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Implications of altered iron homeostasis for age-related macular degeneration

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1. ABSTRACT

Reactive oxygen species (ROS) may contribute to the pathogenesis of age-related macular degeneration (AMD) and they can be produced in the Fenton reaction catalyzed by Fe^{3+} ions. Therefore, altered homeostasis of iron in the retina may be the source of ROS and its damage resulting in clinically detectable AMD symptoms. The results of some *post mortem* research indicate a higher concentration of iron in AMD retinas in comparison with non-affected retinas, although those results do not determine whether iron overload is the reason or a consequence of AMD. However, the results of some other research suggest that iron may contribute to the pathogenesis of AMD. Those include increasing of macular iron level with age, involvement of iron in the pathogenesis of some degenerative diseases linked with AMD, upregulation of transferrin in AMD, developing of AMD-like syndromes in mice deficient in ceruloplasmin and hephaestin, association between polymorphism of the iron homeostasis genes and AMD and others. Better understanding of the role of altered iron homeostasis may be useful in prevention and curing of AMD.

2. INTRODUCTION

Iron overload has been implicated in the age-related neurodegenerations: Alzheimer's disease and Parkinson's disease and iron ions may catalyze the Fenton reaction, leading to the production of reactive oxygen species (ROS), which play an important role in the etiology of another age-related degenerative disease, age-related macular degeneration (AMD) (1). Therefore, iron may be implicated in the pathogenesis of AMD, which was confirmed by some research *post mortem*, pointing out enhanced concentrations of iron in the retina of subjects with AMD (2). Moreover, several symptoms of hereditary iron overload, including aceruloplasminemia, hereditary hemochromatosis, and pantothenate kinase associated neurodegeneration, were associated with pathologic changes in the retina (3). All these pathologic symptoms have some similarity to AMD, and support hypothesis of the iron involvement in the pathologic processes in the macula. The exact mechanism of iron contribution to AMD is not known, and several pathways may be taken into account, because iron is needed in some enzymes taking part in several processes of normal cellular metabolism.

Table 1. Main proteins of human iron homeostasis

Protein (alternative name)	Abbreviation (alternative)	Function
Ceruloplasmin	Cp	Oxidase converting Fe^{2+} to Fe^{3+}
Divalent metal transporter 1 (divalent cation transporter)	DMT1 (DCT1)	Transport of divalent iron ions across the membrane
Erythropoietin	EPO	Regulation of the expression of other iron homeostasis proteins
Ferrireductase	Steap3, Dcytb	Reduction Fe^{3+} to Fe^{2+}
Ferritin	Ft	Iron storage, ferroxidase activity
Ferroportin 1 (Iron regulatory protein 1)	FPN1, Ireg1	Fe^{3+} transport out of the membrane
Heme carrier protein 1	HCP1	Facilitates heme transport across the membrane
Heme exporters	LFLVCR, ABCB6, Bcrp/Abcg2	ATP-dependent and independent iron transport across the cytoplasmic and mitochondrial membrane
Heme oxygenase	HO	Liberates iron from heme
Hemochromatosis protein	HFE	Regulates the interaction of transferrin with its receptors
Hemojuvelin	HJV	Stimulates hepcidin expression
Hemosiderin		Iron storage
Hepcidin	HEP, HAMP, LEAP1	Ferroportin inhibitor
Hephaestin	Hp	Ferroxidase activity and keeping ferroportin at the cell surface
Lipocalin 2		Mediates iron uptake
Mitoferrin	Mfrn, Slc25a37	Iron import in mitochondria
Transferrin	Tf	Binding Fe^{3+}, ligand for TfR1 and TfR2
Transferrin receptor 1	TfR1	Uptake of iron bound to Tf
Transferrin receptor 2	TfR2	Uptake of iron bound to Tf, regulation of hepcidin expression

Iron is a cofactor for the enzyme fatty acid desaturase, which is involved in the retinal membrane biogenesis (4). Several other enzymes also utilize iron to display their activity in the retina (5, 6). Disturbance of the activity of these enzymes, evoked by inappropriate iron binding or its lack, may lead to serious changes in the retina, involving the macula. Several other aspects of perturbations of iron homeostasis may be taken into account in AMD pathogenesis. These perturbations can be hereditary or induced by life style or environmental factors.

Therefore, aspects of iron homeostasis and its disturbance should be carefully considered in the searching for mechanism of AMD pathogenesis.

3. IRON HOMEOSTASIS

Iron is essential for cellular metabolism and aerobic respiration – hundreds of proteins require iron in the cell. Many of these are involved in enzyme catalysis and electron transport, whereas others are needed for oxygen transport and delivery. Iron can shuttle between two thermodynamically stable oxidation states, Fe^{3+} or ferric iron and Fe^{2+} or ferrous iron and the conversion between these states is realized through single electron exchange. Cellular iron overload leads to toxicity and cell death *via* free radical formation and lipid peroxidation, thus, iron homeostasis requires tight regulation (7). Therefore both individual cell and whole organism must strictly regulate their iron influx and efflux. The normal diet contains 15-20 mg of iron but only approximately 10% of it is absorbed to the human organism.

Several proteins are involved in iron homeostasis in humans (Table 1). Ingested iron can be classified as non-heme and heme iron. Non-heme iron is mainly composed of ferric iron and is absorbed into enterocytes through the divalent metal transporter 1 (DMT1) after reduction of Fe^{3+} to Fe^{2+} by duodenal cytochrome *b* (8). Heme-iron is absorbed through a heme carrier protein into enterocytes, where it is transferred from the luminal to the vascular site of the cell, and released into the circulation. Because iron plays an essential role in the cellular metabolism and aerobic respiration, its homeostasis should be under strict control and many proteins are involved in this control (Table 1), as a deficiency of this element may contribute to serious diseases.

There are some common aspects of iron handling that are shared by most cells, but each cell type handles iron in its own specific way to meet its functions (9). The first requirement for iron homeostasis is its proper transport as nearly all absorption of dietary iron occurs in the duodenum, from where it must be transported to the blood. Iron in the blood is incorporated into the plasma protein ferritin (Ft) (9). If there is a need for a cell to accommodate more iron than can be loaded onto Ft, another form of iron storage, hemosiderin consisting of iron oxide clusters and aggregates of degraded ferritin, may be utilized.

In normal conditions, the cell takes most of needed iron through the plasma protein, transferrin (Tf) and its receptors 1 and 2 (TfR1 and TfR2). Each Tf molecule can bind two molecules of Fe^{3+} and after binding, these ferric iron ions can be effectively utilized by the cell (10). In general, Tf may deliver its iron to the cell in several pathways but the mechanism with TfR1 seems to be preferentially utilized (11). The transport is facilitated by the low pH inside endosomes, reductase activity displayed by Steap3 (six-transmembrane epithelial antigen of the prostate 3) and other proteins of the STEAP family and conformational changes in Tf associated with its binding to TfR1 (12-14). Under pathological conditions, some cells may utilize iron from circulating heme, hemoglobin and Ft (15). The polarized absorptive cells have carriers for the influx of iron at their apical, bush-border membranes (DMT1) and carriers for the efflux of iron at the basolateral membranes (ferroportin 1, FPN1) (16, 17).

The functioning of the iron transport systems should be adjusted to the cellular requirements for this element. When there is a high cellular demand for iron, the proteins involved in the iron import are expressed at high levels, in contrary to proteins engaged in iron export.

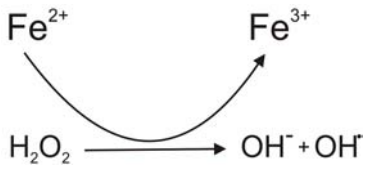

Figure 1. Fenton reaction. Hydrogen peroxide is decomposed to hydroxyl ion (OH^-) and highly reactive hydroxyl radical ($OH^\bullet$) in the presence of ferrous iron (Fe^{2+}), which undergoes oxidation to ferric iron (Fe^{2+}).

Therefore, TfR1 and DMT1 concentrations are increased on the cell surface under iron deficiency and the expression of FPN is decreased. In the case of iron deficiency its virtually whole pool is utilized in the metabolic processes and there is no need to store it in Ft, so its expression is also decreased. When the cell is iron-rich, an opposite pattern of expression should be observed. However, the real pattern of the expression of protein involved in iron transport in the cases of iron deficiency and proficiency strongly depends on the cell type (tissue) and its environment (15).

The key regulator of systemic iron homeostasis is hepcidin, a 25 amino acid peptide, secreted by hepatocytes (18; 19). It binds to FPN1 and induce its internalization and degradation and in this way reduce the export of iron from the cell (20). Hepcidin expression is regulated by HFE, TfR2 and HJV (15). Hepcidin regulation through HJV is probably the main way of controlling its expression and its imposed by the signaling through the BMP (bone morphogenetic protein)/SMAD pathway (21, 22).

The mitochondrion plays a central role in the cellular iron homeostasis and it is main iron reservoir in the cell, because two of the most widespread iron centers, heme and iron-sulfur clusters, are predominantly synthesized in mitochondria (23). The mechanism of transport of iron from endosomes to mitochondria is not fully understood. There are some hypotheses on this, including sequestering by ligands of low molecular weight or proteins and direct contact between endosomes and mitochondria (24). Iron is transported to mitochondria through mitochondrial membrane by mitoferrin (Mfrn) (25).

4. PATHOGENIC POTENTIAL OF IRON OVERLOAD

Iron in aqueous solution can be oxidized from ferric to ferrous state in the reaction, known as Fenton reaction (Figure 1).

In this reaction hydrogen peroxide is decomposed to hydroxyl ion and highly reactive hydroxyl radical ($OH^\bullet$) in the presence of ferrous iron (OH^-). Moreover, the superoxide, one of ROS, can reduce Fe^{3+} to Fe^{2+} with the production of oxygen:

$$H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + OH^- + OH^\bullet$$

Superoxide may react with hydrogen peroxide in the presence of iron in iron-catalyzed Haber-Weiss reaction (26):

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^\bullet$$
$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

This set of processes illustrated by the reactions represent a viscous circle-like situation. Namely, even in a small amount of hydrogen peroxide, which can be formed even in physiological conditions, the presence of unbound ferric ions may lead to the production of ROS, which, in turn, may reduce ferrous to ferric ions. This means that ROS, including hydroxyl radical, may be progressively produced if iron ions are accessible. This can take place if there is an excess of iron in a local environment. The overproduction of ROS may have expansive character, so this initially local character may change into more common. This also has serious implications for diseases, which are induced with the involvement of ROS. Such disease may continuously progress due to the increased production of ROS, which were the reason of their occurrence.

In general iron is absorbed in the intestine, but is exerted from the organism in a lesser degree. Therefore, it is accumulated in the organism with age. The increased amount of iron is associated with the increased need of its control and this challenge is not always taken up by the organism, which may lead to age-related, iron-associated diseases. Iron overload was implicated in several age-related neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease (1).

From the molecular point of view, hydroxyl radicals may damage lipids, proteins and nucleic acids. The reaction of the radical with DNA may produce 8-oxo-7,8-dihydroguanine (8-oxoG), a DNA damage with a high carcinogenic potential (27). The reaction of hydroxyl radical with lipids may yield lipid hydroxyl-peroxide (ROOH) and lipid peroxidative product such as malondialdehyde and 4-hydroxy-2-nonenal are accumulated in iron overload conditions, leading to the production of lipid-based radicals ROO-(alkyl oxyradical) and RO-(alkoxy radical), which have longer half lives than hydroxyl radical. Therefore, lipid-based radicals may have directly be involved in chronic cell/organ/organism toxicity.

Pathological states associated with body iron overload are designated as iron overload syndromes, and iron deposition causes organ dysfunction including cell death, fibrosis, and carcinogenesis (28). Iron overload syndromes are classified as genetic and secondary and hereditary hemochromatosis is the most common genetic disorder in Western countries, which is manifested by iron deposition in the main organs of the organism (29). The most common pathological secondary conditions associated with iron overload occurs in patients who require long-term blood transfusion due to severe anemias. This is the exogeneous iron, given with transfusion, which is responsible for severe symptoms, including liver failure,

cardiac failure and diabetes mellitus (7). Apart from these severe conditions there are several diseases associated with mild iron deposition or dysregulation of body iron distribution, including chronic hepatitis C, alcoholic liver disease, non-alcoholic steatohepatitis (28).

Iron is important in normal retinal function as an essential metabolic component of both heme- and non-heme-containing proteins, including retinal pigment epithelium-specific protein 65 kDa, RPE65, if dysregulated it can cause oxidative stress in the retina (30). Retinal levels of iron are high and increase with age in AMD (31). However, iron cannot freely move from the bloodstream to the retina.

5. IRON IN THE RETINA

In the retina, the forming of new photoreceptor disc membranes requires fatty acid desaturase, an enzyme containing iron (4). A growing body of evidence indicates the damaging potential of excess of iron in the retina and its strict regulation in this organ (32). Iron injected locally in the eye may diffuse through the vitreous body and retina and induce the degeneration of photoreceptor and RPE cells (33-35). Rats with deficient phagocytosis of photoreceptor outer segments, had an increased amount of iron in RPE and photoreceptor cells as compared with control animals, which underlies an important role of authopagy and iron in the diseases of the retina, including AMD (36-38). Retinal degeneration was observed in patients with hereditary diseases causing retinal iron overload, aceruloplasminemia, Friedreich's ataxia, and pantothenate kinase-associated neurodegeneration (39-41). A higher level of iron in RPE and photoreceptor cells was observed in AMD retinas as compared with age-matched control retinas (42, 43).

As previously mentioned, iron is not freely exchanged between the systemic circulation – a barrier between blood and retina can be considered. This barrier may have two essential and distinct components: one, at the level of the RPE in the outer retina and a second at the level of the retinal capillary endothelial cells in the inner retina (44). The complex formed by RPE cells that form the outer component of the blood-retinal barrier is documented to be involved in the pathogenesis of AMD (45). The RPE cells form an outer obstruction to free movement of polar solutes and large macromolecules that escape the choriocapillaris. This effect is underlined by building up tight junctions which plumbs up the paracellular space in close vicinity of the apical border of the RPE (46). The inner retinal component of the blood-retinal barrier is a continuous capillary wall consisting of endothelial cells sealed with tight junctions at all their contacts. What are the mechanisms underlying the penetration of the retina by iron ions? TfR-mediated transcytosis was reported to be the main mechanism for iron to cross the inner blood-retina barrier, and the source of iron for the inner retina under normal conditions (47). However, some contradictory results were obtained as well (48). Therefore, further studies are needed to establish exact mechanism of iron transport through the blood-retina barrier.

Transferrin, the main carrier of iron in the bloodstream, has been also found in the retina and the RPE is a main site of its synthesis in this organ (49). Iron is transported from the RPE to the photoreceptors by the mechanism underlined by the interaction between Tf and its receptor (49). TfR in RPE cells is a monomer protein of 93 kDa, located on the basolateral and apical surfaces of the cells (50). Another iron carrier, DMT1, was detected in several regions of the retina, including photoreceptor inner segments, but its functional significance is not known (51). An auxiliary role in retinal iron transport could be associated with Dextras1, a protein belonging to the G family and interacting with Peripheral Benzodiazepine Receptor Associated Protein (PAP7), which binds to DMT1 (52). Iron in the retina is stored in ferritin and ceruloplasmin is the main protein responsible for the export of iron from retina (49, 51, 53). Another protein playing a role in the export of iron, hephaestin, was detected in the retina (42, 51). Ferroportin is also present in the retina with the main localization in the RPE, photoreceptor inner segments and plexiform layers (42). The expression of hepcidin in the RPE is also well documented in the retina and it seems to have a cell-specific character (54). Therefore, it is evident that iron homeostasis in the retina is maintained by proteins, which are involved in the general iron homeostasis and disruption of these protein and/or their dysfunction may result in pathological conditions of the retina including hemochromatosis, aceruloplasminemia, age-related macular degeneration.

6. IRON IN AMD

Age-related macular degeneration is the leading cause of irreversible blindness in the western countries in people over the age of 65 (55, 56). In early stages of AMD, the disease becomes clinically relevant, when drusen can be seen. As the disease progresses, large areas of geographic atrophy may form in nonexudative, or dry AMD, or areas of choroidal neovascularization may develop in exudative, or wet AMD. The pathogenesis of AMD is not completely known, but it is generally accepted that the disease is a results of some age-related vascular and structural changes influenced by genetic predisposition and some environmental factors (57). The latter are mainly oxidative stress, smoking, blue light and UV radiation. The oxidative stress seems to play a special role in the AMD pathogenesis, because both smoking and radiation may produce such stress, which, in turn, may be related to the process of aging (58). Also some dietary compounds may contain agents promoting the stress and dietary supplementation with antioxidants and zinc significantly reduced the progression to advanced AMD (59, 60). Therefore, important and recognized factors in AMD pathogenesis: age, smoking, diet and radiation may be related to oxidative stress associated with the overproduction of ROS, including H_2O_2, which may be decomposed to highly reactive hydroxyl radical in the Fenton reaction in the presence of iron. For that reason, iron overload may potentiate the consequences of oxidative stress associated with aging and environmental factors.

As mentioned above, *post morten* AMD-affected eyes were reported to have an excess of both chelatable and non-chelatable iron in the RPE and Bruch membrane, including drusen (42). Surely, these results do not indicate definitely whether elevated concentration of iron contributes to pathogenesis of AMD or it is a consequence of the disease. However, the results of several studies mutually support the former option.

As mentioned above, transferrin, the main protein involved in iron transport, is expressed at high yields in the retina. It was observed that mean transferrin mRNA levels were elevated 3.5- and 2.1-fold in dry and wet AMD retinas, respectively as compared with retinas without evidence of the disease (61). Also a 2.1-fold increase in the transferrin protein level in the AMD-affected eyes was observed in that study. Those results along with the report on elevated levels of iron in the AMD-affected retinas, support the hypothesis of a significant role of disturbed iron homeostasis in the pathogenesis of AMD.

Hepcidin, described above, is an iron regulatory hormone, which is upregulated by inflammation, leading to increased storage of iron in tissues (62). On the other hand, several pieces of evidence, linking AMD with genetic polymorphism in complement cascade factors, suggest that inflammation may be involved in AMD (63-67). Iron may fuel inflammation through oxidative cellular damage or just by activation of the complement cascade (68). Iron is reported to be implicated through inflammation in cardiovascular disease and Alzheimer disease, which are epidemiologically linked to AMD (69, 70).

It is well established that sex is a major risk factor in AMD, with females being at a higher risk than males (45). Interestingly, an increase in iron levels in retina was observed with age and women had more retinal iron at all ages (71). This was not the case when similar studies were conducted on mice, but the genetic and hormonal differences may explain that contrast (72). Therefore, there is a direct correlation between AMD, age, sex and iron content in the retina.

It was shown that mice deficient in ceruloplasmin (Cp) and its homolog hephaestin (Heph) had a striking, age-dependent increase in retinal pigment epithelium and retinal iron (42). Ferritin was also increased in those mice. After retinal iron levels had increased those mice had age-dependent retinal pigment epithelium hypertrophy, hyperplasia and death, photoreceptor degeneration and subretinal neovasclarization. Therefore such mice provided a model of some features of human AMD, indicating an important role of iron in this disease.

Recently we showed that the of the *25129A>C* polymorphism of the *NRF2* gene may be associated with the increased risk of the occurrence of both dry or wet form of AMD (73). The *NRF2* gene encodes enzymes involved in the generation and removal of iron-mediated reactive oxygen species. In a similar study we examined an association between AMD risk and polymorphisms of the genes encoding enzymes involved in the generation and removal of iron-mediated oxidation: NAD(P)H:quinine oxidoreductase 1 *NQO1* (609C>T), nitric oxide synthase 3 *NOS3* (894G>T) and nuclear factor erythroid2-related factor 2 *Nrf2* (25129A>C) (unpublished data). We found an association between the C/C genotype of the 25129A>C polymorphism and the occurrence of AMD. We also observed an association between dry AMD and the C/C genotype of the 25129A>C polymorphism as well as the T/T genotype of the 894G>T polymorphism. The A/C genotype of the 25129A>C polymorphism decreased the risk of AMD as well as dry AMD. Moreover, we noted that the interaction between genotypes of the *NQO1, NOS3* and *Nrf2* polymorphisms may contribute to higher prevalence of AMD. These results confirm the pathogenic role of iron in AMD and the mechanism of such pathogenicity – increasing the oxidative stress.

All these reports suggest that iron may contribute to the pathogenesis of AMD. However, it cannot be treated as an independent risk factor, because it may be linked with oxidative stress, potentiating it by the production of hydroxyl radical and other radicals when there is an excess of hydrogen peroxide, a major by-product of the oxidative stress. Therefore, iron is probably not the primary case but rather play a role in AMD.

7. PERSPECTIVE

The most straightforward action to reduce pathological consequences of iron overload is to reduce the amount of iron in the organism. This can be reached by the limitation of iron in the diet, serial phlebotomy and iron chelation (74). It is tempting to focus on the diet, as it can be easily modified. However, reduction in dietary iron may be associated with a decrease of its levels and levels of other elements below required in some organs. Chelation is another attractive perspective, because it is targeted selectively to iron. Deferoxamine mesylate (DFO) is an iron chelator which is used for the treatment of chronic iron overload in patients requiring regular transfusions (75). DFO has a high affinity for ferric iron and it removes iron from hemosiderin, ferritin and transferrin (76). However, iron chelation with DFO may evoke retinal toxicity (77-79). Other than DFO iron chelators have been tested as potential therapeutics in ocular diseases. Those include salicylaldehyde isonicotinoyl hydrazone, isonicotinic acid [2-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)-benzylidene]-hydrazide and others and the results obtained seem to be promising (80, 81). Therefore, iron chelation may be useful therapeutic strategy in AMD. This is supported by the reports on usefulnes of such strategy in some neurological diseases, including Alzheimer disease and Parkinson disease, and Huntington disease and Friedrich ataxia (82-85).

Iron may induce insolubility of glycoproteins , which may contribute to drusen formation, and this process can be inhibited *in vitro* by dibasic amino acids L-arginine and its precursor L-citrulline (86, 87). Therefore, further study with those amino acids may create a new perspective in AMD therapy.

As we mentioned, an apparent easiest way to fight with iron overload, but eliminating some iron-rich products or decreasing their amount, may not always be

proficient. However, diet rich in red meat should be avoided, also for many other reasons and there is no doubt, that individuals with ocular problems taking diet supplements containing iron should stopping this, unless there is not a medical recommendation for doing so.

The mechanism leading to iron overload in AMD is not fully understood and genetic variability in the genes of iron homeostasis may contribute to it. Therefore, searching for the association between AMD and polymorphism of the genes encoding proteins involved in iron homeostasis, may be a promising direction to better understanding mechanisms of AMD pathogenesis and curing this disease, including gene therapy. Working out of microarray "Iron in AMD" would be important for both scientific and clinical aspects.

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