Iron and colorectal cancer: evidence from in vitro and animal studies

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Iron is a vital trace element essential for mammalian life. It is involved in numerous biological and cellular processes such as oxygen transport, oxidative phosphorylation, and DNA synthesis, as well as cell cycle progression and growth. Normal and neoplastic cells have similar qualitative requirements for iron. In addition, research shows that iron promotes cancer cell growth. An adequate balance of iron is, therefore, critical for health. In states of iron deficiency, anemia can develop, whereas iron excess increases oxidative stress in body tissues, leading to lipid, protein, and DNA damage via the Fenton reaction, which results in the synthesis of hydroxyl radicals and other oxidants. It is thought that some of these processes are implicated in the pathogenesis of colorectal cancer. This review provides the clinician with an up-to-date summary of the recent advances in this field using established in vitro and animal models.

INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females, with more than 1.4 million new cases and 694 000 associated deaths estimated to have occurred in 2012 worldwide. There is geographical variation in prevalence, with colorectal cancer being more common in the developed world and rare in less affluent countries. The prevalence of colorectal cancer in Western countries has been linked mainly to environmental factors and lifestyle patterns. Specific components of a Western lifestyle such as diet, physical activity, obesity, and smoking are likely to have a major impact on colorectal cancer development.

Lifestyle modifications can result in a substantial reduction in cases of colorectal cancer. Adoption of a Westernized diet has been shown to increase the incidence of colon cancer in several populations, as exemplified by migration studies (e.g., Japanese who migrated from Japan to Hawaii). Diets high in red and processed meat and animal fat, excess consumption of alcohol, smoking, and high body mass index are all risk factors for colon cancer, while an inverse association exists with dietary fiber.

There is evidence to support the hypothesis that excess iron is associated with increased risk of colorectal cancer, implying that both dietary iron and body iron status increase cancer risk. There are two distinct routes of exposure of colonic tissue to iron: 1) excess dietary iron (exogenous) passing through the gastrointestinal tract, which may have a local colonic effect directly from the lumen, and 2) excess body iron stores and elevated serum iron (endogenous), which may have deleterious effects on target organs and cells. In a normal individual, the amount of iron absorbed is usually no more than 10% of the amount of iron ingested. Consequently, a significant fraction of dietary iron remains unabsorbed in the small intestine and may enter the colon. A number of human and animal studies have further suggested that iron can be a factor in the development of colorectal cancer.
studies have demonstrated an association between dietary and total body iron stores, with both being risk factors for the development of colorectal cancer.\textsuperscript{7,11–13} These studies have been reviewed elsewhere in the literature and will not be covered in detail here.\textsuperscript{13} Collectively, they support the belief that excess iron is associated with an increased risk of colorectal cancer; however, the source of excess iron (i.e., exogenous or endogenous) is still not clear.

IRON ABSORPTION

Dietary iron exists in two forms, heme (found almost exclusively in meat) and nonheme. The richest sources of nonheme iron are cereals, vegetables, nuts, eggs, fish, and meat. Heme iron is more efficiently absorbed than nonheme iron.\textsuperscript{14}

Divalent metal transporter 1 (DMT1) at the apical membrane of enterocytes takes up inorganic iron from the lumen of the duodenum after duodenal cytochrome B reduces ferric iron (Fe\textsuperscript{3+}) to ferrous iron (Fe\textsuperscript{2+}), as illustrated in Figure 1. This step is a critical determinant of available iron as there is no active mechanism for iron excretion.\textsuperscript{15} The mechanism of heme iron absorption remains unclear. In the enterocyte, iron is then either stored within ferritin or exported into the circulation through the basolateral iron transport protein ferroportin (FPN)\textsuperscript{16} and reoxidized by hephaestin.\textsuperscript{15} Hepcidin, which is another important protein of systemic iron homeostasis, inhibits the release of iron from absorptive enterocytes into the circulation by binding to and promoting the internalization and subsequent degradation of FPN.\textsuperscript{17}

STUDIES DEMARCATING SYSTEMIC IRON FROM LUMINAL IRON

A number of animal studies have suggested that luminal iron is the specific source of increased colonic neoplasia. Lund et al.\textsuperscript{18} demonstrated that a four-fold increase in dietary iron supplementation in rats had a significant effect on luminal iron concentration, and this was associated with an increase in the epithelial cell proliferation rate in the rat colon. This demonstrates that elevated luminal iron is a direct consequence of an iron-rich diet. The same group also showed that oral iron supplementation in healthy human volunteers increases the concentration of fecal iron and the formation of free radicals through reactive oxygen species (ROS).\textsuperscript{19} The ROS are unstable species produced as byproducts of normal aerobic metabolism as well as by macrophages and neutrophils.\textsuperscript{20,21} They help signal transduction when present at low levels.\textsuperscript{21,22} However, higher levels of ROS can generate an inflammatory response, leading to oxidative stress.\textsuperscript{20,23} This has been shown to damage DNA, resulting in genomic instability associated with carcinogenesis.\textsuperscript{21,24}

Ilsley et al.\textsuperscript{25} showed that elevated dietary iron also induced a significant increase in the size and multiplicity of adenomas in the colons of mice treated with the colonotropic carcinogen, azoxymethane. In a model of inflammatory bowel disease (cyclic dextran sulfate sodium murine model) iron-enhanced colorectal tumorigenesis was prevented when mice were treated with parenteral iron, suggesting that excess luminal iron is key to the generated oxidative stress.\textsuperscript{26} Radulescu et al.\textsuperscript{27} demonstrated that excess luminal iron drives tumorigenesis in adenomatous polyposis coli (Apc)-deficient cells and that tumorigenesis was suppressed in mice fed an iron-deficient diet. The results of this study suggested that systemic iron replacement had no effect on intestinal tumorigenesis in a background of an aberration in Apc. It is also clear from this study that Apc-deficient cells appear to require precise levels of iron for efficient tumorigenesis.

IN VITRO STUDIES

The molecular mechanism by which iron specifically enhances colorectal carcinogenesis is not well understood. The environmental and dietary factors that impact the colonic mucosa at the cellular level are discussed here.
Regulation of iron uptake in colorectal cancer

The continuous proliferation of tumor cells requires an enhanced supply of iron relative to the needs of non-transformed cells. Iron in serum is transported while bound to transferrin (Tf), which interacts with Tf receptor 1 (TfR1) on the membrane of cells that take up iron. Gatter et al. have shown that TfR1 distribution is limited in normal tissues compared with malignant tissues. In the latter, distribution was found to be enhanced in carcinomas and sarcomas and in tissue samples from patients with Hodgkin's disease. Okazaki et al. investigated the rhythmic variation in TfR1 expression in colon cancer–bearing mice. They confirmed that TfR1 showed a clear 24-h oscillation, and its expression in colon cancer cells was controlled by c-Myc. This helps to potentiate the antitumor effects of cytotoxic agents by choosing the most appropriate time of day for their administration. The 6-transmembrane epithelial antigen of prostate (STEAP) family of proteins participates in iron uptake by reducing ferric iron to ferrous iron. The only exception in this family is STEAP1, which has no reductase activity. STEAP1 and STEAP2 are overexpressed in several types of human cancers, highlighting these as potential immunotherapeutic targets against cancer.

Iron storage in cancer

In addition to the need to acquire iron, normal and malignant cells differ in their intracellular distribution and storage. Previous animal studies conducted using rat fetal hepatocytes and hepatoma cells established that neoplastic cells direct more of their iron into metabolic functions than into storage. In normal cells, the regulation of intracellular iron homeostasis is mainly controlled by two messenger RNA (mRNA)–binding molecules known as iron-regulatory proteins 1 (IRP1) and 2 (IRP2). Iron-responsive elements (IREs) are short conserved stem loops that are found in the untranslated regions of various mRNAs that have products involved in iron transport. The IRPs can bind to IREs and either stabilize the expression of the mRNA against degradation or inhibit translation (Figure 2). These mechanisms seem to be crucial in the regulation of iron homeostasis.

The c-Myc proto-oncogene has been shown to repress expression of the heavy subunit of the protein ferroportin (H-ferritin) and to stimulate expression of IRP2. H-ferritin stores iron in soluble, nontoxic form, and its downregulation makes intracellular iron more readily available. This effect can be augmented by IRP2 upregulation because IRP2 binds to an IRE in the H-ferritin RNA and inhibits its translation, thus further decreasing H-ferritin levels. Furthermore, IRP2 enhances iron import by binding to IREs in the Tf receptor RNA and inhibiting its degradation.

Iron transport proteins in colorectal cancer

Brookes et al. demonstrated increased expression of iron import proteins (duodenal cytochrome b, DMT1, and TfR1) and a block in iron export due to decreased expression and abnormal localization of hephaestin and ferroportin FPN, respectively, in human colorectal cancer specimens. The net effect of these changes was increased intracellular iron loading, which favors cell proliferation through modulation of cell cycle proteins and induction of ROS. They also reported the effect of iron loading on E-cadherin, a transmembrane glycoprotein that mediates epithelial cell-to-cell adhesion and is commonly repressed in epithelial malignancies including colorectal cancer. The researchers found significant downregulation of E-cadherin mRNA expression following iron loading of the Caco-2 and SW480 cell lines.

Role of hypoxia-inducible factor in colon carcinogenesis

Hypoxia-inducible factors (HIFs) are heterodimeric transcription factors that consist of an alpha subunit (HIF-1α or HIF-2α) and a beta subunit (aryl hydrocarbon nuclear translocator). HIF-1α and HIF-2α have structurally similar DNA binding and dimerization domains but they differ in their transcriptional response. Hydroxylases continuously modify HIF-1α in the presence of oxygen. HIF-1α is hydroxylated by at least 3 prolyl-4-hydroxylases and 1 asparaginyl hydroxylase. Following hydroxylation, HIF is recognized by the von Hippel–Lindau ubiquitin ligase complex (pVHL-E3) and targeted for proteasomal degradation. When prolyl-4-hydroxylation is inhibited, e.g., in hypoxic situations, HIF-1α is not degraded and translocates to the nucleus where it dimerizes with HIF-1β to form the HIF-1 complex, which then binds to the hypoxia-responsive element in the promoters of target genes to activate their expression (Figure 3). The target genes of HIF-1α have important roles in many physiological and pathological events such as angiogenesis, erythropoiesis, energy metabolism, iron transport, cell proliferation, survival and apoptosis, and tumor progression. HIF hydroxylases are iron-, oxygen-, and ascorbate-dependent oxygenases. Thus, to an extent, HIF-hydroxylase activity is regulated by
physiological or pathological changes in cellular Fe$^{2+}$ availability. In iron-depleted cells, these enzymes are readily inhibited, leading to failure of hydroxylation of specific proline residues in HIF-$\alpha$. Hydroxylation of HIF-$\alpha$ permits binding to the von Hippel–Lindau ubiquitin ligase complex, which results in proteasomal degradation of HIF-$\alpha$. Thus, in iron-deficient cells, HIF-$\alpha$ is stabilized, leading to activation of HIF transcriptional activity.

Hepcidin is a hypoxia-regulated, small polypeptide produced in hepatocytes; it consists of 25 amino acids and plays a vital role in the maintenance of systemic iron homeostasis. Its significance in regulating iron stores was accidentally discovered by Nicolas et al. in an upstream stimulatory factor 2 knockout mouse that was found to exhibit severe iron overload. The researchers concluded that the iron overload phenotype in this mouse was associated with the lack of hepcidin rather than upstream stimulatory factor 2 deficiency. Hepcidin facilitates the degradation of FPN, thereby suppressing intestinal iron uptake from enterocytes and release from hepatocytes and macrophages. Hypoxia suppresses hepcidin, thereby augmenting intestinal iron uptake and release from internal stores. Using a mouse model, Liu et al. showed that hepcidin suppression in hypoxia is not directly regulated by HIF and found that HIF-mediated suppression of the hepcidin gene Hamp1 required erythropoietin induction (Figure 4). Growth differentiation factor 15 is secreted from maturing erythroblasts and suppresses hepcidin in hepatocytes under conditions of stimulated erythropoiesis.

A retrospective analysis of 64 patients in Japan who underwent curative resection of colorectal liver metastasis between 2000 and 2008 showed that HIF-$1\alpha$ was an independent risk factor for recurrence after curative resection. It has been shown that intestinal HIF-$2\alpha$ is a critical regulator of iron absorption. HIF-$2\alpha$ is the central transcription factor required for the regulation of intestinal iron-absorptive genes, and its deletion in enterocytes decreases the severity of tissue iron loading in hepcidin knockout mice (murine model of hemochromatosis).

Recent data demonstrate that the activation of HIF-$2\alpha$ potentiates intestinal inflammation and colon cancer. Shah et al. demonstrated that a chronic increase in HIF signaling (through inactivation of the Vhl gene) in colon epithelial cells initiated an increase in colonic inflammation in murine models; this was mediated by HIF-$2\alpha$ activation of the pro-inflammatory gene macrophage migration inhibitory factor. Activation of HIF signaling (through disruption of Vhl) augmented colon tumorigenesis in the Apc$^{min/+}$ (adenomatous polyposis coli mouse model with multiple intestinal neoplasia). These effects were prevented with disruption of both Vhl and HIF-$2\alpha$, demonstrating that HIF-$2\alpha$ may be a critical factor in the development of colon cancer.
following \( \text{Apc}^{\text{min/þ}} \) mutation. There was significant induction of DMT1 expression in the colons of intestinal \( \text{HIF-2a} \)-overexpressing mice (\( \text{Vhl}^{\text{DIE}} \)) and mice with an \( \text{Apc}^{\text{min/þ}} \) mutation compared with wild-type littermates.57

**Role of the stem cell marker promin-1/CD133 in colorectal tumorigenesis**

The five-domain transmembrane surface protein CD133 has been widely studied for its role in colorectal tumorigenesis.58 Recently, Bourseau–Guilmain et al.59 investigated a possible function of CD133 in endocytosis. They showed a significant increase in cellular uptake of Tf in CD133\(^{\text{low}}\)-Caco-2 cells. Further experiments using chemical inhibitors of known endocytic pathways supported the finding that Tf accumulation within Caco-2 cells was mainly due to clathrin-mediated transport. In addition, CD133 interacts with intracellular cholesterol and inhibits Tf endocytosis, as evidenced by CD133 upregulation of Tf uptake after cholesterol extraction (methyl-\( \beta \)-cyclodextrine treatment). A reduction in Tf uptake was also seen when nondifferentiated Caco-2 cells were treated with the AC133 antibody. Effects of iron supplementation and deprivation showed a dose-dependent downregulation of CD133 expression, which suggests CD133 is regulated by iron. A discrepancy in the CD133-Tf-iron network can lead to loss of fine control of iron accumulation and possibly iron-induced carcinogenesis.

**Iron in Wnt signaling**

At the molecular level, there are number of pathways by which iron may be involved in colorectal carcinogenesis. Inappropriate activation of the Wnt pathway contributes to the development of many epithelial cancers.60 This signaling is initiated by secreted Wnt proteins, which bind to Frizzled receptors, a class of seven-pass transmembrane receptors.61 In the absence of a Wnt, the cytoplasmic protein \( \beta \)-catenin binds to a destruction complex formed by axin, adenomatosis...
polyposis coli (APC), glycogen synthase kinase 3β, and casein kinase 1α (CK1α) and is phosphorylated by glycogen synthase kinase 3β and CK1α. The phosphorylated β-catenin is subsequently degraded by the β-TrCP–mediated ubiquitin–proteasome pathway.

When Wnt proteins are present, they bind to the Frizzled receptor and to low-density lipoprotein-related receptors 5 and 6. This results in dissociation of β-catenin from the APC/axin/glycogen synthase kinase 3β destruction complex, leading to accumulation of unphosphorylated-β-catenin in the cytoplasm and translocation to the nucleus. Within the nucleus, β-catenin interacts with T-cell and lymphoid-enhancing factors to activate the transcription of Wnt/β-catenin–mediated target genes (Figure 5).

Mutations of APC are present in more than 80% of sporadic colon cancers, and mutations of β-catenin are present in approximately 10% of colon cancers. APC mutations represent one of the earliest genetic aberrations and are prevalent in all familial adenomatous polyposis patients. These mutations lead to increased β-catenin/T-cell factor complexing and target gene activation.

Brookes et al. studied in vitro models of colorectal cancer to determine whether excess iron could induce Wnt signaling and if this was APC dependent. They demonstrated that inorganic iron and heme could induce Wnt signaling in malignant cell lines that contained an APC mutation. However, in wild-type APC- and β-catenin–containing cell lines, iron-induced Wnt signaling could only occur once the β-catenin destruction complex had been abrogated. Further studies showed that iron could induce a Wnt signal in a wild-type APC cell line that harbored an active mutant β-catenin molecule. From these studies, it appears that iron can only augment a Wnt signal in a setting in which β-catenin escapes the destruction complex through mutations in APC or activating mutations in β-catenin. The Wnt–β-catenin pathway has gained recognition as an alluring molecular target for therapeutic interventions in human cancers.

**CHELATOR STUDIES**

Tumor cells are more susceptible to iron deprivation because of their high demand for iron. In this context, the use of iron chelators for cancer treatment has recently been considered.

Iron depletion results in G1/S arrest, DNA damage, and apoptosis. The most convincing explanation of
these effects is that iron depletion results in inhibition of the iron-containing enzyme ribonucleotide reductase, which is critical for DNA synthesis. This enzyme mediates the conversion of ribonucleotides to 2'-deoxy-ribonucleotides, thereby providing the precursors necessary for both synthesis and repair of DNA. Cancer cells rapidly proliferate, and this is revealed by the fact that they have a higher number of TfRs on their cell surface, mediating high iron intake. Ribonucleotide reductase is also upregulated in cancer cells, facilitating increased production of DNA precursors necessary for replication. Iron chelation, achieved by targeting ribonucleotide reductase enzyme, is one therapeutic option in the treatment of cancer.

Iron depletion also leads to increased expression of the growth and metastasis suppressor, N-myc downstream-regulated gene-1. It was previously shown that transforming growth factor-β (TGF-β) was increased in various cancer cell types and that this plays an important role in the process of metastasis via induction of the epithelial–mesenchymal transition, which is the initial step for cancer cell migration, invasion, and metastasis. Recent studies have shown that cellular iron depletion inhibits the TGF-β-induced epithelial–mesenchymal transition via upregulation of N-myc downstream-regulated gene-1 in colon cancer HT29 and prostate cancer DU145 cell types. In addition, iron depletion mediated by iron chelators resulted in induction of growth arrest and DNA damage 45α gene (GADD45α, members of the GADD gene family) expression. Iron depletion also led to upregulation of p53, decreased cyclin D1 expression, downregulation of p21 protein, and downregulation of Bcl-2.

Roh et al. previously demonstrated that downregulation of β-catenin inhibits the proliferation of colon cancer cell lines grown in vitro and the tumorigenic
growth of carcinoma xenografts implanted into nude mice. The Wnt–β-catenin signaling pathway is an outstanding method for identifying new drugs, as Wnt signaling participates in multiple processes and abnormal β-catenin signaling occurs in many cancers. Iron chelators such as acetylhydrazones were found to act downstream of the β-catenin destruction complex and were able to block Wnt signaling and growth in APC and β-catenin mutant colorectal cancer cells. 79

Screening for cell-based small molecules may be useful for helping to discover new compounds that not only inhibit subsets of β-catenin targets but also have additional antiproliferative effects. HQBA (N-((8-hydroxy-7-quinolinyl) - (4-methylphenyl)methyl)benzamide) is a newly identified iron chelator that chelates Fe^{2+}, inhibits Wnt–β-catenin signaling in a subset of colon cancer cell lines, and was shown to block the progression of spontaneous cancer in two distinct genetically engineered mouse models. 80

Inositol hexaphosphate (phytic acid, IP6), which is abundantly present in legumes, cereals, oilseeds, and nuts, 81 is a natural iron chelator. In addition to cellular functions such as signal transduction, cell proliferation, and cell differentiation, phytic acid has a broad spectrum of biological activities that include regulation of cellular responses to external stimuli as well as mediation of enzyme activity. 82–84 Phytates have both in vivo and in vitro anti-cancer activity. 84,85 It has been proposed that phytates reduce colon cancer through chelation of iron and suppression of iron-related initiation and promotion of carcinogenesis. Administration of phytic acid was found to inhibit colon cancer in rodents and to significantly lower the mitosis rate in azoxymethane-induced colorectal tumors in rats. 86–88 A recent study showed that administration of IP6 not only suppressed tumor incidence in rats treated with azoxymethane, it also resulted in less expression of β-catenin and cyclooxygenase-2 at the mRNA level, suggesting that IP6 may have a therapeutic role in colorectal cancer and may contribute to new strategies in the prevention and treatment of the disease. 89 Phytic acid may exert its antimetastatic activity through inhibition of interleukin-1β–induced matrix metalloproteinases and tissue inhibitors of matrix metalloproteinase gene expression at the transcriptional level in Caco-2 cells, thereby preventing cancer cell migration and invasion. 83 TGF-β appears to have a dualistic role, whereby the suppressor functions are found in early tumorigenesis and the oncogenic effects are seen in a later (metastatic) phase of tumor progression. 90 Kapral et al. 84 showed that phytic acid can inhibit tumor development in the early stage by enhancing expression of TGF-β and by increasing TGF-β receptor expression to prevent cancer progression. In addition, phytates were found to enhance the activity of natural killer cells in a rat dimethylhydrazine-induced colon tumor. 91

Curcumin, the yellow pigment of turmeric, is an iron chelator that has been used in India and China for centuries as a spice and as a medicine. Turmeric used in Indian cooking may contribute to the lower incidence of large-bowel cancers seen in Indians. 92,93 Its therapeutic efficacy was investigated in human colorectal carcinoma HCT-15 cells, and these experiments demonstrated that curcumin inhibited HCT-15 cell proliferation and induced apoptosis. 74 The ability of turmeric to induce apoptosis in cultured cancer cells has generated interest in its therapeutic potential in the management of colon cancer. As a result of the promising findings in animal studies, several controlled clinical trials in humans designed to evaluate the effect of oral curcumin supplementation on preneoplastic colorectal lesions, such as adenomas, are under way. In a phase Ia clinical trial, curcumin at a dose of 2 or 4 g was administered over a 30-day period to 44 eligible smokers with 8 or more aberrant crypt foci. Results showed that curcumin at a dose of 4 g significantly reduced the number of aberrant crypt foci by 40% (P < 0.005), whereas the number of aberrant crypt foci was not reduced by the 2-g dose. 95 The evidence for a role of iron chelation in cancer therapeutics is expanding, indicating this will remain an important area for future investigations.

CONCLUSION

Recent in vitro and animal studies have shown that luminal iron together with Apc loss can promote intestinal carcinogenesis. Cancer cells exhibit altered mechanisms of iron acquisition that would allow continued multiplication within the host. This appears to be accommodated by colorectal cancer cells via the overexpression of proteins involved in iron uptake, leading to increased entry of iron into cells. These iron-rich colonicocytes proliferate through modulation of cell cycle proteins, and alongside this, iron appears to mediate ROS, resulting in DNA adduct formation, especially on a background of loss of APC. Recent data suggesting iron can induce Wnt signaling in the presence of an Apc or β-catenin mutation offers an unprecedented advance in understanding of the mechanistic associations between iron and colorectal cancer. It is possible that iron accumulation in intestinal cells can promote the Wnt signaling pathway; therefore, chelation of excess iron is an important area of future investigation. In previous experiments, iron chelators demonstrated potent antineoplastic properties in a number of cancers in vitro. These agents have yet to be used in clinical practice, although finding a chelating agent with the fewest possible side effects and without lowering body iron stores.
will be a challenge. Magnetic nanoparticles carrying chemotherapeutic drugs have recently provided a new tool for solid tumor-targeted therapy. It has been shown that adequate quantities of 5-fluorouracil could be selectively concentrated into the tumor mass along with a hyperthermia effect using nanoformulation. This may constitute a potential candidate for combined antitumor therapy against colon cancer.

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