The Murine Misty Mutation: Phenotypic Effects on Melanocytes, Platelets and Brown Fat

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Manuscript received June 6, 1997
Accepted for publication September 15, 1997

ABSTRACT

Although the recessive murine mutation misty (m) is well known, its phenotype has never been reported beyond brief descriptions of a dilution of coat color and white spotting of the belly and extremities, suggesting a developmental mutation. A report in abstract has also suggested effects on white fat and body weight. Here, we report effects of the homozygous misty mutation on an unusual combination of three cell types: melanocytes, platelets, and brown fat. Brown fat appeared to be completely absent from all expected locations in neonatal m/m mice. A prolonged bleeding time was observed; platelet count and platelet serotonin and ATP levels were normal, but the level of ADP in m/m platelets was low. Primary cultures and immortal lines of melanocytes from m/m mice showed several abnormalities. There was a marked deficiency in net proliferation, suggesting that the color dilution and spotting in vivo may result from reduced numbers of melanocytes and their precursors. m/m melanocytes were also hyperdendritic in morphology, overproduced melanin, and had deficient responses to the CAMP agonist cholera toxin and melanocyte-stimulating hormone, which normally promote melanin production. The misty gene product may be involved in adenine nucleotide metabolism or signaling.

MELANOCYTES are ideally suited to genetic analysis, because germline mutations affecting pigmentation are often nonlethal, readily visible and thus easily selected. About 85 coat-color loci are now known in the mouse (Silvers 1979; Bennett 1993; Doolittle et al. 1996; Mouse Genome Database (MGD), June 1997. Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, ME, http://www.informatics.jax.org/). Many of these are involved in the function of melanocytes, but around 33 act in their development (Bennett 1993; Mouse Genome Database); these are the spotting loci, which reduce the area of skin and hair that is pigmented, while not usually affecting eye color (Silvers 1979). The technique of melanocyte culture, pioneered with human (Eisinger and Marko 1982) and mouse (Sat o et al. 1985; Bennett et al. 1987; Tamura et al. 1987) cells, has proved valuable in molecular studies of color genes, for example, the albino and brown loci, encoding the melanosomal enzymes tyrosinase and TRP1 (Yamamoto et al. 1989; Bennett et al. 1990; Jackson and Bennett 1990). The sites of a number of the developmental (spotting) mutations have been cloned. These include the loci Sl, W, Is, s, Sp and mi, encoding, respectively, the growth factor SCF (stem cell factor) and its tyrosine kinase receptor, the cytokine endothelin 3 and its receptor Ednr b, and the transcription factors Pax-3 and Mi/MITF (Bennett 1993; Baynash et al. 1994; Jackson 1994). Most of these genes have also proved to be loci for human genetic defects, including piebald trait (W/KIT), Hirschsprung’s disease (s/EDNRB) and Waardenburg’s syndrome types 1 (Sp/PAX3) and 2 (mi/MITF) (Bennett 1993; Baynash et al. 1994; Tassabehji et al. 1994).

Another such spotting locus is misty (m). Although it is well known, as an anchor locus in chromosomal mapping, little is understood about this mutation. It arose spontaneously in the DBA/J strain of mice over 50 years ago (Woolley 1941, 1945). It is located on chromosome 4, 7.2 cm distal to brown. There is no molecular information on this locus, although several PCR-based sequence markers have been mapped near to misty (Fiedor et al. and Kay 1994), and a recent abstract has suggested a deficiency in body weight and white fat in older m/m mice (Truett et al. 1996). Homozygous misty mice typically show paler pigmentation on black, brown and agouti backgrounds, interpreted as a dilution of eumelanin (black melanin), but also have a white tail-tip and feet and a belly streak or spot, suggesting reduced numbers of melanocytes or their precursors (Silvers 1979). As no illustration of these coat features has been published to our knowledge, we include one here (Figure 1).

We became interested in misty as a candidate developmental mutation that might affect late melanoblast development. Because so little is known of the misty...
phenotype, we initially performed a general histological survey of neonatal misty mice to check for gross abnormalities other than in color. As another general study, we tested whether misty formed one of the group of 14 pigmentary mutations, such as beige, ruby-eye and pale ear (Swank et al. 1991; Swank et al. 1996; Bennett 1993), that also produce platelet storage pool deficiency (Rao 1990) and, generally, lysosomal abnormalities as well. Similar disorders occur in humans (Spritz and Hearing 1994; King et al. 1995). For example, Chediak-Higashi syndrome is homologous to the murine beige mutation (Barbosa et al. 1996; Nagle et al. 1996; Perou et al. 1996), while pale ear was recently reported to be a homolog of Hermansky-Pudlak syndrome (Feng et al. 1997). Lastly, we prepared cultures of m/m melanoblasts and melanocytes to test for any effect on cell proliferation, differentiation, survival or other property of this lineage, with striking results.

MATERIALS AND METHODS

Materials: Tissue culture plastics (Nunc) and fetal calf serum (FCS) were from GIBCO Europe (Uxbridge, UK). 12-O-tetradecanoyl phorbol acetate (TPA), cholera toxin (CT), mitomycin C, 2-mercaptoethanol (2-ME), phenylthiourea (PTU; also known as phenylthiocarbamid) and [4-Nle,7-d-Phe]-α-melanocyte stimulating hormone (here abbreviated to N-MSH) were from Sigma Chemical (Poole, UK). Basic fibroblast growth factor (bFGF) (bovine) was supplied by Peninsula (St. Helens, UK). All protein factor stock solutions were prepared in phosphate-buffered saline (PBS) with bovine serum albumin (1 mg/ml) as carrier and stored at −70°C.

Histology: Mice homozygous for the misty mutation but on an outbred background (C3H/HeH × 101/H) carrying variable agouti (A, a/a) and brown (B, b) alleles were obtained from Beechey (MRC Radiobiology Unit, Didcot, UK). misty was separated from the other mutations and combined with non-agouti (black: a/a, B/B), a standard genetic background for study of color mutations, by backcrossing to the C57BL/6j strain every two generations, alternated with sibling mating and selection for the misty nonagouti phenotype. After about eight backcross generations, when no other color alleles were being segregated, a/a, m/m litters were obtained from parents of this genotype. One-day-old mice were killed and fixed whole in formal-calcium (formalin with calcium acetate). They were paraffin embedded, and transverse 7 μm sections were cut at various body levels. These were dewaxed, stained with Ehrlich’s alum hematoxylin and eosin, and mounted in Histomount for bright-field microscopy and photography.

Feeder cells: Keratinocyte feeder cells were from the immortal XB2 murine keratinocyte line, provided by Jim Rheinwald (Rheinwald and Green 1975), and were prepared as described previously (Bennett et al. 1989), using mitomycin C treatment for 3 hr followed by resuspension and freezing. Feeder cells were thawed and plated, usually 1 day before use or earlier on the same day.

Primary cultures of melanoblasts and melanocytes: Primary melanocyte cultures were made from trunk skin of neonatal a/a, m/m mice (obtained as above) and control C57BL/6j (a/a +/+ ) mice, by procedures described previously (Sviderskaya et al. 1995). In short, the skin was split with trypsin, epidermal sheets were pooled and dissociated briefly with trypsin and EDTA, and the resulting cell suspension was plated on to XB2 immortal keratinocyte feeder cells. The volume of culture prepared per mouse was 12 ml in six 3-cm dishes for growth/differentiation experiments or 8 ml in two 5-cm dishes for immortalization. The basic growth medium was RPMI-1640 supplemented with penicillin, streptomycin, glutamine (2 mm) and FCS (5%; 10% for cell lines once free

Figure 1.—Coat color phenotype of misty mice. A typical m/m mouse of the C57BL/6j black strain is compared to a +/+ mouse of the same strain. The dorsal view (A) shows the slightly paler fur, normal eyes and white feet of the m/m mouse (right), while ventrally (B) the typical white belly spot is visible in the m/m mouse (left). The white tail-tip is also visible. Pale fur around the nipples (seen in +/+ mouse, which is female) is normal in nonagouti mice.
of fibroblasts). Other supplements, where specified, were added to primary cultures after cell attachment or 1 day later. Incubation was with 10% v/v CO\textsubscript{2} at 37\textdegree, which gives a suitable pH of 7.0–7.1.

**Serial passage of melanocytes:** Cultures for the preparation of cell lines were grown in RPMI-1640 growth medium, with various supplements as discussed (see results). New feeder cells were added after 2–3 wk if a culture was not passaged by then. Cells were subcultured by one rinse in Dulbecco’s PBS lacking calcium and magnesium chlorides (PBSA) and one rinse in PBSA containing 250 μg/ml trypsin and 200 μg/ml EDTA; they were incubated until completely detached and resuspended in growth medium for dilution as required. Misty cell lines, once established, were grown in RPMI growth medium with 10% FCS, 200 nM TPA and 100 μM 2-ME. The tyrosinase inhibitor PTU was also added at 100 μM for a few days before freezing and after thawing cell stocks in liquid nitrogen, and at 200 μM to the frozen stocks; this reduced melanin content and appeared to improve viability of stocks. Indeed, we are now similarly using PTU for freezing of all pigmented melanocyte lines.

**Melan-a (a/a) and melan-c (albino, c/c) melanocytes** were grown as described (Bennett et al. 1989). Mel-18 and mel-29 are melanocyte lines obtained from reversion spots on W\textsuperscript{ro} + mice (De Sepulveda et al. 1995). These cells had lost the W\textsuperscript{ro} allele by somatic recombination and are thus wild-type (De Sepulveda et al. 1995). These were provided by J. Pantier and grown in RPMI-1640 growth medium supplemented with FCS (10%), 2-ME (100 μM) and TPA (200 nM).

**Cell proliferation and melanin assays:** Triplicate cultures were plated at the specified density on 3-cm dishes for counting or 3–10-cm dishes for melanin assays and cultured as specified. Media were renewed twice weekly. Cells were harvested separately from each plate by trypsinization. Triplicate cell counts from each suspension were made by hemocytometer.

**Primary cultures for growth/differentiation experiments** were plated in 3-cm dishes on XB2 feeder cells and were grown in the media and for the times specified. Counts of melanocytes and melanoblasts were done on formaldehyde-fixed cells as before (Sväder skyä et al. 1995).

For melanin assays, at least two experiments for each cell line were performed. Cells were plated at 1.5 × 10\textsuperscript{4} cells/ml and grown until they were about 75% confluent (about 7–9 days for a/a lines, 11–14 days for m/m lines). Melanin assays were carried out as described (Friedmann et al. 1990). After removal of an aliquot for counting, the remaining cells were pelleted; pellets were resuspended in 100 μl of 1 M NaOH and diluted with water (400 μl). The OD\textsubscript{470} was measured and converted to melanin content via a standard curve using synthetic melanin (Sigma). This was normalized to cell number. To exclude photometric absorbance not due to melanin, we performed assays on melan-c albino (unpigmented) melanocytes in the same way, and the mean absorbance per melan-c cell was subtracted from the mean values for other lines.

**Bleeding times and platelet analyses:** For bleeding time and platelet analyses, misty mice of either the C57BL/6 or the C57BLKS/ strain were purchased from the Jackson Laboratory (Bar Harbor, ME) together with appropriate control mice of the same strain, and breeding colonies of each were maintained at Roswell Park Cancer Institute. No significant differences between the two misty strains were noted in any hematological parameter. Also, no differences were noted between male and female mice. Mice 6–12 wk old were used.

Bleeding times were determined by tail bleeding time essentially as described (Dejana et al. 1979; Novak et al. 1988). A 2-mm portion of the tail was severed with a sharp razor blade and the tail was immersed in a beaker of physiologic saline at 37\textdegree. Each mouse was maintained in a horizontal position in a restrainer with the tip of the tail 4–5 cm below the body. The time required for the small stream of blood to stop abruptly was taken as the bleeding time. Statistical analyses of bleeding times and other parameters were performed with the Student’s t-test.

Platelets were counted manually (Br ccker and Cronkite 1950). Platelets were collected for serotonin and adenine nucleotide assays as described (Swank et al. 1991). For serotonin analyses, platelets were lysed in 1 ml of distilled water and assayed fluorometrically as described by Crost i and Lucchelli (1962). Platelet adenine nucleotides were determined by a luciferin-luciferase system (Novak et al. 1981).

**RESULTS**

**General histology, deficiency of brown fat:** Organs were compared between m/m and +/+ C57BL/6j mice using sections through 1-day-old mice of both genotypes at similar levels. Abnormalities were found in only two organs. One was the skin, where the deep portions of hair follicles were mostly well pigmented in wild-type mice but poorly or unpigmented in neonatal misty mice, consistent with the overall m/m phenotype of diluted coat color. The other was the brown (multilocular) fat that forms interscapular and lateral thoracic (auxiliary) aggregations or pads in mice and other rodents (Blooom and Fawcett 1986); these fat pads were clearly visible in the wild-type mice (Figure 2, A and C). In m/m mice they were absent, being replaced by a thin layer of fibrous connective tissue in the interscapular location (Figure 2B) and by a compact, almost epithelioid layer of roundish cells in the auxiliary position (Figure 2D). It was possible that these roundish cells were brown (multilocular) adipocytes lacking lipid, because either immature adipocytes (Néchad 1986) or brown adipocytes depleted of lipid following hormonal or thermal stimulation (Blooom and Fawcett 1986) have a somewhat similar epithelioid appearance. No brown fat was detected in m/m mice in other expected positions either: in the perirenal area or around the aorta and great vessels of the neck (Blooom and Fawcett 1986).

The outbred misty mice initially obtained seemed relatively small at birth, but after backcrossing to the inbred C57BL/6j strain for several generations, the birth weight of m/m mice (1.44 ± 0.08 g, n = 8) was similar to that of +/+ mice of this strain (1.42 ± 0.02 g, n = 90). Litter sizes were noted because mice in larger litters tend to be smaller. Mean sizes of the litters examined were similar however: m/m, 8.38: +/+ , 6.80. Body weight in older mice was not studied.

**Misty melanoblasts and melanocytes in primary culture:** To study the general properties of misty melanoblasts and melanocytes, primary cultures were prepared from neonatal m/m skin in “melanocyte medium” containing TPA and choleratoxin (CT), a cAMP agonist that strongly stimulates growth and differentiation of wild-type cultures (Sväder skyä et al. 1995). In this me-
Figure 2.—Transverse histological sections through C57BL/6J m/m and +/+ neonatal mice at thoracic level, showing altered brown fat and hair follicle pigmentation. In the dorsal region, the interscapular brown fat of +/+ mice (A) is absent and replaced by a layer of fibrous connective tissue in m/m mice (B) (straight arrows). The bases of most hair follicles were well pigmented in +/+ neonatal skin (curved arrow, although this was difficult to show photographically), but not in m/m skin. The typical lateral (axillary) brown fat of +/+ mice (arrow, C) was also lost in m/m mice (D), but replaced in this region mainly by a somewhat epithelioid tissue (arrow) (see text). Scale bar, 100 μM.
of wild-type melanoblasts fell by around 90%; this was mainly through differentiation to melanocytes, as judged both by microscopy, and because the ratio of melanocytes to melanoblasts increased, for example, from about 10:1 (14 days) to about 40:1 (21 days) with CT (Figure 4). Conversely, the numbers of m/m melanocytes and melanoblasts remained steady between 14 and 21 days, either with or without CT, as did their ratio (about 10:1). Thus, neither growth nor differentiation of the m/m primary cultures was significantly stimulated by CT (Figure 4).

Establishment of immortal lines from misty melanocytes: The deficient proliferation of misty melanocytes caused prolonged difficulties with the establishment of cell lines, but eventually three such lines were isolated from misty cultures: melan-m1, melan-m2, and melan-m3, as follows. These lines were from simultaneous but separate primary cultures and were kept separate throughout. All were grown with XB2 keratinocyte feeder cells, which were replaced regularly (see material and methods). Initially, a variety of culture supplements were tested, including those used for melanoblasts grown without serum by Hirobe (1992). However, under our conditions no improvement was seen over the medium supplemented with TPA (200 nM) and CT (200 pM), which was therefore used from 3 wk onward. Poor cell growth with much cell death continued. Moreover, microscopy of the cultures suggested that the misty melanocytes were more darkly pigmented than normal. Because melanin intermediates can be toxic at high concentrations, the tyrosinase inhibitor PTU (50 \( \mu \)M) was added to all dishes from the sixth week of culture to reduce melanin synthesis. Growing colonies of cells were now observed. Two months later the concentration of PTU was increased to 100 \( \mu \)M. After 5 mon of culture and subculture, melanocytes appeared to be growing without fibroblasts, so the medium was supplemented with 10% FCS instead of 5%. Later 2-ME (100 \( \mu \)M) was added, and the concentration of CT was reduced to 20 pM after preliminary indications that these changes improved growth. Ultimately, three independent immortal cell lines were obtained in quantities sufficient for experimentation and freezing, although the time to this stage was long in all three cases (7–9 mon). PTU and CT were now omitted from the medium, except that PTU was added for a few days before and after preparation of frozen stocks (a procedure we now use for all pigmented melanocytes). The appearance of the three lines is shown in Figure 5.

Growth characteristics of misty melanocyte lines: Because melanocyte growth and responses to CT appeared deficient in primary misty cultures, we studied the growth of the three m/m lines and their responses to CT and MSH (melanocyte-stimulating hormone, another cyclic AMP agonist) in comparison to three melanocyte lines wild-type at this locus (Figure 6). Growth
rate varied between lines of either genotype, but growth of all three m/m lines in standard melanocyte medium with TPA was slower than normal; on average, wild-type lines produced fourfold more cells in 14 days. Cell yields in the presence of TPA were not significantly increased in wild-type immortal lines by CT or MSH (a difference from primary cultures); in fact, greater relative proliferative responses to these agents (about 50% more cells with either CT or MSH than without) were seen in the misty lines. m/m cells cultured with CT and MSH also gave higher yields than without these agents in the absence of TPA, possibly indicating an effect on cell survival, because cell number fell with time in control cultures with none of these supplements, for example, from 3.00 (day 0) to 2.00 \pm 0.09 \times 10^4 \text{ cells/ml} in melan-m1 cells grown for 7 days; parallel cultures reached 28.96 \pm 0.35 with TPA, 4.00 \pm 0.07 with CT, 3.78 \pm 0.14 with MSH and 2.35 \pm 0.10 with 20 pM bFGF (units of 10^6 cells/ml, mean and SEM of triplicate dishes). The proliferative response to TPA at different concentrations was examined in melan-m1 cells and seemed normal, apart from growth rates being low throughout (data not shown); the optimal concentration was 200 nM, the same as for the wild-type line melan-a (Bennett et al. 1987).

**Hyperpigmentation of misty melanocyte lines:** The melanin contents of three wild-type and three misty melanocyte lines and the effects of CT and MSH are shown in Figure 7. Melanin contents varied markedly among the m/m lines, but in normal medium with TPA, all three misty lines contained more melanin than any wild-type line, on average over threefold more, a highly significant difference. After growth with CT, melanin content was significantly (twofold) higher in wild-type and 1.2-fold higher in misty lines on average (not significant); the latter numerical increase was due to a response by only one m/m line, melan-m3, whereas all wild-type lines responded (Figure 7). Similarly, all wild-type lines contained more melanin after growth with MSH (1.8-fold on average), whereas only melan-m3 did so among the misty lines.

**Bleeding times and platelet analyses:** A significant increase (3.3-fold) in bleeding time was noted in mice of homozygous misty genotype (Table 1). Prolonged bleeding times occurred despite normal platelet counts and normal platelet serotonin levels. Platelet ADP levels were, however, reduced to 41% of normal levels. The small apparent decrease in platelet ATP levels was not significant. Resulting ATP/ADP ratios were about two-fold higher in misty platelets (Table 1). Results similar to the reported values for +/+ mice were obtained when heterozygous (m/+) mice were analyzed (data

![Figure 4](image-url)
not shown), indicating that the bleeding and ADP effects, like the effect on coat color, are recessive.

**DISCUSSION**

The misty phenotype presents an unusual and intriguing pattern of biological effects. It is a spotting mutation, which suggests an abnormality in a regulatory pathway in melanocytes rather than in their function. Yet there is also a bleeding disorder. To date the existence of a set of loci at which mutations affect color, bleeding time, and kidney function has been attributed to evolutionary relationships among the melanosome (pigmentary organelle), lysosome, and platelet dense granule (Orlow 1995). These organelles have some proteins in common, which are expected to include the products of many of these loci. This framework would suggest instead that the misty locus encodes an organelle protein.

The deficient proliferation and apparently increased death rate of m/m melanocytes in culture suggest that the pigmentary defects in vivo may reflect an insufficient number of hair-follicle melanocytes. The poor growth is consistent with a developmental effect such as a lesion in a receptor or other signaling molecule. It could also be consistent with a defect in an organelle protein, as with silver, which encodes a melanosomal enzyme (Chakraborty et al. 1996), and yet is associated with deficient growth in vitro and reduced numbers of melanocytes in vivo (Spanakis et al. 1992). In that case, poor cell growth was speculated to be associated with a disturbance in melanogenic pathways, possibly in superoxide metabolism (Chakraborty et al. 1996). A similar effect may be occurring in misty melanocytes, which clearly overproduce melanin; this might well lead to accumulation and leakage from melanosomes of toxic melanin precursors such as dihydroxyindole (Pawelek and Lerner 1978). This is consistent with subjective impressions of improved net growth of long-term cultures after addition of PTU to inhibit melanogenesis.

Primary cultures of m/m melanoblasts and melanocytes showed no significant response to the cAMP agonist CT, which strongly stimulates growth and differentiation of +/+ murine diploid melanocytes. In general, cAMP and its agonists are well known as stimu-
lators of both melanogenesis and dendrite formation in pigment cells, and are believed to act through protein kinase A (PKA), at both the post-transcriptional (e.g., Wong and Pawelek 1975; Presto et al. 1987) and transcriptional levels (e.g., Bertolotto et al. 1996). This finding raises the specific possibility of an abnormality somewhere in the cAMP signaling pathway. The overproduction of melanin and dendrites by m/m cells suggests overactivity of some component, leading to maximal or near-maximal activity of the pathway and hence lack of stimulation by CT. This could happen if misty encoded a subunit of adenylate cyclase (such as an inhibitory G subunit of the G1, G0 or G2 families; Wilkie et al. 1993), a subunit of PKA, or a PKA substrate such as a transcription factor. Another class of explanation is suggested by the observations on platelets (see below). Whatever the mechanism, the observed partial restoration of responsiveness to CT in immortal m/m melanocytes, especially noticeable in line melan-m3, could result from a reversion, compensating

Figure 6.—Proliferation of wild-type and misty melanocyte lines. Cells were plated at 1.5 × 10^4 cells/ml and grown in RPMI growth medium with supplements as shown: TPA (200 nM), CT (200 pM) or MSH (1 nM). Cells were harvested and counted at about 75% confluency (at times varying between cell lines). To facilitate comparison, doubling times were obtained from cell counts for each line on the assumption of exponential growth (adequately correct under these conditions) and were used to calculate the expected cell number after 7 days (shown). Wild-type lines melan-a, mel-18, and mel-25 were compared to the three misty lines. Data are the means and SEM of six separate experiments per genotype (two for each cell line, with triplicate dishes in each experiment). Differences in cell proliferation between misty and wild-type lines were significant by Student’s t-test under all three conditions (P < 0.05).

Figure 7.—Melanin contents of wild-type and misty melanocyte lines. Cells were plated at 1.5 × 10^4 cells/ml and harvested at about 75% confluency, i.e., after 7 days (melan-a), 9 days (melan-m1 and melan-m3) and 14 days (melan-m2) in culture. Cells were grown in RPMI growth medium (materials and methods), with TPA (200 nM) and either with or without CT (200 pM) or MSH (1 nM). Shown are means and SEM of six cultures from each cell line (triplicate dishes from each of two experiments). To exclude photometric absorbance not due to melanin, we subtracted the mean absorbance per melan-c (albino) melanocyte from the values shown here (materials and methods). There was variability among individual lines, but the difference in mean melanin content between misty and wild-type lines was significant by Student’s t-test under all conditions: TPA only (P < 0.01), TPA with CT (P < 0.02) and TPA with MSH (P < 0.05). The increase in overall mean melanin content with CT or with MSH was highly significant for wild-type lines (P < 0.01) but not significant for misty lines.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal (+/+)</th>
<th>misty (m/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding time (min)</td>
<td>4.0 ± 0.63 (11)</td>
<td>13.2 ± 0.83 (16)*</td>
</tr>
<tr>
<td>Platelets/ ml (10^9)</td>
<td>0.62 ± 0.04 (6)</td>
<td>0.55 ± 0.03 (6)</td>
</tr>
<tr>
<td>Serotonin (µg/10^9 platelets)</td>
<td>1.57 ± 0.17 (8)</td>
<td>1.35 ± 0.13 (8)</td>
</tr>
<tr>
<td>ATP (µmol/10^{11} platelets)</td>
<td>4.66 ± 0.27 (11)</td>
<td>3.67 ± 0.34 (11)</td>
</tr>
<tr>
<td>ADP (µmol/10^{11} platelets)</td>
<td>3.22 ± 0.24 (11)</td>
<td>1.32 ± 0.20 (11)*</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.44</td>
<td>2.78</td>
</tr>
</tbody>
</table>

Data are the mean and SEM of values from the number of mice in parentheses. *P ≤ 0.001.
mutation or change in expression of the misty gene product or an interacting one.

It is possible that overproduction of cAMP in multilocular adipocytes (brown fat cells) could lead to the delipidation or atrophy of this tissue, because cAMP appears to be a signal for lipolysis in this tissue, and atrophy can follow prolonged lipolysis (for review, see Nicholls and Locke 1984). Interestingly, it was recently reported in an abstract that older m/m mice of the C57BL/6 strain also have reduced amounts of white fat and (from the second week) reduced body weight (Truett et al. 1996). We did not detect this because we examined only neonatal mice of this strain. It seems plausible that the misty mutation may affect both brown and white fat by similar mechanisms.

The observation of reduced levels of platelet ADP provides a likely explanation for the prolonged bleeding in misty mice. It is well known that ADP is an important physiological agonist that stimulates platelet aggregation (Holmsen 1989). ADP released from the platelet dense granule pool during aggregation is in turn a potent platelet agonist that interacts with an ADP receptor on adjacent platelets to stimulate them further and ultimately to form a platelet plug that arrests bleeding. Our data rule out thrombocytopenia and platelet serotonin deficiency, which cause inherited prolonged bleeding times in human thrombocytopenic and storage pool deficiency syndromes, respectively (Newman and Poncz 1995).

Another possible explanation of the prolonged bleeding time in misty mice derives from the known interactions between ADP and cAMP. In platelets, application of external ADP has been shown to block the ability of other agents to stimulate the synthesis of cyclic AMP by adenylate cyclase (MacFarlane 1986). If it is assumed that the misty defect in ADP is in the platelet granule pool, less ADP would be released from misty platelets at injury. This would lead to higher-than-normal production of cAMP in surrounding platelets, inhibition of aggregation, and the observed prolonged bleeding. Alternatively, the lowered intraplatelet ADP levels observed in misty mice may directly stimulate cAMP production, which would lead in turn to inhibition of platelet aggregation and the observed prolonged bleeding. Increased levels of cAMP within platelets result in the inhibition of most platelet pharmacological responses, including aggregation (MacFarlane 1986).

It is of interest that Callan et al. (1995) have reported a similar example of prolonged bleeding time associated with apparently normal platelet serotonin levels and lowered platelet ADP in the American cocker spaniel. The unique aspect of the platelet abnormality in both the misty mouse and the cocker spaniel is that the storage pool deficiency is specific to ADP with no effect on serotonin. To our knowledge, there are no comparable cases of storage pool deficiency in humans, though Weiss et al. (1979) describe several platelet storage pool deficiency patients having markedly lowered ADP accompanied by mild reductions in serotonin. Additional studies will be required to determine whether the lowered platelet ADP in misty platelets is strictly in the dense granule storage ADP pool, as would be expected for a platelet storage pool deficiency syndrome, or whether the mutant platelet metabolic pool is also affected.

An interesting related issue is whether ADP levels are lowered in melanocytes and fat as they are in platelets. If ADP is depressed and if cAMP production is likewise modulated by ADP in these cells as it is in platelets, a reasonable explanation for the overproduction of melanin and dendrites in melanocytes and for the delipidation or atrophy of mutant brown and white fat is available. These possibilities are under investigation.

We are indebted to Jean-Jacques Pantier (Ecole Nationale Vétérinaire d’Alfort) for melanocyte lines mel-18 and mel-29, to Simon Hill and Madonna Reddington for skilled technical assistance, and to George Mewis for expert preparation of histological sections. This work was supported by Wellcome Trust grants 386603/Z/92/Z and 046038/Z/95/Z, by grant HL-31698 from the National Heart, Lung and Blood Institute and by Roswell Park Cancer Institute core grant P30 CA 16056.

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Communicating editor: R. E. Ganschow