The Mouse Pale Ear Pigment Mutant as a Possible Animal Model for Human Platelet Storage Pool Deficiency

By Edward K. Novak, Sek-Wen Hui, and Richard T. Swank

The mouse pigment mutant pale ear, ep/ep, which has a defect in kidney lysosomal enzyme secretion, had prolonged bleeding on experimental injury. Platelet counts and platelet protein did not differ from normal. There was, however, a deficiency in the platelet dense granule contents, serotonin, ATP, and ADP. Furthermore, a marked reduction of platelet dense granules was observed by electron microscopy. The results suggest that pale ear is a useful animal model in the study of platelet storage pool disease. Studies on this mutant and other pigment mutants have established that one gene can regulate at least three subcellular organelles, including the melanosome, the lysosome, and the platelet dense granule.

Another animal with altered pigmentation, the fawnhooded rat, has likewise been suggested to be an animal model for platelet storage pool disease. We, therefore, examined the pale ear mouse, a pigment mutant with defective secretion of lysosomal enzymes, for abnormalities in bleeding. The results indicate that the pale ear mouse has a bleeding problem caused by a deficiency in dense granules and dense granule contents. We propose the pale ear mouse as a promising animal model for the study of human platelet storage pool diseases, such as Hermansky-Pudlak syndrome.

MATERIALS AND METHODS

Animals

Male C57BL/6J +/-, C57BL/6J ep/+ , and C57BL6J ep/ep mice were obtained from the Jackson Laboratories, Bar Harbor, Maine and later bred at the animal facilities of Roswell Park Memorial Institute. Female mice (3-4 mo old) were used for bleeding times. Male mice (4-6 mo old) were used in all other studies.

Platelet Collection

Platelets were collected by a modification of the method of Holland. Blood was collected through a 22-gauge needle in a 1 ml syringe containing 0.1 ml of 3.8% sodium citrate and was transferred to plastic centrifuge tubes at 4°C. The supernatant from centrifugation at 150 g for 10 min (platelet rich plasma) was used for electron microscopy. For other studies, the blood was brought to 3.0 ml with 0.85% NaCl and platelet rich plasma, platelet poor plasma, and platelet pellets were collected according to Holland. The washed platelet pellet was either stored at -20°C or used immediately for adenine nucleotide assay.

Platelet Counts

Platelets were counted after dilution in 1% ammonium oxalate according to Brecher and Cornkite.

Bleeding Time

A 2 mm portion of the tip of the tail of unanesthetized mice was severed using a sharp razor blade. The tail was blotted with filter paper at 15-sec intervals until bleeding stopped.

Platelet Serotonin Assay

Platelet pellets were lysed in 1 ml of distilled water and assayed for serotonin by the method of Crosti and Luchelli.
0.85% NaCl was added. Thrombin (1.25 U) in 0.1 ml 0.85% NaCl solution (minus calcium plus magnesium). To control tubes extracellular levels (5% of intracellular levels) of the soluble enzyme platelets were washed twice and resuspended in 1.0 ml Tyrodes ethanol-EDTA solution was added directly to platelet pellets or 0.1 ml cold ethanol-EDTA solution (9 volumes of ethanol + 1 volume of 0.1 M EDTA). Dessicated firefly tails (Sigma) (100 mg) were immersed in the cold. Platelet pellets were removed by centrifugation and both pellet and supernatant were mixed with ethanol-EDTA solution. Aliquots were either removed for determination of radioactivity or were stored at -20°C for adenine nucleotide assay. Under these secretion conditions there was no increase in the lactic dehydrogenase.

Platelet Adenine Nucleotides

Platelet adenine nucleotides were determined by a modification of the method of Holmsen, Storm, and Day.22 Fresh platelet pellets were lysed in 0.5 ml H2O at 4°C followed by addition of 0.5 ml of cold ethanol-EDTA solution (9 volumes of ethanol + 1 volume of 0.1 M EDTA). Dessicated firefly tails (Sigma) (100 mg) were stirred in 10 ml of 0.5 M sodium arsenate buffer pH 7.4 according to Beutler and Baluda23 for at least 2 hr before use. Standards of ATP, ADP, and ATP plus ADP were included in all experiments. Luminescence intensity was determined using an Amino fluoro-colorimeter to which was attached a Chem-glow reaction chamber (American Instrument Company). Similar results were obtained if ethanol-EDTA solution was added directly to platelet pellets or platelet rich plasma.

Thrombin Stimulated Platelet Secretions

To platelet rich plasma 10 µCi of 3H serotonin (Amersham Searle) was added and allowed to incubate for 30 min at 37°C. The platelets were washed twice and resuspended in 1.0 ml Tyrodes solution (minus calcium plus magnesium). To control tubes 0.1 ml 0.85% NaCl was added. Thrombin (1.25 U) in 0.1 ml 0.85% NaCl was added and incubated at 37°C 3 min in a shaking water bath. After 3 min 50 µl of 0.1 M EDTA was added and the tubes were immersed in the cold.24 Platelet pellets were removed by centrifugation and both pellet and supernatant were mixed with ethanol-EDTA solution. Aliquots were either removed for determination of radioactivity or were stored at -20°C for adenine nucleotide assay. Under these secretion conditions there was no increase in the extracellular levels (5% of intracellular levels) of the soluble enzyme lactate dehydrogenase.

### RESULTS

When mice homozygous for the pale ear gene were experimentally injured, the time required to stop bleeding was much longer than normal. Six of seven mice bled more than 15 min, while normal mice bled 3-4 min (Table 1). Bleeding times in heterozygous mice were not significantly different from normal mice.

A deficiency of platelets could not account for these prolonged bleeding times, since platelet counts were equal in normal and pale ear mice (Table 1). This result, plus reports linking albinism to platelet storage deficiency,5 encouraged us to test normal and pigment mutant platelets for a deficiency in dense granule contents.

In platelets of pale ear mice, serotonin concentration was only 20% of normal (Table 2), suggesting a deficiency in the storage pool of dense granules. The serotonin concentration of ep/+ heterozygous mice was not significantly different from normal mice. Also, serotonin concentration of platelet poor plasma was negligible in normal and pale ear mice (not shown). As a further test of a possible dense granule deficiency in pale ear mice, the adenine nucleotides of normal and pale ear platelets were measured. Total

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Serotonin (µg/10^9 Cells)</th>
<th>ATP + ADP (µmole/10^11 Cells)</th>
<th>ATP (µmole/10^11 Cells)</th>
<th>ADP (µmole/10^11 Cells)</th>
<th>ATP/ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>5.29 ± .51</td>
<td>17.5 ± 1.48</td>
<td>11.9 ± 1.20</td>
<td>5.58 ± 0.78</td>
<td>2.14</td>
</tr>
<tr>
<td>ep/ep</td>
<td>1.05 ± 1.16*</td>
<td>7.81 ± 0.78*</td>
<td>5.91 ± 0.51†</td>
<td>1.89 ± 0.43†</td>
<td>3.13</td>
</tr>
<tr>
<td>ep/+</td>
<td>5.86 ± .54</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean ± SEM for platelets from 8-10 male mice except for 3 male ep/+ mice.

*p < 0.001.

†p < 0.01.
Table 3. Thrombin Stimulated Release of Dense Granule Contents

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Thrombin Units</th>
<th>ATP Released (% of Total)</th>
<th>ADP Released (% of Total)</th>
<th>ATP:ADP Released</th>
<th>$^3$H Serotonin (% of Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
<td>3.13% ± .86%</td>
<td>8.7% ± .92%</td>
<td>4.0% ± 5.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>62.3% ± .89%</td>
<td>64.9% ± 8.7%</td>
<td>2.05</td>
<td>91.0% ± 1.1%</td>
</tr>
<tr>
<td>Pale ear</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>5.8% ± .85%</td>
<td>75.0% ± .85%</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>75.0% ± .85%</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM for 4 normal and 2 pale ear mice.

Adenine nucleotides (ATP plus ADP) were twofold greater in normal mouse platelets than those of pale ear (Table 2). ATP values were twofold greater in normal mice. However, ADP concentrations were threefold greater in normal mouse platelets than pale ear platelets. This resulted in a shift of the ATP/ADP ratio from 2.14 in normal mice to 3.13 in pale ear mice. The shift in this ratio is thus in the direction characteristic of platelet storage pool disease.

To test whether the deficiency in adenine nucleotide levels was due to a deficiency in the granule pool or cytoplasmic pool, normal and pale ear mouse platelets were stimulated to secrete their dense granule contents with thrombin. Under conditions (Table 3) in which more than 90% of the $^3$H serotonin CPM were released in normal platelets, over 60% of both ATP and ADP were released. The ratio of ATP to ADP in the secreted pool was 2.05. Thus the secretable (granule) pool of adenine nucleotides in murine platelets has a significantly higher ratio of ATP:ADP as compared with the value of 0.67 found in human platelets.

When pale ear platelets were treated in a similar fashion, 75% of $^3$H serotonin was released while only negligible amounts of ATP and ADP were released, consistent with a lowered granular pool of adenine nucleotides. Thus most of the difference of adenine nucleotides between normal and pale ear platelets can be accounted for by a deficiency in the secretable granular pool.

Since dense granule contents were reduced in platelets of pale ear mice compared to normal mice, we directly examined dense granules in platelets of normal and pale ear mice by electron microscopy (Figure 1A and 1B). Air-dried platelets appeared

Fig. 1. Typical electron micrographs of instantaneously air-dried and unstained whole platelets from (A) normal $+/+$ mouse and (B) pale ear $ep/ep$ mouse. Both dense granules (large arrow) and small, bead-like particles (small arrow) are visible (A). Bar represents 1 nm.
deficiency that has symptoms in common with certain enzymes, \( \beta \)-glucuronidase and \( \beta \)-galactosidase. The platelets were tested for two lysosomal enzymes and serum, and platelets were observed. There were no differences in dense granules per platelet in normal and mutant platelets. The number of granules in normal mice and in mutant platelets was noted (Fig. 2). The number of granules in normal mice was widely distributed with a mean of 6.4 dense granules per platelet. However, many platelets in pale ear mice had no dense granules and a mean of 1.2 dense granules per platelet was observed. Therefore, the platelets from pale ear mice are deficient in both dense granule storage contents and in the actual number of dense granules.

Since pale ear was previously characterized to have altered lysosomal enzyme concentration in kidney and serum, platelets were tested for two lysosomal enzymes, \( \beta \)-glucuronidase and \( \beta \)-galactosidase. The platelet concentration of both enzymes were normal in pale ear mice (Table 4).

**DISCUSSION**

The pale ear mouse has a platelet storage pool deficiency that has symptoms in common with certain human platelet storage pool diseases. These characteristics are a bleeding tendency that is accompanied by a marked decrease in the number of dense granules and dense granule contents with no effect on platelet number. Serum clotting factors, which are normal in human platelet storage deficiency, have not been tested in pale ear mice.

In two human platelet storage pool diseases, the Chediak-Higashi syndrome and the Hermansky-Pudlak syndrome, there is an accompanying abnormality in pigmentation and in lysosome function. Abnormal kidney lysosome function has been described in the pale ear mouse. Chediak-Higashi patients have diluted pigmentation accompanied by giant melanosomes. They likewise have giant lysosomes in many tissues. Hermansky-Pudlak patients are tyrosinase-positive albinos, like Chediak-Higashi patients, but the giant subcellular granules typical of the latter have not been reported. In both diseases, defective lysosomal enzyme secretion from platelets has been reported.

Pale ear, a tyrosinase-positive mutation on chromosome 19 of the mouse, has diluted pigmentation at the extremities of the ears and tail, possibly caused by abnormally small melanosomes. As in Chediak-Higashi and Hermansky-Pudlak patients, the mutation is recessively inherited and has accompanying lysosomal abnormalities. In particular, pale ear mice have defective secretion of kidney lysosomal enzymes and a two- to threefold elevation of serum lysosomal enzyme concentrations. However, recent studies from this laboratory have established that the pale ear mouse lacks the giant lysosomes typical of Chediak-Higashi cells and of cells of the beige mouse, an animal model for this disease. The pale ear mouse thus more closely approximates the abnormalities present in Hermansky-Pudlak patients.

Patients with the Hermansky-Pudlak syndrome have a relatively mild bleeding tendency. This is consistent with effects of the pale ear gene. We are not aware of overt pathologic manifestations of the pale ear gene although we have not studied mice greater than 8 mo old. The finding of normal lysosomal enzyme concentrations in platelets of pale ear mice despite greatly reduced dense granule contents suggests that in mice as in humans, serotonin and adenine nucleotides reside in subcellular organelles separate from those containing lysosomal enzymes. Platelet lysosomal enzyme concentrations are likewise normal in the human storage pool diseases, Chediak-Higashi syndrome and Hermansky-Pudlak syndrome.

The molecular basis of the storage pool defect in patients or in animal models is unknown. In storage pool disease, abnormalities in plasma membrane glycoproteins and in lipids have been reported in
members of one family. In Chediak-Higashi patients and/or animal models of this disease, abnormalities in membrane fusion, cyclic nucleotide levels, microtubule polymerization, and a deficiency of the leukocyte enzyme elastase have been reported. Which, if any, of these defects is the primary lesion in the disease remains to be established. The availability of genetically defined animal models like the pale ear and beige mutants are congenic to C57BL/6J, having been backcrossed to the parent strain 20 times, the possibility is very high that the effects on the three subcellular organelles are caused by a single mutation at the pigment gene.

The conclusion seems inescapable, therefore, that there are master genes that simultaneously affect the structure and/or function of several different intracellular organelles. The conclusion is supported by reports of defective platelet dense granules in human albinos and in the fawn-hooded rat and of defective lysosomal function in six mouse pigment mutants in addition to the pale ear and beige mutants. Thus mutations which simultaneously affect pigmentation, lysosome function, and platelet dense granule function have been found in humans and animals. The analysis of genetically defined animal models should aid our understanding of the actions of these genes in normal cellular processes and in disease states.

ACKNOWLEDGMENTS

We thank Charlotte Abraham and Cynthia Stewart for skillful technical assistance and Cynthia Bell and Gail Walsh for secretarial aid.

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