Review Article

Role of iron in carcinogenesis: Cancer as a ferrotoxic disease

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Iron is abundant universally. During the evolutionary processes, humans have selected iron as a carrier of oxygen inside the body. However, iron works as a double-edged sword, and its excess is a risk for cancer, presumably via generation of reactive oxygen species. Thus far, pathological conditions such as hemochromatosis, chronic viral hepatitis B and C, exposure to asbestos fibers, as well as endometriosis have been recognized as iron overload-associated risks for human cancer. Indeed, iron is carcinogenic in animal experiments. These reports unexpectedly revealed that there are target genes in iron-induced carcinogenesis and that iron-catalyzed oxidative DNA damage is not random *in vivo*. Several iron transporters and hepcidin, a peptide hormone regulating iron metabolism, were discovered in the past decade. Furthermore, a recent epidemiological study reported that iron reduction by phlebotomy decreased cancer risk in the apparently normal population. These results warrant reconsideration of the role of iron in carcinogenesis and suggest that fine control of body iron stores would be a wise strategy for cancer prevention. (*Cancer Sci* 2009; 100: 9–16)

Body iron stores accumulate insidiously with aging due to the fact that intake exceeds loss and no biological mechanisms exist for excretion of iron in excess of physiological requirements.[1] It was first reported in 2008 that iron reduction by phlebotomy decreased cancer risk in a supposedly normal population that had peripheral arterial disease.[2] Despite some criticisms, this paper is a highly significant observation supporting other epidemiological studies,[3,4] and one should not underestimate the role of iron in carcinogenesis.

'Redox cycling' is a characteristic of transition metals such as iron (Fe[III] ↔ Fe[II]) and copper (Cu[II] ↔ Cu[I]). Iron is an essential metal involved in oxygen transport mediated by hemoglobin in mammals and in the activity of various enzymes including catalase. Both deficiency and overload may cause such serious conditions in humans as anemia and hemochromatosis, respectively. Thus, iron metabolism is finely regulated. Recently, the understanding of iron metabolism entered a new era by the discoveries of several iron transporters, post-transcriptional regulation of 'iron metabolism-associated' genes, and hepcidin, a peptide hormone produced by hepatocytes. Furthermore, there is a growing body of evidence that suggests a role of iron in carcinogenesis. Our laboratory has established and been studying animal models of iron-induced carcinogenesis. The present review describes recent new findings associated with iron metabolism and focuses further on the mechanisms of how iron induces cancer. A novel concept called 'oxygenomics' is proposed based on these findings.

Fenton chemistry and 'catalytic' iron

Iron present in heme, in iron–sulfur clusters or closely associated with proteins plays a pivotal role in a variety of physiological cellular functions such as oxygen transport, energy metabolism, electron transport, and modulation of H_2O_2 levels. However, non-protein-bound 'free' or 'catalytic' iron functions to damage biomolecules.[5] Iron is the most abundant transition metal in the human body (approximately 2–6 g).[6] Redox cycling of iron is closely associated with the generation of ROS. A British chemist, Fenton, reported as early as 1894 that ferrous sulfate and H_2O_2 cause the oxidation of tartaric acid, resulting in a beautiful violet color on the addition of caustic alkali.[7] This was the basis for the discovery of the Fenton reaction, which produces hydroxyl radicals ($^{\cdot}OH$), the most reactive chemical species in biological systems (Eqn 1).

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\cdot}OH + OH^- \quad \text{(Eqn 1)}$$

In order to understand the involvement of this chemical reaction, in the biological system the concept of catalytic or free iron proposed by Gutteridge *et al.* is essential.[5] Catalytic iron consists of the following two characteristics: (1) redox activity, and (2) diffusibility. In biological environments at neutral pH, the reduction potential of Fe(III) is +772 mV, close to that of the water–oxygen couple, which is +818 mV.[8] However, Fe(III) can dissolve in water at an extremely low concentration (10^{-17} mol/L) at neutral pH. Most Fe(III) precipitates as iron hydroxides at neutral pH.[9] However, Fe(III) chelated with citrate or phosphates such as ADP, ATP, and GTP can remain as 'catalytic' iron at neutral pH.[10] In the iron chelates suggested above, at least one of the six ligands of iron is left free to exert catalytic activity.[11] It was previously reported that the fewer the number of ligands involved in chelation, the higher the preservation of catalytic activity for ROS production.[12] This is further related to the redox potential of each iron chelate; in the redox potential between +460 mV and –160 mV, the ferrous state gives a Fenton reaction whereas the ferric state can be reduced by $^{\cdot}O_2^-$.[13]

Catalytic iron in the biological environment

Only a limited amount of data is currently available concerning the localization of catalytic iron in cells due to a deficiency in appropriate methods. It is believed that there exists a minute cellular labile pool of iron that is solubilized by chelating to low molecular weight biomolecules.[14] This pool of iron is considered at least partly responsible for the pathological generation of ROS.

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Abbreviations: C/EBP, CCAAT/enhancer-binding protein; DCT 1, divalent cation transporter 1; DMT 1, divalent metal transporter 1; Fe(II), ferrous ion; Fe(III), ferric ion; Fe-NTA, ferric nitrilotriacetate; HAMP, hepcidin antimicrobial peptide; Nramp 2, natural resistance-associated macrophage protein2 RCC, renal cell carcinoma; ROS, reactive oxygen species; SLC11A2, proton-compled divalent metal ion transporter member 2; TBP, thioredoxin-binding protein.

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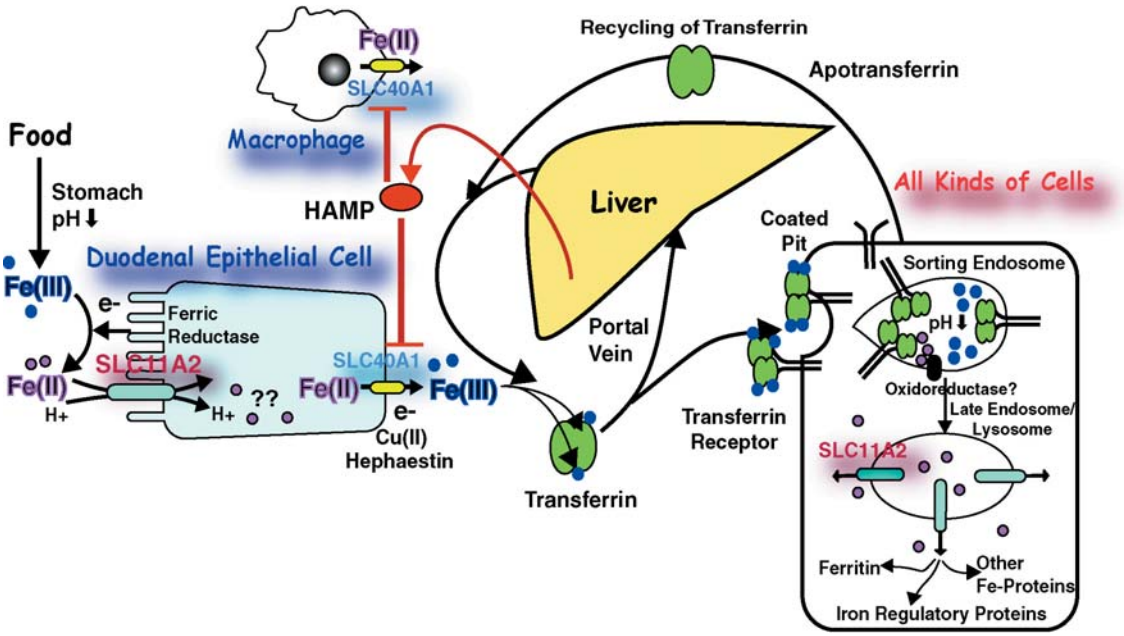

Fig. 1. Summary of iron metabolism. Mechanisms and regulation of iron absorption and transport in mammals. HAMP, hepcidin antimicrobial peptide; SLC11A2, natural resistance-associated macrophage protein 2, divalent metal transporter 1, or divalent cation transporter 1; SLC40A1, iron-regulated transporter 1 or ferroportin 1. Refer to the text for details.

In contrast, abundant data are available regarding extracellular free iron. The clinical significance of 'non-transferrin plasma iron' (catalytic iron) has been well discussed. Plasma transferrin acts as a considerable reserve for handling increasing amounts of incoming iron via iron transporters. However, in acute iron poisoning, catalytic iron concentrations ranging from 128 to over 800 µmol/L have been documented, exceeding several times the total iron-binding capacity of transferrin.[15] Similarly, in severe hemochromatosis and Bantu siderosis, acute episodes of abdominal pain and shock have been observed in individuals with extremely high serum iron measurements exceeding 2000 µmol/L.[16]

Another important hypothetical concept regarding iron-catalyzed oxidative damage was that of a 'site-specific' mechanism that was raised in the 1980s. Fe(III) bound loosely to biological molecules such as DNA and proteins may undergo cyclic reduction and oxidation. This concept is different from that of catalytic iron described above, in that the iron is not diffusible, and it may explain the accumulation of free radical damage to specific sites and possible 'multihit' effects on the molecules at such sites.[17] This issue will be further discussed in the animal cancer model section.

Iron transporters and hepcidin

Until recently, there was little molecular information available on the mechanisms of iron ion absorption into the circulation in mammals. The uptake and transport of iron under physiological conditions require specific mechanisms as Fe(III) has very low solubility at neutral pH.[9] Thus, reduction of iron to Fe(II) has been considered essential for iron absorption. Although the process of transferrin receptor-mediated endocytosis has been well established,[18] this is not the pathway by which iron in the diet is taken up into circulation.

In 1997 solute carrier family 11 (proton-coupled divalent metal ion transporter, member 2; SLC11A2; Nramp2, natural resistance-associated macrophage protein 2; DMT1, divalent metal transporter 1; DCT1, divalent cation transporter 1) was identified as an iron transporter using a genetic approach, studying *mk* mice that develop microcytic anemia. The anemia of *mk* mice was unresponsive to increased dietary iron, and iron injections did not reverse the anemia, suggesting a block of iron entry into red blood cell precursors.[19] It was of interest that Nramp2 is a homolog of Nramp1, which mediates the natural resistance to infection of intracellular parasites, affecting the capacity of macrophages to destroy ingested intracellular parasites early during infection.[20] Independently, this gene was identified with an expression cloning technique from a duodenal cDNA library prepared from rats with iron deficiency. This insightful approach was based on the idea that mRNA for iron transporter must be overexpressed in such a situation. These experiments further revealed that SLC11A2 transports not only Fe(II) but also Zn(II), Mn(II), Cu(II), Co(II), Cd(II), and Pb(II). Although this transporter is expressed almost ubiquitously, in this case of iron deficiency, the highest abundance was in the duodenum.[21] SLC11A2 is also present on the membranes of endosomes and lysosomes, allowing the transport of iron from the transferrin receptor to the cytosol (Fig. 1).

Intestinal epithelial cells have two different iron transporters: one in the apical membrane (SLC11A2) and one in the basolateral membrane. This was determined in *sla* mice that demonstrate normal uptake of iron into the villus cells, via a process mediated by SLC11A2, but show impaired release of iron into the bloodstream. In 2000, a novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the portal vein, was cloned.[22] This transporter was identified independently in two laboratories as ferroportin 1,[23] and metal transporter protein 1,[24] and has now been renamed SLC40A1 (Fig. 1).

The hormone that regulates the activity of SLC40A1 is hepcidin autimicrobial peptide (HAMP). HAMP, first recognized as an antimicrobial peptide in serum and urine, is produced in hepatocytes and interacts with its receptor, SLC40A1, when the complex is taken up by endocytosis, followed by degradation in lysozomes.

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With the complicated structure of its active-form peptide, hepcidin-25, mass spectrometric measurements are necessary.[25] Also, the serum concentration of hepcidin-25 does not correlate with hepcidin mRNA expression.[26] SLC40A1 is the only iron exporter from cells, so its deficiency causes severe iron depletion concomitant with iron accumulation in enterocytes, macrophages, and hepatocytes.[27] HAMP-producing large liver adenomas are associated with iron-refractory anemia.[28] It is possible that overproduction of HAMP is at least partly responsible for anemia in a variety of cancers.[29]

The expression of many proteins that modulate the iron metabolism of mammalian cells is controlled by intracellular iron concentration and other factors such as nitric oxide or oxidative stress. Interestingly, this regulation is mediated mainly at the post-transcriptional level, namely by specific mRNA–protein interactions in the cytoplasm. Particular hairpin structures, called iron-responsive elements, in the respective mRNA are recognized by trans-acting proteins, called iron-regulatory proteins, that can control the efficiency of mRNA translation and stability. This has been reviewed elsewhere.[30]

Hemochromatosis

Hereditary hemochromatosis is a genetic iron overload disorder that in the past was difficult to diagnose until the progressive accumulation of iron, mainly in the form of ferritin and hemosiderin, caused solid organ injury, particularly to the liver, heart, and endocrine pancreas (especially insulin-secreting β-cells). Recently, *HFE*, the gene responsible for hereditary hemochromatosis, was identified by a positional cloning approach (OMIM + 235200). *HFE* is related to major histocompatibility complex class I proteins, and is mutated in hereditary hemochromatosis.[31] Two point mutations, C282Y and H63D, have been linked to the majority of disease cases.[32] Hereditary hemochromatosis is usually inherited in an autosomal-recessive manner. Unfortunately, the exact role of *HFE* is still unclear at present. A mutation in the gene encoding SLC40A1 (OMIM #606069) is associated with an autosomal-dominant type of hemochromatosis.[33] It is of note that defects in HAMP (OMIM #602390) or in transferrin receptor 2 (OMIM #604250) also induce hemochromatosis.

Major causes of death today in hereditary hemochromatosis are due to either hepatic failure with cirrhosis or hepatocellular carcinoma.[34] Indeed, 219, 240, and 92.9 times greater risk was shown for primary hepatocellular carcinoma in hemochromatosis patients compared with the age-matched control population in three independent studies.[35–37] In general, hepatocellular carcinoma is preceded by cirrhosis. A high incidence of cancers originating from other organs (esophageal cancer, skin melanoma, etc.) have been reported.[37–40] Furthermore, cases of hepatocellular carcinoma have been reported in the absence of cirrhosis,[41] and after reversal of cirrhosis with therapy.[42] These reports suggest that irreversible genetic alterations occur early in the course of the disease.

Iron overload and human cancer

Hemochromatosis is not the only human disease with complications of iron overload-induced cancer. Table 1 summarizes other human pathological conditions associated with iron overload-induced cancer. Persistent damage to hepatocytes reduces the production of HAMP, which promotes iron absorption and deposition irrespective of the iron stores.[43] Thus, hepatic iron is increased significantly in patients with chronic viral hepatitis. In this situation, phlebotomy appears an efficient and inexpensive medical strategy to prevent hepatocellular carcinoma.[44,45] A recent study using transgenic mice for hepatitis C virus polyprotein suggests that induced oxidative stress in hepatocytes may downregulate hepcidin transcription through inhibition of C/EBPα DNA binding activity by C/EBP homology protein.[46]

Asbestos fibers have been used heavily in industry from World War II to the present because of their durability, heat resistance, and low cost.[47] However, in 1987, the International Agency for Research on Cancer (IARC) designated asbestos fibers as a group I (definite) carcinogen for humans (http://monographs.iarc.fr/ENG/Classification/crthgr01.php). Epidemiological studies show that asbestos fibers that contain iron (a transition metal that catalyzes free radical generation) are more carcinogenic.[48] Our recent study suggests that local iron overload, whether endogenous or accumulated by other mechanisms, is important for asbestos-induced carcinogenesis.[49]

Several epidemiological studies have suggested an association of endometriosis and ovarian cancer, demonstrating a high risk of ovarian cancer in women with a long-standing history (>10 years) of ovarian endometriosis.[50] Atypical endometriosis was observed in the surrounding tissue in 22.6% of endometrioid and 36.0% of clear cell adenocarcinomas of the ovary, but in only 1.7% of cases of ordinary ovarian endometriosis.[51] Our recent study revealed that ovarian endometriotic cysts are rich in catalytic iron, leading to increased oxidative DNA damage of the epithelia of those cysts.[52]

Animal models of iron-induced cancer

Carcinogenicity of iron compounds is demonstrated clearly in animal experiments. Animal models of iron-induced cancer are summarized in Table 2. The oldest reported experiment of iron-induced carcinogenesis is that mice exposed to iron oxide dust, which caused pulmonary tumors.[53] Subsequently, soft tissue sarcoma was induced by injecting iron dextran.[54] Finally, renal cell carcinoma models were produced by intraperitoneal injection of iron chelates.[55–57] The later models were distinct from the former ones in that the injection sites and the tumor sites were different. Indeed, an injection of these iron chelates causes a Fenton reaction in the renal proximal tubules.[58–60] It is well established that inhalational, intraperitoneal, and intrapleural administration of asbestos fibers causes mesothelioma and lung cancer.[61–64] Success in producing malignant mesothelioma was accomplished by repeated intraperitoneal injection of ferric

Table 1. Pathological conditions associated with iron overload-associated cancer in humans

Condition	Associated cancer
Hemochromatosis (OMIM + 235200, #602390, #604250, #606069)	Hepatocellular carcinoma
Chronic viral hepatitis B and C	Hepatocellular carcinoma
Asbestos exposure	Malignant mesothelioma, lung carcinoma
Ovarian endometriosis	Ovarian carcinoma (clear cell and endometrioid adenocarcinoma)

Refer to the text for details.

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Table 2. Animal models of iron-induced cancer

Iron compound	Species	Cancer	Year
Iron oxide (inh, inhalation of dust)	Mouse	Lung adenocarcinoma, fibrosarcoma	1940
Iron dextran, im	Rat	Spindle cell sarcoma	1959
Amosite, crocidolite, inh	Rat	Lung cancer	1974
Crocidolite, inh	Rat	Pleural mesothelioma	1974
Ferric nitrilotriacetate, ip	Rat	Renal cell carcinoma	1982
Amosite, ip	Rat	Peritoneal mesothelioma	1982
Amosite, ip	Mouse	Peritoneal mesothelioma	1984
Crocidolite, ipl	Rat	Pleural mesothelioma	1984
Ferric nitrilotriacetate, ip	Mouse	Renal cell carcinoma	1987
Ferric saccharate, ip	Rat	Malignant peritoneal mesothelioma	1989
Ferric ethylenediamine-N,N′-diacetate, ip	Rat	Renal cell carcinoma	1994

Im, intramuscular; inh, inhalation; ip, intraperitoneal; ipl, intrapleural. Amosite and crocidolite are iron-rich asbestos fibers. Refer to the text for details.

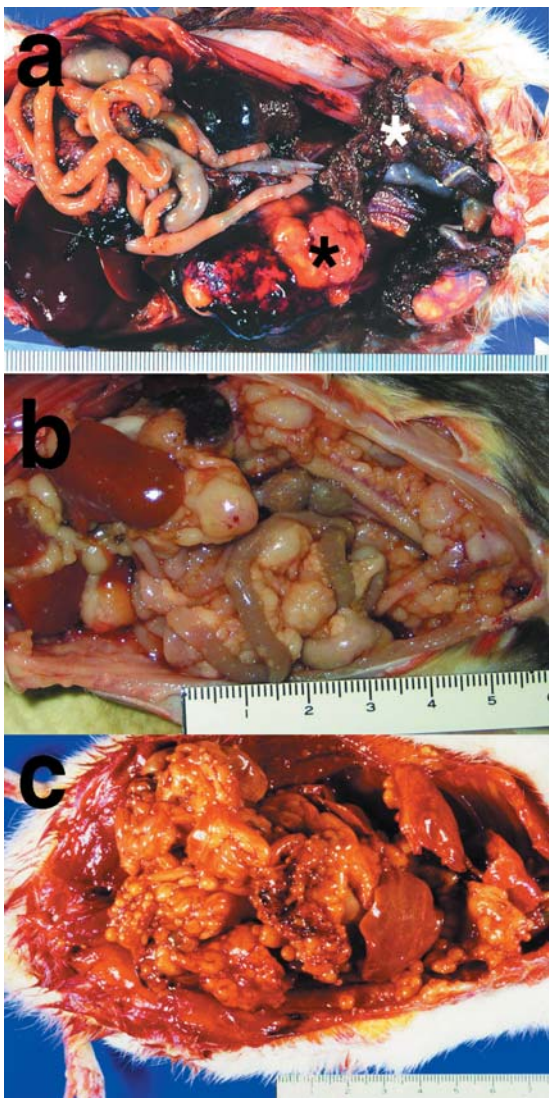

Fig. 2. Macroscopic appearance of iron-induced animal cancer models. (a) Ferric nitrilotriacetate-induced renal cell carcinoma (black asterisk) in rat. Mesothelioma (white asterisk) is also observed around the testes. (b) Amosite (asbestos)-induced malignant peritoneal mesothelioma in rat. Numerous white tumors are observed all over the peritoneal cavity. (c) Ferric saccharate-induced malignant peritoneal mesothelioma in rat.[65]

saccharate.[65] Representative macroscopic appearances of these animal models are shown in Figure 2.

Redox regulation in Fenton reaction-induced cancer

It is of interest to identify potential target genes in Fenton reaction-induced cancer. The significance of oxidative stress in carcinogenesis has been established in the past decade, and is summarized in Figure 3. Of note is the fact that mutation and persistent activation of the new signaling pathways for proliferation are cooperative.[66] Selected mutations of oncogenes generate new signaling pathways for continuous cellular proliferation; consequently, increased proliferation further enhances the mutation rate. Another potential hypothesis is that of a 'mutator phenotype', in which inactivation of caretaker genes leads to a higher mutation rate, leading to the proposal that the mutator genes are the first targets.[67] In a sense, carcinogenesis might be compared to evolution, with the difference that carcinogenesis is impatient with regard to time and is associated mostly with somatic cells. Recently, there has been much interest in epigenetic alterations during carcinogenesis in terms of histone modification (acetylation and methylation) and methylation of CpG islands of the promoter region.[68] Although there is still no convincing data available on the association of oxidative stress and epigenetic alterations, it is believed that such interactions should exist considering the close association between oxidative stress and carcinogenesis and the frequent involvement of epigenetic shutting-off mechanisms of tumor-suppressor genes during carcinogenesis.[69] For example, cells may use controlled DNA oxidation with lysine-specific demethylase (LSDI), a nuclear homolog of amine oxidases, to demethylate the promoter region of estrogen-responsive genes.[70]

Redox regulation is one of the key mechanisms for adaptation to a variety of stresses, including oxidative stress.[71] Recently, it was reported that the thioredoxin antagonist TBP-2 (also known as vitamin D_3 upregulated protein-1)[72] is downregulated in cancers including human adult T-cell leukemia,[73] human gastric cancer,[74] and Fe-NTA-induced RCC in rats.[75] The mechanism of inactivation is methylation of the promoter region. TBP-2 is expressed at higher levels in non-metastatic melanomas than in metastatic melanomas.[76] Studies of a TBP-2-null mutant mouse[77] provided evidence that loss of TBP-2 results in enhanced sulfhydryl reduction and dysregulated carbohydrate and lipid metabolism, namely hyperinsulinemia, hypoglycemia, hypertriglyceridemia, and increased levels of ketone bodies, in the liver and pancreatic β-cells.[78] Loss of TBP-2 appears to be advantageous to cancer cells because it ultimately results in facilitation of the glycolytic pathway via enhancing the enzymatic activity of thioredoxin, which facilitates cancer cell growth in a lower-oxygen

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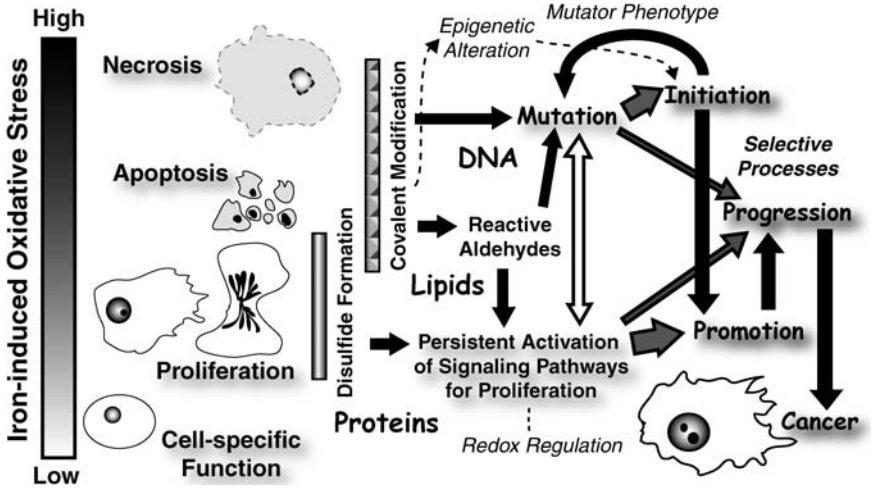

Fig. 3. Significance of iron-mediated oxidative stress in carcinogenesis: Schematic view.

environment as they form a larger mass.[79] Indeed, cancer cells in general take up large amounts of glucose.[80]

Fenton reaction and the genome

Most biochemists assume that free radical reactions present little specificity based on *in vitro* experiments, in contrast to selective interactions, such as antigen–antibody interactions. Indeed, the second-order rate constant for the reaction of hydroxyl radical with guanine is $\sim 1.0 \times 10^{10}$/mol/L/s.[81] Thus, it was generally hypothesized that the genome is damaged at random and that there are no specific 'target' genes or signaling pathways during oxidative stress-induced carcinogenesis. However, it is time to reconsider this assumption. Our laboratory has recently challenged this hypothesis by using a Fe-NTA-induced rat RCC model.[82] At an early stage of this carcinogenesis model, increased numbers of oxidatively modified DNA bases, including 8-oxoguanine,[83] and a major lipid peroxidation product, 4-hydroxy-2-nonenal, and its modified proteins[84–86] are observed. We further showed using gpt delta transgenic mice that deletion and single nucleotide substitutions at G : C sites are the preferred mutations in the kidney after Fe-NTA administration.[87]

In order to clarify whether or not there is a target tumor-suppressor gene, we used a genetic strategy (microsatellite analysis) in F_1 hybrid rats between two different strains. This study revealed that the $p15^{INK4B}$ (*p15*) and $p16^{INK4A}$ (*p16*) tumor-suppressor genes are among the major target genes, which were either homozygously deleted or methylated at the promoter region. This was the first report showing the presence of a target gene in oxidative stress-induced carcinogenesis.[69] Indeed, iron-mediated oxidative damage appears to attack one of the most critical loci of the genome, a crosspoint of the TP53 and retinoblastoma protein pathways.[88,89] Our laboratory later showed that allelic loss of p16 occurs as early as 1 week after the start of the animal experiment and is *p16* specific.[90] This led to our proposal of the existence of 'fragile' sites in the genome that are susceptible to oxidative stress.

We recently used gene expression microarray and array-based comparative genome hybridization analyses to identify target oncogenes in Fe-NTA-induced RCC. At the common chromosomal region of amplification (4q22) in rat RCC, we found that *ptprz1*, a tyrosine phosphatase (also known as protein tyrosine phosphatase ζ or receptor tyrosine phosphatase β), is highly expressed in RCC. In this model, iron-mediated oxidative stress induced genomic amplification of *ptprz1*, resulting in activation of β-catenin pathways in the absence of Wnt signaling during carcinogenesis.[91] Thus, iron-mediated persistent oxidative stress not only confers an environment for gene deletion but also for gene amplification.

Oxygenomics

Studying the localization of oxidative DNA damage in comparison to genome information and fundamental cellular structure is becoming increasingly important. There are a number of published reports on oxidative DNA damage *in vitro* using purified DNA or cultured cells. Based on these data, it has been proposed that certain specific sequences, including telomeres,[92,93] are vulnerable to oxidative damage. However, at present, only limited data are available regarding which part of the genome is susceptible to oxidative damage *in vivo*.

Our laboratory proposes that such studies are now possible given the completion of the genome projects of humans, mice, rats, and other species (http://www.ncbi.nlm.nih.gov/Genomes/). Studies have been carried out to make libraries of ~1-kb DNA fragments that contain one or more 8-oxoguanine,[94] or acrolein-modified adenine,[95] residues by applying the technique of immunoprecipitation[96] (Fig. 4). It must be taken into account that genomic DNA in association with histone proteins is integrated into the chromatin structure, and that some parts of the chromatin structure are open for transcription. Genome information is not continuous, but is divided into many pieces that form chromosomes, although chromosomal structure cannot be observed during interphase. Recently, the concept of 'chromosome territory' has been established.[97,98] This concept proposes that genome information corresponding to each chromosome is located at a rather fixed site in the nucleus even during interphase, and can be divided into nuclear central or nuclear peripheral regions. This localization appears to be different for different kinds of cells.[99] In mouse experiments, the genome corresponding to the nuclear peripherally located chromosome was found to be more susceptible to modification by a lipid peroxidation-associated aldehyde, acrolein.[96] Such a difference might help explain the different signaling pathways that each type of cancer has acquired. A proposal is put forth to call this novel research area 'oxygenomics'. Oxygenomics is defined as a research area studying the localization of oxidative DNA damage in the genome of living cells.

Conclusion

Iron plays an important role in oxidative tissue damage and subsequent carcinogenesis. Clinical features of iron overload are

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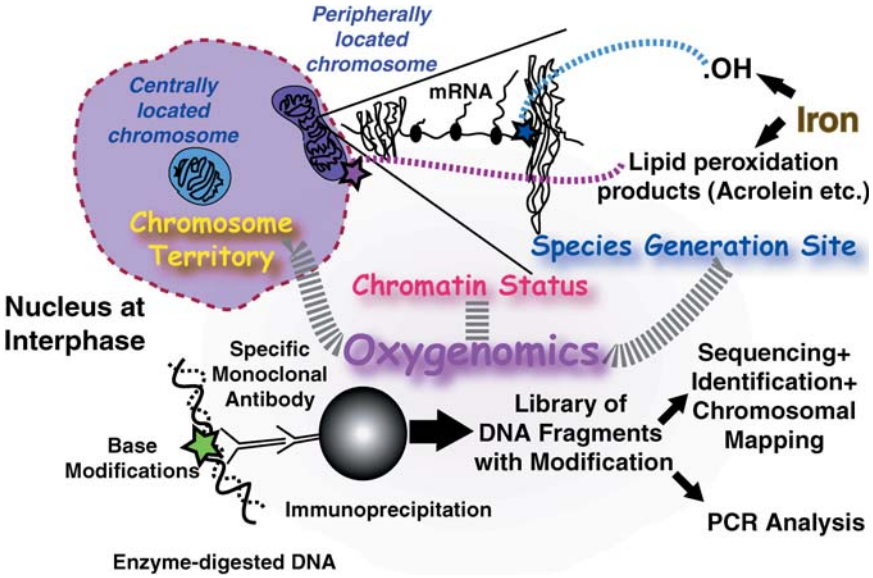

Fig. 4. Concept of oxygenomics to understand the non-random nature of oxidative DNA damage. PCR, polymerase chain reaction.

typical in hemochromatosis. In the past few years, our understanding of iron metabolism, the molecular mechanism of hemochromatosis, and iron-induced carcinogenesis has expanded enormously. Our laboratory has introduced the novel concept of oxygenomics. This concept may also be extended to protein modifications by aldehydes, another key reaction of Fenton reaction-associated covalent modification. Considering the recent report that iron reduction by phlebotomy decreased cancer risk in a supposedly normal population,[2] complete understanding of iron-induced carcinogenesis should be considered a high priority for efficient cancer prevention.

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