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Iron in Skin of Mice with Three Etiologies of Systemic Iron Overload

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Abstract

In human hemochromatosis, tissue toxicity is a function of tissue iron levels. Despite reports of skin toxicity in hemochromatosis, little is known about iron levels in skin of individuals with systemic iron overload. We measured skin iron and studied skin histology in three mouse models of systemic iron overload: mice with a deletion of the hemochromatosis (*Hfe*) gene, mice fed a high iron diet, and mice given parenteral injections of iron. In $Hfe^{-/-}$ mice, iron content in the epidermis and dermis was unexpectedly the same as in $Hfe^{+/+}$ mice, and there were no histological abnormalities detected after 30 wk. A high iron diet produced increased iron in the epidermis of both normal and $Hfe^{-/-}$ animals; a high diet increased iron in the dermis only in $Hfe^{-/-}$ mice. Increased skin iron was not associated with other histological changes, even after 19 wk. Parenteral administration of iron produced increased iron in the epidermis and dermis, and gave the skin a bronze hue. These results show that the amount and distribution of iron in the skin depends on the etiology of iron overload. It appears that neither *Hfe* deletion nor elevated skin iron alone can account for cutaneous manifestations reportedly seen in humans with hereditary hemochromatosis.

Keywords

hemochromatosis; skin; iron; diet; ferritin

Skin from humans with hemochromatosis reportedly shows increased iron (Magnuson and Raulston, 1942), and an epidermis that is hyperkeratotic, atrophic, and pigmented (Cawley *et al*, 1969; Chevrant-Breton *et al*, 1977). These conclusions are drawn from surveys that made no distinction between genetic, dietary, or hemolytic causes for hemochromatosis. The textbook descriptions of skin in “bronze diabetes” are based on these and similar data, which were accumulated decades ago in patients who often had end-stage disease. Questions of whether changes in skin were caused by local effects of iron or by distant effects of iron toxicity on internal organs were usually not considered, with the exception of the pigmentary changes (Cawley *et al*, 1969).

Iron is thought to be a cofactor or mediator of skin toxicity in a variety of pathological situations including sunburn (Bissett *et al*, 1991), porphyria cutanea tarda (Menon *et al*, 1991; Takeshita *et al*, 2004), inflammation (Gira *et al*, 2003), and skin cancer (Bhasin *et al*, 2003). If the *amount* of tissue iron is what determines its role in skin toxicity, then it becomes critical to know which forms of hemochromatosis are associated with increased iron in the skin.

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Nearly 25% of absorbed iron is normally eliminated from the body by exfoliation of epidermal cells (Weintraub *et al*, 1965; Jacob *et al*, 1981). Yet little is known about the effect of systemic iron overload on iron in the skin or epidermis. Although it is now recognized that the regulatory defect responsible for the most common form of genetic hemochromatosis occurs at the level of iron absorption from the gut, phenotypic expression of the hemochromatosis genotype is unexpectedly variable (Pietrangelo, 2004). Environmental and/or additional genetic (Levy *et al*, 2000; Fleming *et al*, 2001) influences will certainly account for most of that variability. Theoretically, variations in the iron accumulation in epidermis and loss through desquamation could also modulate body stores of iron.

Because of earlier recognition and treatment of hemochromatosis, it may never again be possible to determine whether different causes of human hemochromatosis result in the same or different manifestations in skin. Fortunately, systematic evaluation of the epidermis and skin in hemochromatosis is now possible through the availability of genetically defined animal models of iron overload (Levy *et al*, 1999; Hentze *et al*, 2004). Inactivation of the *Hfe* gene in mice alters iron homeostasis and produces changes in tissue iron distribution that are similar to changes found in humans with *HFE* mutations (Zhou *et al*, 1998; Levy *et al*, 1999). In order to identify biochemical and histological changes in the epidermis that are associated with defined types of systemic iron overload, we studied the cutaneous effects of diet, parenteral injection of iron, and inactivation of the *Hfe* gene in mice.

Results

Skin in hereditary hemochromatosis

In preliminary experiments, we established that the iron content in the epidermis and hair was indistinguishable in male and female littermates for 129/Sv $Hfe^{+/+}$ and 129/Sv $Hfe^{-/-}$ mice. By contrast, the iron content of internal organs is known to vary with sex and is generally higher in females than males, a finding we confirmed in livers of 129/Sv $Hfe^{+/+}$ and 129/Sv $Hfe^{-/-}$ mice. Therefore, only females were analyzed for these longitudinal studies.

Iron content was measured in the epidermis from various locations in groups of animals ranging in age from 4 to 30 wk (Fig 1). Epidermal iron varied significantly during the first 2 mo of life. It was highest at 4 wk and lowest at 8 wk, at all locations of the epidermis sampled. Epidermal iron also varied with location. In adults it was highest in the abdominal (ventral) epidermis and lowest in the tail epidermis. At most time points and most locations, epidermal iron was slightly higher in 129/Sv $Hfe^{-/-}$ animals than in wild-type 129/Sv $Hfe^{+/+}$ animals. But differences in epidermal iron between hemochromatosis and control animals were very small and not statistically significant. Iron content of hair varied over time, but the iron in hair of 129/Sv $Hfe^{-/-}$ animals was the same as that in control 129/Sv $Hfe^{+/+}$ animals. Iron in the dermis from which the epidermis and fat had been removed was the same in 129/Sv $Hfe^{-/-}$ mice and in 129/Sv $Hfe^{+/+}$ mice.

Iron in internal organs of our animals showed differences between $Hfe^{+/+}$ mice and $Hfe^{-/-}$ mice as previously described in the literature (Levy *et al*, 1999; Lebeau *et al*, 2002; Turoczi *et al*, 2003). Iron in livers of 129/Sv $Hfe^{-/-}$ mice was 2−3-fold greater than iron in livers of 129/Sv $Hfe^{+/+}$ mice (Fig 1). Hepatic iron gradually increased in the control animals over time, but in the hemochromatosis animals hepatic iron reached a plateau at 8−10 wk of age. Comparing 129/Sv $Hfe^{-/-}$ mice to 129/SV $Hfe^{+/+}$ mice at 30 wk, there were small, statistically significant increases in the iron in pancreas (253 ± 9 *vs* 207 ± 9 μg per g dry wt) and kidney (742 ± 15 *vs* 652 ± 18 μg per g dry wt); iron in the hearts of these groups of animals was the same.

Histological sections from the back, tail, and ear skin were examined for findings that have been reported in humans with hemochromatosis: thickening of the stratum corneum; epidermal

atrophy; evidence for iron deposition or changes in melanin. At 30 wk of age, no differences in the dorsal epidermis or skin could be identified in H&E (Fig S1*a* and *b*) or Fontana Masson (Fig S1*i* and *j*)-stained sections from 129/Sv $Hfe^{-/-}$ mice compared with 129/Sv $Hfe^{+/+}$ mice. In sections stained for iron, there were an increased number of iron-containing histiocytes in the fat and especially in the panniculus carnosus in the hemochromatosis compared with the control animals. In the lower dermis there was a slight increase in the number of iron-stained histiocytes in hemochromatosis compared with control animals (Fig S1*e* and *f*). Stainable iron was not identified in fibroblasts or keratinocytes, even in the hemochromatosis animals. The highly localized collections of increased iron in the lower dermis were clearly not sufficient to affect the biochemical content of iron in the dermis (from which fat and muscle had been removed).

Skin in dietary hemochromatosis

In the absence of significant changes in the skin of animals with hereditary hemochromatosis, we asked whether dietary iron might influence the expression of the genotype in the epidermis. Animals were maintained on either a basal iron or high iron diet for 5 wk after weaning. The high iron diet resulted in significantly increased iron in the epidermis and hair in 129/Sv $Hfe^{-/-}$ mice (Fig 2). Surprisingly, similar increases were observed in 129/Sv $Hfe^{+/+}$ mice on the high iron diet (Fig 2). The high iron diet caused a 2−4-fold increase in the iron content of the epidermis and hair at all sites examined both in control and in hemochromatosis animals. By contrast, the high iron diet increased iron only in the dermis of 129/Sv $Hfe^{-/-}$ mice; diet had no effect on dermal iron in the controls. The high iron diet also caused a 2-fold increase in hepatic iron both in the control and $Hfe^{-/-}$ animals.

Animals on the high iron diet had elevated iron in the epidermis, but had no grossly obvious phenotype compared with animals on the basal iron diet. Animals with increased iron in the epidermis showed no evidence of the epidermal atrophy or ichthyosiform changes that have been reported in human hemochromatosis (Fig S1*c* and *d*). There was no stainable iron in the epidermis, but the number of iron-laden histiocytes in the lower dermis and fat of 129/Sv $Hfe^{-/-}$ mice was increased (Fig S1*g* and *h*). No gross or microscopic changes in pigmentation were appreciated (Fig S1*k* and *l*). In a separate experiment, 129/Sv $Hfe^{+/+}$ mice received either a basal iron or high iron diet for 19 wk, to test whether diet alone would lead to progressive changes in the skin. Epidermal iron showed the same 2−4-fold increase, compared with animals on a control diet, as was observed in the 5 wk diet experiment. Even after 19 wk of elevated iron in the epidermis there were no identifiable histological changes in the skin, other than increased iron in histiocytes (data not shown).

Increased epidermal iron was associated with one expected biochemical change in these animals. Ferritin, which was barely detectable in epidermal extracts from animals on the basal iron diet, was increased in the epidermis of animals on the high iron diet (Fig 3).

Skin in parenteral iron overload

Tissue distribution of iron in secondary hemochromatosis can differ significantly from that in the common form of hereditary hemochromatosis (Andrews, 1999), and parenteral injections of iron have been used to demonstrate toxic effects of iron in skin (Rezazadeh and Athar, 1997; Bhasin *et al*, 2003). Hairless animals were given repeated intraperitoneal injections of iron dextran, and after several weeks iron was measured in the epidermis, dermis, and several internal organs. Doses ranged from 0.1 mg per injection, an amount equivalent on the basis of body mass to that expected in patients with thalassemia major, to 5 mg per injection, the amount used in experimental carcinogenesis studies (Rezazadeh and Athar, 1997; Bhasin *et al*, 2003). Injections of iron led to a dose-related increase in iron in all tissues examined (Fig 4). Epidermal iron increased 3−4-fold in animals receiving iron injections compared with control

animals, but only at the highest dose administered. In distinction to $Hfe^{+/+}$ mice challenged with dietary iron overload, dermal iron also increased (4−8-fold) in animals receiving parenteral iron. The dose response experiment shows that the threshold for tissue iron accumulation was lowest for the liver and spleen, higher for the dermis and highest for the epidermis.

In sharp contrast to animals with exclusively epidermal iron overload, animals receiving the higher doses of parenteral iron had an obvious bronze or rusty appearance (Fig 5*A*). Animals receiving 0.1 mg looked no different than controls, but animals receiving more than 1 mg per injection were bronze colored. Animals with parenterally induced iron overload had large numbers of iron-laden histiocytes in the dermis and greatly increased numbers of hemosiderin granules, which stained positively with Prussian blue (Fig 5*B*). These animals had no identifiable histological changes in the epidermis and no stainable iron in the epidermis. Stain for melanin showed no difference between controls and iron-overloaded animals.

Discussion

We produced systemic iron overload in mice by three different methods, and then monitored morphological and biochemical changes in the skin. The results demonstrate four chief findings. First, in mice with hereditary hemochromatosis ($Hfe^{-/-}$) on a basal iron diet, iron was not increased in the epidermis and there were no identifiable histological changes, compared with wild-type littermates. Second, in mice that got systemic iron overload from diet, iron was increased in the epidermis of $Hfe^{+/+}$ as well as $Hfe^{-/-}$ animals; only $Hfe^{-/-}$ animals had increased iron in the dermis. Third, in mice that got iron overload from parenteral injection, iron was increased in the dermis and in the epidermis. Fourth, 5 mo of chronically elevated iron in the epidermis caused no identifiable gross or histological effects on skin.

Quantitative and qualitative considerations both contribute to the observed differences in cutaneous iron accumulation produced by the three types of systemic iron overload. Quantity matters: the amount of iron in the diet was the sole cause of elevated iron in the epidermis of 129/Sv $Hfe^{+/+}$ control animals on the high iron diet. In animals given parenteral iron, there was a clear threshold below which iron did not accumulate in either the dermis or epidermis. Quality maters: different causes of systemic iron overload result in different distributions of skin iron. The threshold for iron accumulation following parenteral administration is lower for the dermis than the epidermis. By contrast, the threshold for iron accumulation following dietary overload was lower for the epidermis than the dermis. Preferential accumulation in reticuloendothelial cells of the dermis might account for dermal accumulation following parenteral injection of iron, similar to reticuloendothelial accumulation in the spleen after parenteral injections of iron (Andrews, 1999). One limitation of a biochemical measure of iron is failure to detect highly localized changes. Clearly increased stainable iron in the histiocytes of $Hfe^{-/-}$ animals on a normal diet was not extensive enough to significantly increase total iron in the dermis.

The amount of hepatic iron overload does not always correlate with the amount or location of iron in the skin. Hepatic iron was elevated to about the same extent in $Hfe^{-/-}$ animals on a regular diet, in $Hfe^{+/+}$ animals on a high iron diet, and in SKH/129/Sv $Hfe^{-/-}$ animals receiving biweekly injections of 1 mg iron dextran. Yet epidermal iron was elevated only in the $Hfe^{+/+}$ control animals on a high iron diet. In these three groups of animals with equal hepatic iron, dermal iron was increased only in the animals receiving injections.

These studies are systematic and longitudinal investigations of skin in animals with systemic iron overload. In addition, they add to the growing body of evidence indicating that, in hereditary hemochromatosis, the amount of dietary iron is an important factor in iron accumulation in tissues, even in primary target organs such as the liver (Ajioka *et al*, 2002).

Little iron accumulates in the kidneys of $Hfe^{-/-}$ animals on a basal iron diet; on a high iron diet both $Hfe^{-/-}$ and $Hfe^{+/+}$ animals have increased iron in their kidneys (Lebeau *et al*, 2002). This effect of diet on renal iron is similar to our findings in the epidermis.

Why do we not see evidence of tissue pathology in the skin of our animals? Tissue pathology and dysfunction, which are readily identified in humans with systemic iron overload, have been more difficult to demonstrate than simple iron accumulation in $Hfe^{-/-}$ mice. Fibrosis is not seen in the iron-overloaded liver (Lebeau *et al*, 2002) or heart (Turoczi *et al*, 2003) in $Hfe^{-/-}$ animals. But in a cardiac ischemia–reperfusion experiment, injury is exaggerated in $Hfe^{-/-}$ mice on a high iron diet (Turoczi *et al*, 2003). Similarly, $Hfe^{-/-}$ mice with hepatic iron overload do not accumulate porphyrins in their livers, unless they also have an inactivating mutation in one allele of the uroporphyrinogen decarboxylase gene (Phillips *et al*, 2001).

Absence of identifiable pathology in our animals that have increased epidermal iron may simply mean that additional factors are needed to reveal the toxic effects of elevated iron in the epidermis. Parenterally induced iron overload shortens the time of onset and increases the number of skin tumors in experimental chemical carcinogenesis (Rezazadeh and Athar, 1997; Bhasin *et al*, 2003). Although iron was increased 3−4-fold in the normal skin of animals with tumors (Rezazadeh and Athar, 1997; Bhasin *et al*, 2003), the reported measurements were of whole skin, not the epidermis. Our data establish that the parenteral route of iron administration increases iron in the epidermis as well as in the dermis.

The findings in skin of mice with systemic iron overload are not completely concordant with reports of skin changes in humans with systemic iron overload. In particular, we were surprised to find no measurable iron accumulation in skin of mice with hereditary hemochromatosis on a normal diet. We also could identify no morphological changes in skin of mice that endured many months of epidermal iron overload. Most papers that described the “classic” skin findings in hemochromatosis lump all causes of iron overload together. Our data show that the source and mechanism of systemic iron overload is a crucial determinant of whether iron accumulates in the skin and where in the skin it will accumulate. In the past, little consideration was given to additive and complementary roles that diet might have in hereditary hemochromatosis. In our mice, diet was the critical determinant of whether iron accumulated in skin in hereditary hemochromatosis.

If humans with hereditary hemochromatosis really exhibit the previously reported changes in skin, then our data provide good reason to believe that these changes are related to diet as much as to genetic deficiency of the Hfe protein. Finally, iron is increased in skin of patients on renal dialysis (Friedlaender *et al*, 1988), and clinicians easily recognize the distinctive pigmentary changes in patients with thalassemia. Analysis of skin in mice that received parenteral injections of iron suggests that cutaneous iron distribution from the so-called secondary hemochromatosis may well be different from the cutaneous iron distribution in hereditary hemochromatosis.

Materials and Methods

Animals

129/Sv $Hfe^{-/-}$ mice have a deletion of exons 3 and 4 (Levy *et al*, 1999). Control or “wild-type” mice were 129S6/SvEvTac (“129/Sv”, Taconic, Germantown, New York). Hairless, hemochromatosis mice were produced by initially crossing hairless SKH (Charles River, Wilmington, Massachusetts) with 129/Sv $Hfe^{-/-}$ mice. Experiments described here were done using hairless mice (SKH/129/Sv $Hfe^{-/-}$**)** that were homozygous for the disrupted *Hfe* allele and which had been through six generations of brother–sister inbreeding. All mouse production

and experimentation was performed in compliance with guidelines of the Yale Institutional Animal Care and Use Committee.

Diet

Breeders and control animals in the diet experiments were fed standard mouse chow (Harlan, Teklad, Madison, Wisconsin) containing 0.226 g per kg iron (diet TD2018). For the high iron diet, chow was supplemented with carbonyl iron to bring final iron to 20.226 g per kg (diet TD04,064).

Iron injection

Some mice were given iron dextran (NDC 30,798−020−10, Durvet, Blue Springs, Missouri), 5 mg intraperitoneally two times each week for 2 wk before analysis.

Genotyping

Wild-type *Hfe* was distinguished from the disrupted *Hfe* by 30 cycles of PCR, using Taq polymerase (M1661, Promega, Madison, Wisconsin) and primers that produce a 202 bp wild-type band and a 190 bp disrupted band as described (Levy *et al*, 2000).

Tissue preparation

Hair was plucked from regions of skin to be harvested and saved for analysis. Fat was trimmed from the dermis, and 1 × 2 cm pieces of skin were treated overnight in dispase (Kitano and Okada, 1983) to separate the epidermis from the dermis. All manipulations were done using iron-free titanium instruments. The epidermis, dermis, the left lobe of the liver and whole pancreas, heart, and kidney were rinsed in PBS, then blotted dry. All tissues were dried overnight at 65°C in tared microfuge tubes.

Iron measurement

Non-heme iron in tissues and cells was measured using the bathophenanthroline sulfonate (BPS, Sigma, St Louis, Missouri) dye binding assay, originally described by Torrance and Bothwell (1980), and adapted to a microtiter plate format. In brief, tissue was digested in the 10% (v/w) acid mixture, and triplicate samples of 12 μL added to wells in 96-well plates containing 200 μL of BPS. Absorbance was read at 565 nm from samples and ferric chloride standards representing 0−75 ng of iron.

Histology

Tissues were fixed in Bouin's solution, embedded in paraffin, sectioned at 5 μm, deparaffinized, and stained with H&E, Perl's stain for iron and Fontana Masson stain for melanin.

Western blot for ferritin

Total ferritin was estimated by western blot of protein separated on denaturing gels. Epidermal sheets were extracted by sonication on ice in 10 mM Tris, pH 7.4 containing 1% SDS and 1 mM dithiothreitol and protease inhibitors. Protein was measured by a modification of the Bramhall assay (Milstone *et al*, 1982), and 20 μg separated by 15% SDS-PAGE. After protein transfer for 3.5 h at 100 mA, the approximately 21 kDa ferritin band was identified using a rabbit anti-horse ferritin antibody at 1:1750 (F-5762, Sigma), a goat anti-rabbit HRP-antibody at 1:5000 (NA934, Amersham, Piscataway, New Jersey), and an ECL kit (RPN2106, Amersham) and X-ray film (870−1302, Eastman Kodak, Rochester, New York).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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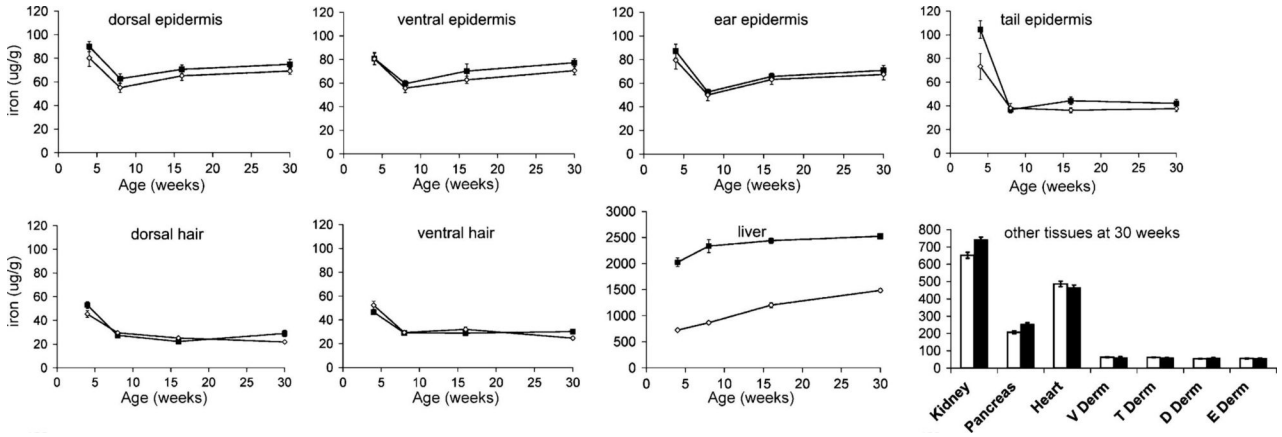

Figure 1. Iron content changes over time in skin, but is unaffected by genotype

Iron was measured (μg per g dry weight) in the epidermis and hair, harvested at various times and from various locations of 129/Sv *Hfe*$^{-/-}$ (*solid square* or *bar*) and 129/Sv *Hfe*$^{+/+}$ (*open diamond* or *bar*) mice. Each point represents mean + SEM of eight to 13 female animals. Last panel shows 30 wk measurements from additional tissues and dermis from ventral (V), tail (T), dorsal (D), and ear (E) skin.

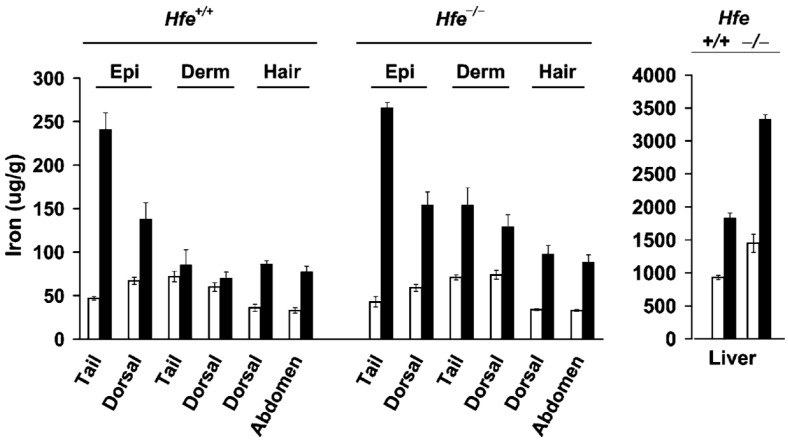

Figure 2. Supplemental dietary iron increases iron in the epidermis, dermis, hair, and liver in 129/Sv Hfe$^{-/-}$ and 129/Sv Hfe$^{+/+}$ mice

Animals were maintained for 5 wk on a normal diet containing 0.226 g per kg iron (*open bars*) or on a diet containing 20.226 g per kg iron (*closed bars*). Iron measurements in the epidermis, dermis, and hair and liver are means ± SEM from five female animals.

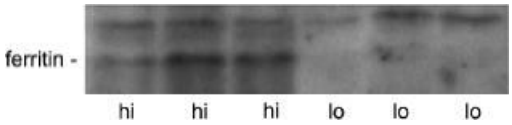

Figure 3. Ferritin increases in the epidermis of 129/Sv *Hfe*$^{-/-}$ mice maintained on a high iron diet

Protein extracts from the epidermis of 129/Sv Hfe$^{-/-}$ mice fed on a normal basal diet (lo) or on a high iron diet (hi) were analyzed for 21 kDa mouse ferritin by western blot. Cross-reacting 28 kDa band on this blot serves as internal loading control.

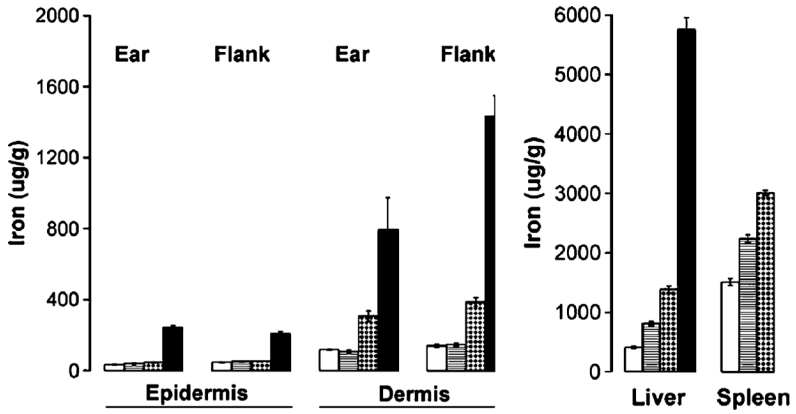

Figure 4. Parenteral injections of iron increase iron in the dermis and epidermis of SKH/129/Sv *Hfe*$^{-/-}$ mice

Iron was measured in tissues of SKH/129/Sv Hfe$^{-/-}$ mice that had received biweekly injections of iron dextran for 2 wk. Data are from groups of five female animals ± SEM saline (□); 0.1 mg ▣; 1 mg ▦; 5 mg (■).

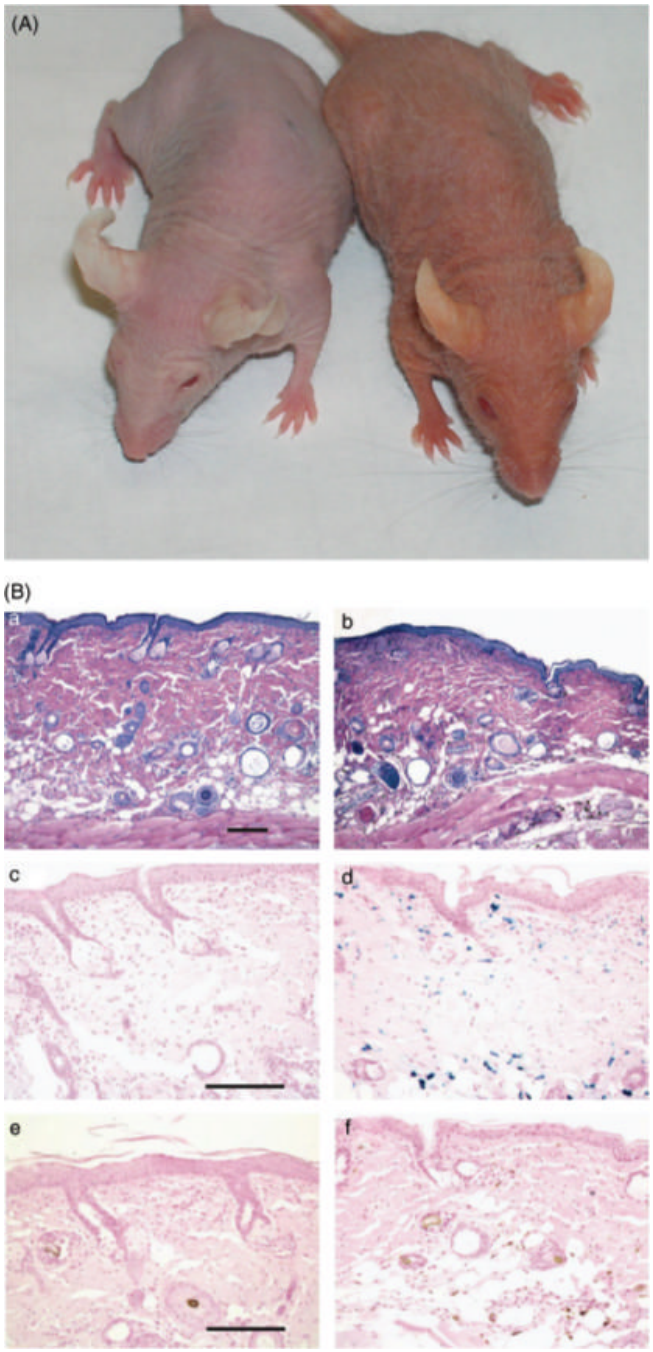

Figure 5. Gross and histological appearance of SKH/129/Sv Hfe$^{-/-}$ mice with iron overload from parentral injections

(*A*) The hairless, hereditary hemochromatosis mouse on the right was given biweekly injections of 5 mg iron dextran for 2 wk. The littermate on the left was injected with saline. (*B*) Skin sections from ears were stained with H&E, Perl's iron or Fontana Masson. Blue granules of iron are visible only in the dermis of mice receiving iron injections. *Scale bar*=250 μm.