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Iron Deficiency: Lessons From Anemic Mice[b]

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Iron is an essential nutrient, and disorders of iron metabolism are common. Nonetheless, intestinal iron absorption and cellular iron transport are poorly understood. Biochemical approaches to elucidating these processes have yielded little in the past decade. As an alternative approach, we have begun to study spontaneous mouse mutants, that have inherited defects in key steps in iron transport. We have undertaken positional cloning of the gene responsible for microcytic anemia (gene symbol *mk*). This report describes the important characteristics of these mice, and our progress in studying them.

INTRODUCTION

Iron is an essential nutrient, that is required by all organisms for basic cellular functions including oxidative metabolism and DNA synthesis. Vertebrates also require iron as a component of the oxygen carrying molecule, hemoglobin. While it is necessary for life, iron is invariably toxic in excess. Hence, its absorption must be strictly regulated. Iron deficiency is manifest primarily by anemia resulting from insufficiency of iron for hemoglobin production. Iron deficiency anemia affects an estimated 600 million individuals worldwide, primarily women and young children. While iron deficiency anemia is most often due to dietary insufficiency, an interesting subset of patients have inherited defects in iron transport.

At the other extreme, hemochromatosis is a disease of iron accumulation, resulting from inappropriately increased absorption of dietary iron. There are two prevalent genetic forms of hemochromatosis. Hereditary hemochromatosis is the most common genetic disease of individuals of Northern European descent. Approximately one in ten individuals in that population carry at least one copy of a mutant gene, dubbed HFE [1]. Heterozygotes have subclinical iron loading, and untreated homozygotes accumulate up to ten times normal body iron stores by middle age. Without intervention, iron toxicity leads to failure of the liver, heart and endocrine organs.

African iron overload affects many individuals in the sub-Saharan portion of the African continent. This disease is similar to hereditary hemochromatosis, though the pattern of iron deposition differs slightly. African iron overload appears to have a genetic basis [2], but it is unlinked to HFE, and the responsible gene has not been mapped.

Despite the prevalence and severity of these disorders, iron transport remains poorly understood. Figure 1 summarizes the important iron compartments in mammals. Dietary

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[b] Based on a presentation at the Symposium Celebrating 75 Years of Yale Pediatrics

[c] *Abbreviations:* Hbg, hemoglobin; MCV, mean corpuscular volume; SSLP, simple sequence length polymorphism.

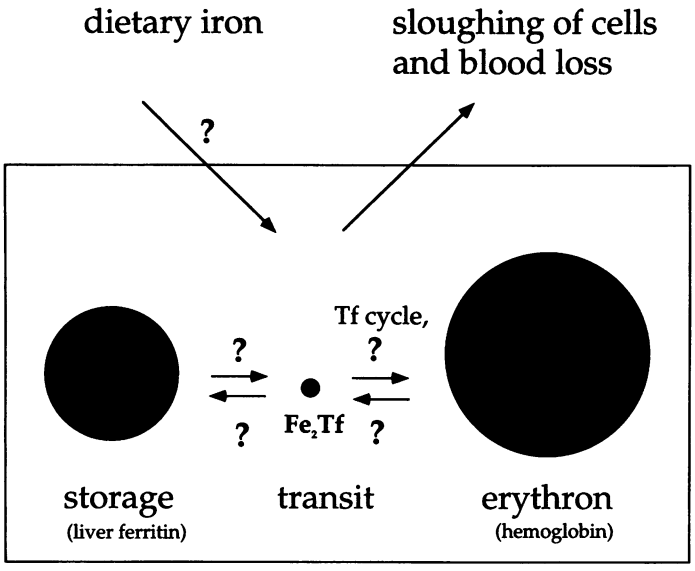

Figure 1. Iron compartments. At the top, arrows indicate the entry of iron into, and exit of iron from the body. Filled circles represent the major iron compartments within the body - storage iron (primarily hepatic ferritin), transit iron (diferric transferrin circulating in plasma) and actively used iron (in the form of hemoglobin, in the erythron). Question marks (?) indicate that little is known about most transport steps. Tf = transferrin.

iron enters the body through the intestinal epithelium. The mechanisms by which iron enters, transits and leaves the enterocyte are unknown. Several models have been proposed, but none has met with widespread acceptance, and they will not be discussed here. There is no physiologic mechanism for the excretion of iron in mammals; iron leaves the body through sloughing of mucosal cells and loss of blood. Iron balance is regulated, therefore, strictly at the level of absorption. Women absorb more iron than men, to compensate for regular menstrual blood loss.

On the serosal side of the enterocyte iron is quickly bound to the chelator protein, transferrin, to enter the transit compartment. Chelation detoxifies iron by making it less reactive. Diferric transferrin delivers iron to the storage compartment, which primarily consists of hepatic ferritin, and to the major sites of use, most notably the erythroid bone marrow. Little is known of how iron enters or leaves hepatic cells, though it is clear that it must be removed from transferrin, and it must remain in an exchangeable form.

Iron entry into the erythron is partially explained by an endocytotic process termed the transferrin cycle. Diferric transferrin binds to specific receptors on the surface of erythroid precursors, and the ligand-receptor complex is endocytosed to form specific endosomes. Next, endosomes are acidified through the action of a proton pump, leading to iron release. These steps have been elegantly described by Klausner, Lodish and their co-workers [reviewed in 3]. Apotransferrin and transferrin receptors are recycled back to the cell surface to repeat the cycle. The fate of the iron, however, is not well understood. Somehow iron must transit the lipid bilayer surrounding the endosome, to gain access to the cytoplasm and ultimately to the mitochondrion where it is incorporated into heme.

We would like to identify the molecules which transport iron across the membranes of the intestinal cell and the red cell. To do this, we have taken a novel genetic approach. The power of this approach becomes apparent from analysis of a patient, and a mutant

mouse, which have identical phenotypes due to defects in iron transport. The patient will be presented briefly, and the mouse model will be discussed in detail.

IRON-RESISTANT IRON DEFICIENCY ANEMIA IN A TODDLER

P.D. was referred to our center for evaluation of "iron-resistant iron deficiency anemia." He was the 4.3 kg product of a healthy mother born in Nigeria after an uncomplicated pregnancy. He did well during his first year and a half, but was noted to be pale at a routine 18 month well-child check. At that time he had a hemoglobin (Hgb)[c] of 7.0 g/dl (normal range: 10.5 - 14.0), and a mean corpuscular volume (MCV) of 49 μm^3 (normal range: 72 - 90). He had no history of blood loss or dietary insufficiency. Oral iron therapy was initiated with no response. Next, a full replacement dose of intravenous iron dextran was given.

Six weeks later, laboratory evaluation showed persistent anemia. In general, even severe iron deficiency anemia will be markedly improved within that period of time after parenteral treatment. P.D., however, had Hgb 7.8 g/dl, MCV 50 μm^3, hematocrit (Hct) 27.2 percent (normal range: 34 - 42), reticulocytopenia, serum ferritin level of 87 µg/l (normal range: 10 - 75), transferrin iron saturation of 5 µg/dl (normal range: 15 - 40) and red cell count of 5.36 x 10^6/ml (normal range: 3.70 - 5.30 x 10^6). Hemoglobin electrophoresis results were reported as normal. We were consulted for a second opinion.

The parents of P.D. are alive and well, and he has a healthy older male sibling. No family members had microcytosis or significant anemia. On physical examination, P.D. was well-developed and appeared to be well-nourished. His height and weight were greater than 90th percentile. He had dry, deeply pigmented skin, and pallor visible in the nailbeds and palmar creases. Auscultation revealed a flow murmur, but no evidence of structural heart disease. He had no organomegaly, adenopathy or bony abnormalities. His blood smear is shown in Figure 2.

In summary, this toddler presented as a well-appearing child with profound hypochromic, microcytic anemia unresponsive to oral iron therapy, and partially responsive to intravenous iron therapy. His anemia persisted after total body iron stores were repleted. Red cell morphology is consistent with severe iron deficiency, but the RBC count was surprisingly high. He appears to have defects in both intestinal iron transport, and red cell iron utilization. No genetic pattern can be discerned, because there is insufficient data. However, similar patients have been described previously by George Buchanan [41 and Kip Hartman [5]; the inheritance in those patients appears to be autosomal recessive.

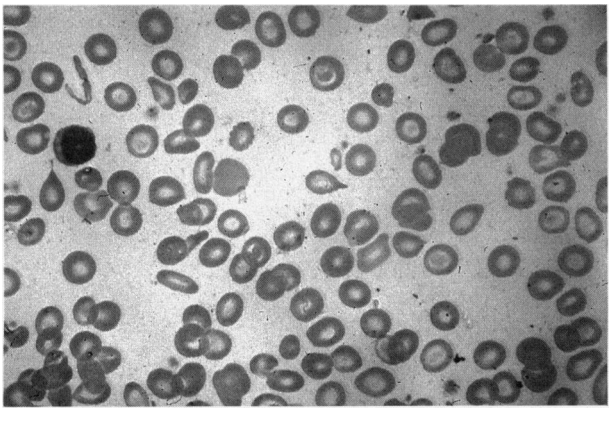

Figure 2. Blood smear from patient P.D. This smear was obtained approximately three months after repletion of body iron stores with intravenous iron dextran. Significant hypochromia, microcytosis, anisocytosis and poikylocytosis are still evident.

MICROCYTIC (*mk*) MICE HAVE A SIMILAR DEFECT

There is a striking similarity between these human patients, and mice with the microcytosis (*mk*) mutation, as noted previously by Robin Bannerman [6]. Homozygous *mk* mice have hypochromic, microcytic anemia with red cell morphology which is indistinguishable from that of the patient, P.D., and a RBC count which is elevated out of proportion to their anemia [7]. These mice fail to respond to oral iron, and respond poorly to intravenous iron. Bone marrow transplantation experiments demonstrate defects at the level of intestinal absorption as well as bone marrow utilization [8]. We hypothesize that the gene defective in *mk* mice encodes a key iron transport molecule. We have undertaken positional cloning to identify and characterize that protein. This approach has several major advantages over biochemical studies of iron transport. First, the functional relevance of the protein has been established by an experiment of nature. Second, we are not limited by the seemingly insurmountable technical difficulties of trying to purify a protein for which no good assay has been devised.

GENETIC LOCALIZATION OF *mk*

In the past five years, technical advances have had great impact on mouse genetics. Dinucleotide repeats of up to 100 basepairs in length (generally with the structure $(CA)_n$) are distributed randomly throughout mammalian genomes. These repeats, termed simple sequence length polymorphism markers or SSLPs, have a convenient property of low grade genetic instability. That is to say, they tend to remain constant in length within inbred mouse strains, but they often vary in length between mouse strains. As a result, they can be used as genetic markers. William Dietrich, Eric Lander, and their colleagues have described over seven thousand SSLP markers, and determined their positions on a high resolution genetic map of the mouse genome [9]. These markers form the framework upon which more precise genetic maps can be constructed. Analysis of these markers is carried out as shown schematically in Figure 3.

To localize the *mk* gene precisely, we set up a large backcross breeding experiment. After nearly one hundred generations of inbreeding, the mice carrying the *mk* mutation are considered a distinct recombinant inbred strain, termed MK/ReJ. Homozygous male

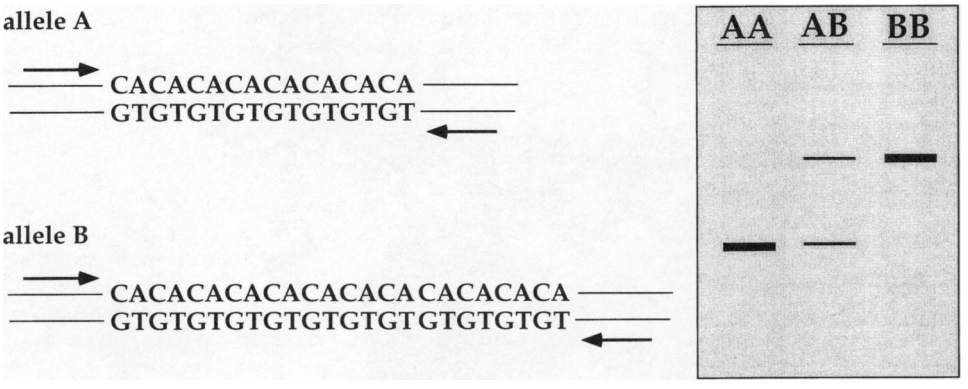

Figure 3. Analysis of SSLP markers. The strategy for PCR-based analysis of simple sequence length polymorphisms (SSLPs) is shown schematically. Arrows represent PCR primers flanking dinucleotide (CA) repeats found in non-coding DNA. The diagram on the right represents an acrylamide gel, fractionating PCR products from the two alleles A and B.

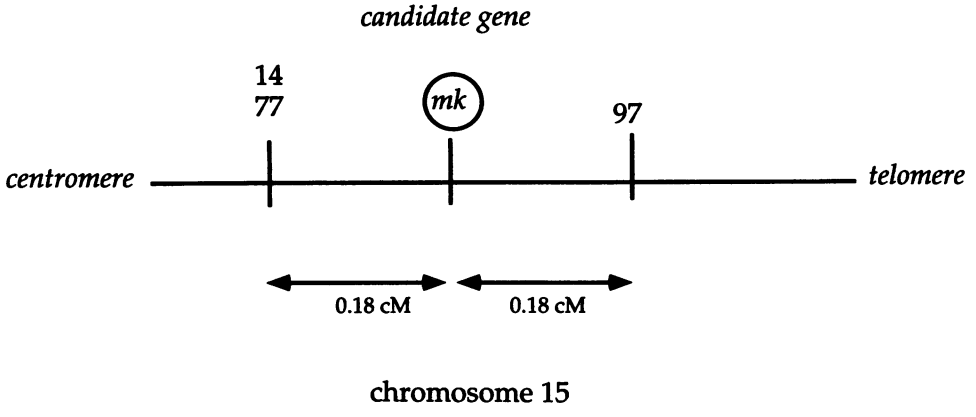

Figure 4. Genetic map of *mk* region on mouse chromosome 15. A genetic map of the *mk* candidate region is shown, with distances indicated in centimorgans (cM). Numerical marker designations refer to D15Mit markers, as described in the text (e.g. 14 is D15Mit14). No recombinations have been observed between the *mk* phenotype and the candidate gene. Flanking markers define a genetic interval of 0.36 cM total; on average, this corresponds to about 700 kb.

mk/mk mice from the MK/ReJ strain were mated (outcrossed) with female mice from either of two strains which differ at most SSLP loci. The offspring from that cross (first filial generation, or F1) derived 50 percent of their genetic makeup from the MK/ReJ father, and 50 percent of their genetic makeup from the outcross mother. The F1 females were then bred (backcrossed) to their MK/ReJ fathers. Meiotic recombinations were apparent in the offspring from this cross, and could be used to map the location of the *mk* gene, through correlation of phenotype (microcytic, hypochromic anemia) and SSLP genotype at various loci. Using this strategy, we confirmed classic genetic mapping of *mk* to mouse chromosome 15, and determined a small region near the telomere which must contain the *mk* gene.

At the time of the symposium, we had analyzed 551 backcross animals. These data were used to generate a genetic map of the region surrounding *mk*, as shown in Figure 4. The *mk* phenotype is flanked by two SSLP markers (Dl5Mit markers 14 and 77) towards the centromere, and one SSLP marker (Dl5Mit 97) towards the telomere. The entire candidate region was less than 0.4 centimorgans. A fourth marker, derived from the 3' untranslated region of a cDNA mapping to that region, was invariably linked to the phenotype, and showed no recombinations in the backcross panel. This was considered a potential candidate gene. We now have strong data indicating that that cDNA from the Nramp2 gene encodes the mk protein; those results will be published elsewhere.

Three genes known to map to mouse chromosome 15 encode proteins have features which led us to consider them as candidates for *mk*. Two transcription factors that have been shown to be important for red blood cell development, p45 NF-E2 [10] and CP2 [11], are encoded by genes residing on the portion of human chromosome 12q which is syntenic with the *mk* region of mouse chromosome 15. Peters et al. previously suggested that p45 NF-E2 was the *mk* gene [12], but this was later shown to be incorrect [13-15]. We have also ruled out CP2, based on more precise mapping.

A third gene, encoding the protein Rab5b, also maps to this region. Rab5b is a cytoplasmic ras-like protein, which co-localizes with the transferrin receptor in an endosomal compartment [16]. While this protein seemed like a plausible candidate for the *mk* gene product, further map data have excluded that possibility.

The ultimate proof of the identity of the *mk* gene will involve functional reconstitution of iron transport in cells carrying the *mk* defect. To that end, we have established immortalized erythroid precursor cell lines from animals homozygous for the *mk* mutation, and their heterozygous (unaffected) littermates. This was achieved by infecting disaggregated fetal liver cells with a retrovirus carrying *myc* and *raf* oncogenes, as described previously [17]. Five cell lines were obtained from mutant animals, and five cell lines were obtained from unaffected animals. Preliminary studies have shown that these lines can be induced to differentiate into more mature erythroid forms by treatment with erythropoeitin. They express mRNA encoding alpha and beta globin proteins. Cell lines from *mk* homozygotes appear to make less ferritin mRNA than controls. We are in the process of designing a reliable assay for iron transport in these cells.

OTHER IRON DEFICIENT MOUSE STRAINS

In addition to *mk*, there are other mouse mutants which have defects in iron transport pathways. Mice homozygous for the hemoglobin deficit (*hbd*) trait have increased serum iron, but poor red cell iron utilization [18]. The *hbd* gene maps to a chromsomal position distinct from that of *mk*, transferrin, ferritin, or the transferrin receptor. We are currently analyzing a large backcross to precisely localize the *hbd* gene. Mice homozygous for the flexed tail (*f*) mutation have fetal siderocytic anemia, apparently due to a prenatal defect in red cell iron utilization [19]. We have just begun to map the *f* mutation. Hypotransferrinemic (*hpx*) mice have a splicing defect which alters the mRNA encoding transferrin, and consequently make little or no functional transferrin protein. These mice have the surprising phenotype of severe iron deficiency anemia in the setting of systemic iron overload. These mouse strains, and others, offer a great deal of information about iron transport.

CONCLUSIONS

The advent of high resolution chromosomal mapping in the mouse has greatly facilitated positional cloning efforts. We have taken advantage of this, to use a genetic approach to work out the details of iron transport pathways. Several spontaneous mouse mutants with defects in key transport steps have been characterized by mouse breeders. Their observable phenotype is hypochromic, microcytic anemia, resulting from insufficiency of iron for hemoglobin production. We are studying several of the most informative of these mutants. Microcytic anemia (*mk*) mice have poor absorption of dietary iron through the intestine, and poor uptake of plasma iron by red cell precursors, indicating both intestinal and hematopoietic cell defects. Hemoglobin deficit (*hbd*) mice have normal intestinal iron absorption, but defective red cell iron uptake. Flexed tail (*f*) mice have an isolated defect in red cell iron utilization, that causes them to form abnormal iron granules in the cytoplasm of red cell precursors. We plan to clone and characterize each of these defective genes. Our long term goal is to define all molecular steps in iron transport, from the intestine to the site of hemoglobin synthesis in the erythron. The mutant mice described above, and others yet to be studied, will be enormously instructive in this endeavor. Once key proteins have been identified, biochemical and genetic interaction studies should help identify other components of the pathways.

It is likely that iron transport mechanisms are similar among mammals, and that lessons learned from mice will lead to an understanding of human iron transport. A small group of Hematology Clinic patients, followed at Children's Hospital, have clinical features that are remarkably similar to the genetic iron transport disorders seen in *mk*, *hbd* and other mouse mutants. We will soon be in a position to test whether the human patients

have mutations in genes homologous to those affected in the mice. In the long term, we hope that improved understanding of iron transport will lead to novel therapies for iron deficiency and iron overload disorders.

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