Platelet Kinetic Studies in Patients with Hyperlipoproteinemia: Effects of Clofibrate Therapy

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SUMMARY Studies of platelet and fibrinogen kinetics in 27 patients with hyperlipoproteinemia and 28 control subjects demonstrated shortened platelet survival and increased platelet turnover in seven patients with type III and 10 patients with type IV-V hyperlipoproteinemia (p < 0.01). There was no correlation between platelet survival time and specific lipid levels, vascular disease, sex or age. Platelet kinetics were not significantly altered from control values in eight patients with familial hypercholesterolemia. Platelet aggregation studies and fibrinogen kinetic measurements did not differ in any of the hyperlipoproteinemic groups of patients from those in control subjects. Despite significant changes in plasma lipids induced by clofibrate, platelet survival was significantly extended only in patients with type IV-V hyperlipoproteinemia (p < 0.05). These results are consistent with the hypothesis that atherogenesis in patients with types III-V hyperlipoproteinemia may be associated with a process of endothelial desquamation, and type IIa hyperlipoproteinemia may involve non-desquamating endothelial injury.

THE AHEROGENIC MECHANISM of hyperlipoproteinemia is not well understood. Since endothelial cell injury is implicated experimentally in the development of atherosclerosis induced by hyperlipidemia in monkeys, we have measured platelet survival and turnover as possible indicators of vascular injury in selected hyperlipoproteinemic patients.

Methods

Patient Studies

The 28 control subjects were adult human volunteers, 14 males and 14 females, mean age 52 years (range 30-80 years) who were free of acute illness. The hyperlipidemic subjects were 12 males and 15 females, mean age 47 years (range 30-67 years), selected from subjects referred to the atherosclerosis prevention clinic of the Northwest Lipid Research Clinic. The lipoprotein pattern of each hyperlipidemic subject was classified according to published criteria. All subjects heterozygous for familial hypercholesterolemia had tendinous xanthomata and at least one first-degree relative with the same lipoprotein pattern. In addition, two LDL-receptor negative homozygous familial hypercholesterolemic patients, ages 15 and 24 years, were studied. Clinical atherosclerosis was assessed by history, physical examination, ECG and treadmill exercise testing with ECG monitoring. None of the heterozygous familial hypercholesterolemic or type II hyperlipoproteinemic subjects had clinical atherosclerosis. Two of the 10 with types IV-V had evidence of clinical sequelae of atherosclerosis.

Laboratory Studies

Platelet counts were measured with an electronic particle counter on peripheral blood collected in EDTA. The platelet count of 100 normal subjects was 250,000 ± 40,000/μl (± 1 sd). Platelet survival was determined from the disappearance of radioactivity from blood sampled 8–10 times over 6 days after injection of autologous 51Cr-labeled platelets. Platelet survival in the 28 age-matched control subjects was 9.0 ± 1.0 days. Platelet survival time was determined by computer fitting to a gamma function. The proportion of labeled platelets remaining within the systemic circulation after infusion (i.e., recovery) was calculated from the platelet activity per milliliter of whole blood at zero time, multiplied by the estimated blood volume, and divided by the platelet 51Cr activity injected. Immediate recovery of freshly labeled platelets in the control subjects was 65 ± 5%. Platelet consumption, measured as platelet turnover per microliter of blood per day, was calculated from the peripheral platelet count divided by the platelet survival time in days and corrected for recovery. Platelet turnover was 35,000 ± 4600/μl per day in the control subjects.

For calculating fibrinogen turnover, the concentration of fibrinogen was estimated spectrophotometrically by a modification of Jacobsson's method, in which the optical density of thrombin-clottable protein is determined after collection on a glass rod and subsequent solution in alkaline urea. The fibrinogen concentration in 87 normal subjects was 2.85 ± 0.22 mg/ml. Labeling of normal fibrinogen with 111In from a single hepatitis-free donor was performed by the method of Takeda. This technique involves repeated precipitation of fibrinogen with 25% ammonium sulfate saturation, followed by solution in 0.005 M sodium citrate, labeling with 111In and removal of un-
bound $^{131}$I. Fibrinogen survival (the average time in circulation) was calculated from the half-time of disappearance divided by the natural logarithm of 2.

Platelet aggregation was estimated from changes in optical transmission of 0.02 M sodium-citrate plasma at 37°C with a concentration of 300,000 platelets per μl. Aggregation was observed in 1-ml samples after the addition of ADP (0.5, 1.0, 1.5, 2.0, and 10 μM), epinephrine (0.1, 1.0, 5.0, 10, 25 and 50 μg) and collagen (10, 50, 100 and 200 μg obtained from Hormon-Chemie, Munich). Plastic equipment was used throughout; platelet-rich plasma, freshly drawn and capped, was kept at room temperature during the 30–70 minutes before testing. During this interval platelet reactivity and pH were shown to remain constant. We compared the concentrations of aggregating agents that produced a 50% change in maximum aggregation. These values were estimated from dose-response plots (logarithm of the dose vs. the extent of aggregation). ED$_{50}$ for ADP was 0.75 μM and 0.1 μM for epinephrine.

### Lipid Measurements

All studies were conducted at the Clinical Research Centers of Harborview Medical Center or University Hospital. During studies of hyperlipidemic subjects isocaloric liquid formula diets of 40% fat (half butterfat and half corn oil), 45% carbohydrate, and 15% protein were administered. When applicable, clofibrate was given in a dosage of 1 g twice daily for at least 10 days before the platelet survival studies.

All blood specimens were drawn by antecubital venipuncture after a 12–14-hour overnight fast. The cholesterol and triglyceride contents of such specimens were analyzed in whole plasma, very low-density lipoprotein (VLDL) low-density lipoproteins (LDL), and high-density lipoproteins (HDL), according to standardized Lipid Research Clinic ultracentrifugal, polyanionic precipitation, and AutoAnalyzer II (Technicon Corp, Tarrytown, New York) techniques. In addition to having VLDL (d < 1.006) which were both cholesterol-enriched and β or slow pre-β migrating, all subjects with type III patterns were lacking in isopropanol lipoprotein E$_a$.

Results are shown as mean ± SD and the significance between means was estimated using the nonparametric Wilcoxon rank sum test.

### Results

#### Platelet Kinetic Measurements

In the hyperlipoproteinemic patients as a group, platelet survival time was modestly but significantly shortened (7.8 days ± 1.9; p < 0.01 compared with 9.0 days ± 1.0 for the age-matched control group, table 1). Also, platelet turnover was reciprocally increased (47,000 plat/μl/day ± 16,000, compared with 35,000 plat/μl/day ± 5000, p < 0.01).

Of greater interest, however, are the results of platelet studies in each hyperlipoproteinemic class (fig. 1, table 1). Eight subjects with type IIa patterns and familial hypercholesterolemia showed neither a significant shortening in their platelet survival (8.6 ± 1.1 days) nor an increase in platelet turnover (42,000 ± 16,000 plat/μl/day). Even the two homozygous familial hypercholesterolemic patients with cholesterol levels exceeding 600 mg/dl demonstrated little shortening of platelet survival (8.0 and 7.6 days).

Both platelet survival and platelet turnover were modestly but significantly (p < 0.01) altered in seven subjects with type III hyperlipoproteinemia and 10 subjects with type IV-V hyperlipoproteinemia (fig. 1, table 1). The distribution of platelet survival determinations in patients with hypertriglyceridemia was either broad or included a subset of patients with significantly shortened platelet survivals, five of the 10 observations lying > 2 standard deviations outside of the control group, i.e., < 7.0 days (fig. 1). There was no correlation with clinical atherosclerosis (p > 0.5).

There was no correlation observed between shortened platelet survival and the specific lipoprotein levels, the presence of vascular disease, sex or age (table 1).

Fibrinogen survival time, concentration and turnover were not significantly different in any of the groups of hyperlipoproteinemic patients from control subjects (4.7 ± 0.4 days, 2.91 ± 0.37 mg/μl, and 0.62 ± 0.07 mg/ml/day, compared with 4.9 ± 0.04, 2.87 ± 0.29 mg/ml, and 0.60 ± 0.06 mg/ml/day, respectively; p > 0.10).

#### Effect of Clofibrate Therapy

Clofibrate had no effect on platelet kinetics in 10 normal subjects (table 2, p > 0.75). Similarly, clofibrate did not alter platelet survival time in sub-

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**Figure 1. Distribution of platelet survival measurements in patients with different types of serum lipoprotein patterns. Mean values are shown by horizontal lines.**
jects with familial hypercholesterolemia and the type IIA lipoprotein pattern despite significant reductions in both cholesterol and triglycerides (table 2). There were no significant changes in either the lipid pattern or platelet survival in patients with broad β disease (type III) during this short-term treatment. However, in patients with hypertriglyceridemia (type IV-V), significant reduction in triglycerides was produced in association with significant prolongation in platelet survival times (p > 0.05; table 2). No correlation was demonstrable between the change in triglyceride levels and alteration in platelet survival time (p > 0.10).

**Platelet Aggregation Studies**

In a blinded analysis of control subjects and hyperlipoproteinemic patients for ADP-, epinephrine- and collagen-induced aggregation comparing the concentrations that produced a 50% change in maximum aggregation achievable, no significant differences were found in any patient group preceding or during clofibrate therapy (p > 0.10).

**Discussion**

Hyperlipidemia is clearly associated with the development of premature atherosclerotic vascular disease. There is increasing evidence that platelets are important both in the complications of established atherosclerosis and in the genesis of the lesions. Several lines of evidence suggest that hyperlipidemia may enhance platelet thrombus formation. For example, Carvalho et al. demonstrated that the platelets of patients with type IIA hyperlipoproteinemia aggregate in vitro in the presence of concentrations of epinephrine, ADP, and collagen which are insufficient to aggregate normal platelets. The degree to which platelet sensitivity to aggregating agents increased in IIA patients varied considerably, and some of the patients had functionally normal platelets. However,

**Table 1. Platelet Kinetic Measurements in Hyperlipoproteinemic Patients**

<table>
<thead>
<tr>
<th>Subjects (n)</th>
<th>Concentration (plat/μl)</th>
<th>Recovery (% injected)</th>
<th>Survival time (days)</th>
<th>Turnover (plat/μl/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (28)</td>
<td>250,000 ± 40,000</td>
<td>65 ± 5</td>
<td>9.0 ± 1.0</td>
<td>35,000 ± 5000</td>
</tr>
<tr>
<td>Type IIA (FHC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygotes (8)</td>
<td>269,000 ± 63,000</td>
<td>68 ± 9</td>
<td>8.6 ± 1.1</td>
<td>42,000 ± 16,000</td>
</tr>
<tr>
<td>Homozygotes (2)</td>
<td>242,000; 279,000</td>
<td>69; 73</td>
<td>7.6; 8.0</td>
<td>40,000; 42,000</td>
</tr>
<tr>
<td>Type III (7) (BB1)</td>
<td>274,000 ± 33,000</td>
<td>75 ± 21</td>
<td>7.4* ± 0.9</td>
<td>47,000 ± 14,000</td>
</tr>
<tr>
<td>Type IV-V (10) (FHTG)</td>
<td>284,000 ± 81,000</td>
<td>72 ± 16</td>
<td>7.5* ± 2.8</td>
<td>51,000* ± 18,000</td>
</tr>
<tr>
<td>All hyperlipidemic patients (27)</td>
<td>276,000 ± 65,000</td>
<td>72 ± 15</td>
<td>7.8* ± 1.9</td>
<td>47,000* ± 16,000</td>
</tr>
</tbody>
</table>

*p <0.01, Wilcoxon rank sum test.

**Table 2. Effects of Clofibrate on Platelet Survival**

<table>
<thead>
<tr>
<th>Subjects (pairs)</th>
<th>Total Cholesterol (mg/dl)</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>223 ± 59</td>
<td>10.0 ± 5.8</td>
<td>148 ± 50</td>
<td>53.0 ± 15.9</td>
</tr>
<tr>
<td>Treated</td>
<td>177 ± 26</td>
<td>8.4 ± 6.2</td>
<td>121 ± 21</td>
<td>47.3 ± 10.3</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IIA (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>384 ± 55</td>
<td>26.9 ± 16.0</td>
<td>302 ± 48</td>
<td>54.8 ± 25.0</td>
</tr>
<tr>
<td>Treated</td>
<td>340 ± 42</td>
<td>17.6 ± 10.5</td>
<td>271 ± 49</td>
<td>51.4 ± 18.9</td>
</tr>
<tr>
<td>p</td>
<td>0.0244</td>
<td>0.0751</td>
<td>0.0711</td>
<td>0.472</td>
</tr>
<tr>
<td>Type III (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>436 ± 41</td>
<td>276 ± 59</td>
<td>130 ± 14</td>
<td>30.5 ± 14.2</td>
</tr>
<tr>
<td>Treated</td>
<td>351 ± 104</td>
<td>188 ± 95</td>
<td>126 ± 15</td>
<td>37.2 ± 7.7</td>
</tr>
<tr>
<td>p</td>
<td>0.245</td>
<td>0.247</td>
<td>0.709</td>
<td>0.387</td>
</tr>
<tr>
<td>Type IV-V (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>258 ± 42</td>
<td>116.3 ± 99.2</td>
<td>116 ± 99.2</td>
<td>36.3 ± 23.3</td>
</tr>
<tr>
<td>Treated</td>
<td>242 ± 55</td>
<td>90.7 ± 91.3</td>
<td>113 ± 58.2</td>
<td>38.1 ± 21.9</td>
</tr>
<tr>
<td>p</td>
<td>0.368</td>
<td>0.272</td>
<td>0.586</td>
<td>0.522</td>
</tr>
</tbody>
</table>

Patients reported in this table were selected from those shown in table 1. Wilcoxon rank sum test was used to determine statistical significance.
there was a general correlation between the cholesterol-phospholipid ratio of platelets and their degree of sensitivity to epinephrine-induced aggregation. Reversal was observed after clofibrate therapy. However, in subjects with the same type IIa lipoprotein pattern and clear-cut familial hypercholesterolemia, we could not show increased sensitivity to platelet aggregation induced by epinephrine, ADP, or collagen in our hyperlipidemic patients. The reason for these differences is not clear.

The atherogenic mechanism of hyperlipidemia has been postulated to involve endothelial cell injury. For example, diet-induced hyperlipidemia in subhuman primates has been associated with endothelial denudation, then platelet-mediated intimal proliferation of smooth muscle cells and a subsequent accumulation of cholesterol. However, other recent studies using rigorous scanning electron microscopy techniques reported little denudation until after intimal lesions had already developed. However, increased endothelial cell turnover has been reported and this finding implies desquamation. Platelet survival measurements have been used as an in vivo indicator of nonendothelialized surfaces exposed to circulating blood. Intimal lesion formation after the desquamation of endothelium in experimental models has been induced by balloon catheter de-endothelialization, and indwelling catheter injury and homocysteinemia, and studies of platelet kinetics in monkeys with diet-induced hyperlipidemia demonstrate a decreased platelet survival associated with a loss of endothelium.

Shortened platelet survival in some patients has been interpreted to reflect platelet consumption by vascular subendothelium. For example, platelet survival is shortened in some patients with generalized atherosclerotic vascular disease and angiographically demonstrated coronary artery disease, cerebrovascular disease, and small vessel disease. Indeed, recent observations suggest a relationship between serum lipids and platelet survival time in men with coronary disease. In that study men with coronary disease and increased serum levels of cholesterol (hyperbetalipoproteinemia) or triglycerides (hyperprebetalipoproteinemia) have more abnormal average values for platelet survival and a greater frequency of shortened platelet survival time than men with coronary disease who do not have hyperlipidemia. Dietary-induced decreases in serum triglyceride were associated with increases in platelet survival, while increases in serum triglyceride were associated with decreases in platelet survival time. Pharmacologically mediated decreases in serum cholesterol extended platelet survival in men with hypercholesterolemia.

In the present study of patients with classically defined type III and type IV-V hyperlipoproteinemia, only a minority of whom had clinical evidence of atherosclerosis, we demonstrated modest but significant shortening of platelet survival and reciprocally increased platelet turnover compared with control subjects. However, in contrast with the in vitro studies of Carvallo et al. and the hyperlipidemic coronary arteriosclerotic patients of Steele and Rainwater, subjects with familial hypercholesterolemia, including two homozygotes with this disorder, failed to show significantly altered platelet aggregation in vitro or shortened platelet survival. Finally, the administration of clofibrate to type IV-V patients resulted in a significant reduction in total triglycerides and a prolongation in platelet survival, presumably through the effect on serum lipids (table 2). In type IIa subjects, despite significant reductions in cholesterol and triglyceride

<table>
<thead>
<tr>
<th>Table 2. (Continued)</th>
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<tbody>
<tr>
<td>Triglycerides (mg/dl)</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>109 ± 38</td>
</tr>
<tr>
<td>83 ± 27</td>
</tr>
<tr>
<td>140.4 ± 64.7</td>
</tr>
<tr>
<td>100.1 ± 50.6</td>
</tr>
<tr>
<td>0.037</td>
</tr>
<tr>
<td>618 ± 264</td>
</tr>
<tr>
<td>428 ± 171</td>
</tr>
<tr>
<td>0.378</td>
</tr>
<tr>
<td>999 ± 1113</td>
</tr>
<tr>
<td>659 ± 963</td>
</tr>
<tr>
<td>0.047</td>
</tr>
<tr>
<td>7.5 ± 2.0</td>
</tr>
</tbody>
</table>
levels, clofibrate had no measurable effect on platelet survival; no effect was demonstrated in four subjects with type III patterns.

These data are consistent with the hypothesis that type IV-V hyperlipoproteinemia may be associated with endothelial cell loss and that the defect in the integrity of the endothelium may be pathogenetically important, but this formulation does not explain the disparity between normal platelet survival times and the predisposition for atherosclerosis in subjects with familial hypercholesterolemia expressed as a type IIa lipoprotein pattern; it is possible that intimal lesion formation may be mediated by a non-platelet derived growth factor. One hypothesis states that the endothelium may produce a mitogen31 in response to sublethal injury mediated by hypercholesterolemia in a manner analogous to increased PGI2 production by endothelial cells after thrombin injury in vitro.32

Acknowledgment

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References

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