Iron Accumulation in Mammary Tumor Suggests a Tug of War between Tumor and Host for the Microelement

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Abstract. *Iron is indispensable for the metabolism and proliferation of both normal and malignant cells. Recycling from senescent erythrocytes in the liver and spleen is critical for iron supply to all tissues. In the liver and spleen from MMTV-neu (erbB-2) mice bearing a mammary carcinoma, we noticed the scarcity of hemosiderin pigment and its abundance in the stroma of the tumor. Thus iron (III) was investigated with the Perls' reaction in tissues from normal and MMTV-neu mice. With respect to normal animals, in MMTV-neu mice, staining for iron was almost absent in the liver and scarce in the red pulp of the spleen. By contrast, iron was abundant in stromal and tumor cells in the invasion, angiogenic, necrotic and hemorrhagic regions and also in the interstitial fluid. These observations suggest that the tumor subverts iron recycling to its own advantage, by directly utilizing iron released from erythrocytes and dead tumor cells. Our findings are in keeping with the development of iron chelating drugs as chemotherapic agents.*

Iron is a micronutrient indispensable for the metabolism and proliferation of both normal and tumor cells (1). Proteins containing iron, either heme- or non-heme-bound, catalyze key reactions such as gas transportation (*e.g.* hemoglobin, myoglobin), electron transport and energy metabolism (*e.g.* aconitase, NADH dehydrogenase, succinate dehydrogenase, cytochromes), DNA synthesis (*e.g.* ribonucleotide reductase), antioxidant and pro-oxidant functions (*e.g.* catalase, peroxidase, myeloperoxidase, lactoferrin) (2-3). Furthermore, oxygen sensing, important for tissue adaptation and response to hypoxia, is an iron-dependent process (*e.g.* iron-dependent modification of hypoxia-inducible factor 1α (HIF-1α) by a prolyl hydroxylase) (4).

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Recycling of iron from senescent erythrocytes is crucial for the homeostasis of this essential micronutrient and for its supply for the biosynthesis of vital iron proteins such as hemoglobin, myoglobin and catalase. The phagocytosis of effete erythrocytes by cells of the reticuloendothelial cells in liver and spleen contributes to bio-availability of iron to the whole organism (5-6). Iron liberated from heme can be stored as ferritin or hemosiderin (7), or else released to the circulation bound to transferrin to be delivered to bone marrow for re-incorporation into red cells and other iron-demanding cells (8). Liver and spleen contain the body's largest amounts of ferritin and hemosiderin (8). Hemosiderin can be detected as golden and refractile pigment on H&E-stained sections.

Whole body iron metabolism is altered by cancer (9-10). In particular, tumor cells are far more sensitive than normal cells to iron depletion, presumably since they have increased iron requirements that are reflected by marked expression of transferrin receptor 1 (TfR1) and express high levels of ribonucleotide reductase (1, 11). Studies using iron chelators, such as desferrioxamine (DFO), have shown that iron deprivation results in G1/S arrest and apoptosis (1, 12). Indeed, a new approach of experimental cancer therapy consists of trying to stop tumor cells from depleting iron using iron-chelators (13).

Female MMTV-*neu* (*erbB-2*) mice are a transgenic mouse lineage overexpressing the activated rat *neu* oncogene in the mammary gland under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) (14). These mice develop breast tumors which present several features in common with human mammary tumors, such as the slow growth in an immunocompetent host and the ability to metastasize to the lungs. When studying the morphology of the liver of MMTV-*neu* (*erbB-2*) mice, we could not help noticing the almost complete absence of hemosiderin pigment. This suggested that iron storage in the liver might be altered under the influence of the tumor. Furthermore, the pigment was very scarce in the spleen whereas it was abundant in the tumor, especially in stromal cells in the invasion, perihemorrhagic, perinecrotic and angiogenic

regions. We previously reported that, notwithstanding hepatic metastases were not observed, the liver of these animals was markedly altered. In particular, when the mammary tumors were in the angiogenesis phase, the liver and the spleen showed extramedullary hemopoiesis. By contrast, in animals where lung metastases were already present, hemopoiesis in the liver (and spleen) was scarce and an oval cell reaction and other pre-carcinogenic features (large cell dysplasia and gamma-glutamyltranspeptidase activity) were observed in the liver (15); no pigment was noticed in liver sinusoidal cells.

Aware that iron is an essential nutrient for unrestricted tumor cell proliferation (16), we investigated non-heme iron stores using the Perls' Prussian blue stain for ferric iron, not only in the normally erythrophagocytic organs liver and spleen but also in the tumor of MMTV-*neu* (*erbB-2*) mice, with respect to normal animals.

Materials and Methods

Experimental model. As the experimental model, MMTV-*neu* transgenic female C1 mice bearing an activated rat neu oncogene engineered at the Institute of Biomedical Technologies, CNR-ITB, University of Milano, Italy were used. This is a transgenic mouse lineage in which overexpression of the activated rat oncogene was directed to the mammary gland by the mouse mammary tumor virus long terminal repeat (MMTV-LTR) (14). The use and care of the animals was approved by the Italian Ministry of Health and by the University Commission for Animal Care following the criteria of the Italian National Research Council.

In this study, ten MMTV-*neu* female transgenic mice aged 3-4 months (average weight 33 g for 3 month tumor-bearing mice and 41 g for 4 month tumor-bearing mice) with vascularized mammary tumors but incipient metastization into the lungs were considered. Five non-transgenic female C1 mice aged 5 months (average weight 33 g) were taken as controls. Small pieces (5-mm thick) of liver, spleen and mammary tumor were fixed in 2% *p*-formaldehyde, dehydrated in graded alcohol and embedded in Paraplast (Sigma, St Louis, MO, USA). The fixed tissue samples were cut with a rotary microtome (Leica Microsystems, RM 2125, Nussloch, Germany) to make sections of 8-μm thickness.

Localization of non-heme ferric iron with Perls' Prussian blue reaction. Briefly, the sections were dewaxed in xylene, rehydrated through graded alcohol and incubated for 30 minutes in a freshly prepared 2% aqueous potassium ferrocyanide-hydrochloric acid incubating solution (composed of equal volumes of 4% aqueous potassium ferrocyanide and 4% aqueous hydrocloric acid). Slides were then washed twice in distilled water, counterstained with hematoxylin for 8 min, washed first in running tap water and then in distilled water, lightly stained for 10 s with eosin and then dehydrated and mounted in Entellan (Merck, Darmstadt, Germany).

Microscopy and photomicrography. The slides were observed with a Zeiss Axioscop 2 Plus light microscope (Carl Zeiss Microimaging, Jena, Germany) equipped with differential interference contrast (DIC). Images were captured with an Olympus 4.1 megapixel C-4040 Zoom digital camera (Tokyo, Japan). Digital images were processed with Corel Paint Shop Pro (Ottawa, Ontario, Canada).

Results

The Perls' reaction was performed on samples from normal liver and spleen and on the liver, spleen and mammary tumor of MMTV-*neu* (*erbB-2*) mice. In the latter animals, the growth phase corresponding to extensive extramedullary hemopoiesis (15) was selected. A later phase of metastatization to the lungs, where liver hemopoiesis regressed, large cell dysplasia with ductular reaction was observed (15) and spleen fibrosis occurred (unpublished observations), was not taken into consideration.

Normal mouse liver. In hepatocytes, detectable iron occurred as diffuse cytoplasmic staining (blush reactivity) of ferritin-iron complexes and as granules of hemosiderin aligned along the bile canaliculi in centrolobular and midzonal areas (acinar zones 2 and 3) (Figure 1).The highest reactivity was seen in sinusoidal cells, mainly in Kupffer and/or endothelial-like cells in all lobular areas, although occasional positive stellate (Ito, fat-storing) cells were also noticed (Figure 2). Positive macrophages in portal tracts were also present.

Liver of mammary carcinoma-bearing MMTV-neu mice. Abundant hemopoietic clusters with morphology similar to granulopoietic/monopoietic and erythropoietic series and isolated cells of the thrombopoietic series were seen in portal tracts and in the periportal and midzonal areas (acinar zones 1 and 2) (Figure 3). No Perls' reactivity was observed in hepatocytes with the exception of occasional ballooned, necrotic cells (inset in Figure 4); sinusoidal cells with stainable iron were also rare (Figure 3). Stainable iron was observed in stromal cells in portal tracts (Figure 4).

Normal mouse spleen. The red pulp was characterized by heavy staining for iron especially in macrophages; macrophages positive for Perls reaction were also seen along blood vessels in the white pulp (Figures 5-6). In the red pulp large, negative, megakaryocytes were occasionally observed (Figure 6).

Spleen of mammary carcinoma-bearing MMTV-neu mice. The red pulp was occupied by abundant hemopoietic clusters and megakayocytes, all negative for Perls reaction. Erythrophagocytosis, assessed in terms of staining for iron, though present was much less intense than in spleens from normal animals (Figures 7-8).

Mammary carcinoma. As already described (14), the alveoli and ductules were filled by proliferating cells (carcinoma *in situ*). The entire lobule proliferated and enlarged, and solid lobular masses tended to coalesce and were separated from one another by thin fibrous trabeculae. The interlobular stroma and fat were infiltrated and replaced by the neoplastic

growth. Most of the solid masses of tumor cells displayed some evidence of tubular morphology. The largest solid areas of tumor often became necrotic. The neoplastic cells were usually uniform in shape and size. Mitoses were a common finding. The tumor was surrounded by hypercellular granulation-like tissue. Hemorrhagic areas close to vessels invaded by the tumor were present. Edema, herein named "tumor interstitial fluid" (TIF) was seen at the tumor edge (close to enlarged lymph vessels) along necrotic areas and fibrous septa. The necrotic zones were often infiltrated by inflammatory cells.

In contrast with the very low storage levels for iron seen in the liver and spleen, Prussian Blue staining indicative of Fe (III) was prominent in several tumor compartments (Figures 9-14). In particular, intense staining was seen in the granulation tissue (Figures 9, 10). Iron (III) storage in tumor cells was only observed around hemorrhagic regions (Figure 11). Furthermore, stainable iron was present in stromal cells in angiogenic areas (Figures 10, 13) and in TIF infiltrations (Figures 12, 13). In the granulation tissue iron-loaded cells with morphology of fibroblasts, mononucleated cells and mast cells were observed (Figure 14).

Discussion

Storage of iron (III) as ferritin and hemosiderin is an index of red blood cell destruction, mainly in hemocatheretic organs such as liver, spleen and bone marrow, but also in hemorrhagic zones. Ferritin and hemosiderin can be visualized with acid ferrocyanide solutions, giving the Prussian blue reaction (Perls' reaction) with high sensitivity and specificity (17). Ferritin dispersed in the cytosol produces a diffuse bluish tint in the cytoplasm (known as blush iron), whereas dark blue granules are due to ferritin and hemosiderin stored together within siderosomes (18).

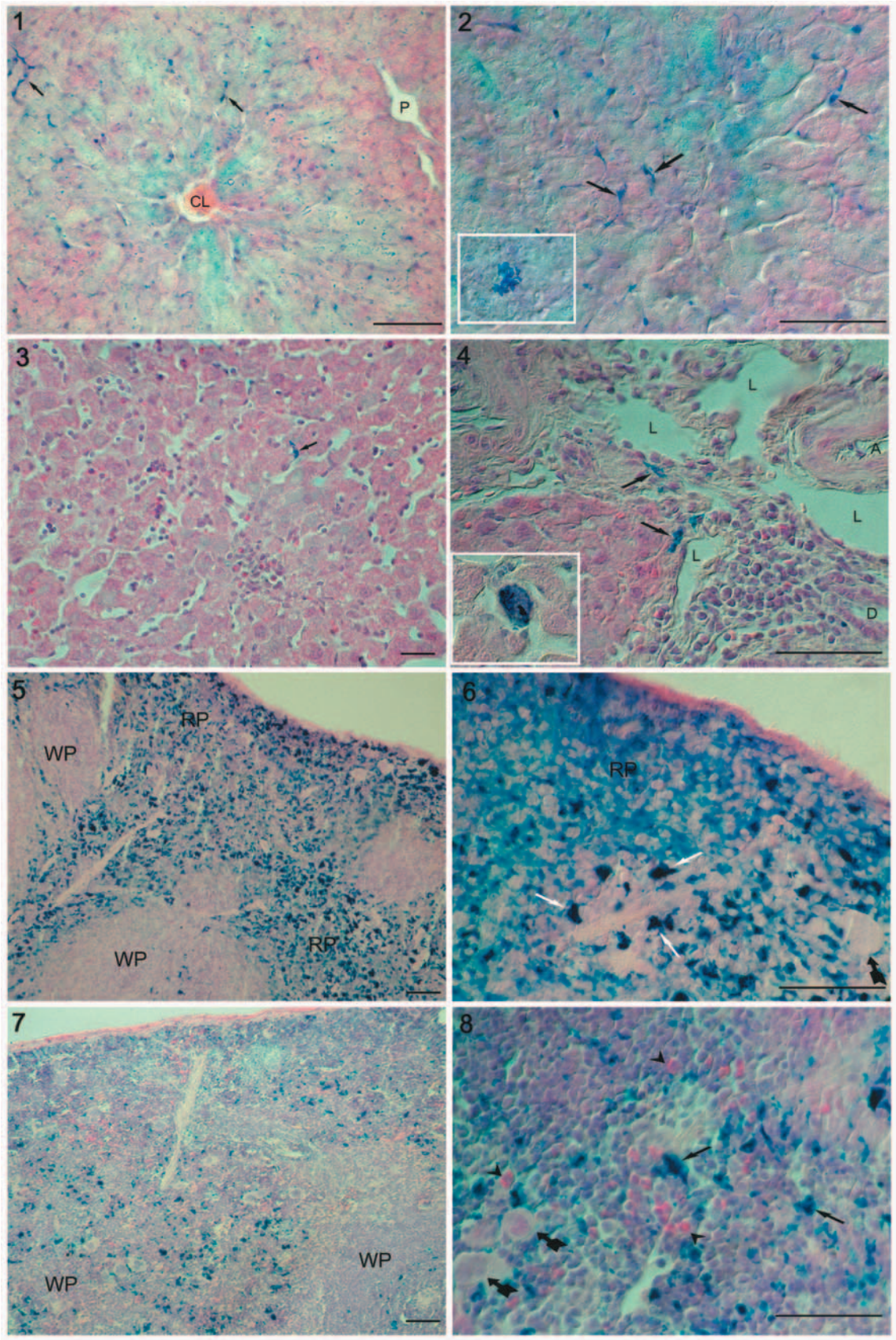
In our study, the distribution of Perls' reaction as diffuse and granular products in normal liver and spleen (Figures 1-2 and 5-6) is in keeping with the normal distribution of ferritin and hemosiderin respectively, in these normally hemocatheretic organs. In contrast, the practical disappearance of stainable iron from the liver and spleen in the tumor-bearing animals (Figures 3-4 and 7-8) indicated that these organs, under the influence of the mammary tumor and, in particular, involved in extramedullary hemopoiesis, were poorly engaged in the destruction of senescent or damaged erythrocytes, thus providing a low amount of iron for erythropoiesis. Indeed, as previously mentioned (15), Giemsa-stained smears of peripheral blood and marrow of these animals indicated megaloblastic anemia.

The distribution of Perls' positive stromal cells (Figures 9-14) was fully in keeping with that of pigment-laden macrophages (also known as siderophages) detected in H&E stained sections (see in particular the inset in Figure 13). These cells were close not only to hemorrhagic foci but also to necrotic tumor cells and invaded normal tissue. The abundance of iron-laden macrophages is considered as a host attempt to withhold or withdraw the metal scavenged from invaded tissues from tumor cells (16). Iron-deprived neoplastic cells may remain viable by maintaining anaerobic glycolysis but are unable to synthesize DNA, to express mitochondrial aconitase activity or to transport electrons *via* mitochondrial NADH:ubiquinone (complex I) and succinate:ubiquinone (complex II) oxidoreductases (16).

It is noteworthy that Perls' positive tumor cells were seen in the immediate vicinity of hemorrhagic foci, whereas those surrounding necrotic foci were negative (Figure 11). This is in keeping with the much higher iron-carrying potential of spent erythrocytes with respect to dead tumor cells. Indeed, mammary tumors from both rats and humans were found to have significantly increased levels of non-heme iron (19-20). That dead cells contained stainable iron was confirmed by the necrotic hepatocyte stained by Prussian blue shown in the inset in Figure 4.

Stainable iron was also seen in the tumor interstitial fluid (TIF) (Figures 12, 13). The TIF is derived from blood plasma oozing at the tumor surface from host vessels that are stimulated to become hyperpermeable due to the secretion of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) by tumor and stromal cells (21-22). In their seminal study, Gullino's group quantified total protein, nonprotein nitrogen, urea, free amino acids, glucose, lactic acid, total sterols, lipid phosphorus, chloride, sodium, and potassium in the TIF from several animal tumor models but did not investigate iron nor iron-related proteins (21). This point was clarified recently through the proteomic characterization of the TIF perfusing a human breast cancer that revealed the presence of transferrin and ferritin therein (23). The presence of iron-chelating proteins in body fluids (except in urine) is believed to be an effective first line defense against microbial cell invaders that need iron for proliferation (24).

Notwithstanding the likely attempts of the host to withdraw iron from malignant cells, tumor growth reveals that neoplastic cells have efficient strategies for extracting iron, in particular through the overexpression of transferrin receptor 1 (1). In our model, with the exception of tumor cells surrounding hemorrhagic regions, most other malignant cells were not stained with Perls' reaction. A possible explanation for this might be that Fe (III) released from transferrin after receptor-mediated endocytosis is separated from transferrin in acidic endosomes and released in the cytoplasm as Fe (II) to be incorporated in heme- or non heme Fe-containing proteins (1), which do not react with ferrocyanide to give Prussian blue (6). Normal and neoplastic cells are believed to differ not only in the mechanism for iron acquisition but also in their intracellular deposition of the metal: whereas normal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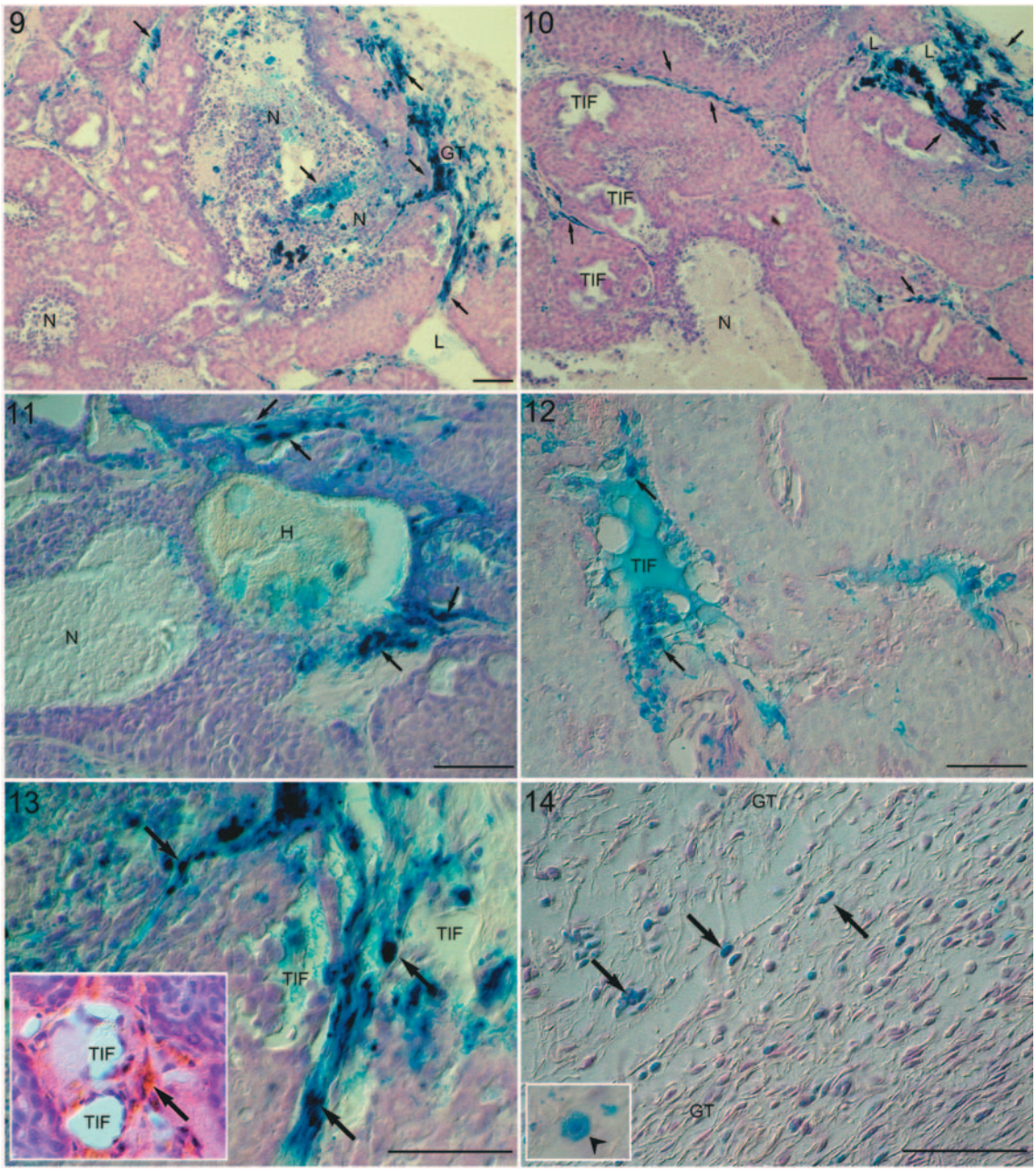

Figures 1 and 2. *Photomicrographs of normal mouse liver stained using Perls' technique for non-heme Fe (III) iron. In hepatocytes detectable iron occurs as diffuse, nongranular cytoplasmic staining (blush reactivity) of ferritin-iron complexes and as granules (hemosiderin) in centrolobular and midzonal areas (acinar zones 2 and 3). The highest reactivity is observed in sinusoidal cells, mainly in Kupffer and endothelial-like cells in all lobular areas (arrows). Occasional positive stellate (Ito, fat-storing) cells can be seen (inset in Figure 2). P: portal vein; CL: centrolobular vein. The photomicrograph in Figure 2 was obtained with differential interference contrast (DIC). Scale bar: 50 µm.*

Figures 3 and 4. *Photomicrographs of the liver of mammary carcinoma-bearing MMTV-neu mice stained using Perls' technique. Extramedullary hemopoiesis is present. With the exception of a few necrotic cells that show intense iron staining (inset in Figure 4), the Perls' reaction is negative in hepatocytes. Sinusoidal cell staining is a rare event (arrow in Figure 3). In the portal stroma, positive stromal cells are seen close to hemopoietic foci and dilated lymph vessels (arrows in Figure 4). D: bile duct, L: lymph vessel. Figure 4 was obtained with DIC. Scale bar: 50 µm.*

Figures 5 and 6. *Photomicrographs of normal mouse spleen stained using Perls' technique for non-heme Fe (III) iron. The reaction is extremely intense in the red pulp (RP), especially in cells with macrophage morphology (arrows). The large black arrow shows a megakaryocyte. A few positive cells along blood vessels in the white pulp (WP) are also visible. Figure 6 was obtained with DIC. Scale bar: 50 µm.*

legends continued

Figures 7 and 8. *Photomicrographs of the spleen of mammary carcinoma-bearing MMTV-neu mice stained using Perls' technique. Extramedullary hemopoiesis is present in the areas occupied by the red pulp in normal animals. Iron staining, much less extensive than in normal animals, is seen in a few macrophage-like cells in the hemopoietic region. This contains myeloid-like hemopoietic clusters, negative for the reaction. Large arrows show megakaryocytes and arrow heads eosinophilic cells. Figure 8 was obtained with DIC. Scale bar: 50 μm.*

Figures 9-14. *Photomicrographs of mammary carcinoma in MMTV-neu mice stained using Perls' technique for non-heme Fe (III) iron. Under low magnification (Figures 9-10) heavy iron staining (arrows) is observed in the granulation tissue (GT) at the tumor invasion edge containing dilated lymph vessels (L), in inner necrotic areas (N) and along tumor blood vessels. Infiltrations of tumor interstitial fluid (TIF) are seen at the necrosis edge, at the tumor periphery (Figures 10) and along fibrous septa (Figures 11 and 13). Under higher magnification stainable iron can be seen in stromal cells on fibrous septa (arrows in Figures 11 and 13). Figure 11 shows iron staining in a hemorrhagic zone and in the surrounding layer of tumor cells. In Figure 12, iron is present in a TIF infiltrate and in adjacent macrophages (arrow). The inset in Figure 13 shows the sharp correspondence between Perls' staining patterns and the yellow pigment in H&E stained sections. In the granulation tissue (GT) at the invasion edge (Figure 14), staining for iron is seen in fibroblasts and mononucleated cells (arrows) and in mast cells (inset, arrow head). Figures 11-14 were obtained with DIC. Scale bar: 50 μm.*

cells apparently convey a higher percentage of acquired iron into ferritin deposition, neoplastic cells consign a greater proportion to metabolic tasks (16).

In the literature, TIF is viewed mainly as a barrier to the delivery of therapeutic agents to tumor cells (25). However, and as previously proposed by our group, rather than being a mere tumor epiphenomenon, blood serum-derived TIF might be a key determinant of tumor growth (26-28). The findings herein reported strengthen this speculation, as TIF percolates not only at the tumor surface but also in inner tumor areas, in particular along the perinecrotic region. It could in theory deliver iron not only to tumor cells but also to inflammatory and stromal cells. It should be remembered that the expression of the transferrin receptor, ubiquitously expressed on normal cells, is increased on cells with a high proliferation rate (29).

A solid tumor such as a breast carcinoma contains not only aberrant epithelial cells and stroma but also recruited blood vessels, activated fibroblasts and infiltrating macrophages, lymphocytes and leukocytes. In particular, the recruitment of macrophages was proven to be important for breast tumor progression, with macrophage infiltration correlating with a poor prognosis (for a recent review see (30)). Iron is therefore likely needed for the proliferation, metabolism and specialized function of all these cell types. Fibroblasts need iron for collagen biosynthesis, in particular for the hydroxylation of prolyl and lysyl residues in the endoplasmic reticulum (31), macrophages use the element for NADPH oxidase, peroxidase and catalase activity (3) and serotonin-producing mast cells need iron for the tryptophan hydroxylase reaction (32). Ferritin-like particles have been detected in macrophages and associated mast cells in a variety of normal tissues, suggesting a collaboration between these two inflammatory cell types for reducing the effective concentration of iron in the extracellular fluid (33).

In conclusion, our findings support the rationale for the use in cancer chemotherapy of iron chelators such as desferroxamine (DFO), especially the newer generation drugs that have been shown to have a greater antiproliferative effect on tumor cells than DFO (9, 34) and even to up-regulate the expression of a growth inhibitory and metastasis suppressor gene (35). It should however be remembered that iron depletion can support angiogenesis by stabilizing HIF-1α factor, which is a critical regulator of the production of angiogenic factors, including VEGF. Indeed, it has been suggested that the final balance between pro-angiogenesis or proliferation inhibition will depend on the iron depletion capacity of the chelators used (36). In favor of the approaches for depleting cancers of iron, it is worthwhile mentioning recent research showing that blockade of the transferrin receptor (TfR) by two anti-TfR monoclonal antibodies inhibited breast cancer cell proliferation, up-regulated the expression of HIF-1α and HIF transcription factors, down-regulated TfR expression and down-regulated cellular labile iron pools (37). The authors tested the effects of physiological concentrations of ascorbate (vitamin C), necessary for the full catalytic activity of prolyl hydroxylase enzymes, on viability and HIF-1α levels on cells treated with the anti-TfR antibodies, demonstrating that ascorbate suppressed HIF-1α protein levels and HIF transcriptional targets while not suppressing the antiproliferative effect of the antibodies.

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