
<td>9,000 ± 70</td>
<td>3.0 ± 0.2</td>
<td>147 ± 23</td>
<td>352 ± 43</td>
<td>279,000 ± 2,114</td>
</tr>
<tr>
<td>Type IV (12)</td>
<td>287,000 ± 6,000</td>
<td>21,500 ± 1,950</td>
<td>7.5 ± 0.6</td>
<td>992 ± 320</td>
<td>345 ± 34</td>
<td>266,000 ± 643</td>
</tr>
</tbody>
</table>

Electrophoresis of the clear top layers showed extremely faint beta bands. In the two patients with Type II hyperlipoproteinemia the counts of the sedimented platelets were 373,000 and 335,000; those of the top plasma layers were 6,000 and 5,000, while those of the mixed PRPs were 390,000 and 340,000, respectively. Electrophoresis of the cloudy top layers displayed heavy beta bands of the low density lipoproteins. In the two patients with Type IV disease in whom the platelet counts in the mixed PRPs were 280,000 and 390,000, the counts in the top plasma layers were 15,000 and 12,000 and those in the sediments were 260,000 and 380,000, respectively. Electrophoresis of the “milky” top layers disclosed a typical pre-beta band migration of the very low density lipoproteins.

The results of platelet studies in the eleven normal volunteers fed 65 g of fat as breakfast after an overnight fast are shown in table 2 and figure 1, together with concurrently determined plasma triglyceride and total cholesterol concentrations. The curve representing mean serum triglyceride changes in response to fat ingestion in these normal subjects paralleled that of mean platelet counts in the top layers. When compared with the mean postabsorptive value, the mean platelet counts in the top layers of plasma at two to five hours following the fat load rose significantly ($P < .001$). Concurrently, the number of sedimented platelets decreased, reaching significance ($P < .05$) at two and three hours. The mean plasma total cholesterol values, calculated at the same intervals as those for plasma triglycerides and platelet counts, did not change significantly after the fat load. All fasting plasmas were clear. Turbidity was seen at two and up to five hours after breakfast. During this time interval and following high speed centrifugation (10,000), the plasmas appeared creamy at the top, cloudy at the middle and clear at the bottom. This distinct separation vanished slowly after the fourth hour following the fat ingestion and in eight of the volunteers, the plasmas again were clear throughout the tube at the end of the experiment. Electrophoresis of the top layers carried on on all the patients at the peak of hyperlipemia disclosed a nonmigrating pattern with a heavy concentration at the origin, suggesting the presence exclusively of chylomicrons.

The plasma triglyceride concentrations in the postabsorptive state in the 22 normal subjects, the seven patients with Type II and twelve patients with Type IV hyperlipoproteinemia displayed a significant degree of correlation with the platelet counts in top layers of their respective plasmas (fig. 2). In contrast, there was no significant correlation in the same subjects between plasma total cholesterol and platelet counts in top layers of plasma (fig. 3).

Table 2
Platelet Counts, Plasma Cholesterol and Triglyceride Concentrations in 11 Normal Adults Following Ingestion of 65 g of Fat

<table>
<thead>
<tr>
<th>Hours</th>
<th>PRP (mean ± se)</th>
<th>Top layer (mean ± se)</th>
<th>Top layer % of PRP (mean ± se)</th>
<th>Plasma triglycerides (mean ± se)</th>
<th>Plasma total cholesterol (mean ± se)</th>
<th>Platelets in sedimented plug resuspended to original plasma volume (mean ± se)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>385,909 ± 13,017</td>
<td>4,300 ± 760</td>
<td>0.9 ± 0.1</td>
<td>91.5 ± 11.7</td>
<td>193.6 ± 4.3</td>
<td>382,000 ± 12,160</td>
</tr>
<tr>
<td>1</td>
<td>375,545 ± 12,596</td>
<td>7,300 ± 1,647</td>
<td>1.7 ± 0.3</td>
<td>110.5 ± 15.0</td>
<td>193.4 ± 4.8</td>
<td>369,000 ± 12,016</td>
</tr>
<tr>
<td>2</td>
<td>338,636 ± 11,891</td>
<td>11,400 ± 1,614</td>
<td>3.0 ± 0.4</td>
<td>161.8 ± 20.0</td>
<td>186.7 ± 5.9</td>
<td>348,000 ± 11,957</td>
</tr>
<tr>
<td>3</td>
<td>361,363 ± 12,614</td>
<td>13,400 ± 2,191</td>
<td>3.6 ± 0.5</td>
<td>200.2 ± 27.5</td>
<td>187.0 ± 4.5</td>
<td>348,000 ± 12,372</td>
</tr>
<tr>
<td>4</td>
<td>339,000 ± 10,379</td>
<td>11,100 ± 2,428</td>
<td>2.8 ± 0.5</td>
<td>202.4 ± 31.5</td>
<td>188.9 ± 5.7</td>
<td>369,000 ± 10,375</td>
</tr>
<tr>
<td>5</td>
<td>382,727 ± 10,008</td>
<td>9,200 ± 1,781</td>
<td>2.3 ± 0.3</td>
<td>183.3 ± 36.0</td>
<td>190.3 ± 5.3</td>
<td>374,000 ± 9,577</td>
</tr>
<tr>
<td>6</td>
<td>383,750 ± 3,145</td>
<td>3,750 ± 834</td>
<td>0.9 ± 0.2</td>
<td>112.6 ± 18.0</td>
<td>200.0 ± 1.8</td>
<td>380,000 ± 2,886</td>
</tr>
</tbody>
</table>

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The platelets in the top layers of plasma were identified clearly by transmission electron microscopy. The buoyant platelets from normal subjects (fig. 4) and patients with Type II disorder displayed their characteristic granules, few pseudopods and an open canalicular system which contained osmophilic material indicating the presence of lipid. No osmophilic structures were seen between the platelets. The floating platelets from patients with Type IV disease (fig. 5) displayed a similar ultrastructure except for the presence of more lipid inclusions in their open canalicular system and more pseudopods; also, osmophilic structures were scattered outside the platelets. The sedimented platelets from normal subjects displayed a smooth contour with occasional excrescences, while those from patients with Types II and IV hyperlipoproteinemia disclosed a striking formation of pseudopods (fig. 6).

Figure 1
Changes (mean ± se) in plasma triglyceride (a) and cholesterol (c) concentrations and platelet counts in top plasma layers (b) after high speed centrifugation in eleven normal young adults prior to and at intervals up to six hours following the ingestion by each subject of 65 g of fat.

Figure 2
Semi-log plot showing a significant degree of correlation between triglyceride concentrations and platelet counts in top layers of plasma after high speed centrifugation. The subjects involved in these correlations were 22 normals, seven with Type II and twelve with Type IV hyperlipoproteinemia.

Figure 3
Semi-log plot showing a lack of correlation between plasma total cholesterol concentrations and platelet counts in top layers of plasma after high speed centrifugation. The subjects involved in these correlations were 22 normals, seven with Type II and twelve with Type IV hyperlipoproteinemia.
Discussion

The results indicate that the sedimentation behavior of platelets can be influenced by the state of the plasma lipids. It is apparent that a certain proportion of platelets “floats” in normal plasma during high speed centrifugation. This proportion increases significantly in plasmas from patients with Type II disease. However, in our series the most striking increase in the proportion of floating platelets was observed in the plasma from patients with Type IV hyperlipoproteinemia. Thus, the presence in the plasma of increased concentration of beta-lipoprotein and to a larger extent of pre-beta lipoprotein appears to promote “floating” of platelets. The striking correlation between the top layer: PRP platelet ratio and serum triglycerides, and the lack of correlation between such a ratio and plasma total cholesterol suggest that triglyceride-rich lipoproteins are involved in the platelet “floating” phenomenon.

The hyperchylomicronemia induced by oral fat loads in the normal subjects was associated with an increase in the proportion of platelets in the top layer of plasma suggesting again that hypertriglyceridemia is involved in the flotation of platelets. It is possible that the lipoprotein particles, when present in inordinate concentrations in the plasma (hyper-beta and hyper-pre-beta lipoproteinemias and hyperchylomicronemia), physically adhere to a certain type of platelet thus rendering them buoyant. This possibility also could explain the occurrence of increased flotation of platelets in the plasma from patients with Type II disease. However, since the degree of platelet floating in these patients was significantly lower than that in patients with Type IV disease, the buoyant effect on the platelets of the pre-beta-lipoproteins, on the basis of physical interaction, appears to be more pronounced than that of the beta-lipoproteins. Surface interactions of platelets have been described with a variety of macromolecules and molecular aggregates including polymerizing fibrin,21 antigen-antibody complexes, and uncoated latex particles and those coated with protein.22 Moreover, isolated human beta lipoproteins have been shown to enhance platelet aggregation in the presence of adenosine diphosphate or thrombin.10 The extent to which such in vitro phenomena may be pertinent to modification of platelet sedimentation by the lipoproteins cannot be ascertained.

Another possible mechanism for the accelerated buoyancy of platelets derived from patients with hyperlipoproteinemia may relate to a changed density of the platelets. A well known physiologic behavior of

Figure 4
Platelets from top layer of ultracentrifuged plasma from a normal subject during postabsorptive state. Platelets display their characteristic dense bodies, granules, and open canalicular system. Three of four platelets are disc shaped. There are only two sections of pseudopods. Note osmophilic material (arrow) in canalicular system. No osmophilic round structures in background. (× 7,200.)

Figure 5
Platelets from top layer of ultracentrifuged plasma from a patient with Type IV hyperlipoproteinemia during postabsorptive state. Platelets display their characteristic dense bodies, granules, and open canalicular system. No disc shaped platelets are seen. There are several sections of pseudopods. Note increased osmophilic material (arrow) in canalicular system. Numerous osmophilic round structures are seen in background. (× 7,200.)
platelets is their ability to phagocytize a variety of particulate material,\textsuperscript{22, 23} including emulsions of lipid.\textsuperscript{24} Also, it has been shown that the platelets in animals rendered hyperlipidemic by dietary means undergo a change in the fatty acid pattern of their lipids in accordance with the nature of the dietary fatty acids. For example, when butter was the major component of dietary fat, a larger proportion of saturated fatty acids was observed in the platelets.\textsuperscript{25} A similarly increased proportion of saturated fatty acids was demonstrated in platelets derived from patients suffering from coronary heart disease.\textsuperscript{26} Thus, it may be assumed that at least some of the fat entering the platelets is derived from the plasma lipoproteins or the chylomicrons. As shown in this study, the ultrastructure of the buoyant platelets from normal subjects and patients with hyperlipoproteinemia reveals the presence of osmophilic material, presumably lipid, within the platelet open canalicular system. The presence of these inclusions in the floating platelets may explain, in part, the mechanism and degree of platelet buoyancy. It is noteworthy that the buoyant platelets from normal subjects and patients with Type II hyperlipidemia displayed a slight degree of pseudopod formation, while those from patients with Type IV disorder disclosed an enhanced formation of pseudopods. Studies have shown that formation of pseudopods by platelets may be a manifestation of their response to a variety of stimuli.\textsuperscript{27} It is possible that the uptake of lipid by the platelets may induce such a structural response. This mechanism also may explain pseudopod formation in the sedimented platelets from patients with hyperlipidemia. However, other factors than lipid uptake by the platelets must play a role in their buoyancy, since the majority sediment. One such factor is a change in the density of the plasma consequent to an increased concentration of lipoproteins. Also, the non-lipid components of the platelets may differ in the buoyant and the sedimented platelets. Further investigation is needed to ascertain the extent to which hyperlipidemia is associated with a change in platelet behavior, in terms of increased aggregation or interaction with the arterial endothelium.

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