Tumor Cell Heme Uptake Induces Ferritin Synthesis Resulting in Altered Oxidant Sensitivity: Possible Role in Chemotherapy Efficacy

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ABSTRACT

Neovascularization and hemorrhage are common features of malignant tumors. We wondered whether hemoglobin derived from extravasated RBC deposits heme-derived iron into the tumor, which could modulate the sensitivity of cancer cells to oxidant-mediated injury.

A brief exposure (1 h) of "Cr-radiolabeled breast cancer cells (BT-20) but not colon cancer cells (Caco-2) to heme (10 μM) or FeSO₄ (10 μM) significantly enhances cytotoxicity mediated by 0.5 mM hydrogen peroxide (H₂O₂). Associated with Caco-2 resistance, these cells were found to be enriched in the endogenous iron chelator, ferritin. If cellular ferritin is even further increased through 24 h incubation (24 h prior to H₂O₂ exposure) of both cell types with heme, FeSO₄ or exogenous spleen apoferritin itself (24 h), marked resistance to H₂O₂-mediated cytotoxicity is manifest.

Under several conditions, the sensitivity of tumor cells to oxidant-mediated lysis is inversely proportional to their ferritin content. Pretreatment of BT-20 and Caco-2 cells with heme or FeSO₄ rapidly increases H-ferritin mRNA but only slightly increases L-ferritin mRNA; nevertheless, large increases in overall ferritin content of iron-exposed cells result. Data analogous to those with H₂O₂-mediated cytotoxicity were obtained in studies of bleomycine-engendered DNA strand breakage and cell damage, i.e., brief treatment of BT-20 cells with both heme or FeSO₄ significantly increases their sensitivity to bleomycine (100 μg/ml), whereas treatment followed by 24 h incubation with media alone significantly protects against bleomycine toxicity.

We speculate that acute exposure of tumors to iron (e.g., derived from heme-proteins in hemorrhagic cancerous lesions) may increase sensitivity of some cancer cells, particularly those relatively low in endogenous ferritin, to oxidant-mediated lysis. In contrast, repeated, more chronic, exposure may result in resistance of various tumors to oxidant-producing immune effector cells or chemotherapeutic agents, an effect derived from their increased synthesis and accumulation of the intracellular iron scavenger, ferritin.

INTRODUCTION

Iron represents an essential element for the growth and viability of all cells including neoplastic tissues (1, 2). It plays a crucial role in respiratory and oxidative cell metabolism as well as in DNA synthesis. On the other hand, "free (low molecular weight) iron" may play a catalytic role in the generation of tissue-damaging activated oxygen species, such as the hydroxyl radical, via the Haber-Weiss reaction (radicals capable of damaging DNA, lipids and proteins) (3–6). Under physiological conditions, cellular iron metabolism is seemingly tightly controlled by mechanisms that: (a) regulate cellular iron uptake through modulation of transferrin receptors (7, 8); and (b) store excess iron in a nontoxic form as ferritin (9, 10).

Neoplastic cells have qualitative needs for iron similar to those of normal cells (11). In fact, the role of iron in cell proliferation is thought to represent an important factor in the clonal expansion of cancer cells; thus, in a variety of tumors, including breast cancer, transferrin receptors are increased relative to their minimal expression on surfaces of nonmalignant cells in the same tissue (12–14). This has led to the speculation that such receptor up-regulation may be advantageous for tumor proliferation by supplying iron for DNA synthesis (15), and, indeed, neoplastic cells have an increased tendency to incorporate iron into metabolically active compounds (11). Moreover, patients with cancer also tend to harbor increased amounts of storage iron in liver, spleen, and bone marrow with corresponding elevated serum ferritin levels (16). Furthermore, different cancer cells exhibit increased amounts of intracellular ferritin (1, 17–19) that is either poorly iron saturated (1) or well saturated (18). Malignant breast tissue has a 7-fold increase in cytosolic ferritin compared to benign lesions of the breast (19).

Since tumor-engaging immune cells and diverse chemotherapeutic agents act via oxidant-mediated toxicity, it seems reasonable to question whether the quantity and quality of cellular iron in tumor cells might modulate their susceptibility to reagent oxidants, such as H₂O₂, or to prototype oxidant chemotherapeutic agents, e.g., bleomycin. Such inquiry seems germane since neovascularization and hemorrhage are common features in human neoplasms. We reasoned that leakage of RBC from fragile vessels may deposit "heme-derived iron" into tumors. In addition, many neoplasms are chronically bathed in blood; most notably, colonic tumors may be exposed to luminal hemoglobin for long periods during their evolution to a more malignant phenotype. Heme, which is readily released from methemoglobin (20, 21), represents an ubiquitous iron-containing compound which is potentially toxic when it escapes from the intracellular space. In fact, high concentrations of the specific heme-binding protein, hemopexin, are present in plasma and have been shown to diminish heme-driven cytotoxicity (22–24). In recent studies (22), we have shown that free heme, if unbound to hemopexin, readily incorporates into the hydrophobic domains of endothelial cells where, acutely, it markedly aggravates cytotoxicity induced by H₂O₂ or activated polymorphonuclear leukocytes. In contrast, more prolonged exposure of endothelium to the same amount of heme renders it completely resistant to oxidant-mediated injury, a paradox shown to reflect the marked induction of synthesis of the potent iron-chelating storage protein, ferritin (10).

Although these previous studies were performed to provide insights into mechanisms of vascular damage (10, 21, 22), in the present studies, which have been reported in preliminary form elsewhere (25), we questioned whether an excess of heme-driven iron might affect the biology of cancer cells. We demonstrate significant and dichotomous effects of brief versus prolonged exposure of two different cancer cell lines to heme or FeSO₄ on their sensitivity to oxidant-mediated cellular damage.

MATERIALS AND METHODS

Reagents. Hemin (hemin chloride) was obtained from Sigma Chemical Co. (St. Louis, MO), dissolved in 10 mM NaOH (EM Science Inc., Cherry Hill, NJ) as a 1 mM stock solution, and diluted to the desired final concentration in DMEM.

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The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; HIVEC, human umbilical vein endothelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide.
DMEM, HBSS, and FCS were obtained from Gibco (Grand Island, NY). Desferrioxamine mesylate was from CIBA-Geigy, Ltd. (Basel, Switzerland), and ferrous sulfate (FeSO₄) was from Mallinckrodt, Inc. (Paris, KY). Sodium phosphate was obtained from JT Baker, Inc. (Phillipsburg, NJ).

₅¹CrO₄ (as the sodium salt), as well as other radiolabeled compounds and nylon membranes, were from Amersham (Arlington Heights, IL). All other reagents, including horse spleen apoferritin and MTT, were obtained from Sigma unless otherwise specified.

Cell Cultures. Cancer cell lines were established cell lines from human breast adenocarcinoma (BT-20) and from human colon adenocarcinoma (Caco-2). The cells were purchased from the American Type Culture Collection (Rockville, MD).

The cell lines were grown in DMEM + 10% heat-inactivated FCS supplemented with l-glutamine, penicillin, and streptomycin and were used from passages 3 to 10 after reaching confluence. HUVEC were prepared and grown as described previously (21, 26).

Cell Treatments and the Cytotoxicity Assay. Confluent BT-20, Caco-2 cells, and HUVEC grown in 24-well (2 cm²/well) tissue culture plates were radiolabeled with 2 μCi/well Na₅¹CrO₄ in DMEM plus 10% FCS. After 16 h, the wells were washed 3 times with HBSS, and H₂O₂ (in desired concentration) was added in HBSS for 4 h. Specific cytotoxicity values were calculated as described previously (22). Spontaneous ⁵¹Cr release was below 10% in all experiments performed.

In studies of sensitization to free radical-mediated injury, the cells were incubated with hemin (10 μM) in DMEM for 1 h, washed once with HBSS followed by a 1-h incubation with hemin-free media, and then challenged with H₂O₂. For inducing cytoprotection against free radical damage, the cells were pretreated with hemin (10 μM) in DMEM for 1 h, washed with HBSS followed by a 24-h incubation with hemin-free media, and then challenged with hemin and H₂O₂ as described above. In some experiments, FeSO₄ (100 μM) was preincubated with fresh human serum for 30 min at 37°C and added to the cells (10 μM final concentration) for the same time periods as hemin (1 or 24 h). Iron-pyridoxal isonicotinoyl hydrazone chelate (27) (200 μM iron content) was added to the cells for 24 h prior to the cytotoxicity assay. Control studies using 10% human serum without added iron were performed concurrently.

Ferritin Assays. Ferritin content was measured in cells by the Stratus fluorometric enzyme immunoassay system (28) after cell solubilization (10). The results were expressed as ng ferritin/mg cell protein (assessed by BCA protein assay; Pierce, Rockford, IL).

Ferritin mRNA Analysis. Ferritin mRNA content was analyzed in BT-20 and Caco-2 cells after treatment with hemin or FeSO₄. Cellular mRNA was isolated by the RNAzol method (Tel-TEST, Inc., Friendswood, TX). Aliquots (20 μg) of total RNA were electrophoresed in a 1% agarose gel and transferred onto nitrocellulose membranes (29). In each gel the position of the 28S and 18S ribosomal RNA was determined in a separate lane by ethidium bromide staining. The size estimates for the signals were checked by the use of a ³²P-labeled complementary DNA ladder. The membranes were then hybridized at 42°C with nick-translated ³²P-labeled complementary DNA probes for human H- and L-ferritin (obtained from Dr. H. N. Munro, Tufts University, Boston, MA). Autoradiographs were obtained and quantified by computer-assisted videodensitometry (30).

MTT Dye Cell Viability Assay. The MTT cell viability assay was performed as described previously (31, 32). Briefly, the BT-20 cells were grown in a 96-well tissue culture plate (1 × 10⁵ cells/well), and after preincubation with various reagents for sensitization or cytoprotection periods, the cells were exposed to 100 μM bleomycin (Nippon Kayaku, Tokyo, Japan) for 4 consecutive days. MTT dye reagent was dissolved in phosphate-buffered saline at a concentration of 2 mg/ml and added to the cells (50 μl/well) at the end of the incubation period for 4 h; medium was then replaced with 150 μl of 100% dimethyl sulfoxide to solubilize crystals of the dye, and after vigorous shaking, the absorbance was read at 540 nm in a THERMOmax microplate reader (Molecular Devices Corp., Menlo Park, CA).

The viability of the cells in each sample was expressed as a percentage of absorbance of untreated control cells incubated for the same time period. The viability of cells treated for 4 days with various reagents without bleomycin was 96–102% of untreated cells.

DNA Strand-Breaks Detection. DNA single-strand breaks were detected using the method described by Birnboim and Jevcak (33). The BT-20 cells (2 × 10⁶/ml), pretreated with different reagents and incubated with bleomycin (100 μg/ml) for 4 h, were trypsinized; and after repeated washing and cell lysis, the cell lysate was exposed to alkali solutions permitting partial unwinding of the DNA (the degree of unwinding reflecting DNA single-strand breakage). After sonication, the fluorescent dye ethidium bromide, which preferentially binds to double-stranded DNA, was added and the intensity of fluorescence was measured in a fluorospectrophotometer (Perkin-Elmer 605–103; excitation 520 nm, emission 590 nm). The results were expressed as the percentage of double-stranded DNA remaining in cell samples compared to total double-stranded DNA fluorescence in samples not exposed to alkali.

RESULTS

Effect of Hemin or FeSO₄ on H₂O₂-mediated Cytotoxicity. Caco-2 and BT-20 tumor cells or endothelial cells (HUVEC) undergo dose-related lysis when exposed to increasing H₂O₂ concentrations, but the former cells were generally less sensitive to oxidant injury (Fig. 1). For all further experiments, we used 0.5 mM H₂O₂ which killed ~20–30% of cancer cells after 4 h exposure.

As shown in Fig. 2A, compared to incubation with H₂O₂ alone (Fig. 2A, Column 1), both hemin (Fig. 2A, Column 2) and FeSO₄ (Fig. 2A, Column 3), when added to BT-20 cells for a 1-h sensitization period, significantly enhance H₂O₂-mediated cytotoxicity (hemin, P < 0.0001; FeSO₄, P < 0.01). In contrast, if added for 1 h (24 h prior to sensitization), hemin (Fig. 2A, Column 4) or FeSO₄ (Fig. 2A, Column 5) virtually prevents H₂O₂-mediated cytolysis (P < 0.0001 for both reagents). Preincubation of BT-20 cells with a cell permeant Iron-pyridoxal isonicotinoyl hydrazone chelate (27), which is known to increase extracellular ferritin content (10), results in marked protection against subsequent hemin plus H₂O₂ challenge (42.2 ± 4.8% control versus 28.1 ± 4.8%). That induction of ferritin synthesis underlies this cytoprotection is likely; i.e., addition of exogenous horse spleen apoferritin itself to BT-20 cells for 24 h markedly inhibits oxidant damage (Fig. 2A, Column 6). In results not shown and similar to our previous data with endothelial cells (10), apoferritin was rapidly taken up into the cytoplasm of BT-20 cells as detected by immuno-histochemical methods. Iron is necessary for the induction of ferritin in BT-20 cells since 24-h pretreatment of BT-20 cells with 10 μM iron-free stannoprotoporphyrin IX does not induce ferritin or afford protection against cytotoxicity (42.2 ± 1.3% control versus 40.9 ± 3.8%).

As shown in Fig. 2B, Caco-2 cells respond somewhat differently than BT-20 cells; thus, their brief exposure to hemin or FeSO₄ for a 1-h
sensitization period fails to enhance peroxide-mediated killing. Nevertheless, as with the breast cancer cells, a 1-h exposure to hemin or FeSO₄ added 24 h prior to the sensitization period, as well as a 24-h exposure to exogenous horse spleen apoferritin, inhibits oxidant-mediated lysis (P < 0.01 for all three reagents).

Analysis of Cellular Ferritin Content. As noted above, we speculated that cellular ferritin content may be critical in determining the sensitivity of cells to oxidants. In support of this hypothesis, Fig. 3 shows that endogenous ferritin contents of resting HUVEC, BT-20, and Caco-2 cells are different; Caco-2 cells contain greater amounts of ferritin than BT-20 cells or HUVEC and are intrinsically more resistant to H₂O₂-mediated cytotoxicity (see Fig. 1). As shown in Table 1, after 24 h a 1-h hemin exposure increases the ferritin content of both cancer cell lines (Table 1, line 2); as previously reported (10), a similar increase is observed in HUVEC. Analogously, a 1-h incubation with FeSO₄ increases ferritin levels of both tumor cell types (Table 1, line 3) unless the iron chelator desferrioxamine is added concomi-

**Table 1** Induction of ferritin synthesis in BT-20 and Caco-2 cells by hemin or FeSO₄.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>BT-20 cells</th>
<th>Caco-2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43.21 ± 5.17</td>
<td>68.77 ± 6.74</td>
</tr>
<tr>
<td>Hemin</td>
<td>246.57 ± 34.20</td>
<td>315.52 ± 56.06</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>198.82 ± 27.17</td>
<td>177.71 ± 35.87</td>
</tr>
<tr>
<td>FeSO₄ + DFO</td>
<td>16.19 ± 1.41</td>
<td>22.13 ± 2.02</td>
</tr>
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*a DFO, desferrioxamine.

**Fig. 3.** Ferritin content in untreated BT-20 cells, Caco-2 cells, and HUVEC. Results represent mean ± SE of 5 experiments (in duplicates). Bars, SD.

**Fig. 4.** Effect of different incubation time periods with hemin (10 μM) on ferritin content (ng/mg cell protein) in BT-20 cells. A representative experiment of duplicate samples is shown.

Significant ferritin synthesis is first detected in heme-exposed BT-20 cells after about 2–4 h and progressively increases thereafter for at least 24 h (Fig. 4). After 24 h, large amounts of granular-appearing deposits of ferritin are noted with immunofluorescent techniques, in contrast to the paucity of immunofluorescent-staining ferritin in resting BT-20 cells (data not shown).

**Fig. 2.** Effect of pretreatment of BT-20 cells (A) and Caco-2 cells (B) with different reagents for different time periods on H₂O₂-mediated cytotoxicity. BT-20 and Caco-2 cells were treated with either hemin (10 μM) or FeSO₄ (Fe) (10 μM) for 1 h before H₂O₂ cytotoxicity assay (0.5 mM H₂O₂ for 4 h). For induction of cytoprotection, the cells were pretreated with hemin (10 μM) or FeSO₄ (10 μM) for 1 h, 24 h prior to hemin exposure, as well as with exogenous spleen apoferritin (2 mg/ml) for 24 h before addition of hemin + H₂O₂. Results represent mean percentage of cytotoxicity of 5 experiments (in duplicates). Bars, SE.
Ferritin mRNA Analysis. As shown in Fig. 5, Northern blot analysis of H-ferritin mRNA harvested from BT-20 cells after 1-h treatment with hemin (10 μM) or FeSO₄ (10 μM) followed by 3 h incubation with media alone reveals an ~7-fold increase in H-ferritin mRNA level after exposure to hemin and an ~3-fold increase after exposure to FeSO₄. Incubation of Caco-2 cells with hemin (10 μM) or FeSO₄ (10 μM) results in an ~7- and 5-fold enhancement in H-ferritin mRNA level. In contrast, exposure of BT-20 or Caco-2 cells to hemin or FeSO₄ does not affect L-ferritin mRNA level.

Effect of Hemin or FeSO₄ on Bleomycin-induced Cytotoxicity. To assess the relevance of cellular iron on oxidant-dependent chemotherapeutic efficacy, we examined the effect of pretreating BT-20 cells with hemin, FeSO₄, or horse spleen apoferritin on cytotoxicity induced by bleomycin, an agent known to damage DNA through iron-driven oxygen radicals. In studies shown in Fig. 6, cell viability was assessed with a MTT dye assay after a 4-day incubation with bleomycin. Augmented bleomycin cytotoxicity (loss of viability) was noted in cells treated with hemin added for 1 h before bleomycin exposure (Fig. 6, Column 2) (P < 0.01). In contrast, pretreatment with either hemin or FeSO₄ added for 1 h (24 h prior to bleomycin exposure), as well as exogenous apoferritin added for 24 h, induces resistance to bleomycin as shown by improved viability of the pretreated breast cancer cells (hemin and apoferritin, P < 0.05; FeSO₄, P < 0.01).

Concordantly, as shown in Fig. 7, a 1-h pretreatment of BT-20 cells with 10 μM FeSO₄ followed by 24-h incubation of the cells with media alone decreases bleomycin-mediated DNA breakage. That is, bleomycin causes DNA single-strand breaks, noted by loss of double-stranded material (Fig. 7, Column 2); such loss is inhibited by inorganic iron added 24 h prior to the addition of bleomycin (Fig. 7, Column 3).

DISCUSSION

The present studies provide two potential insights concerning tumor sensitivity to oxidant-mediated damage, namely: that endogenous ferritin content may inversely correlate with tumor cell susceptibility to oxidant stresses; and that exposure of tumor cells to heme or inorganic iron dichotomously alters oxidant sensitivity (acute exposure enhancing, but chronic exposure suppressing, damage). The latter, in turn, seems to reflect induction of ferritin synthesis. Using endothelial cells as targets, we demonstrated previously the mechanism of heme-driven, ferritin-mediated cytoprotection (10). Thus, free heme (if not scavenged by hemopexin) rapidly incorporates into hydrophobic cellular domains and renders cells hypersusceptible to subsequent oxid-
erythroleukemia cells have been shown to manifest increased levels of synthesis at the translational level has been demonstrated (49), and role for heme is also possible. In fact, direct control by heme of ferritin was demonstrated that it might protect rapidly proliferating cells from toxicity of free in vivo, the administration of a single dose of iron to rats results in a slight increase in L-ferritin mRNA isolated from rat liver; this peak occurred within 2-4 h (43). We find it surprising, therefore, that in both tumor cell lines used in the present studies H-ferritin mRNA significantly increased after 6 h incubation with hemin or FeSO₄ (Fig. 5). Since H-ferritin binds iron rapidly, induction of ferritin synthesis by exposure of both cell types to either iron scavenger, ferritin, contribute to the resistance of various tumors to oxidant-producing immune effector cells or to chemotherapeutic agents that require iron for their cytotoxic effect. It may not be fortuitous that Caco-2, a cell line derived from colon adenocarcinoma, is particularly enriched in cellular ferritin (and concomitantly resistant to oxidant damage), since colon neoplasms are likely to encounter heme from mucosal hemorrhage and from ingested heme proteins. On the other hand, a briefer exposure to heme-derived iron may conversely increase tumor cell susceptibility to oxidant-mediated lysis. This suggests that release of "free" iron from ferritin by appropriate reducing agents (53–56) might be beneficial, especially if it is immediately followed by exposure to oxidant-mediated chemotherapeutic agents.

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