Influence of Plasma Lipoproteins on Platelet Aggregation in a Normal Male Population

David G. Hassall, Leslie A. Forrest, K. Richard Bruckdorfer, Christine B. Marenah, Peter Turner, Claudio Cortese, Norman E. Miller, and Barry Lewis

Aggregation tests were performed on platelet-rich plasma from healthy male volunteers to determine the minimum concentration of adenosine diphosphate (ADP), epinephrine, collagen, or thrombin required to induce secondary aggregation. Platelets were also analyzed for cholesterol and phospholipid, and in some cases their membrane fluidity was determined by fluorescence depolarization of the probe, diphenylhexatriene. Concentrations of the major lipoprotein fractions in the plasma were measured and related to the sensitivity of platelets to the four agonists. Low density lipoprotein (LDL) and total cholesterol concentrations, but not high density lipoproteins (HDL) or very low density lipoproteins (VLDL), were positively correlated with sensitivity to aggregation by epinephrine, but not by other agonists. By arrangement of the lipoprotein concentration into quintiles, the effect of LDL was most striking in the lower two quintiles, where the sensitivity to adrenaline and ADP were much diminished. The middle and upper two quintiles showed a similar sensitivity. Lower platelet cholesterol/phospholipid ratios were also associated with a reduced sensitivity to epinephrine or ADP, but only at the lower end of the range. Membrane microviscosity was correlated negatively with collagen sensitivity and with VLDL cholesterol concentrations, but positively with HDL cholesterol concentrations. Platelet behavior appears, therefore, to be influenced by lipoprotein concentrations within the range found in a healthy population. (Arteriosclerosis 3:332–338, July/August 1983)

A pathogenic role has been ascribed to platelets in atherosclerosis and thrombotic disorders. With an appropriate stimulus (e.g., damaged endothelium and exposed collagen), the platelet undergoes a rapid shape change and aggregates with other platelets. This aggregation can be simulated in vitro in platelet-rich plasma and the technique is now widely used to study platelet responses.

Platelets from patients with Type IIa hyperlipoproteinemia are more sensitive to epinephrine and adenosine diphosphate (ADP) than platelets from normal persons and this may be significant because the increased concentration of cholesterol-rich low density lipoproteins (LDL) associated with this disorder has been linked with atherogenesis. This greater sensitivity was associated with an increase in the cholesterol/phospholipid ratio in platelet membranes. The mechanism of the cholesterol effect is uncertain, although changes in membrane fluidity have been postulated; enhanced synthesis of thromboxane has also been suggested.

It was the purpose of this study to investigate the range of sensitivities of platelet-rich plasma to aggregating agents in a normal male population, and to determine whether these variables were related to the serum concentrations of very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Relationships were also sought between these observations and the cholesterol/phospholipid ratios and membrane fluidity of
platelets. Such relationships were demonstrated for LDL, platelet cholesterol/phospholipid ratios, and platelet sensitivity to epinephrine.

Methods

Epinephrine bitartrate, ADP, and thrombin were purchased from Sigma Chemical Company U.K., and solubilized collagen was obtained from Hormonchemie Gesellschaft mit beschränkter Haftung, Munich, Germany. Total serum cholesterol concentration was initially measured in a random population sample of 300 apparently healthy working males aged 25 to 55 years who were not taking drugs known to influence lipid metabolism and who gave informed consent for this study. Diabetics were excluded from the study. From this population, a sample of 150 representatives of the lowest, modal, and highest quintiles of plasma cholesterol (age-adjusted) were recalled. Further blood samples from fasted men were taken for lipoprotein preparative ultracentrifugation and differential precipitation using heparin and manganese to determine VLDL (d < 1.006 g/ml) and HDL cholesterol concentrations. The LDL cholesterol was then calculated by difference. At the same time, samples of blood were collected from 130 subjects into acid-citrate-dextrose and centrifuged directly at 200 g for 10 minutes at room temperature to produce platelet-rich plasma. After leaving the samples 1 hour for the platelets to recover, we used this to study platelet aggregation. The tests were completed within 3 hours. The platelet counts were within the normal range in all cases, but were not adjusted to the same value, to avoid dilution of the plasma lipoproteins.

Aggregation tests were performed with an aggregometer (Payton Associates, Scarborough Ontario Canada, Model No 300 BD-5) fitted with 0.1 ml cuvette holders using the method of Born. The sensitivity of the platelets to epinephrine, ADP, collagen, and thrombin was determined using a series of concentrations depending on the sensitivity of individual samples. The threshold concentration was the minimum concentration sufficient to produce a complete secondary aggregation at 37°C for each agonist within 2 minutes.

In some cases platelets were washed free of plasma by centrifugation at 800 g with Tyrode’s buffer (pH 7.4). The washed platelets were incubated for 1 hour with diphenylhexatriene, and the fluorescence polarization P was determined at 20°C and 37°C on a microviscosimeter model MV1-A (Elscint Limited, Crawley, U.K.).

From P the fluorescence anisotropy r is calculated:

\[ r = \frac{2P}{3 - P} \tag{1} \]

The relative microviscosity (RMV) at 20°C or 37°C is calculated:

\[ \text{RMV} = \left( \frac{r^o}{r} \right)^{-1} - 1 \tag{2} \]

where \( r^o \) is the limiting anisotropy for diphenylhexatriene. The relative microviscosity is used as an index of membrane fluidity.

In all cases, samples of platelet suspensions were counted and the lipids were extracted using chloroform/methanol mixtures. Cholesterol was determined using cholesterol oxidase E.C. 1.1.3.6 (Boehringer Mannheim Limited, Lewes, England). Phospholipid phosphorus was assayed by the method of Bartlett. The cholesterol/phospholipid molar ratio was then calculated. Spearman’s test was used to obtain correlation coefficients, whereas differences between the first and higher quintiles of LDL, HDL, and VLDL concentrations were evaluated using Student’s t test.

Results

Volunteers from the modal and extreme quintiles of the total cholesterol range were selected to provide a wide range of lipoprotein concentrations. There was some regression to the mean of serum cholesterol values between the two samplings, and cholesterol and lipoprotein cholesterol concentrations in the second samples were related to platelet behavior. The range of values (not age-adjusted) were: total cholesterol 3.2 – 9.6, LDL cholesterol 1.4 – 7.6, HDL cholesterol 0.87 – 2.55, and VLDL cholesterol 0.01 – 2.78 mmol/liter. The HDL concentrations were higher, on the average, than those recorded in some other Western populations. A wide range of sensitivities to the aggregating agents was observed. Platelets from 10 volunteers showed no response to epinephrine, as has often been observed. This has been shown in some cases to be an inherited trait. These were excluded from the statistical analyses relating to epinephrine sensitivity. The sensitivity to epinephrine may be somewhat lower than that found by other workers who collect blood in 0.38% citrate, which yields a higher final pH.

Relationships between Platelet Sensitivity and Lipoprotein Cholesterol

In Table 1, the relationships between total lipoprotein cholesterol concentrations and platelet sensitivities to the aggregating agents are shown. Significant, but weak, correlations were found between the epinephrine sensitivity and total cholesterol or LDL cholesterol concentration, hence some 20% of the variance may be attributed to differences in total or LDL cholesterol concentration. The sensitivity increased (i.e., the threshold concentration of epinephrine required to cause aggregation was lowered) as total or LDL cholesterol increased. There was no correlation between sensitivity to epinephrine and HDL or VLDL cholesterol concentration.
Table 1. Correlation Coefficients Between Lipoprotein Cholesterol Concentrations and Platelet Aggregation In Male Volunteers

<table>
<thead>
<tr>
<th>Aggregating agent</th>
<th>Total cholesterol</th>
<th>VLDL cholesterol</th>
<th>LDL cholesterol</th>
<th>HDL cholesterol</th>
<th>% LDL of total</th>
<th>% HDL of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (n = 130)</td>
<td>-0.112</td>
<td>-0.013</td>
<td>-0.093</td>
<td>-0.183*</td>
<td>-0.021</td>
<td>-0.02</td>
</tr>
<tr>
<td>Epinephrine (n = 118)</td>
<td>-0.362†</td>
<td>-0.107</td>
<td>-0.395†</td>
<td>-0.046</td>
<td>-0.304†</td>
<td>-0.236*</td>
</tr>
<tr>
<td>Collagen (n = 128)</td>
<td>-0.028</td>
<td>-0.138</td>
<td>-0.026</td>
<td>-0.013</td>
<td>-0.093</td>
<td>-0.003</td>
</tr>
<tr>
<td>Thrombin (n = 130)</td>
<td>-0.072</td>
<td>-0.059</td>
<td>-0.062</td>
<td>-0.159</td>
<td>-0.033</td>
<td>-0.025</td>
</tr>
</tbody>
</table>

Aggregation tests were performed on 0.1 ml samples of platelet-rich plasma from each volunteer to which a series of concentrations of agonists was added. The minimum concentrations required to induce aggregation (sensitivity) was determined in each case. A negative $R_s$ therefore, indicates a lower concentration is required (higher sensitivity) as lipoprotein concentrations increase.

Sensitivity to ADP, collagen, or thrombin was not significantly related to lipoprotein cholesterol concentration, except for a weak negative correlation between HDL concentration and ADP threshold concentration. HDL cholesterol concentration was unrelated to total and LDL cholesterol, but was inversely related to VLDL concentration ($R_s = -0.48$).

When lipoprotein cholesterol concentrations were expressed as a percentage of the total cholesterol, there was still a negative correlation between the threshold concentration for epinephrine and LDL. A positive correlation for HDL also appeared. However, the percentage of HDL correlated negatively with LDL concentration ($R_s = -0.72$), more strongly than with that of HDL in the opposite direction ($R_s = 0.60$).

The data were further analyzed by arranging the lipoprotein cholesterol concentrations in quintiles and determining the mean values of the corresponding epinephrine or ADP sensitivities in each quintile. The results in Figure 1 show that the sensitivity to epinephrine in the first quintile for total cholesterol, LDL cholesterol, and VLDL was significantly lower than in quintiles 3, 4, and 5. In the case of total cholesterol and LDL, the difference was of an order of magnitude; the sensitivity in the second quintile occupied an intermediate position. In the case of VLDL, the second quintile had a similar sensitivity to adrenaline as that of quintiles 3, 4 and 5. There were no significant differences in sensitivity between the HDL cholesterol quintiles.

Sensitivities to ADP showed a similar pattern except that the differences were less marked, particularly in the case of VLDL. In the fourth and fifth quintiles of the HDL range, the mean sensitivities to ADP were lower than that in the other quintiles, but this was not statistically significant. A comparable treatment for the data on collagen and thrombin sensitivity did not show any relationships.

We also studied the effect of age on the lipoproteins and platelet behavior. As expected, a significant positive correlation existed between age and total cholesterol ($R_s = 0.27$) and LDL cholesterol ($R_s = 0.29$), but not HDL cholesterol. A weak correlation was shown between age and the epinephrine concentrations required to produce secondary aggregation ($R_s = -0.186$), suggesting that sensitivity increased slightly with age. This was not the case with the other aggregating agents.

Table 2. Correlation Coefficients Between the Relative Microviscosity (RMV) of Isolated Platelets and Plasma Lipoprotein Concentrations on the Sensitivity to Various Agonists of Platelet-Rich Plasma from Male Volunteers (n = 57)

<table>
<thead>
<tr>
<th>Lipoprotein cholesterol</th>
<th>RMV at 20°C</th>
<th>RMV at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>-0.142</td>
<td>-0.141</td>
</tr>
<tr>
<td>VLDL</td>
<td>-0.383†</td>
<td>-0.456‡</td>
</tr>
<tr>
<td>LDL</td>
<td>-0.025</td>
<td>-0.067</td>
</tr>
<tr>
<td>HDL</td>
<td>+0.307*</td>
<td>+0.454‡</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Platelet sensitivity:</th>
<th>RMV at 20°C</th>
<th>RMV at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>-0.197</td>
<td>-0.218</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>-0.055</td>
<td>-0.041</td>
</tr>
<tr>
<td>Thrombin</td>
<td>+0.112</td>
<td>+0.030†</td>
</tr>
<tr>
<td>Collagen</td>
<td>-0.402‡</td>
<td>-0.527‡</td>
</tr>
</tbody>
</table>

RMV of diphenylhexatriene was measured by fluorescence depolarization in isolated platelets. Aggregation tests were performed on 0.1 ml samples of platelet-rich plasma of each volunteer to ascertain the minimum concentration required to induce aggregation (sensitivity). A negative $R_s$ indicates an increasing sensitivity as RMV increases.

$p < 0.05$.

†$p < 0.01$.

‡$p < 0.001$. 
LIPOPROTEINS AND PLATELET AGGREGATION

Hassall et al.

Platelet Cholesterol Content and Sensitivity to Agonists

The cholesterol/phospholipid (C/P) molar ratio in platelets has a mean value of 0.65, close to the value frequently cited. There was a correlation between the C/P ratio and LDL cholesterol concentration ($R_p = 0.337$). However, no significant relationship existed between C/P and VLDL or HDL cholesterol concentration. Very similar correlations were obtained if the value for cholesterol/10^8 platelets was substituted for C/P, but no correlation existed with phospholipid content which was less variable. There was a significant correlation between the C/P and the sensitivity of platelets to epinephrine ($R_p = -0.318$), so that the threshold concentration decreased as C/P increased. No such correlation existed with the other aggregating agents.

The mean sensitivities of each quintile of the C/P values were calculated. The lowest quintile of the C/P ratios exhibited a significantly lower sensitivity to epinephrine than the other four quintiles, which were similar to each other (Figure 2). A similar pattern of distribution also existed with ADP, but not with collagen or thrombin, for which all quintiles were similar in sensitivity.

Figure 1. The sensitivities to aggregation by epinephrine and adenosine 5'-diphosphate (ADP) of platelet-rich plasma from male volunteers by quintile of total serum and individual lipoprotein cholesterol concentrations. The means ± se are of the lowest concentrations of agonists required to cause secondary aggregation for the individuals in each quintile of cholesterol concentration. A significant difference of a quintile from quintile 1 is represented by *p < 0.05; **p < 0.001.
Figure 2. The sensitivities to epinephrine, adenosine 5’-diphosphate (ADP), collagen, and thrombin of platelet-rich plasma from male volunteers by quintile of cholesterol/phospholipid molar ratio. The means ± SE are of the lowest concentrations of agonist required to cause secondary aggregation for the individuals in each quintile of cholesterol concentration. A significant difference of a quintile from quintile 1 is represented by *p < 0.05, **p < 0.01.

**Platelet Membrane Fluidity**

In approximately one-half of the samples, the fluorescence polarization P of the probe, diphenylhexatriene, was determined, and from this the relative microviscosity (RMV) was calculated as an index of fluidity (RMV increases as fluidity decreases). At 20°C or 37°C there was no correlation between RMV and the platelet C/P or the cholesterol content. Furthermore, fluidity was not correlated with either total plasma cholesterol or LDL cholesterol concentration (Table 2). However, a negative correlation existed between VLDL concentration and RMV at both temperatures (Table 2). RMV was positively correlated with HDL cholesterol concentration. Microviscosity at both temperatures was found to be negatively correlated to collagen aggregation and not to the platelet sensitivity to other agents (Table 2).

**Discussion**

Carvalho et al. found that the sensitivity to epinephrine of platelets from patients with familial Type II hyperlipoproteinemia was increased compared with healthy individuals. Our data show that within a population of healthy males, total and LDL cholesterol concentrations influence the sensitivity of platelets to epinephrine and to a lesser extent to ADP.

The threshold for epinephrine-induced platelet aggregation showed a modest inverse relationship with LDL and total cholesterol concentration. The relationship was nonlinear, the greatest differences in platelet sensitivity occurring in subjects with the lowest plasma cholesterol and LDL cholesterol levels. In a study that used controls with average total plasma and LDL cholesterol concentrations, no differences were found in the aggregation of platelets compared...
to patients with familial Type II hyperlipoproteinemia. More recently, Type II patients were shown to have more sensitive platelets than controls, but the mean total cholesterol concentration of the control group fell within the range of the first quintile of cholesterol concentration in our population, as did the controls used by Shattil et al. It seems likely, therefore, that discrepancies in the literature may depend on the plasma cholesterol concentration of the control group rather than on that of the patients. On the other hand, two patients with abetalipoproteinemia were found to have platelets that were normal with respect to ADP, but slightly hyperreactive with epinephrine and collagen. These platelets contained unusually high concentrations of cholesterol.

In one study, patients with Type IV hyperlipoproteinemia were found to have platelets that were more sensitive to epinephrine alone. In our study, VLDL cholesterol concentration had little effect on platelet sensitivity, except for a reduced epinephrine response in the lowest quintile of VLDL concentrations.

In a study of 26 normal males, Nordoy et al. found no effect of HDL concentration on platelet aggregation, but discovered a positive correlation between platelet factor 3 and HDL. This lack of effect of HDL on platelet aggregation was essentially confirmed in our work, although some very weak correlations were observed.

These findings on the effect of lipoproteins are supported by studies on isolated platelets. Low concentrations of LDL had little effect on platelet aggregation until concentrations were above 4 mM LDL cholesterol, when LDL enhanced aggregation by epinephrine and ADP. HDL also sensitized the platelets, but only at concentrations above the physiological range.

As other studies showed, LDL concentrations were found to be age-dependent; however, VLDL and HDL were not. Only the epinephrine response showed an increase with age, but this association was very weak. It is, therefore, difficult to attribute any of the effects described above to age. The mechanism of action of LDL on platelets is still uncertain, but it has been proposed that it may increase the cholesterol content of the platelets. Many cell types, including platelets, are known to bind LDL, although platelets do not appear to internalize and degrade the lipoproteins. The C/P ratio and the cholesterol content of the platelets were correlated with the LDL cholesterol concentration, but not with HDL or VLDL cholesterol concentrations. In contrast, LDL did not appear to influence the cholesterol content of lymphocytes obtained during this study. It was in the lowest quintile of observed C/P ratios that a striking reduction in platelet responsiveness to epinephrine and ADP was observed (Figure 2).

The relationship between cholesterol content and platelet sensitivity described here and for Type II hyperlipoproteinemia does not always hold. The C/P ratio is increased in Tangier disease, but the sensitivity to epinephrine is decreased. In abetalipoproteinemia, a dramatic increase in C/P ratio is not accompanied by striking changes in platelet responsiveness. We have found that a high C/P ratio in platelets from patients with liver disease did not increase their sensitivity. This was possibly attributable to other factors such as fatty acid composition or lecithin/sphingomyelin ratio.

Other workers have proposed that the increased cholesterol content of platelets from patients with hypercholesterolemia may modulate adenyl cyclase activity or increase the synthesis of thromboxane A2, which would promote the aggregation of the platelets. On the other hand, malondialdehyde production was only slightly increased in Type IIa hyperlipoproteinemia. In recent studies with isolated platelets in vitro we have found that LDL overcame the inhibitory action of prostacyclin on platelet aggregation.

It has been proposed that changes in membrane fluidity may underlie the effects of cholesterol on platelet aggregation. It was shown that manipulation of the platelet cholesterol content with liposomes brought about parallel changes in fluidity as measured by fluorescence polarization of diphenylhexatriene. In our study of platelets with a wide range of cholesterol contents, this relationship was weak, just outside the 95% confidence limit. Cholesterol is known to reduce membrane fluidity (increase microviscosity), but other factors can also play an important role. This may account for the stronger correlation between membrane fluidity and VLDL or HDL cholesterol concentrations, since phospholipid composition and fatty acid profiles may be affected by these lipoproteins. The fluidity, when measured at 20°C or 37°C, was related only to the platelet sensitivity to collagen and not to epinephrine, ADP or thrombin. This may reflect the different receptors which exist for these agonists.

In conclusion, it can be said that the concentration of low density lipoproteins may influence the response of platelets to some aggregating agents, but not to others. The effect of the lipoproteins is particularly noticeable over the lower range of LDL concentrations. Such low concentrations of LDL are common in countries in which the incidence of coronary heart disease is low. The physiological significance of some of these agents, particularly epinephrine, is still a matter of debate, and further work is required to understand the mode of action of LDL on platelet behavior and its biological significance.

Acknowledgments

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