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Invited Review

Aquisition, Mobilization and Utilization of Cellular Iron and Heme: Endless Findings and Growing Evidence of Tight Regulation

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TAKETANI, S. *Aquisition, Mobilization and Utilization of Cellular Iron and Heme: Endless Findings and Growing Evidence of Tight Regulation.* Tohoku J. Exp. Med., 2005, **205** (4), 297-318 —— Iron is fastidiously utilized by living cells, since it is an essential element, but is toxic in excess. Cells take up iron via a transferrin-transferrin receptor-dependent endocytotic process. The iron thus taken up is used for essential biological functions including oxygen transport, electron transfer, and DNA synthesis. The intracellular level of iron is tightly controlled, through regulation of the cellular uptake of iron and the sequestering of low molecular labile iron into the storage protein ferritin. The known proteins of iron transport and storage, transferrin, transferrin receptor and ferritin, have been recently linked with a number of newly identified proteins that are responsible for inherited diseases of iron metabolisms and play critical roles in the maintenance of iron homeostasis. These proteins are involved in regulation of intracellular levels of iron, iron transport, and heme transport and the oxygen-dependent regulation of gene expression. On the other hand, most iron is transported into mitochondria and immediately used for the biosynthesis of heme in erythroid cells. The heme biosynthesis in mitochondria is coupled with the supply of iron, and the heme, exported from mitochondria, is utilized as prosthetic groups of hemeproteins. Furthermore, non-erythroid and erythroid cells possess the different regulatory systems for the biosynthesis of heme; iron positively regulates the biosynthesis in erythroid cells while heme negatively regulates it in non-erythroid cells. Because of the toxicity and insolubility of heme, the intracellular level of uncommitted heme is maintained at a low concentration ($< 10^{-9}$ M). The influx and efflux of heme also help to prevent cytotoxicity. Finally, heme-binding transcriptional factors such as Bach1 and NPAS2 regulate the transcription of several genes involved in the synthesis and degradation of heme-hemeproteins. The discovery of new molecules related to disorders of iron and heme metabolism is ascribable to a complete mechanistic understanding of the cellular network of iron homeostasis. The network of interactions that link iron and heme metabolisms with functions of cellular regulation involving oxidative stress and inflammations contributes to new insights into clinical aspects of disorders. ———— Iron; heme; transferrin; transport; mitochondria

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The iron taken up by cells is essential for cell proliferation and respiration. Dietary iron is primarily absorbed by the duodenum, and mainly transported into the liver where it is stored. Iron is changed to functional forms such as heme and the iron-sulfur cluster, accompanied by a transition between ferrous and ferric ions. Iron is, however, a dangerous metal despite being an essential element. Hydrogen peroxide undergoes the Fenton reaction in the presence of ferrous ions to produce the hydroxyl radical, an extremely reactive radical (Harrison and Arosio 1996). The resulting ferric ions are reduced by reductants such as superoxide, ascorbate, and others, after which the ferrous ions produced are rereduced. Therefore, the ligands for ferric ions, including transferrin and ferritin, may function to sequester free ferric ions from chemicals in the biological fluids. Although free iron must remain at quite low concentrations, radical reactions are initiated by reactive oxygen species (ROS) mainly produced from mitochondria, where ATP is formed by reacting oxygen with 4 electrons and 4 protons resulting in the formation of water. When oxygen accepts one electron, superoxide is produced: this reaction often occurs in mitochondria. Two superoxides are converted by superoxide dismutase into oxygen and hydrogen peroxide, which reacts with ferrous ions as above. The hydroxyl radical that is generated attacks lipids, DNA and proteins, sometimes leading to cell death or cancer.

Heme is synthesized in mitochondria by an enzyme, ferochelatase, which catalyzes the insertion of ferrous ions into protoporphyrin IX. Iron not only is an element of heme but can also function in competition or co-operation with heme in many cases. Both are transferred and metabolized independently and components of compounds essential for life. Heme reversibly binds to oxygen and is highly reactive to various compounds by transport of electrons. As heme is insoluble, proteins to resolve the heme-moiety are required for the catalysis of the oxide-reduction reaction. On the other hand, the reactivity of heme with oxygen can lead to toxicity due to the destruction of the porphyrin ring by hydroxyl radicals, similar to the toxicity of iron. Alternatively, heme-binding ligands protect against damage to proteins and lipids, and prevent cytotoxicity. Heme oxygenase (HO, now classified as HO-1 and HO-2), a rate-limiting enzyme of heme catabolism, serves as a regulator to maintain the intracellular level of heme. The iron formed by HO is reutilized.

Research has provided insight into the transport of iron and heme independently of the biological membrane. The tight regulation of the metabolism of these compounds by each other, and their efficient functions as a toxin and as a regulator when internalized are becoming established. In this review, we focus on the transport and utilization of iron and heme in cells. The relation of genetic defects in iron and heme metabolism to disorders is discussed.

The uptake, utilization and regulation of iron by cells

The serum glycoprotein transferrin recognizes transferrin receptors (now classified as transferrin receptors 1 and 2) at the cell surface, binds them and then enters the cells via an endocytotic process. The uptake of differic transferrin via transferrin receptors has been investigated intensively (Richardson and Ponka 1997; Ponka et al. 1998). To synthesize hemoglobin, erythropoietic cells require a large amount of iron. The number of transferrin receptors increases during erythroid differentiation. Most iron incorporated is utilized for the synthesis of heme. On the other hand, 60-70% of the iron taken up is incorporated into ferritin in hepatocytes, the remainder iron is transported into mitochondria and used to produce heme. Ferritin functions as a storage protein in a variety of cells. Although the delivery of iron from transferrin via transferrin receptors is reasonably well understood, the mechanism and regulation of the mobilization to cellular components such as heme, iron-sulfur cluster and ferritin are not.

Regulation of the expression of transferrin receptors occurs in three steps. First, the number of receptors at the cell-surface differs among cells. In proliferating cancer cells, 3 in 10 cellular transferrin receptors are located at the cell-surface, compared to 0.5-1.0 in 10 in hepatocytes

(Richardson and Ponka 1997). Only the receptors at the cell-surface have the ability to take up iron into the cells. Second, the expression of transferrin receptors is increased at the transcriptional level in growing cells. The receptors are synthesized at the G_2-S phase of cell division. Therefore, mitogenic stimulation of peripheral blood lymphocytes as well as the regeneration of liver causes the synthesis of transferrin receptors. The proximal region in the 5'-promoter of the human transferrin receptor 1 gene, including a putative AP-1-, and Sp1-binding sites, is responsible for the control of cell division. On the other hand, there are several erythroid-related elements, NF-E2 and CREB/AP-1 like-motif in the promoter region of the transferrin receptor gene, which may be responsible for the expression of the transferrin receptor with concomitant expression of other genes related to hemoglobin synthesis. Furthermore, in iron-depleted cells, an increase in the transcription of the receptors was observed in a hypoxia inducible factor-1 (HIF-1)-dependent manner (Lok and Ponka 1999). The increase in the expression of the transferrin receptors responds to the necessity of to utilize iron to augment erythropoiresis under hypoxic conditions.

Third, post-transcriptional regulation of the expression of transferrin receptor 1 was demonstrated. Namely, several investigators have reported that the levels of transferrin receptor 1 and ferritin were tightly regulated by the level in a labile iron-pool of cells (Klausner et al. 1993; Richardson and Ponka 1997). Hentze et al. (1987) first demonstrated the presence of a novel sequence, iron-responsive element (IRE), in the 5´-noncoding region of ferritin mRNA. This was also found in 5 sites of the 3´-noncoding region of the transferrin receptor 1 mRNA (Klausner et al. 1993). IRE-binding protein (IRP) binds to IRE and stabilizes the transferrin receptor 1 mRNA from attack by nucleases. In iron-deficient cells, IRP binds to IRE, and the synthesis of transferrin receptor 1 protein increases while IRP is released from IRE and the receptor 1 mRNA decreases. Otherwise, the binding of IRP to IRE of ferritin and erythroid-specific δ-aminolevulinic acid synthase (ALAS2) mRNAs blocks the translation of the RNAs under iron-deficient conditions, where the levels of these proteins decrease, while under iron-loaded conditions, the release triggers the synthesis of proteins. This system regulates iron homeostasis in cells to sequester toxic free iron when the intracellular level of iron increases. In contrast, a decrease in the iron level leads to a de-

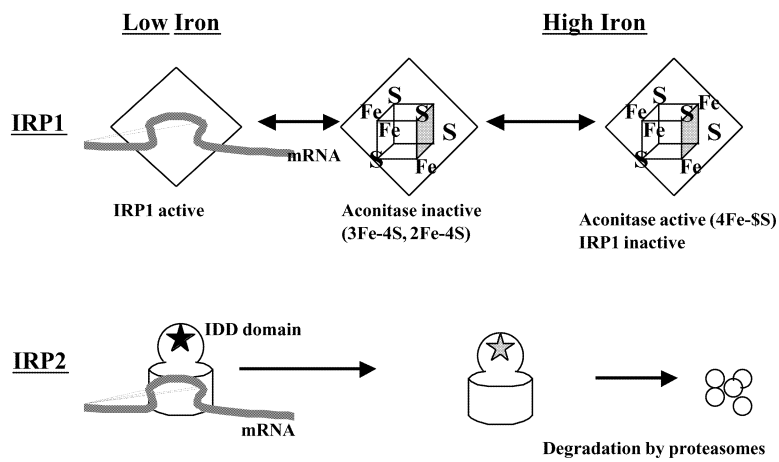

Fig. 1. Regulatory mechanisms of IRP1 and IRP2. IRP1 contains a 4Fe-4S cluster, and shows aconitase activity at high levels of iron. At a decreased iron level, a loss of the cluster is accompanied by a loss of the aconitase activity and IRP1 becomes active to bind mRNAs of iron-regulated genes. On the other hand, IRP2 is degraded by proteasomes, but a decrease in the level of iron leads to the protection of IRP from degradation, in an IDD-dependent fashion, and binding to corresponding mRNA.

crease in the synthesis of ferritin and increase in receptors.

The amino acid sequence of IRP1 shows homology with mitochondrial aconitase which contains a 4Fe-4S cluster (Rouault et al. 1990). Cytoplasmic IRP1 also has a 4Fe-4S cluster and retains the aconitase activity (Ponka and Lok 1999) (Fig. 1). When the level of intracellular iron is reduced, IRP1 loses its aconitase activity, which is accompanied by the loss of an iron from the cluster (i.e. 3Fe-4S), and then binds to IRE. On the other hand, the 4Fe-4S-containing IRP1, which exhibits aconitase activity, cannot bind to IRE. Furthermore, once heme irreversibly binds to IRP1, IRP1 is rapidly degraded, suggesting that the redox state of iron and heme regulates the function and amount of IRP1. The expression of IRP1 can be regulated by nitric oxide (NO) since NO attenuates the aconitase activity, with a comcomitant increase in the binding of IRP1 to IRE (Kim and Ponka 2002). These results suggest that NO regulates the expression of proteins involved in iron metabolism at the post-transcriptional level.

The other IRE-binding protein IRP2, a homologue of IRP1, is expressed in a variety of tissues, but does not exhibit aconitase activity (Iwai et al. 1995). Although the level of IRP1 protein seems to be constant, the expression of IRP2 is regulated dependent on the intracellular level of iron. IRP2 has an iron-degradation domain (IDD), consisting of 73 amino acids. IDD is involved in the ubiqutination of IRP2, after which it is degraded by proteasomes when the intracellular level of iron increases (Fig. 1). The recent finding that the heme-mediated oxidation of the IDD is required for the ubiquitination of IRP2, indicates that IRP2 functions mainly in the regulation of iron homeostasis (Iwai et al. 1998). This may be supported by the observation that IRP1-knock out mice did not exhibit a clear phenotype, but that the accumulation of iron in neural tissues and an increase in ferritin content were observed in IRP2-knock out mice (Meyron-Holtz et al. 2004). In erythroid cells, the synthesis of the transferrin receptor 1 and ALAS2 increased although the intracellular levels of iron and heme were high (Ponka and Lok 1999). The regulation of the expression of transferrin receptor 1 in erythroid cells is independent of the IRE/IRP-dependent regulation in non-erythroid cells since the number of receptors in erythroid cells did not decrease on treatment of the cells with iron. Further studies are required to clarify the specific control of the expression of transferrin receptors in erythroid cells.

Ferritin is a ubiquitous protein whose only clearly defined function is the sequestration and storage of iron. Mammalian ferritin consists of a multimeric protein shell with 24 light (L) and heavy (H) chain subunits that can accommodate up to 4,500 atoms of iron in a ferric hydroxide core (Harrison and Arosio 1996). The best characterized regulatory system of ferritin expression is the post-transcriptional, iron-dependent machinery based on the interaction of IRP with IRE on the H- and L-chains mRNAs (Klausner et al. 1993). This regulation system is sensitive not only to the availability of iron, but also to the oxidative status of the cell. In many respects, ferritin can be viewed as a member of the group of proteins that respond to stress and inflammation (Harrison and Arosio 1996). Inflammatory cytokines, tumor necrosis factor α and interleukin-1β, positively regulate ferritin synthesis in various cells including mesenchimal cells, hepatocytes, and monocyte-macrophages (Miller et al. 1991). Most of the stimuli related to inflammation and directed at the synthesis of ferritin seem to upregulate H-chains preferentially over L-chains, thus resulting in an increase in catalytic sites and a reduction in the availability of iron (Kwak et al. 1995).

The H subunit is the main regulator of ferritin activity. Overexpression of the H-chain in MEL cells led to an iron-deficient phenotype with a decrease of the iron labile iron pool, and decrease of hemoglobin synthesis (Picard et al. 1996). These cells reduced generated fewer ROS upon exposure to hydrogen oxide or hemin. HeLa cells overexpressing the H-chain also showed resistance to oxidative stress, suggesting regulation of the redox status of the cells, by removing the potentially toxic ferrous ions (Epsztejn et al.

1999). Thus, the biological evidence for an anti-oxidative role of ferritin is now compelling.

Functions and regulation of iron metabolism in mitochondria

In hepatic mitochondria, 30-50% of mitochondrial iron is heme, and the remaining 50-70% is non-heme iron. Of the mitochondrial non-heme iron 40% is found in iron-containing proteins, including iron-sulfur cluster-containing proteins and the remaining 60% is known as labile iron and utilized for heme biosynthesis. The labile iron pool in mitochondria varied with the concomitant change in production of heme. The administration of griseofulvin, an inhibitor of heme biosynthesis, to rats caused an increase in porphyrins and mitochondrial iron in liver (Tangeras 1986). The main route of metabolism of iron in mitochondria in hepatic cells seems to be similar to that in erythroid cells and other tissues. It has been considered that the iron content of mitochondria could change markedly dependent on the intracellular iron level (iron-deficient or -overload). However, the recent development of techniques for the isolation of mitochondria have clarified that the amount of lysosomal iron, but not mitochondrial iron, changed with the load of iron in the cells (Tangeras 1986; Richardson and Ponka 1997).

Mitochondria take up iron when they are incubated with various iron compounds such as ferric chloride, ferric citrate, ferric sucrose and ferric pyrophosphate. When transferrin and ferritin were used as the ligands, the iron was incorporated into heme (Nilsen and Romslo 1985). Thus, the true ligand for iron in cells is unclear. As discussed later, molecules related to iron transfer and metabolism were recently identified as a result of studies on the abnormal metabolism of iron in human inherited diseases and yeast mutants. Generally, studies on iron metabolism in vitro face obstacles such as the rapid oxidization of ferrous ions, and the insolubility and non-specific absorption of ferric ions. Therefore, in vitro research on the control of iron is difficult.

Mitochondria play a major role especially in erythroid cells, producing a large amount of heme. The metabolism of mitochondrial iron can be independent of the biosynthesis of heme since the uptake of iron by mitochondria was observed even when the synthesis of heme stopped (Ponka et al. 1998). The main form of labile iron in mitochondria has been unclear, but a recent report demonstrated the presence of mitochondrial ferritin (Mt-ferritin), as one of the iron-storage proteins (Levi et al. 2001). Mt-ferritin exhibits homology to H-ferritin (heavy chain) and is expressed as a monopolymer. The over-expression of Mt-ferritin led to an accumulation of iron in mitochondria, decrease of cytosolic ferritin and increase in the expression of transferrin receptor 1. Considering that Mt-ferritin levels increased in patients with characteristics of iron accumulation (i.e. sideroblastic anemia), Mt-ferritin may have a protective effect against iron-toxicity (Cazzola et al. 2003). In contrast to storage-type mitochondrial iron, most labile iron in mitochondria can be readily used for heme synthesis. Moreover, newly synthesized heme is rapidly exported outside of the mitochondria into the cytosol and endplasmic reticulumn, and associates with apo-hemoproteins (Taketani and Tokunaga 1980; Senjo et al. 1985). Especially in erythroid cells, most iron taken up by mitochondria is exported to the cytosol in the form of heme, and the level of uncommitted heme in mitochondria can be quite low (Ponka et al. 1998; Taketani et al. 1998a). The accumulation of iron in mitochondria and ferritin is not observed in erythroid cells when the synthesis of heme is inhibited. Considering that a large proportion of the iron taken up is utilized to produce heme in erythroid cells, a specific system controlling iron and heme may be present in erythroid cells. A recent study proposed that a transient interaction between iron-rich endosomes and mitochondria takes place to facilitate the transport of iron aquired from transferrin to ferrochelatase (Zhang et al. 2005). On the other hand, the regulation of the metabolism of iron in non-erythroid cells is different from that in eryhtroid cells since excess iron in non-erythroid cells is stored in ferritin (Picard et al. 1996). Thus, the interaction among organelles and molecules may be dependent on the functions of the cell.

It is known that copper transport is tightly coupled with iron-transport and heme biosynthesis since reticulocytes in copper-deficient animals exhibit a reduction in not only heme biosynthesis but also iron uptake by mitochondria (Cox et al. 1994; Li and Kaplan 2004). In disorders of heme biosynthesis including anemia with ALAS2-deficient, ring-sideroblasts and hemosiderin characteristics of iron accumulation appear (May and Bishop 1998; Bekri et al. 2003). These phenotypes are distinguished from iron-deficient anemia. Four reasons for the appearance of sideroblasts are: (A) mitochondria of erythroblasts are committed to targeting organelles of iron, (B) protoporphyrin, a substrate of heme synthesis, is not produced, (C) heme, a negative feedback product of iron uptake is not synthesized and iron comes to be accumulated and (D) mitochondrial iron is not exported before the iron is used as heme. Sideroblasts isolated from patients with Pearson disease normally produce heme, but show a reduction of ferric ions due to abnormal mitochondrial DNA, resulting in the accumulation of iron. (Muraki et al. 2001) Deletions or mutations of mitochondrial DNA with aging affect indirectly the metabolism of iron and trigger a disorder of iron utilization in the case of primary-acquired sideroblastic anemia classified as myelodysplastic syndrome (Muraki et al. 2001). On the other hand, it is unclear why iron is accumulated in acquired-sideroblastic anemia since there is no evidence of defects in the production of protoporphyrin.

Friedreich's ataxia is a neurodegenerative disorder typically caused by adeficiency of frataxin, a mitochondrial protein involved in the metabolism of iron in mitochondria (Pollard et al. 2004). The first intron of the human frataxin gene contains 6-42 GAA repeats in normal subjects and 200-1,700 GAA repeats in patients with Friedreich's ataxia. The three dimensional structure of frataxin is now known and the protein has a pocket to bind iron (Bulteau et al. 2004). A frataxin homologue was found in yeast and the loss of yeast frataxin causes an accumulation of iron in mitochondria (Chen et al. 2004). The frataxin-deficient mutant was very sensitive to oxidative stress, a phenotype which was reversed by the expression of human frataxin. The uptake of iron by mitochondria is normal in the yeast mutant, but the flow or efflux of iron in mitochondria can be decreased. Since decreases of mitochondrial and cytosolic iron-sulfur-containing proteins in the frataxin-deficient mutant are observed, frataxin plays a role in iron homeostasis in mitochondria. Recently, frataxin was found to interact with aconitase to reduce the level of oxidant-induced inactivation, and converted the inactive 3Fe-4S enzyme into the active 4Fe-4S form (Bulteau et al. 2004). In mitochondria, more than 10 subunits of enzymes involved in respiratory chain (Complexes I, II and III) exist as the iron-sulfur cluster-containing proteins, and require iron, suggesting that frataxin is an iron-chaperone protein that protects these enzymes from disassembly and promotes enzyme reactivation (Fig. 2). Moreover, it is found that DNA damage-inducible nuclear genes were highly expressed in cells lacking frataxin, suggesting that frataxin has a protective role in the nucleus and mitochondria to detoxify reactive oxygen species (Karthikeyan et al. 2002).

Atm1p in yeast was first identified as an ATP-binding cassette (ABC) transporter involved in iron metabolism in mitochondria (Csere et al. 1998). The Atm1p-deficient yeast mutant accumulated iron in mitochondria and was sensitive to oxidative stress. Since a decrease in the cytosolic proteins containing the iron-sulfur cluster was observed in yeast Δatm1 cells, Atm1p plays a role in the export of iron compounds and the maturation of cytosolic Fe-S proteins (Lill and Kispal 2001). Five half-type ABC transporters are localized in mitochondria. The human homologues of yeast Atm1p are ABC7 and MTABC3 (Mitsuhashi et al. 2000). These two proteins functionally complement the deficiency of yeast Atm1p. In humans, defects in ABC7 cause iron to accumulate in mitochondria, in the inherited disease sideroblastic anemia/ataxia, an X-linked recessive disease (Bekri et al. 2000). Since the function of ABC7 or MTABC3 is similar to that of yeast Atm1p, these pump proteins are involved in the export of iron from mitochondria (Lill and Kispal

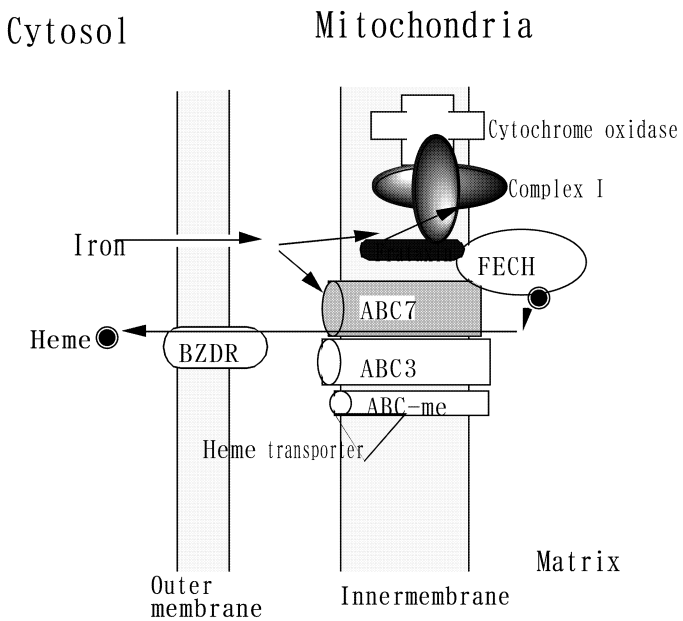

Fig. 2. Biosynthesis and transport of heme in mitochondria. At the last step of heme biosynthesis, several proteins involved in respiration, ferric reduction and transporters are tightly coupled. Iron is imported into mitochondria and reaches molecules catalyzing ferric reduction, including Complex I and cytochrome oxidase. Reduced iron is immediately used for heme synthesis, by ferrochelatase (FECH) and then transferred outside of mitochondria, possibly mediated by peripheral-type benzodiazepine receptors (BZDR). ABC type-pump proteins are also involved in export of iron and heme.

2001). We recently demonstrated the association of ABC7 and ferrochelatase, a mitochondrial iron-sulfur containing protein (Taketani et al. 2003). The increases in ferrochelatase activity and hemoglobin synthesis with the expression of ABC7 led us to conclude that mammalian ABC7 plays a role in the maturation of mitochondrial iron-sulfur-containing proteins, in addition to cytosolic iron-sulfur-containing proteins (Fig. 2). Another mitochondrial ABC transporter, ABC-me, also promotes hemoglobin synthesis and erythroid differentiation (Shirihai et al. 2000).

Biosynthesis and regulation of heme metabolism in mitochondria

The first and last three steps in the biosynthesis of heme take place in mitochondria. ALAS [EC 2.3.1.37] is the first enzyme of the heme biosynthetic pathway and catalyzes the condensation of glycine and succinyl-CoA to form ALA (Sassa 1988). The enzyme forms a homodimer and is located in the innermembrane of mitochondria in mammals. ALAS activity is found in a variety of cells. A high level of enzyme activity and inducible expression of enzyme by chemical treatments or by physiological stimuli are found in two tissues as follows: bone marrow cells and liver. Many studies have demonstrated that ALAS is the rate-limiting enzyme of heme biosynthesis in liver and negatively regulated by heme (this subject is also discussed later) (Sassa 1988). On the other hand, ALAS from erythropoietic cells is apparently distinct from that from hepatic cells: the erythroid enzyme is not induced by porphyrogenic chemicals. The molecular weight and immunoreactivity of the erythroid enzyme were different from those of the enzyme from liver. Then, the cDNA and gene structure of the erythroid enzyme (ALAS2) from human (chromosome Xp11.2) and mouse were demonstrated (Sassa and Nagai 1996). ALAS2 mRNA is markedly expressed during erythroid differentiation. Characterization of the promoter region of the human ALAS2 gene revealed several putative binding sites for the ery-

throid-specific transcription factors GATA-1 and NF-E2. In addition, as discussed before, the IRE motif of ALAS2 mRNA in the 5´-protein noncoding region is involved in regulating the translation of mRNA to protein (Bekri et al. 2003). Mice lacking ALAS2 died in the embryonic stage (Nakajima et al. 1999). Male hemizygous null mutants could not support fetal hematopoiesis and were not viable after embryonic stage E11.5. The heterozygous females exhibited ring sideroblasts in the marrow, thereby indicating that ALAS1 cannot rescue the ALAS2 deficiency.

The house keeping isoform ALAS1 is ubiquitously expressed and is encoded by a gene located on human chromosome 3 at q21 (Roberts and Elder 2001). The enzyme produces heme for a variety of hemoproteins. The level of intracellular uncommitted heme is extremely low since increased levels of free heme are toxic, so heme biosynthesis is tightly regulated at the step of ALAS1 by multiple mechanisms such as (1) preventing the transfer of ALAS1 precursor to the mitochondria, (2) decreasing the stability of ALAS1 mRNA, and (3) repressing the transcription of ALAS1 mRNA in mammals (Munakata et al. 2004). On the other hand, the drug-responsive elements that mediate the direct activation of the transcription were identified in the promoter region of the murine, chicken and human ALAS1 genes (Fraser et al. 2002; Podvinec et al. 2004). The binding of nuclear receptors such as constitutive androstane receptor and pregnane X receptor to the drug-responsive elements led to activation of the transcription, the mechanisms being similar to those for the transcriptional activation of drug-inducible cytochrome P-450s (Podvinec et al. 2004).

As the final step in the biosynthesis of heme, ferrochelatase catalyzes the insertion of iron into the protoporphyrin IX ring to form protoheme. An understanding of the enzyme at the molecular level is needed to elucidate how the metabolism of heme and iron metabolism is regulated (Taketani 1993). The mammalian enzyme is located in the innermembrane of mitochondria and faces the active site of the matrix (Taketani 1993). The mammalian enzyme contains a 2Fe-2S cluster in the carboxyl terminal region (Wu et al. 2001; Ohgari et al. 2005). The iron-sulfur cluster is essential for promotion of the high enzyme activity although the cluster is not part of the binding region of the ferrous ion substrate (Furukawa et al. 1995; Taketani et al. 2000, 2003). Given that the expression of ferrochelatase is regulated by the level of intracellular iron, via the iron-sulfur cluster, and that an iron-transporter ABC7 interacting with the Fe-S cluster region in ferrochelatase regulates the level of ferrochelatase protein, ferrochelatase is an iron sensor in mitochondria (Taketani et al. 2000, 2003). Furthermore, the cellular content of heme decreased when erythroid and non-erythroid cells were treated with an iron chelater, desferioxamine, indicating that the decrease in ferrochelatase activity causes the decrease in heme biosynthesis (Taketani et al. 2000). However, protoporphyrin did not accumulate in iron-deficient cells or patients with ABC7 deficient-sideroblastic anemia/ataxia. Conversely, in patients with erythropoietic protoporphyria caused by ferrochelatase deficiency protoporphyrin but not iron is accumulated (Taketani 1993). Thus, the de novo synthesis of porphyrin is coupled to the availability of iron as a result of a translational induction of ALAS2 by iron (Ponka et al. 1998). Considering that a deficiency of ALAS2 causes a phenotype very similar to that caused by ABC7, the biosynthesis of heme is tightly coupled with the homeostasis of iron in erythroblasts. In addition, NO-induced loss of heme was observed in primary cultures of hepatocytes where ALAS1 as well as ferrochelatase was reduced (Kim et al. 1995). Intracellular iron is a major target of NO, which impairs the metabolism of mitochondrial iron, indicating that the hepatic synthesis of porphyrin is also regulated by iron.

Ferrous ion is only a substrate of the ferrochelatase reaction in biological systems. The reduction of ferric ion is necessary to produce heme. Then, the reason for the return of the heme-biosynthetic pathway to mitochondria from cytoplasm is considered to be the requirement for a considerable amount of ferrous ion for the final reaction. In this connection, several studies showed that yeast ferrochelatase formes a com-

plex with more than 10 mitochondrial peptides, and NADH dehydrogenase in mammalian mitochondria reduces ferric ion to ferrous ion, which is directly utilized for the ferrochelatase reaction (Taketani et al. 1986; Camadro and Labbe 1988). More recently, it was shown that subunit I of cytochrome oxidase is able to reduce ferric ion and a mutation of this in patients with acquired idiopathic sideroblastic anemia led to the accumulation of ferric ion, suggesting that subunit I is involved in the reduction of ferric ion (Gattermann et al. 1997). Although the site of iron reduction is not clear, the complex involved in iron transport, iron reduction and heme production is universally responsible for the main function of mitochondria.

Transport and utilization of heme in cells

Heme is insoluble and generally requires specific extracellular and intracellular proteins as transient carriers. Extracellular heme in plasma is taken up by various cells including hepatocytes. There are several reports on the direct internalization and utilization of heme in cultured cells. The heme taken up by the cells plays roles in the prompt proliferation and differentiation of cells (Sassa 1988). The main pathways of the uptake of heme and utilization of internalized heme are shown in Fig. 3. Human erythroleukemia K562 and murine erythroleukemia (MEL) cells differentiate into erythroid-like cells on treatment with hemin (Sassa 1988). Heme also promotes the differentiation of white cells. Heme and cobalt-protoporphyrin which inhibits heme oxygenase induce macrophage-dependent mitogenesis of human T lymphocytes (Stenzel et al. 1981). The neural differentiation of PC12 cells occurs in response to hemin (Zhu et al. 2002). Apart from the uptake of iron via the transferrin-mediated system, the mechanisms involved in the uptake of heme by these cells are poorly understood.

There have been difficulties with experiments in vitro on heme binding due to the insolu-

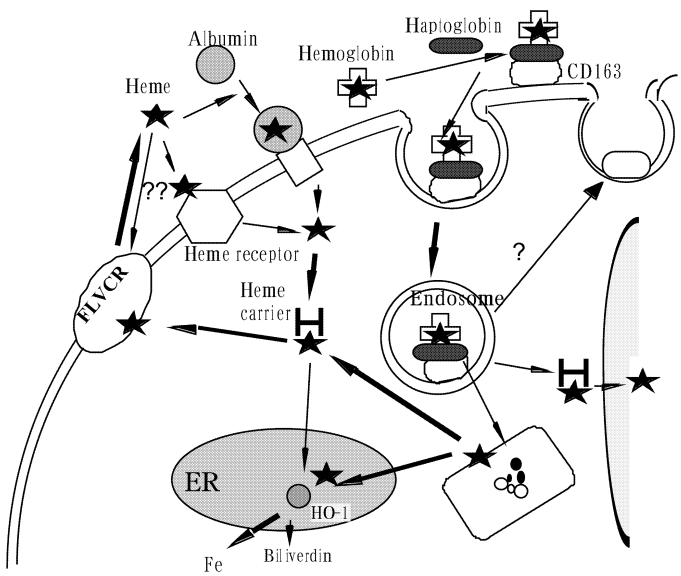

Fig. 3. Uptake and utilization of heme by cells. Plasma heme binds to albumin and hemopexin, and enters the cells in a receptor-mediated fashion. Heme taken up by the cells binds to several cytosolic heme carriers such as p22 HBP, SOUL and ligandin, and is utilized directly for the maturation of hemeproteins. Heme is also transported to endoplasmic reticulumn (ER) and mitochondria. Then, excess heme is degraded to iron and biliverdin by HO. Hemoglobin released from erythrocytes rapidly associates with haptoglobin, and the resulting complex is then recognized by CD163 (haptoglobin receptor) on macrophages. The hemoglobin-haptoglobin complex is taken up by the cells via an endocytotic pathway. After hemoglobin is lysed in lysosomes, the heme-moiety is transported to the ER, then degraded.

ble and sticky nature of heme (hemin). It is likely that all the specific proteins that bind heme have been characterized. The heme-binding proteins in blood and culture medium are serum proteins, albumin and hemopexin. Albumin binds various compounds of low molecular weight and 1 mol heme/ mol of albumin with a Kd of 10^{-8} M, and several sites with lesser affinity (Muller-Eberhard and Fraig 1993). Hemopexin specifically binds 1mol heme/mol hemopexin with an extremely high affinity (Kd: $\sim 10^{-12}$ M). Since hemopexin exhibits high affinity for heme, hemoglobin-heme (Kd: $\sim 10^{-8}$ M) released from disrupted erythrocytes can be transferred to apo-hemopexin (Muller-Eberhard and Fraig 1993). Hemopexin-heme activates HO-1 and metallothionein genes after being taken up by cells in a receptor-mediated manner (Ren and Smith 1995). There are several early studies on the hemopexin receptors from various tissues such as rat liver and human placenta, but studies on properties of the receptors have not been made (Taketani et al. 1987; Ren and Smith 1995). On the other hand, although the induction of HO-1 and repression of ALAS1 was observed in hemin-treated chicken and rat hepatocytes in culture (Sinclair et al. 1988), the addition of apo-hemopexin to the medium cancelled these effects, indicating that hemopexin binds free heme and suppresses the uptake of heme (Sinclair et al. 1988; Taketani et al. 1998b). Thus, the hemopexin-mediated uptake of heme by the cells has been confused. Finally, hemopexin-deficient mice did not show any impairment of heme metabolism but were sensitive to oxidative stress, as playing a role as anti-oxidants for exogenous insults (Tolosano and Altruda 2002). Along with hemopexin receptors, the presence of heme receptors was reported on the surface of MEL cells, Caco cells and hepatocytes (Uc et al. 2004). Very recently it was found that the expression of FLVCR, a cell surface receptor for feline leukemia virus group C, was inversely proportional to the intracellular level of heme (Quigley et al. 2004). The export of heme from the cells increased with the expression of FLVCR. Thus, FLVCR exports cytoplasmic heme and the expression decreases during erythropoiesis, indicating that FLVCR plays an important role in the early stages of erythropoiesis. The relation of FLVCR to the cell-surface receptors for heme is a subject of future investigations. It is possible that FLVCR is the same as previously isolated heme or hemopexin receptors.

When hemoglobin is released from erythrocytes due to injury, a serum protein haptoglobin captures the released hemoglobin, and the resulting complex is internalized into the liver after which globin is hydrolyzed in lysosomes. The heme moiety is, on the other hand, degraded by the microsomal HO, to produce biliverdin and iron. The iron that is released is incorporated into ferritin and then re-utilized. In 2001, CD163, a cell marker of macrophages and monocytes, was identified as a specific receptor for the hemoglobin-haptoglobin complex, thereby indicating that the hemoglobin-haptoglobin complex in hepatic circulation is predominantly taken up via CD163, by Kupper cells (Kristiansen et al. 2001).

Heme synthesized inside the innermembrane of mitochondria is utilized as a prosthetic group by cytochromes in mitochondria, microsomal hemoproteins such as cytochromes b_5 , and P-450, a peroxisomal catalase. Hemoglobin and myoglobin in the cytosol also readily associated with heme. Isoforms of glutathione transferase, ligandin and Yb_2Yb_2, have been considered to be intracellular carriers of heme which transfer it to the endoplasmic reticulumns (Senjo et al. 1985). Z-type fatty acid-binding proteins also promote the removal of heme from mitochondria (Vincent and Muller-Eberhard 1985). Furthermore, cDNA for HBP23 was first isolated from rat liver, and HBP23 showed heme-binding ability (Iwahara et al. 1995). On the other hand, it was found that yeast and mouse homologues of HBP23 act to protect against oxidative stress rather than as a heme-binding protein (Ishii et al. 1993). They are known as peroxiredoxins (Prdx), and show a peroxidase activity which is independent of heme-binding (Wang et al. 2003). Finally, Perx1 acted as an antioxidant and tumor-suppressor in Perx1-knock out mice although the role of heme-bound Prdx1 is not clear (Neuman et al. 2003). p22 HBP, SOUL, a homologue of p22HBP and other

candidates were also identified as soluble heme-binding proteins, but their precise roles are not clear (Taketani et al. 1998a; Sato et al. 2004).

Recent advances in techniques for the expression of isolated cDNAs into *E. coli* and simple purification of expressed protein are now resolving the obstacles to the characterization of heme-binding properties. Findings with novel diseases and knock-out mice involving the metabolism of heme will highlight the functions of heme-binding proteins in physiological conditions. Important roles for HO in controlling cellular responses to stress or sensing oxygen have been recently demonstrated. To evoke HO activity at the endoplasmic reticulumn, it is essential that the substrate (heme) reach the active center of the enzyme. Therefore, resolving the mechanisms for the transport of heme or hemoproteins by endoplasmic reticulumn will facilitate the development of clinical applications.

Heme-dependent regulation of hemoprotein biogenesis

The expression of yeast respiratory enzymes such CYC-1p and CYC-2p in mitochondria is controlled by the intracellular level of heme. Heme activating factor-1 (HAP-1) was first demonstrated as a heme-dependent transcription factor and regulates the expression of CYC-1p and CYC-2p (Kwast et al. 1998: Zhang et al. 1998). HAP-1p and HAP-2/3/4/5p contain a DNA-binding domain recognizing the promoter region of these genes and a heme-binding domain where six copies of consensus amino acid sequence K/RCPV (CP-motif) are present (Zhang et al. 1998; Ogawa et al. 2001). Yeast coproporphyrinogen oxidase, catalyzing the 6th step in the biosynthesis of heme, is the rate-limiting enzyme and also regulated by the HAP system (Amillet et al. 1995).

Dependent on the increase in the heme pool in mammalian cells, the induction of HO-1, the repression of ALAS1 and an increase in the expression of cytochrome P-450 (b-type) were observed. The regulation of ALAS1 expression also occurs at the post-transcriptional level. Namely, the translation of ALAS1 mRNA is inhibited by heme (Munakata et al. 2004). The translocation of the ALAS1 precursor into mitochondria is also inhibited by heme. This inhibition is clearly explained by the fact that the CP-motif is found in the leader peptide of the ALAS1 precursor and can bind heme (Munakata et al. 2004). Furthermore, the stability of ALAS1 mRNA and ferrochelatase mRNA is regulated by heme (Fujita et al. 1991; Fukuda et al. 1993). However, for a long time, the mechanisms involved in the heme-dependent regulation of expression at the transcription level had not been understood. The transcription factor Bach1 was found as a partner of the small Maf proteins including MafK, MafF and MafG, and as the first mammalian factor exhibiting heme-binding (Ogawa et al. 2001). The Bach1-Maf protein heterodimer represses the transcription of the target genes by binding to the Maf-recognition (MARE) sites in the enhancer region of HO-1. Details of how Bach1 is involved in the heme-induced activation of the HO-1 gene are given in the excellent review by Shibahara (2003).

Red blood cells have a massive requirement for heme as a prothestic group of hemoglobin. Then there is an unique association between heme and globin synthesis. It is well known that heme up-regulates β-globin synthesis during erythroid differentiation. Previous studies have shown that heme regulates hemoglobin synthesis at the transcription and translation of the β-globin mRNA (Sassa and Nagai 1996; Crosby et al. 2000). A heme-regulated elF-2α kinase (HRI) is responsible for the regulation of β-globin mRNA. The binding of heme to HRI inhibits the phosphorylation of elF2α, resulting in promotion of the translation of globin and other proteins in erythroid cells (Crosby et al. 2000). On the other hand, when heme content was decreased, the level of β-globin mRNA significantly decreased (Tahara et al. 2004b). Exogenously added hemin reversed the suppression. The human globin cluster spans a region of 70 kb containing five developmentally regulated genes ε, γ_G, γ_A, δ, and β. The entire region is controlled by the microlocus control region (μLCR) (Crossley and Orkin 1993). The heme-dependent expression of β-globin is a tran-

scriptional event and the expression of the human globin gene promoter containing μLCR is regulated by the intracellular level of heme (Tahara et al. 2004b). The control of the μLCR-β-globin gene promoter activity was dependent on DNase-hypersensitive site 2(HS2), which contains the MARE sites. The MARE site has been identified as the binding site of a transcriptional activator NF-E2. An increase in NF-E2-binding was observed during erythroid differentiation. The decrease of Bach1-binding to MARE sites and concomitant increase in NF-E2-binding was dependent on the intracellular level of heme, indicating that heme acts as a positive regulator of β-globin expression by inhibiting the repressor activity of Bach1. Similar to the mode of the activation of the β-globin gene by heme, the expression of the human α-globin mRNA was also regulated by intracellular heme, in a Bach1-dependent manner (Tahara et al. 2004a). It is thus concluded that mechanisms involved in the up-regulation by heme of hemoglobin synthesis in erythroid cells contribute to the huge quantities of the oxygen carrier hemoglobin in every tissue.

The core clock of circadian rhythms comprises PAS (PER-Arnt-Sim) domain proteins and the PAS domains of several bacterial proteins operate as oxygen sensors via a heme prosthetic group (Shearman et al. 2000: Hardeland et al. 2003). Mammlian NPAS2 (neuronal PAS domain 2) was first identified as a member of the basic helix-loop-helix (bHLH) family of transcription factors. Clock exhibiting a sequence similar to NPAS2 was then characterized as a crucial regulator of circadian rhythms. NPAS2 and Clock regulate the activating or inactivating portions of the circadian transcriptional feedback cycle by forming heterodimeric complexes with other bHLH transcription factors, BMAL1, PER1, PER2 and CRY. Of the major transcription factors, NPAS2 was found to bind heme. NPAS2 heterodimerizes with BMAL2 and binds to the E-box of the target gene while CLOCK competes with the dimerization with BMAL2. The transcriptional activity of the NPAS2-BMAL2 complex is regulated in a CO-heme- dependent manner (Gilles-Gonzalez and Gonzalez 2004). CO is formed in neurons predominantly by HO-2 whose location partially overlaps with the distribution of NPAS2. Thus, it is possible that HO-2 produces a low concentration of CO in the brain, which is available to regulate the DNA-binding activity of NPAS2. Recent studies have identified a novel role of HO-2 for oxygen sensing (Adachi et al. 2004; Williams et al. 2004), suggesting that oxygen controls the NPAS2-dependent transcription, via the HO-2 activity.

A recent study demonstrated that NPAS2 and PER2, another heme-binding factor, control the circadian rhythms of heme biosynthesis (Kaasik and Lee 2004). Namely, in mice deficient in PER2 the expression of NPAS2 is modulated. Conversely, PER2 is a positive regulator of BMAL1-NPAS2 transcription activity and NPAS2 regulates the transcription of the ALAS1 gene, encoding a rate limiting enzyme of heme biosynthesis (Kaasik and Lee 2004). Heme biosynthesis is coordinated with the expression of circadian controlled genes that encode hemoproteins such as NO synthase, guanylyl cyclase and other enzymes (Gilles-Gonzalez and Gonzalez 2004). An increasein the level of intracellular heme induces the expression of HO-1 producing CO, biliverdin and iron. CO inhibits the binding of the heme-bound NPAS2-BMAL1 to DNA, causing the transcription to stop, and PER2 further modulates the decrease in transcription. The decrease in heme eventually reaches a nadir, which allows for the binding of NPAS2-BMAL1 to DNA and then the cycle is restarted.

New members involved in the regulation of intracellular levels of iron

In the classic iron delivery system, only transferrin receptor 1 has been identified as a player in the uptake of iron by the cells. Recently, a new human gene has been identified, and named transferrin receptor 2 since it exhibited homology (more than 50% similarity) to transferrin receptor 1 (Fleming et al. 2002). Receptor 2 is highly expressed in liver and in some proliferating cells. Its function is similar to that of receptor 1 though its affinity for transferrin is higher than that of receptor 1, and the expression is regulated in a dif-

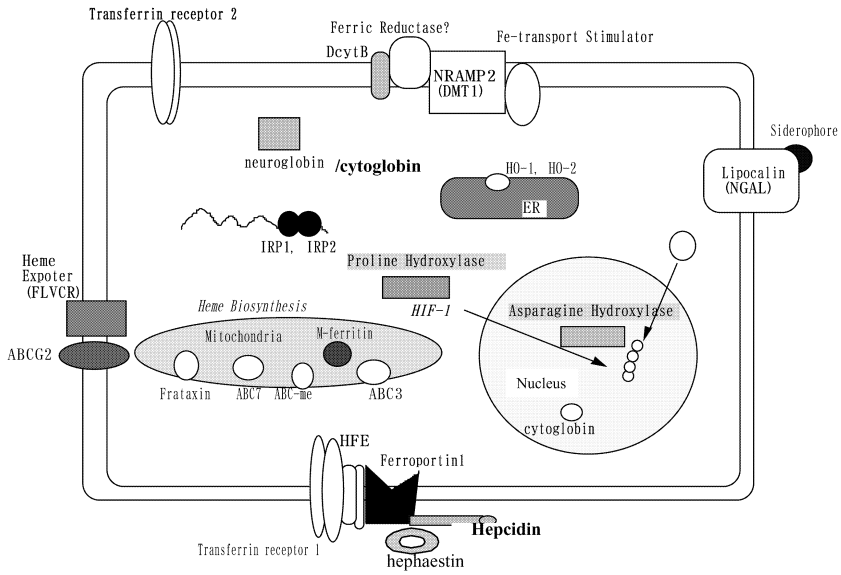

Fig. 4. New members involved in the transport and regulation of iron and heme. HFE, DcytB, hephaestin, transferrin receptor2, Fe-transporter stimulator, ferroportin1, lipocalin and hepcidin are involved in iron metabolism; FLVCR, $ABCG_2$, neuroglobin and cytoglobin are involved in heme transport and regulation; HIF-1α proline hydroxylase and HIF-1α asparagine hydroxylase are regulators as an oxygen sensor.

ferent way. Namely, transferrin receptor 2 mRNA does not contain IRE and may be expressed constantly. Transferrin receptor 1 is absolutely required for life because mice lacking transferrin receptor 1 die in utero, therefore, receptor 2 may have some helpful function in cells actively utilizing iron (Brissot et al. 2004).

Dietry iron enters the body primarily via the most proximal portion of the intestine. Small intestinal cells which do not have transferrin receptors on their luminal surface possess an iron delivery system containing mobilferrin and integrin which play an important role in facilitating mucosal the uptake of iron and other metals. Before the entry of iron into enterocytes, insoluble ferric ion can be reduced by a cytochrome b-like hemoprotein Dcytb at the plasma membrane (Fig. 4) (McKie et al. 2002). A transmembrane protein Nramp2 (also known as DCT1 or DMT1) is expressed on the lumen of the intestine and transports ferrous ion across the membrane. Nramp2 is a proton/divalent metal co-transporter that transfers several transition metals including iron, manganese and cobalt (Gunshin et al. 1997). The Nramp2 mRNA contains IREs and the expression of Nramp2 is regulated post-transcriptionally by the iron level in the body. Defects in Nramp2 (*mk* mouse and Belgrade rat) lead to the impaired transport of iron and the defective endosomal transport of iron (Andrews 2002; McKie et al. 2002). These results indicated that Nramp2 is also involved in the transport of ferrous ion across endosomal membrane into the cytoplasm after the transferrin-iron is released from transferrin in endosomes and reduced by ferrireductase. A transferrin-independent iron transport system in cells expressing transferring receptors has been described. In some cells, a protein named stimulator of Fe transport (SFT) seems to enhance both transferrin and non-transferrin-bound iron transport (Yu and Wessling-Resnick 1998). It is evident that micro-organisms secrete small peptides, siderophores, to trap iron which is taken up by cells. Similar to the bacterial iron uptake system, mammalian neutral gelatinase–associated lipocalin (NGAL/24p3) involved in iron-binding at the cell surface was discovered (Yang et al. 2002; Kaplan 2002). Lipocalins are a large group involved in the transport of chemicals including retinal, fatty acid and fatty acids and ordorants.

NGAL/24p3 binds the bacterial chemoattractant peptide f-Met-Leu-Phe, is secreted from neutrophils and epithelial cells, and then plays a role in the regulation of immune systems. Expression of recombinant NGAL/24p3 revealed that bacterial enterobactin, one of the siderophores, bound iron and the complex was internalized into cells, via an endocytotic process (Yang et al. 2002: Kaplan 2002). Based on observations that internalized iron can regulate the expression of iron-responsive genes and transferrin receptor 1-knock out mice show normal development in the early embryonic stage, differentiating epithelial cells utilize NGAL-mediated iron delivery and this is specifically important in the early development of embryos where the circulation of transferrin and the expression of transferrin receptors are not established (Kaplan 2002).

After the absorption of iron by intestinal Nramp2, the transport of iron across the basolateral membrane to the portal vein occurs. An iron-regulated transporter, IREG1, specially expressed in the duodenal mucosa has been cloned, and functions in the stimulation of iron efflux from the cells (McKie et al. 2002). The expression of IREG1 is increased dependent on the increase in iron absorption and the 5´-uncoding region of IREG1 mRNA contains an IRE. Following the finding of IREG1, a mutant gene was characterized from the zebrafish mutant weissherbst (weh) that causes a hypochromic anemia, apparently through iron deficiency (Donovan et al. 2000). The normal allele of this gene was termed ferroporptin 1. Homologs identified in mouse and human showed that ferroportin 1 mRNA is present in tissues involved in iron export: zebrafish yolk sac and intestine, as well as mouse placenta, intestine and macrophages. The function of ferroportin 1 in iron export was demonstrated by the efflux of radioactive iron incorporated by Xenopus expressing DMT and ferroportin 1. In addition, ceruloplasmin, a plasma protein, is required for the export of iron from non-intestinal cells. Ceruloplasmin oxidizes ferrous ion, exported by ferroportin 1, to ferric ion to facilitate the binding of iron to transferrin (Andrews 2002; Lee et al. 2002). Humans and mice deficient in ceruloplasmin accumulate iron in several cells including macrophages, neural cells and hepatocytes, indicating that serum ferroxidase activity is essential for the smooth mobilization of iron between macrophages and other tissues. Hephaestin is a transmembrane-bound ceruloplasmin homologue that is apparently involved in the passege of iron through the intestinal enterocytes (Anderson 1999; Vulpe et al. 1999; Lee et al. 2002).

A novel regulator of intestinal iron absorption and iron homeostasis in cells has been recently identified. Hepcidin is a peptide hormone that was discovered simultaneously as an antibacterial protein found in human urine (Park et al. 2001) and as a protein preferentially expressed in iron-loaded murine liver. Mice lacking hepcidin exhibited massive iron overloading whereas the transgenic expression of hepcidin led to hypoferremia and the anemia of inflammation (Bridle et al. 2003: Nicolas et al. 2004). In humans, high levels of hepcidin in hepatic adenomas resulted in chronic anemia (Nicolas et al. 2004). These observations indicated that hepcidin is produced as a result of iron-overload in liver and regulates the intestinal absorption of anemia. Most recently hepcidin was found to bind to ferroportin 1 in cultured cells (Nemeth et al. 2004). The hepcidin-bound ferroportin 1 was internalized, and then degraded, resulting in a decrease of iron export. Thus, hepcidin is a key to iron homeostasis that it secreted dependent on the intracellular level of iron and controls the level of ferroporptin 1 on the cell surface.

Dysfunction of molecules involved in iron metabolism

Hereditary hemochromatosis is a common disease whose main feature is the excessive absorption of dietary iron with a relative lack of iron in reticuloendothelial stores (Feder et al. 1996). Actually, the deposition of iron in parenchymal tissues results in cirrhosis of the liver, diabetes mellitus, skin pigmentation and testicular failure. HFE, the protein that is defective in most patients with hereditary hemochomatosis, is a family of major histocompatibility complex (MHC) class I proteins (Bennett et al. 2000). Newly synthesized

HFE forms a complex with β_2-microglobulin, in the same fashion as human HLA (class I) and mouse H-2 antigens and these heterodimers are expressed on the cell surface (Feder et al. 1996; Sheth and Brittenham 2000). Different from immunological antigens, HFE does not show diversity among individuals and does not directly contribute to the immune response. HFE is abundantly expressed in the crypt cells of the duodenal mucosa, the expression being inversely related to the level of Nramp 2 (DMT1) in intestinal cells (Canonne-Hergaux et al. 2001). A missense mutation in the HFE gene is responsible for 60-100% of cases of hereditary hemochromatosis. The most common such mutation in Caucasians corresponds to the substitution C282Y, followed by H63D and then S65C (Table 1). Importantly, 10-15% of patients worldwide demonstrate none of these three mutations, but are not clinically distinguished from others (Sheth and Brittenham 2000; Tomatsu et al. 2003).

The C287Y mutation disrupts a critical disulfide bridge used for interaction with β_2-microglobulin and the mutated protein is retained within the internal organelles. Mice lacking β_2-microglobulin are reported to exhibit an iron-overload similar to hemochromatosis (Ponka and Lok 1999; Sheth and Brittenham 2000). The HFE protein forms a stable complex with transferrin receptor 1, but not transferrin receptor 2, and apparently reduces the affinity of the receptor for transferrin (Tomatsu et al. 2003; Brissot et al. 2004). The complex forms at the cell surface and along the endocytotic pathway for the uptake of transferrin, where HFE and transferrin receptors are co-localized. Overexpression of HFE results in about a 30% reduction in the rate of iron uptake, with a subsequent reduction of intracellular iron and ferritin, thus suggesting that the role of HFE is to inhibit transferrin-dependent uptake of iron in vivo. Then, in cases where HFE is defective, cells would take up excess iron. However, in patients with hemochromatosis and mice deficient in β_2-microglobulin, the crypt cells behave as if starved of iron (Sheth and Brittenham 2000; Ahmad et al. 2002; Waheed et al. 2002). The expression of the molecules required for intestinal iron absorption is upregulated in the mature enterocytes and reticuloendothelial cells from HFE-deficient humans and mice did not accumulate iron in vivo (Sheth and Brittenham 2000; Waheed et al. 2002; Tomatsu et al. 2003). These observations are inconsistent with the dominant function of HFE as a competitive inhibitor of iron uptake. Therefore, it is possible that the proportion of HFE bound to transferrin receptor 1 at cell surface is inversely related to the degree of iron-saturation of transferrin in serum, and free HFE is able to inhibit the function of ferroportin 1. Another possible model of iron-overload is that HFE and transferrin receptor 2 must be key regulators of iron homeostasis, as homozygosity for point mutations in either of the genes encoding these proteins results in iron accumulation since the delivery of iron by transferrin receptor 2 is independent of the level of iron.

In this connection, patients with non HFE-associated type hemochromatosis mapped to

TABLE 1. *Disorders of iron transport and metabolism*

Disorder	Locus	Gene	Defect
Atransfrrinemia	3q21	Transferrin	Unknown
Aceruloplasminemia	3q23-2	Ceruloplasmin	W858X, del2389G
Hemochromatosis (HFE1) (adult)	6p21.3	HFE	C282Y, H63D, S63C
Hemochromatosis (HFE2) (juvenile)	1q	Hepcidin ?	—
Hemochromatosis (HFE3) (adult)	7q22	Transferrin receptor 2	Y250X
Hemochromatosis (neonatal)	2q32	Ferroportin1	Q248H, N144H
Friedreich's ataxia	9q13	Frataxin	GAA repeat
HMOX1 deficiency	22q12	HO-1	del exon2/del in exon3

chromosome 7q22 were found to have a mutation of the gene for transferrin receptor 2 (i.e. Y250X and M172K) (Camaschella et al. 2000; Pietrangelo 2004). Hemochromatosis in Northern Europe is associated with HFE defects, while in most African-American patients with iron-overload diseases and one-third of Italian patients with hemochrmatosis there is no link to the HFE locus. It is possible that a dysfunctional transferrin receptor 2 is responsible for the diseases in a considerable proportion of these patients. Mutations of ferroportin 1 were found in the autosomal dominant form of hemochromatosis (Montosi et al. 2001). Patients with this type of hemochromatosis show particular accumulations of iron in macrophages. The increase in the level of serum ferritin was always accompanied by a slight increase in transferrin saturation, which is different from HEF-linked hemochromatosis, exhibiting a slight elevation of transferrin saturation and normal level of serum ferritin. Since macrophages which highly express ferroportin 1 play a central role in iron metabolism among tissues (Nicolas et al. 2004), a disturbance of iron stores primarily takes place. In the rare human disorder aceruloplasminanemia, mutations in the ceruloplasmin gene are responsible for a deficiency of a copper transport protein and associated with iron overload in tissues (Vulpe et al. 1999; Pietrangelo 2004). Furthermore, two different phenotypes of hemochromatosis with an unidentified gene locus were also reported, and very recently one of them has been found to be a hepcidin defect (Roetto et al. 2004; Papanikolaou et al. 2004). There are many hypotheses on the mechanisms of iron-overload diseases, but they have not yet been proven.

Contribution of iron and heme to oxygen-sensing system

A number of physiologically important genes are expressed following a prolonged exposure to low concentrations of oxygen (hypoxia). Increased production of erythropoietin (EPO), a cytokine required for the formation of red blood cells, and an increase in the number of erythrocytes enhances the delivery of oxygen to tissues (Semenza 2001). Vasucular endothelial growth factor (VEGF) is a key regulator of blood vessel growth (angiogenesis) and tumor angiogenesis. Tyrosine hydroxylase is the rate-limiting enzyme in glomus cells of the carotid body in the neck and enables hypoxic animals to achieve a sustained increase in ventilation. Hypoxia also induces the synthesis of glycolytic enzymes, maintaining the level of energy-rich molecules including ATP. Oxygen sensing and chemical signaling in mammals occur via hypoxic responsive pathways, leading to the activation of a hypoxia-inducible transcription factor (HIF-1). Early studies showed that EPO production is markedly up-regulated by transition metals including cobalt, manganese and nickel, which can be incorporated into protoporphyrin IX, and have suggested that the oxygen sensor involved in the synthesis of EPO is a hemeprotein which binds oxygen (Goldberg et al. 1988, 1990). Furthermore, iron should play an important role in the oxygen sensing system since the increased expression of HIF-1, followed by the induction of EPO synthesis, was observed under iron-depleted as well as hypoxic conditions (Furukawa et al. 2001; Mizutani et al. 2002). Iron can alter levels of chemical messengers in the oxygen-sensing pathway since iron catalyzes the production of ROS by the Fenton reaction. However, until recently, the mechanisms by which cells sense alterations in oxygen tension and subsequently change HIF-1 activity have remained unknown.

The HIF factors are composed of two subunits: HIF-1α (or isoforms HIF-2α and HIF-3α), a hypoxia-regulated subunit; and HIF-1β (arylhydrocarbon receptor nuclear translocator [ARNT]), an oxygen-insensitive subunit (Semenza 2001). Under normoxic conditions, the oxygen-dependent domain (ODD) of HIF-1α is modified by a HIF-prolyl hydroxylase, leading to the recognition of HIF-1α by the von Hippel-Lindau tumor suppressor gene (pVHL) and rapid degradation by the proteasomes (Ivan et al. 2001). Moreover, an asparaginyl hydroxylase in the nucleus independently modifies the carboxyl-terminal transactivation domain of HIF-1α, blocking the interaction of HIF-1 with the transcriptional co-activator

p300 (Lando et al. 2002a, b). Both hydroxylases are oxygen- and iron-dependent. In mammals, prolyl hydroxylase 1, 2 and 3, have been identified and recognize the amino acid motif LXXLAP in HIF-1α protein (Epstein et al. 2001). Hypoxia and iron deficiency block both hydroxylase activities, allowing for the accumulation of HIF-1α, and then its association with HIF-1β. The HIF-1 complex enters the nucleus and binds to p300 located at HIF responsive element, triggering the transcriptions of HIF-1 target genes. Thus, oxygen and iron are absolutely required for the HIF response, but the following questions arise: (1) Are prolyl- and asparaginyl-hydroxylases only oxygen sensors? (2) What signals enhance oxygen-extraction and -diffusion from the cell-surface to inside of cells? (3) Do other hemoproteins like the oxygen-dependent sensor exist in the nucleus? Regarding the last question, two new members of the vertebrate globin family, namely neuroglobin and cytoglobin have been discovered.

Neuroglobin and cytoglobin are monomer and exhibit relatively low homology (20-25%) to myoglobin and hemoglobin (Burmester et al. 2000; Schmidt et al. 2004). Both globin molecules have hexacoordinated iron atoms, the distal histidine binding in the 6th ligands, and bind oxygen reversibly with moderate affinity. Neuroglobin is exclusively expressed in neural cells and up-regulated in response to hypoxia. Considering that the cytosolic localization of neuroglobin is related to that of mitochondria, the protein protects the neurons against hypoxic damage in addition to supplying of oxygen. In contrast to the tissue-specific expression of neuroglobin, cytoglobin is expressed in various tissues. The transcription of the cytoglobin gene is regulated by a HIF-1-dependent pathway. Immunocytochemistry studies showed that cytoglobin is located in the cytoplasm in mouse connective tissues, and nucleus as well as cytoplasm of neurons, implying the respiratory function (Schmidt et al. 2004). Moreover, the nuclear location of cytoglobin in mouse neural and endocrine cells was also reported (Geuens et al. 2003). When human cytoglobin was expressed in Cos7 cells, the protein in some cells was exclusively localized to the nucleus and in others to the cytoplasm and nucleus (J. Matsubara and S. Taketani, unpublished data), indicating that the localization of cytoglobin changed, dependent on conditions of cells. It is unlikely that cytoglobin directly binds to DNA, but this hemoprotein may interact with other transcription factors. There are several identified genes whose expression is heme-regulated, but the regulatory mechanisms are not completely clarified. Furthermore, it is possible that heme-bound transcription factors such as NPAS2 and Bach1 act as partners of cytoglobin because the oxygen-requirement of these transcription factors is unclear. Finally, unknown nuclear factors involved in cytoglobin-mediated oxygen sensing may play an important role in the pleiotrophic functions of cells.

Conclusion

Iron has a central primary role in the proliferation and maintenance of various tissues. The functions of iron and heme-iron are accomplished in part by specific signals which can modulate cell-cell communication and also by direct effects on the growth and differentiation of cells. We have shown how iron is utilized in differentiated or cancerous cells in a different way, how iron can regulate cellular functions in differentiated cells, and how erythroid cells specifically utilize iron and produce heme. However, little is known of the transport of heme inside and outside of cells and trafficking of intracellular iron. Regarding to these issues, new evidence has been emerging. Namely, several disorders including hemochromatosis and Friedreich's ataxia are striking samples of diseases in which an immediate clinical benefit has been obtained from basic discoveries at the molecular level. Phenotypes of gene-targeting mice and yeast mutants, and adaptations of bacteria to changes in environmental conditions often help our understanding of the cellular functions of iron and heme. A combination of systematic research in different fields of study has demonstrated that molecules other than iron and heme are major regulators of iron and heme homeostasis. Many factors exhibiting heme- or iron-binding properties have been identified, but their func-

tions are obscure. There is a need to clarify their biological significance. Iron is indeed currently in the spotlight, connecting research on molecular and cell biology with clinical medicine.

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