Analysis of Atherosclerosis Susceptibility in Mice
with Genetic Defects in Platelet Function

Beverly Paigen, Patricia A. Holmes, Edward K. Novak, and Richard T. Swank

To determine whether platelets contribute to the development of atherosclerosis, we compared the severity of atherosclerosis in susceptible C57BL/6 mice carrying either a normal or a variant phenotype for platelet function. Five genetically distinct mutants with increased bleeding times and abnormal dense granules were used: maroon (ru2), light ear (le), ruby eye (re), beige (bg1), and pale ear (pe). After a 14-week consumption of an atherogenic diet, three mutants had significantly less disease involvement than the control: light ear, maroon, and ruby eye. In contrast, pale ear had lesions similar to control animals. After 48 weeks, the two mutants with the least degree of atherosclerosis at 14 weeks, light ear and ruby eye, showed greater than 50% survival. In contrast, no animals from the beige, pale ear, or the normal C57BL/6 strains survived. To determine whether a specific biochemical component of platelet function is related to atherosclerosis, we measured serotonin found in dense granules. Serotonin showed no correlation with each mutant's atherosclerotic susceptibility. These results indicate that some particular component of platelet function affects atherosclerosis. That component is intact in pale ear, moderately affected in beige and maroon, and severely affected in light ear and ruby eye. The identity of that component remains an interesting question whose answer may provide further insight into the atherosclerotic disease process.

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Several lines of evidence suggest that platelets are involved in the pathogenesis of atherosclerosis.1-4 Laboratory studies show that activated platelets aggregate at damaged blood vessels and release secretory products with atherogenic activity; these products stimulate vascular smooth muscle to proliferate2-6 and cause smooth muscle cells to accumulate cholesterol. Platelets may also exacerbate the atherosclerotic process. By inducing thrombus formation on atheromatous plaques, platelet actions reduce arterial potency and further compromise vascular function.2

Recent experiments have demonstrated that the mouse is a suitable model for atherosclerosis research.7-10 When fed a diet similar to the American diet in fat with a high cholesterol content, the mouse has a lipoprotein profile and atherosclerotic lesion pathology comparable to that observed in humans.9,10 Advanced atheromatous lesions with a fibrous cap overlying cellular debris and cholesterol crystal deposits are found in the aorta and coronary arteries. These lead to occluded coronary arteries, aortic aneurysm, and death after 7 to 9 months' consumption of the atherogenic diet.

In this study, we used murine genetic variants to evaluate the role of platelets in atherosclerosis. The control strain, C57BL/6, is susceptible to experimentally induced atherosclerosis; these animals develop numerous and large atheromatous plaques after consuming a diet enriched in cholesterol and fat.7,8 We compared atherosclerosis susceptibility in C57BL/6 mice with five pigment mutants with abnormal platelet function: beige, maroon, pale ear, light ear, and ruby eye.11-18 Being co-isogenic or congenic with C57BL/6, these mutants share the same genetic information as C57BL/6 animals except for a gene affecting platelet function and, in the case of congenic mutants, a small segment of adjacent DNA. Consequently, any reduction in atherosclerosis susceptibility in the mutant animals can be related to their platelet abnormality.

Each strain carries a single mutation affecting platelet function, but the chromosomal location of this altered gene differs among strains.14 Consequently, each mutant strain has a different biochemical alteration even though phenotypically the mutant strains have much in common. To determine whether these biochemical differences have atherogenic significance, we correlated each strain's susceptibility for atherosclerosis with serotonin, a component of platelet dense granules. Serotonin is significantly reduced in all five platelet mutants but to different degrees.14 As shown in this article, no correlation between the serotonin levels and atherosclerosis susceptibility was found among the five mutant strains.

Methods

Animals

Female mice, ages 2 to 4 months, were used in all experiments. The murine strains included C57BL/6 and

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Its congenics carrying the mutants maroon (ru-2nu), light ear (le/le), ruby eye (ru/ru), beige (bg/bg), and pale ear (ep/ep). All mice were originally obtained from the Jackson Laboratory, Bar Harbor, ME; mutants were bred in Robert T. Swank's animal colony at Roswell Park Memorial Institute. The experiments were approved by the Institute's Animal Care and Use Committee.

**Experimental Design for Evaluating Atherosclerosis Susceptibility**

Fourteen control (C57BL/6) and five to nine mice from each mutant strain were used to measure atherosclerosis susceptibility at 14 weeks. All mice were fed an atherogenic diet containing 15% fat, 1.25% cholesterol, and 0.5% cholic acid; the diet was given ad libitum. The onset of the feeding schedule varied for different mutant groups, according to their availability. Consequently, there were three experimental intervals, each including a new group of C57BL/6 controls. There were no significant differences among the three control populations for atherosclerosis susceptibility, so they were analyzed as a single population. Measurement of food consumption showed no significant differences among strains; the grams of food consumed per gram of mouse per day was 0.11 g for pale ear, 0.13 g for light ear, 0.12 g for beige, 0.11 g for ruby ear, and 0.15 g for the control C57BL/6.

At the conclusion of the feeding schedule, the animals were fasted overnight and were sacrificed by CO2 asphyxiation. In each mouse, the heart and an adjacent segment of aorta between the heart and the aortic arch were dissected, washed in physiological saline, fixed in formalin-saline, and evaluated for atherosclerotic lesions.

For evaluation of mortality caused by the atherogenic diet, 8 to 10 female mice of each strain except maroon (which was not available) were fed ad libitum for 48 weeks. Early in the experiment, one cage of light ear mice was lost due to a water bottle leaking, so that only five light ear mice remained in the experiment. Food consumption was determined during the 10th week of the diet by measuring the food at the beginning and end of the week. Animals were checked daily except weekends, and deaths were recorded. We had planned to evaluate lesions in mice that died but were unable to do so for most mice due to deterioration of the carcasses. Lesions were evaluated in mice that survived the entire 48 weeks.

**Evaluation of Aortas for Atherosclerotic Lesions**

Detailed methods for evaluating the aorta for atherosclerosis are described in previous publications. Briefly, a 350 μm segment of aorta was sectioned on a cryostat; single 10 μm thick sections were collected at 80 μm intervals for a total of five sections. The number of atherosclerotic lesions and their cross-sectional areas were determined in each section. The cross-sectional area was measured in μm² with an eyepiece micrometer (20×20 micrometer disc no. 478, American Optical Scientific Instruments, Buffalo, NY). All sections were coded to conceal their identity and were evaluated by one individual. The results for each strain are reported as the number of lesions per section, their average cross-sectional size, and the total lesion area per section.

The histological reagents were obtained as follows: oil red O, from Aldrich Chemical, Milwaukee, WI; hematoxylin light green SF and O.C.T. compound embedding medium from Fisher Chemical, Santa Clara, CA.

**Determination of Platelet Serotonin**

For determination of serotonin, platelets pooled from three mice from each strain were used; three pools were used for C57BL/6 and two pools for the mutant strains. Mice were different from those used in the histological study but were fed the same atherogenic diet. Platelets were obtained by a modification of the method of Holland. To obtain platelets, animals were euthanized with CO2. Before the heart stopped beating, approximately 1 ml of blood was removed from the heart with a 22-G needle and a 1 ml syringe containing 0.1 ml of 3.8% sodium citrate. The blood samples from each strain were pooled, and platelets were counted after dilution in 1% ammonium oxalate according to the method of Brecher and Cronkite. Plasma was spun for 10 minutes at 150 g in a Sorvall centrifuge. The platelet-rich supernatant was decanted and further centrifuged at 1000 g to produce a platelet pellet. Platelet pellets were washed twice with platelet washing solution and then stored at −70°C until assayed. When platelets are prelabelled with 3H-serotonin, the washing procedure continues until counting shows no further release.

The histological reagents were obtained as follows: oil red O, from Aldrich Chemical, Milwaukee, WI; hematoxylin light green SF and O.C.T. compound embedding medium from Fisher Chemical, Santa Clara, CA.

**Table 1. Atherosclerosis In Strain C57BL/6 and In Five Mutants with Altered Platelet Function**

<table>
<thead>
<tr>
<th>Mice strain</th>
<th>Number of mice</th>
<th>Total lesion area/section (μm² × 10⁵)</th>
<th>Average number of lesions/section</th>
<th>Average lesion size (μm² × 10⁴) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>14</td>
<td>1.02±0.20</td>
<td>0.7±0.09</td>
<td>1.45±0.23 (45)</td>
</tr>
<tr>
<td>Pale ear</td>
<td>5</td>
<td>1.59±0.45</td>
<td>0.6±0.11</td>
<td>2.49±0.55 (16)*</td>
</tr>
<tr>
<td>Beige</td>
<td>4</td>
<td>0.67±0.33</td>
<td>0.6±0.20</td>
<td>1.49±0.64 (9)</td>
</tr>
<tr>
<td>Maroon</td>
<td>8</td>
<td>0.50±0.10*</td>
<td>0.3±0.10*</td>
<td>0.79±0.09 (25)*</td>
</tr>
<tr>
<td>Light ear</td>
<td>8</td>
<td>0.19±0.06†</td>
<td>0.3±0.07†</td>
<td>0.68±0.14 (11)*</td>
</tr>
<tr>
<td>Ruby eye</td>
<td>9</td>
<td>0.16±0.06†</td>
<td>0.3±0.08†</td>
<td>0.60±0.16 (12)*</td>
</tr>
</tbody>
</table>

Statistical evaluation utilized Student's t test. The number of observations for columns 3 and 4 was five times the number of mice, since five sections were used for each mouse. n, the number of observations for column 5, is the total number of lesions in all mice of the strain. Thus, strains that had no lesion at all in many sections, such as light ear and ruby eye, had a small n.

*p < 0.05, †p < 0.005.
Table 2. Serotonin Levels in Mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotonin levels*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal C57BL/6</td>
<td>1.72±0.23</td>
</tr>
<tr>
<td>Light ear</td>
<td>0.63</td>
</tr>
<tr>
<td>Pale ear</td>
<td>0.30</td>
</tr>
<tr>
<td>Beige</td>
<td>0.12</td>
</tr>
<tr>
<td>Ruby eye</td>
<td>0.30</td>
</tr>
<tr>
<td>Maroon</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*Serotonin levels are expressed as µg serotonin per 10⁹ platelets. The standard error of the mean is given for C57BL/6 because three samples of pooled platelets from three mice were tested. The average of two samples of pooled platelets from three mice are provided for the mutant strains.

Results

Table 1 compares atherosclerosis susceptibility for the five platelet mutants and the control C57BL/6 strain. For total lesion area, only the pale ear mutant had as extensive disease involvement as the control. In contrast, mutants light ear and ruby eye had significantly fewer aortic lesions (p<0.005). The total lesion area of beige and maroon mutants was approximately half that of the control, but only maroon was significantly different from C57BL/6 (p<0.05). The failure of beige mutants to reach statistical significance may be due to sample size; four of the eight mice in this group died of an infectious disease during the experimental period. Increased susceptibility to infection has been documented in this mutant.²¹

To determine whether the reduced atherosclerosis susceptibility was due to fewer lesions or to a reduced size of lesions, mutants were compared for number and size of lesions. Of the three mutant strains with significant disease reduction, only light ear and ruby eye had significantly fewer numbers of lesions per section (p<0.05). Their lesions were also smaller than lesions in the control mice. These differences in lesion size, however, were less significant than the differences in lesion number.

The results of the serotonin assays are shown in Table 2. The control C57BL/6 strain had the highest serotonin levels with an average of 1.72±0.23 µg/10⁹ platelets. Platelet serotonin is significantly reduced in all the mutant strains with beige mice expressing the lowest levels. The serotonin levels are similar to those reported earlier by Novak et al.¹⁴ in mice fed normal chow rather than an atherogenic diet. In Figure 1, the total lesion area per section for each mutant is plotted against each mutant's level of serotonin (Figure 1). The results show no correlation between the serotonin levels and atherosclerotic susceptibility.

Figure 1. Correlation of atherosclerosis susceptibility with platelet serotonin levels. The extent of atherosclerotic lesion area/aortic cross-section is correlated with the serotonin levels in platelets. Bars indicate the standard errors. The data are from Tables 1 and 2.

To determine whether an increased bleeding time and reduced platelet function protected against the more advanced stages of atherosclerosis, 8 to 10 mice of each strain (the control C57BL/6 and beige, ruby eye, light ear, and pale ear) were fed the atherogenic diet for 48 weeks. Food consumption measured during the 10th week of the diet did not differ among the groups. Previous unpublished experiments have shown that few control C57BL/6...
mice survive beyond 42 weeks on an atherogenic diet, and the major pathology observed was constriction and occlusion of coronary arteries by atherosclerotic lesions.

The survival curves depicted in Figure 2 show that C57BL/6 and pale ear mice had similar survival curves; no mice survived the entire 48 weeks. No beige mice survived the 48 weeks either, but beige mice began to die much earlier than the other strains, probably due to the increased susceptibility to infection. Light ear and ruby eye, the two mutants with the least degree of atherosclerosis at 14 weeks (Table 1), showed 56% and 60% survival at 48 weeks. The animals surviving at 48 weeks were sacrificed, and both mutants had substantial lesions (Table 3). However, the lesion area was substantially less than lesions in C57BL/6 mice sacrificed at an earlier time point, 39 weeks. The data for C57BL/6 mice are from another experiment, which occurred during the same time period in our laboratory and are included for comparative purposes.

### Discussion

This study used genetic variants of the atherosclerosis-sensitive C57BL/6 strain to evaluate the role of platelets in atherosclerosis. These mutants have a genetic lesion affecting the function of three subcellular organelles: melanosomes, lysosomes, and platelet dense granules.14 Their platelet abnormalities are characterized by prolonged bleeding times and a reduction in the contents of platelet dense granules, such as serotonin.14,15 The mutants are congenic with control C57BL/6 mice. Except for the mutation affecting platelet function and a small section of adjacent DNA equivalent to less than 0.1% of the genome, the genetic background is constant. Therefore, by comparing their atherosclerosis susceptibility to that of the C57BL/6 controls, the effect of essentially single-gene alterations in platelet function on the atherosclerotic process can be determined. These mutants also allow us to test whether the presence of normal platelet activity is essential for the development of atherosclerotic disease.

Our study revealed that three of the five platelet mutants, ruby eye, light ear, and maroon, had significantly increased resistance to experimentally induced atherosclerosis. These results suggest that impaired platelet performance can affect the disease process of atherosclerosis. In contrast, the mutant, pale ear, had extensive disease involvement which was similar to the control, indicating that normal platelet function is not essential for the genesis or exacerbation of atherosclerosis.

The five platelet mutants in this study have genetic lesions that are mapped to different chromosomes.14 As a result, their alterations in platelet function are biochemically distinct. Some, but not all, platelet mutants show reduced atherosclerosis susceptibility. The identity of the biochemical link between platelet function and atherosclerosis susceptibility is unknown. To determine whether the atherogenic agent is associated with platelet dense granules, we correlated a mutant's susceptibility to atherosclerosis with its concentration of serotonin, a dense granule component. Despite the fact that all platelet mutants have significantly reduced serotonin content,14,15 there was no correlation between a mutant's atherosclerosis susceptibility and its platelet serotonin level.

The ability of the mutants to survive the atherogenic diet was more closely related to each mutant's susceptibility to lesion formation than to its decreased platelet function. All of the mutants have similar increases in bleeding time, yet they differed considerably in susceptibility to atherosclerosis and survival on the atherogenic diet. The mutant, pale ear, had atherosclerosis susceptibility and survival curve similar to the control C57BL/6. The mutants, ruby eye and light ear, had reduced susceptibility to atherosclerosis and increased survival. The only mutant that did not fit the pattern was beige, a mutant with reduced atherosclerosis but the poorest survival. We ascribe this poor survival to the fact that beige mice have reduced immune function as a consequence of the mutation.21

These results indicate that some particular component of platelet function affects atherosclerosis susceptibility. That component is intact in pale ear, moderately affected in beige and maroon, and severely affected in light ear and ruby eye. The identity of that component remains an interesting question whose answer may provide further insight into the atherosclerotic disease process.

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