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Anthracyclines Induce Accumulation of Iron in Ferritin in Myocardial and Neoplastic Cells: Inhibition of the Ferritin Iron Mobilization Pathway

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ABSTRACT

Anthracyclines are potent antitumor agents that cause cardiotoxicity at high cumulative doses. Because anthracycline cardiotoxicity is attributed to their ability to avidly bind iron (Fe), we examined the effect of anthracyclines on intracellular Fe trafficking in neoplastic cells and differentiated cardiomyocytes. In both cell types, incubation with doxorubicin (DOX) resulted in a significant ($p < 0.004$) accumulation of Fe in the storage protein, ferritin. Pulse-chase experiments using control cells demonstrated that within 6 h, the majority of ^{59}Fe donated from transferrin was incorporated into ferritin. Over longer incubation periods up to 18 to 24 h, ^{59}Fe was subsequently mobilized from ferritin into other compartments in control cells. However, anthracyclines inhibited ferritin-^{59}Fe redistribution during the 18- to 24-h period, resulting in a significant ($p < 0.0003$) 3- to 5-fold accumulation of ferritin-^{59}Fe compared with control cells. The increase in ferritin-^{59}Fe after a 24-h incubation with DOX could not be correlated with increased ferritin expression, suggesting that ^{59}Fe accumulation occurred in pre-existing ferritin. In addition to DOX, other redox-cycling agents (i.e., menadione and paraquat) also increased ferritin-^{59}Fe levels. Moreover, the intracellular superoxide scavenger, Mn(III) tetrakis(4-benzoic acid)-porphyrin complex, partially prevented the ability of DOX and menadione at inducing this effect. Hence, superoxide generation by these compounds could play a role in causing ferritin-^{59}Fe accumulation. This study is the first to demonstrate the effect of anthracyclines at inhibiting Fe mobilization from ferritin, resulting in marked Fe accumulation within the molecule. This response may have consequences in terms of the cytotoxic effects of anthracyclines.

Anthracyclines are potent antineoplastic agents used extensively in the treatment of a range of cancers (Gianni and Myers, 1992; Gerwirtz, 1999). However, their efficacy is severely hindered by the development of cardiotoxicity at high cumulative doses, resulting in heart failure (Gianni and Myers, 1992; Gerwirtz, 1999). The antineoplastic effects of anthracyclines have been well characterized, yet little is understood regarding the mechanisms responsible for their cardiotoxicity (Gianni and Myers, 1992; Gerwirtz, 1999). To enhance cancer management using anthracyclines, it is important to investigate the mechanisms of both cardiotoxicity and antitumor activity. This knowledge is required for the development of treatment regimens that will enhance the antineoplastic effects and limit the cardiotoxicity of these agents.

There is good evidence that the cardiotoxicity of anthracyclines is caused, at least in part, by their avid interaction with iron (Fe). In fact, it is well known that anthracyclines strongly bind Fe, forming metal ion complexes (Gianni and Myers, 1992). Furthermore, Fe loading has been shown to potentiate the cardiotoxicity of the anthracycline doxorubicin (DOX) (Hershko et al., 1993; Link et al., 1996). It is also of interest that the only clinical intervention for anthracycline-mediated cardiotoxicity is the Fe chelator ICRF-187 (also known as dexrazoxane; Gerwirtz, 1999). In addition, another Fe chelator, desferrioxamine (DFO), which is used clinically for the treatment of Fe overload disease, can reduce the cardiotoxic effects of DOX (Hershko et al., 1993; Link et al., 1996). Collectively, these studies convincingly suggest a role for Fe in anthracycline-mediated cardiotoxicity.

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ABBREVIATIONS: DOX, doxorubicin; DFO, desferrioxamine; Tf, transferrin; TfR1, transferrin receptor 1; IRP, iron-regulatory protein; BSO, buthionine sulfoximine; FAC, ferric ammonium citrate; NAC, *N*-acetylcysteine; SOD, superoxide dismutase; MnTBAP, Mn(III) tetrakis (4-benzoic acid) porphyrin complex; DAU, daunorubicin; EPI, epirubicin; ICRF-187, dexrazoxane [(+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane]; PCIH, 2-pyridylcarboxaldehyde isonicotinoyl hydrazone; 311, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone; PAGE, polyacrylamide gel electrophoresis; GSH, glutathione; ferritin-H, ferritin heavy chain; ferritin-L; ferritin light chain.

Cells obtain Fe via the binding of transferrin (Tf) to the transferrin receptor 1 (TfR1; Richardson and Ponka, 1997). The Tf-TfR1 complex is then internalized into cells by receptor-mediated endocytosis. Acidification of the endosome results in Fe release from Tf, which is then transported across the membrane by Nramp2 (also known as divalent metal ion transporter DMT1; Gunshin et al., 1997). Once Fe enters the cytosol, it becomes incorporated into a poorly characterized compartment known as the intracellular Fe pool. This pool has been suggested to contain low molecular mass Fe complexes or it could be composed of high molecular mass chaperone molecules that bind Fe (Richardson and Ponka, 1997). From this pool, Fe can be incorporated into cytochromes and [Fe-S] proteins, etc., or can be stored in ferritin (Richardson and Ponka, 1997). However, the precise pathways involved in the uptake and release of Fe from proteins such as ferritin remain unknown.

Ferritin consists of two types of subunits, heavy (H) and light (L) chains. Twenty-four subunits are organized into a symmetrical structure (with 4-, 3-, and 2-fold axes), generating a cavity within the protein for Fe storage. At the 3-fold axes of the protein structure, there are channels traversing the protein shell; these are believed to be the main entry routes for Fe(II) and its oxidation to Fe(III) (Harrison and Arosio, 1996). Although the Fe deposition pathway that leads to the incorporation of the Fe core within ferritin has been investigated, there is little information regarding the process of Fe release from ferritin under physiological conditions.

Despite this property of anthracyclines to readily bind Fe, few studies have examined the effect of these drugs on cellular Fe metabolism. The most comprehensive experiments to date have examined the effects of anthracyclines on the iron regulatory proteins (IRPs) (Minotti et al., 1998; Kotamraju et al., 2002; Kwok and Richardson, 2002). The IRPs are involved in post-translational regulation of various molecules involved in Fe metabolism, including ferritin and the TfR1 (Hentze and Kühn, 1996; Richardson and Ponka, 1997). Several studies in vitro have also assessed the ability of anthracyclines to mobilize Fe from purified ferritin (Demant, 1984; Thomas and Aust, 1986; Gianni and Myers, 1992), Tf (Demant and Norskov-Lauritsen, 1986), and microsomal membranes (Minotti, 1990; Gianni and Myers, 1992). However, it is difficult to determine whether the Fe release observed from isolated and purified ferritin is physiologically relevant.

In the present study, we investigated the effect of anthracyclines on intracellular distribution and trafficking of Fe in neoplastic cells and differentiated cardiomyocytes. These cell types were compared to understand the role of Fe in anthracycline-mediated cardiotoxicity and antitumor activity. We demonstrated in control cells that once Fe is internalized, it is mainly incorporated into ferritin, followed by redistribution to other compartments. Significantly, we showed for the first time that anthracyclines result in marked accumulation of ferritin-Fe because of inhibition of Fe mobilization from the protein. The ability of anthracyclines to inhibit cellular Fe redistribution from ferritin may affect the cytotoxicity of these antitumor agents.

Materials and Methods

Cell Treatments and Reagents. Buthionine sulfoximine (BSO), catalase, ebselen, ferric ammonium citrate (FAC), horse spleen ferritin, menadione, *N*-acetylcysteine (NAC), paraquat, and superoxide dismutase (SOD) were obtained from Sigma Chemical Co. (St. Louis, MO). Cisplatin was from Pharmacia and Upjohn (Sydney, Australia). The Mn(III) tetrakis (4-benzoic acid) porphyrin complex (MnTBAP) was obtained from ICN Biomedicals (Aurora, OH). DOX, daunorubicin (DAU), and epirubicin (EPI) were obtained from Pharmacia (Sydney, Australia). DFO was obtained from Novartis Pharmaceutical Co. (Basel, Switzerland). ICRF-187 was purchased from Chiron B.V. (Paasheuvelweg, Amsterdam, The Netherlands). The Fe chelators 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH) and 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (also known as 311), were synthesized by standard techniques (Becker and Richardson, 1999). A polyclonal anti-ferritin antibody was obtained from Roche Diagnostics (Indianapolis, IN). All other chemicals were of analytical reagent quality.

Cell Culture. The human SK-Mel-28 melanoma, SK-N-MC neuroepithelioma, and MCF-7 breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were grown in Eagle's modified minimum essential medium (Invitrogen, Mount Waverley, Australia) containing 10% fetal calf serum (Invitrogen), 1% nonessential amino acids (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 100 U/ml penicillin (Invitrogen), and 0.28 μg/ml Fungizone (Squibb Pharmaceuticals, Montréal, ON, Canada). Cells were grown in an incubator (Thermo Forma, Marietta, OH) at 37°C in a humidified atmosphere of 5% CO_2/95% air and subcultured as described previously (Richardson and Baker, 1992). Cellular growth and viability were assessed by phase contrast microscopy and Trypan blue staining.

Primary cultures of beating neonatal myocardial cells were isolated from 2- to 3-day old rats using well established methods (Link et al., 1985; Hershko et al., 1993; Link et al., 1996; Terman and Brunk, 1998). Briefly, ventricles were minced and incubated in the presence of 0.05% collagenase type II (Worthington, Lakewood, NJ) at 37°C. The cell suspension was centrifuged in a Percoll gradient (1.05 g/ml; Amersham Biosciences AB, Uppsala, Sweden) to purify cardiomyocytes from other cell types, including fibroblasts and red blood cells (Terman and Brunk, 1998). Cells were plated on collagen-coated plates and cultured at 37°C in an atmosphere of 8% O_2/5% CO_2 (Terman and Brunk, 1998). Experiments were performed on day 4 of culture. Purity of cardiomyocyte cultures was confirmed by immunofluorescent staining of the cells using an α-actinin antibody (Sigma; Goncharova et al., 1992). These cells were used because they demonstrate many of the functional characteristics of the intact heart, including contractility, rhythmicity, and automaticity (Link et al., 1985). Furthermore, the effects of anthracyclines, Fe-loading, and Fe chelators on these cells have been well characterized, and this model has been shown to closely mimic the in vivo situation (Link et al., 1985; Hershko et al., 1993; Link et al., 1996).

Preparation of ^{59}Fe-Transferrin. Human apotransferrin (Sigma) and rat apotransferrin (kindly provided by Professor E. H. Morgan, Department of Physiology, University of Western Australia, Perth, Australia) were labeled with ^{59}Fe (PerkinElmer Life Sciences, Boston, MA) to produce $^{59}Fe_2$-transferrin (^{59}Fe-Tf) as described previously (Richardson and Baker, 1992). Unbound ^{59}Fe was removed by exhaustive vacuum dialysis against 0.15 M NaCl buffered to pH 7.4 using 1.4% $NaHCO_3$. Fully saturated diferric Tf was used in all experiments. Human ^{59}Fe-Tf and rat ^{59}Fe-Tf were used to label human neoplastic cells and rat cardiomyocytes with ^{59}Fe, respectively.

Effect of Anthracyclines on ^{59}Fe Efflux from Prelabeled Cells. Iron efflux experiments examining the ability of various agents to mobilize ^{59}Fe from cells were performed using established techniques (Richardson and Milnes, 1997). Briefly, cells were prelabeled with ^{59}Fe-Tf ([Tf] = 0.75 μM; [Fe] = 1.5 μM) for 3 to 18 h at 37°C. This medium was aspirated and the cell monolayer washed six times with ice-cold Hanks' balanced salt solution (Invitrogen). The cells were then reincubated for 3 to 24 h at 37°C with minimum essential medium in the presence or absence of the agents to be

tested. After this incubation, the overlying media containing released ^{59}Fe were collected in γ-counting tubes. The cells were removed from the Petri dishes and placed in a separate set of tubes. Radioactivity was measured in both the cell pellet and supernatant using the γ-scintillation counter (1282 Compugamma; Amersham Biosciences).

Effect of Anthracyclines on ^{59}Fe Uptake from Transferrin by Cells. The ability of various agents to affect cellular ^{59}Fe uptake from ^{59}Fe-Tf was performed using standard procedures (Richardson and Milnes, 1997). Briefly, cells were incubated with ^{59}Fe-Tf (0.75 μM; i.e., [Tf] = 0.75 μM; [Fe] = 1.5 μM) together with the agents of interest for 3 to 24 h at 37°C. This medium was then aspirated and the cell monolayer washed six times with ice-cold Hanks' balanced salt solution. Cells were then harvested on ice using a plastic spatula and placed in γ-counting tubes. As a measure of cellular density, protein concentrations were assessed using the Bio-Rad protein reagent (Bio-Rad, Hercules, CA). The data are expressed as counts per minute of ^{59}Fe/mg of protein. Separate experiments demonstrated that cell number was directly proportional to protein concentration.

Determination of Intracellular Iron Distribution using Native-PAGE-^{59}Fe-Autoradiography. Native-PAGE-^{59}Fe-autoradiography was performed using established techniques (Richardson et al., 1996; Richardson and Milnes, 1997). Briefly, cells were labeled with ^{59}Fe-Tf (0.75 μM) in the presence or absence of anthracyclines and/or other agents and then lysed using 30 μl of ice-cold 1.5% Triton X-100 containing 2 mM phenylmethylsulfonyl fluoride (Sigma), followed by one freeze-thaw cycle. Samples were then vortexed and centrifuged at 14,000 rpm for 45 min at 4°C to separate the stromal-mitochondrial membrane fraction from the cytosol. The protein concentration of the cytosol was determined using the Bio-Rad protein assay. Radioactivity was assessed using the γ-scintillation counter described above.

Samples were loaded onto a 5% native PAGE gel at equal protein concentration (100 μg/lane). Experiments loading equal radioactive counts per sample gave results similar to those obtained using equal protein loading. Electrophoresis was performed at 15 mA per gel for 2 to 3 h at 4°C. Gels were subsequently dried, and autoradiography was performed. Bands on X-ray film were quantified by scanning densitometry using a Laser Densitometer and analyzed by Kodak Biomax I Software (Eastman Kodak, Rochester, NY).

Immunoprecipitation of ^{59}Fe-Ferritin. To measure the amount of ^{59}Fe in ferritin, immunoprecipitation was performed using an anti-human ferritin antibody (Roche Diagnostics) via established procedures (Baker et al., 1985). Briefly, cells were incubated with ^{59}Fe-Tf (0.75 μM) for 18 h at 37°C in the presence or absence of anthracyclines (5 μM). Cell lysates were prepared as described for native-PAGE-^{59}Fe autoradiography. Samples were incubated with or without the anti-human ferritin antibody (Roche Diagnostics) for 24 h at 37°C, using dilutions from 1:10 to 1:50. Samples were then transferred to 4°C for 24 h to allow precipitation of the antibody-antigen complexes and centrifuged at 14,000 rpm for 1 h at 4°C. The supernatant was removed and the pellet washed twice with ice-cold phosphate-buffered saline. The radioactivity in the pellet was measured using the γ-scintillation counter described above.

ATP Assay. Cellular ATP levels were assessed using the Sigma diagnostic kit as per the manufacturer's instructions. In these experiments, three 75-cm^2 flasks of cells were treated with the agents of interest for 18 h at 37°C. Cells were then resuspended in 0.6 ml of phosphate-buffered saline and lysed in an equal volume of 12% trichloroacetic acid. The decrease in the absorbance of NADH at 340 nm was used as a measure of ATP in the sample.

Western Blot Analysis. Western blot analysis was performed essentially as described previously (Kwok and Richardson, 2002). Briefly, cells were lysed using 1.5% Triton X-100 containing complete protease inhibitor (Roche Diagnostics, Mannheim, Germany) and centrifuged at 14,000 rpm for 45 min at 4°C. Protein concentrations of cytoplasmic extracts were then determined using the Bio-Rad protein assay kit (Bio-Rad). Samples (100 μg) containing 20% β-mercaptoethanol were loaded onto a SDS-polyacrylamide gel consisting of 4% stacking and 15% resolving gels. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ) overnight at 4°C.

Membranes were subsequently soaked in methanol, followed by blocking with 3% skim milk and 2% bovine serum albumin in Tris-buffered saline for 2 h at room temperature. After blocking, membranes were probed for 3 h at room temperature using rabbit anti-human ferritin antibody (1:30 dilution; ICN Biomedicals, Aurora, OH) or mouse anti-human β-actin antibody (clone AC; 1:5000 dilution; Sigma). Membranes were then washed four times with Tris-buffered saline containing 0.1% Tween-20 (Sigma). After washing, anti-rabbit antibody (1:5,000 dilution) or anti-mouse antibody (1:10,000 dilution) conjugated with horseradish peroxidase was incubated with the membranes for 1 h at room temperature. Membranes were washed and then developed using the ECL+ Western blot detection reagent (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and exposed to X-ray film. All densitometric data were normalized to β-actin.

Glutathione Assay. Intracellular glutathione (GSH) levels were measured using 5′,5′-dithiobis-(2-nitrobenzoic acid) (Sigma) as described previously (Sedlack and Lindsay, 1968). After incubation of cells for 6 to 24 h at 37°C with or without anthracyclines, cellular GSH levels were assessed. Cells were lysed and equal volumes of sample and 5% metaphosphoric acid (Merck, Darnstadt, Germany) were mixed to precipitate cellular protein before addition of 200 μM 5′,5′-dithiobis-(2-nitrobenzoic acid) and incubation at 37°C for 1 to 2 h. Absorbance readings were performed at 412 nm against a GSH standard curve.

Statistical Analysis. Experimental data were compared using Student's t test. Results were considered statistically significant when $p < 0.05$.

Results

Anthracyclines Induce Ferritin-^{59}Fe Accumulation in Normal and Neoplastic Cells during ^{59}Fe Uptake from ^{59}Fe-Transferrin. Considering the high affinity of anthracyclines for Fe (Gianni and Myers, 1992; Gerwirtz, 1999), it was important to understand their effects on intracellular Fe distribution. This was particularly relevant for understanding the mechanisms involved in Fe-mediated cardiotoxicity and antitumor activity of anthracyclines. To examine this, we used the native-PAGE-^{59}Fe-autoradiography technique that has been used to assess the intracellular distribution of Fe and the identity of intermediates involved in Fe uptake by cells (Richardson and Milnes, 1997; Watts and Richardson, 2002). In the current study, we have concentrated our efforts on examining the effects of anthracyclines on the distribution of ^{59}Fe in cytosolic fraction that constituted the greatest proportion of ^{59}Fe in the cell. Indeed, the amount of ^{59}Fe present within the stromal-mitochondrial membrane fraction represented ~20 to 30% of the total cellular ^{59}Fe.

Initially, our studies compared the human SK-Mel-28 melanoma cells and primary cultures of rat cardiomyocytes, because their Fe metabolism has been well characterized (Link et al., 1985; Richardson and Baker, 1992; Hershko et al., 1993; Kwok and Richardson, 2002). Cells were incubated for 3 to 24 h at 37°C with ^{59}Fe-Tf in the presence or absence of the anthracyclines, and native-PAGE-^{59}Fe-autoradiography was performed. The majority of cellular ^{59}Fe could be detected in three bands: an upper band, the ferritin band, and a diffuse lower band (Fig. 1A). The identity of the ferritin band was confirmed by supershift experiments using an anti-

ferritin antibody (Fig. 1A, lanes 1 and 2) and the fact that it comigrated with purified horse spleen ferritin, as described previously (Watts and Richardson, 2002). The more diffuse band below ferritin comigrated with low molecular mass Fe complexes (e.g., ^{59}Fe-citrate) and could be removed using the Fe chelator DFO as shown previously (Richardson et al., 1996; Richardson and Milnes, 1997). This diffuse band is thought to correspond to ^{59}Fe bound from the lysate by weak low molecular mass chelators in the gel running buffer (e.g., Tris) (Richardson et al., 1996; Richardson and Milnes, 1997). In the present investigation, the band above ferritin and the low molecular mass band will not be considered in detail because they remain undefined and their relevance remains uncertain.

Incorporation of ^{59}Fe into the ferritin of control cells was obvious after a 3-h labeling period with ^{59}Fe-Tf and, after 6 h, had increased by a further 49% (Fig. 1A, compare lanes 3 and 5). However, by 18 and 24 h, ferritin-^{59}Fe incorporation in control cells had decreased to levels slightly less than that observed after a 3-h labeling period (Fig. 1, A, compare lane 3 with lanes 7 and 9, and B). This suggested that in control cells, ^{59}Fe was initially taken up into ferritin and subsequently mobilized from the protein.

In contrast to control cells, incubation with ^{59}Fe-Tf in the presence of 20 μM DOX resulted in a marked time-dependent accumulation of ferritin-^{59}Fe (Fig. 1, A and B). After 3- and 6-h labeling periods with ^{59}Fe-Tf, ^{59}Fe incorporation into ferritin was slightly higher in DOX-treated cells compared with control cells (Fig. 1A, compare lanes 3 and 4 and lanes 5 and 6). However, with longer labeling periods in the presence of DOX, ^{59}Fe incorporation into ferritin continued to increase (Fig. 1, A, lanes 8 and 10, and B). By 24 h, ferritin-^{59}Fe incorporation was 3-fold higher ($p < 0.004$) in DOX-treated cells compared with the relevant control (Fig. 1, A, compare lanes 9 and 10, and B). To confirm the results observed using native PAGE, immunoprecipitation studies were performed with an anti-ferritin antibody. This technique also demonstrated significantly ($p < 0.001$) higher levels of ferritin-^{59}Fe in DOX-treated cells compared with control cells incubated with medium alone (data not shown).

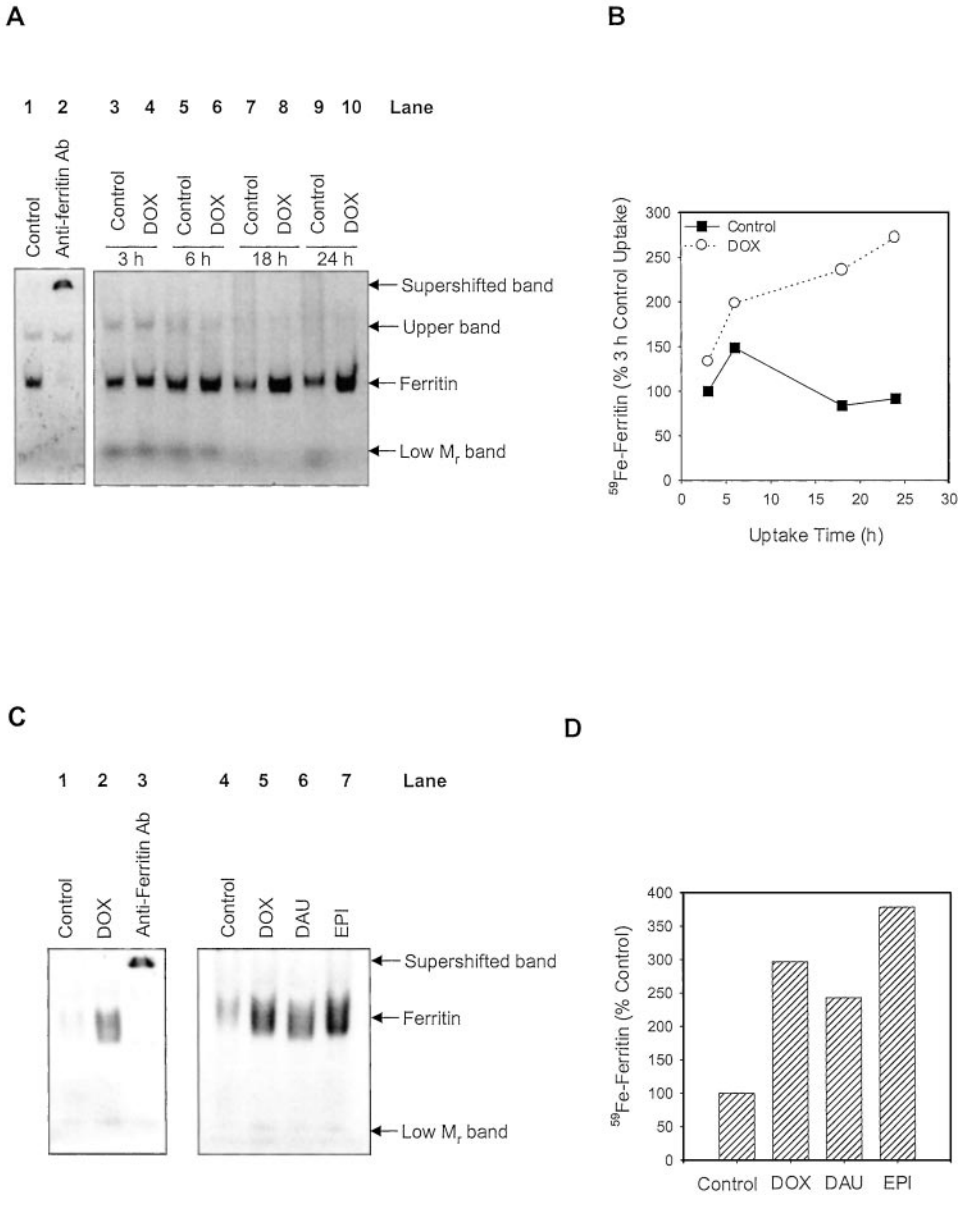


Fig. 1. Anthracyclines result in ferritin-^{59}Fe accumulation during ^{59}Fe uptake from ^{59}Fe- transferrin (^{59}Fe-Tf). A, SK-Mel-28 melanoma cells were incubated with ^{59}Fe-Tf (0.75 μM) for 3 to 24 h at 37°C in the presence or absence of 20 μM DOX. The ^{59}Fe-PAGE autoradiography was then performed (see *Materials and Methods* for details). Supershift experiments were performed by incubating samples with an anti-ferritin antibody for 1 h at 25°C before ^{59}Fe-PAGE autoradiography. The results are a typical experiment from three performed. B, densitometric analysis of the autoradiograph shown in A. C, cardiomyocyte cultures were incubated with ^{59}Fe-Tf (0.75 μM) in the presence or absence of DOX (5 μM), DAU (5 μM), or EPI (5 μM) for 18 h at 37°C and ^{59}Fe-PAGE autoradiography was performed. Supershift experiments were performed as in A. Results shown are a typical experiment from three performed. D, densitometric analysis of autoradiograph shown in C.

These data demonstrated that incubation of cells with DOX resulted in marked accumulation of ^{59}Fe in ferritin.

To determine whether the effect of DOX shown in Fig. 1A was observed only in neoplastic cells, similar experiments were performed using primary cultures of cardiomyocytes (Fig. 1C). Again, DOX (5 μM) resulted in a marked accumulation of ferritin-^{59}Fe compared with control cells (Fig. 1C, compare lanes 4 and 5). Other structurally related anthracyclines, including DAU (5 μM) and EPI (5 μM), had a similar effect on ferritin-^{59}Fe accumulation, suggesting that this was a general anthracycline effect (Fig. 1, C, compare lane 4 with lanes 5–7, and D). Interestingly, the ^{59}Fe-ferritin band observed from cardiomyocytes was not a single well-defined band as seen in melanoma cells (Fig. 1A) or other normal and neoplastic cells (Richardson et al., 1996; Richardson and Milnes, 1997); rather, it seemed to be composed of two overlapping bands (Fig. 1C). Supershift experiments using an anti-ferritin antibody showed that this broad band was immunologically identical to ferritin (Fig. 1C, lanes 1 to 3). This broad ferritin band could be consistent with multiple ferritin species. Previous studies have documented a glycosylated ferritin from heart tissue that is smaller than cellular ferritin and is also cross-reactive with serum ferritin (Campbell et al., 1993). Additionally, cleavage of ferritin within siderosomes has been documented, giving rise to different mobilities of the molecule on native PAGE (Andrews et al., 1987).

Anthracyclines Inhibit ^{59}Fe Mobilization from Ferritin after Prelabeling with ^{59}Fe-Tf. Chase experiments were designed to examine the effect of DOX on cellular ^{59}Fe distribution after the initial labeling of SK-Mel-28 melanoma cells with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C (Fig. 2). After ^{59}Fe labeling, the cells were then washed and reincubated for 0 to 24 h at 37°C in the presence or absence of DOX (20 μM). After 3- and 6-h reincubations of ^{59}Fe-prelabeled cells in control media, marked 3- and 4-fold increases in ferritin-^{59}Fe levels were observed, respectively, compared with the 0 h control (Fig. 2, A, compare lane 1 with lanes 2 and 4, and B). However, with longer periods of reincubation in control media, ferritin-^{59}Fe levels decreased and by 18 to 24 h reached levels comparable with the 0 h time point (Fig. 2, A, compare lane 1 with lanes 6 and 8, and B). In addition, after an 18- or 24-h reincubation in media alone, ^{59}Fe was evident in a range of very diffuse bands above ferritin, suggesting redistribution of ferritin-^{59}Fe to other cellular compartments in control cells (Fig. 2A, lanes 6 and 8).

Incorporation of ^{59}Fe into ferritin after 3- and 6-h reincubations was slightly higher in DOX-treated cells compared with control cells (Fig. 2, A, compare lanes 2 and 4 with lanes 3 and 5, and B). However, in contrast to control cells, ^{59}Fe-ferritin incorporation continued to increase in DOX-treated cells; after an 18- or 24-h reincubation, ferritin-^{59}Fe levels reached 6 to 7 times ($p < 0.0003$) that observed at the 0-h time point (Fig. 2, A, compare lane 1 with lanes 7 and 9, and B). In DOX-treated samples, there was less redistribution of ^{59}Fe from ferritin into other diffuse bands above ferritin compared with control cells (Fig. 2A, compare lanes 6 and 8 with lanes 7 and 9).

The DOX-mediated induction of ferritin-^{59}Fe accumulation was very efficient; 1 μM DOX resulted in a 3-fold accumulation of ferritin-^{59}Fe compared with control cells (Fig. 2, C, compare lanes 1 and 2, and D). The effect of DOX reached a plateau at concentrations between 5 and 20 μM, with ferritin-^{59}Fe accumulation ranging between 540 and 580% of the control value (Fig. 2, C and D). The concentration of DOX in human serum can reach 5 μM (Gerwirtz, 1999). Hence, the experiment in Fig. 2, C and D, demonstrated that the ability of DOX to induce ferritin-^{59}Fe accumulation occurred at clinically relevant concentrations.

Considering these results, it seemed that during cellular ^{59}Fe uptake from ^{59}Fe-Tf and during reincubation after ^{59}Fe uptake, the normal course of cellular Fe distribution involved short-term (3–6 h) incorporation and storage into ferritin (Figs. 1A and 2A). This was followed by a period of ^{59}Fe redistribution from ferritin to other cellular compartments (Figs. 1A and 2A). However, it is clear that when cells were exposed to anthracyclines, this normal redistribution of Fe from ferritin into other compartments was inhibited, resulting in a pronounced accumulation of ferritin-^{59}Fe (Figs. 1 and 2). Hence, the ^{59}Fe that accumulates in ferritin is redistributed from other compartments. It is notable that we are assessing cytosolic ^{59}Fe levels in Fig. 2A. Therefore, it is possible that the ^{59}Fe accumulating in ferritin was coming from the ^{59}Fe-containing stromal-mitochondrial membrane fraction (data not shown).

Further experiments examined the effect of DOX on intracellular Fe trafficking in a number of cell types (Fig. 2, E and F). Cells were labeled for 3 h at 37°C with ^{59}Fe-Tf (0.75 μM), washed, and reincubated for 18 h at 37°C with control media or media containing DOX (5 or 20 μM). The marked accumulation of ferritin-^{59}Fe caused by DOX was observed not only in SK-Mel-28 melanoma cells, but was also found in human SK-N-MC neuroepithelioma cells, human MCF-7 breast cancer cells, and rat cardiomyocytes (Fig. 2, E and F). These results demonstrated that the effect of DOX on cellular Fe distribution was not cell-type–specific.

Doxorubicin Neither Inhibits ^{59}Fe Uptake from Transferrin nor Mobilizes ^{59}Fe from Cells. It could be suggested that the ferritin-^{59}Fe accumulation observed when cells were coincubated with ^{59}Fe-Tf and DOX (Fig. 1, A-D) was caused by an increase in cellular ^{59}Fe uptake. To examine this possibility, SK-Mel-28 cells were labeled with ^{59}Fe-Tf (0.75 μM) for 3 or 24 h at 37°C in the presence or absence of DOX (5–20 μM) (Fig. 3A). Cells were subsequently washed and lysed, and total cellular ^{59}Fe content was assessed. The Fe chelators DFO (0.5 mM) and 311 (50 μM) were used as positive controls and significantly ($p < 0.0001$) decreased ^{59}Fe uptake into cells after both 3- and 24-h incubations with ^{59}Fe-Tf (Fig. 3A), as documented previously (Richardson and Milnes, 1997; Darnell and Richardson, 1999). However, in these studies, there was no increase in ^{59}Fe uptake from ^{59}Fe-Tf in the presence of DOX (5–20 μM), indicating that the accumulation of ^{59}Fe in ferritin was not caused by enhanced ^{59}Fe uptake (Fig. 3A).

Considering the chase experiments described previously (Fig. 2, A and B), it is also possible that the decrease in ferritin-^{59}Fe in control cells over time was caused by ^{59}Fe release from cells. Conversely, the accumulation of ferritin-^{59}Fe in DOX-treated cells after ^{59}Fe-Tf uptake (Fig. 2, A and B) could have been caused by DOX-induced inhibition of ^{59}Fe mobilization from the cell, resulting in ferritin-^{59}Fe accumulation. To examine whether ^{59}Fe in control cells was being mobilized to other compartments from ferritin and not being released from the cell, cellular ^{59}Fe mobilization in the pres-

ence or absence of DOX was assessed (Fig. 3B). Cells were labeled with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C, washed, and then reincubated for 3 or 24 h at 37°C in control media or media containing 5 to 20 μM DOX. Again, the Fe chelators DFO (0.5 mM) and 311 (50 μM) were used as positive controls and significantly ($p < 0.004$) increased ^{59}Fe mobilization from cells after both 3- and 24-h reincubations (Fig. 3B), as demonstrated previously (Richardson and Milnes, 1997; Darnell and Richardson, 1999). However, all concentrations of DOX had no significant effect on ^{59}Fe mobilization compared with control cells (Fig. 3B). These results demonstrate that control and DOX-treated cells had comparable cellular ^{59}Fe release. Furthermore, the accumulation of ferritin-^{59}Fe observed in DOX-treated cells (Figs. 1A and 2A) was not caused by inhibition of cellular ^{59}Fe efflux compared with control cells (Fig. 3B). Rather, it was attributable to inhibition of intracellular ferritin-^{59}Fe distribution to other compartments (Fig. 3A).

Accumulation of Ferritin-^{59}Fe Is Not Caused by Changes in Cellular Ferritin Protein Levels. To determine whether the increase in ferritin-^{59}Fe levels observed upon incubation with DOX was caused by an increase in total ferritin synthesis in DOX-treated cells, Western analysis was performed (Fig. 3C). Briefly, cells were incubated with control media, DFO (100 μM), FAC (100 μg/ml), or DOX (5–20 μM) for 18 h at 37°C. The Fe chelator DFO depletes cellular Fe levels and results in a decrease in ferritin synthesis (Hentze and Kühn, 1996) and was used as a negative control.

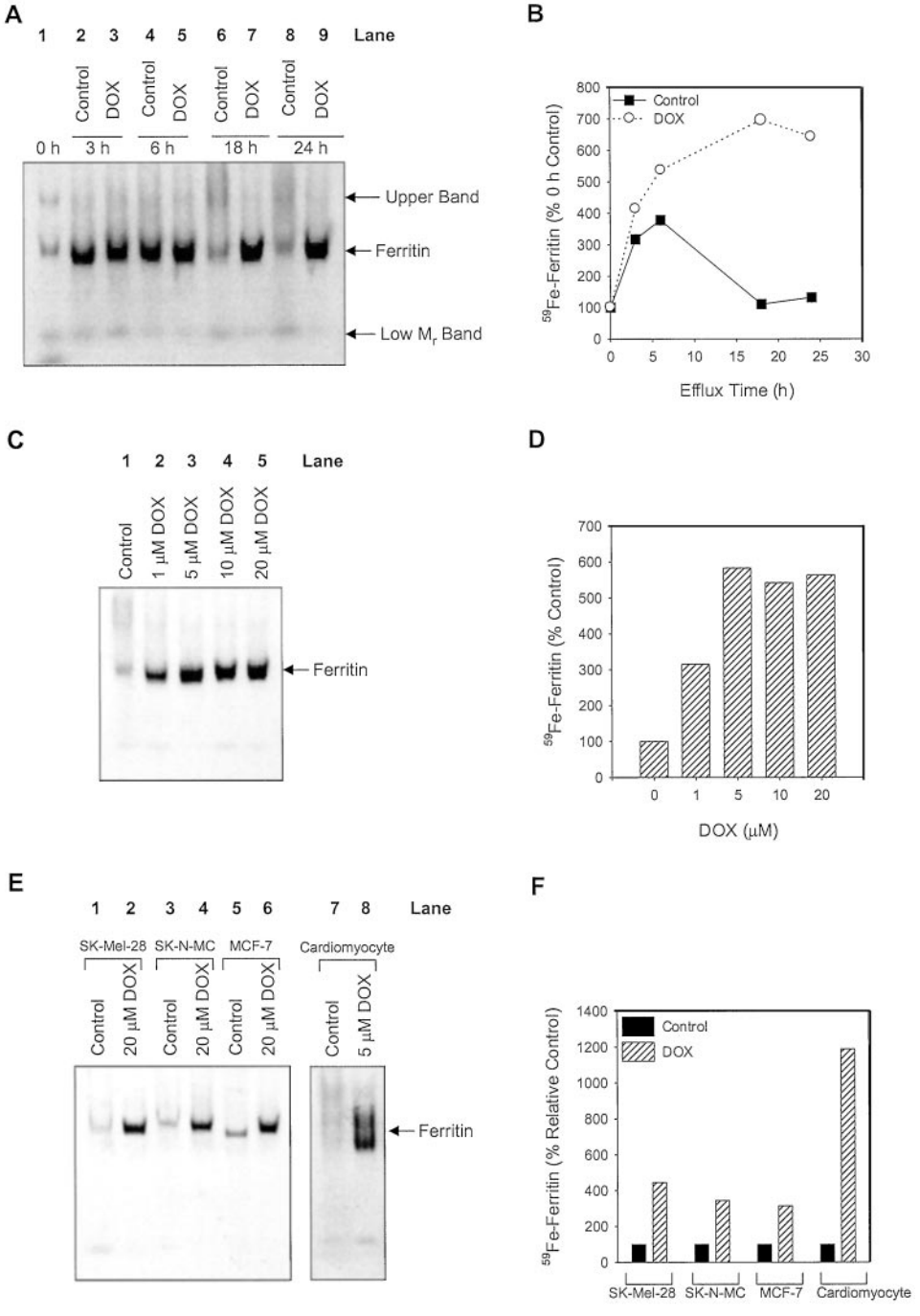


Fig. 2. DOX inhibits ^{59}Fe mobilization from ferritin after prelabeling cells with ^{59}Fe-Tf. A, SK-Mel-28 cells were incubated with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C. Cells were then washed and re-incubated with control media or media containing DOX (20 μM) for 0 to 24 h at 37°C and ^{59}Fe-PAGE autoradiography performed (see *Materials and Methods* for details). The results are a typical experiment from five performed. B, densitometric analysis of autoradiograph shown in A. C, SK-Mel-28 cells were prelabeled for 3 h at 37°C with ^{59}Fe-Tf (0.75 μM). The cells were then washed and reincubated with control media or media containing DOX (1–20 μM) for 18 h at 37°C and ^{59}Fe-PAGE autoradiography was performed. The results are a typical experiment from three performed. D, densitometric analysis of C. E, a range of neoplastic cell types, including human SK-Mel-28 melanoma cells, human SK-*N*-MC neuroepithelioma cells, human MCF-7 breast cancer cells, and rat cardiomyocytes were prelabeled with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C, followed by an 18-h reincubation at 37°C in control media or DOX (5–20 μM). Results shown are a typical experiment from three performed. F, densitometry of autoradiograph shown in E.

Conversely, FAC donates Fe to cells increasing the synthesis of ferritin (Wang and Pantopoulos, 2002) and was an appropriate positive control.

Western blotting using anti-human ferritin antibody detected both ferritin-H and -L subunits (Wang and Pantopoulos, 2002). As expected, cellular Fe depletion using DFO markedly decreased both ferritin-H and -L protein levels to 20% and 10% of that found for control cells, respectively ($p < 0.007$; Fig. 3, C, compare lanes 1 and 2, and D). In contrast, the Fe donor, FAC, significantly ($p < 0.04$) increased the expression of both ferritin-H and -L subunits to 151 and 176% of the control, respectively (Fig. 3, C, compare lanes 1 and 3, and D). Incubation of cells with DOX (5–20 μM) slightly increased ferritin protein levels compared with control cells; this was more notable for the ferritin-H-chain compared with the -L-chain (Fig. 3, C, compare lane 1 with lanes 4–6, and D). However, this increase in ferritin protein was not statistically significant ($p > 0.20$) over three experiments (Fig. 3, C and D). This result suggested that the observed increase in ferritin-^{59}Fe after incubation with DOX (Figs. 1 and 2) was not caused by any change in cellular ferritin synthesis but rather by an increase in ^{59}Fe accumulation within pre-existing ferritin after DOX treatment. Time course experiments incubating cells from 3 to 24 h with DOX (20 μM) also demonstrated little change in ferritin protein levels compared with cells treated with media alone (data not shown).

The Cytotoxic Agent, Cisplatin, was Far Less Effective than DOX at Increasing Ferritin-^{59}Fe Accumulation. The accumulation of ferritin-^{59}Fe induced in DOX-treated cells could be caused by an indirect effect of the cytotoxicity of this agent. To examine this possibility, the effect of anthracyclines were compared with the cytotoxic agent cisplatin (Fig. 4, A and B). The SK-Mel-28 cells were labeled with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C, washed, and then re-incubated for 18 h with control media, DOX, DAU, and EPI (20 μM) or cisplatin (10 or 20 μM). As expected, all three anthracyclines resulted in marked accumulation of ^{59}Fe-ferritin (Fig. 4, A, compare lane 1 with lanes 2 to 4, and B). In contrast, cisplatin had no effect at 10 μM (Fig. 4, A, compare lanes 1 and 5, and B) and at 20 μM only slightly increased ferritin-^{59}Fe incorporation (Fig. 4, A, compare lanes 1 and 6, and B).

We also considered whether the ability of DOX to inhibit ^{59}Fe mobilization from ferritin was caused by its effects on ATP levels. The SK-Mel-28 cells were incubated with or without DOX (5–20 μM) for 18 h at 37°C, and ATP levels were assessed. In these studies, the metabolic inhibitor so-

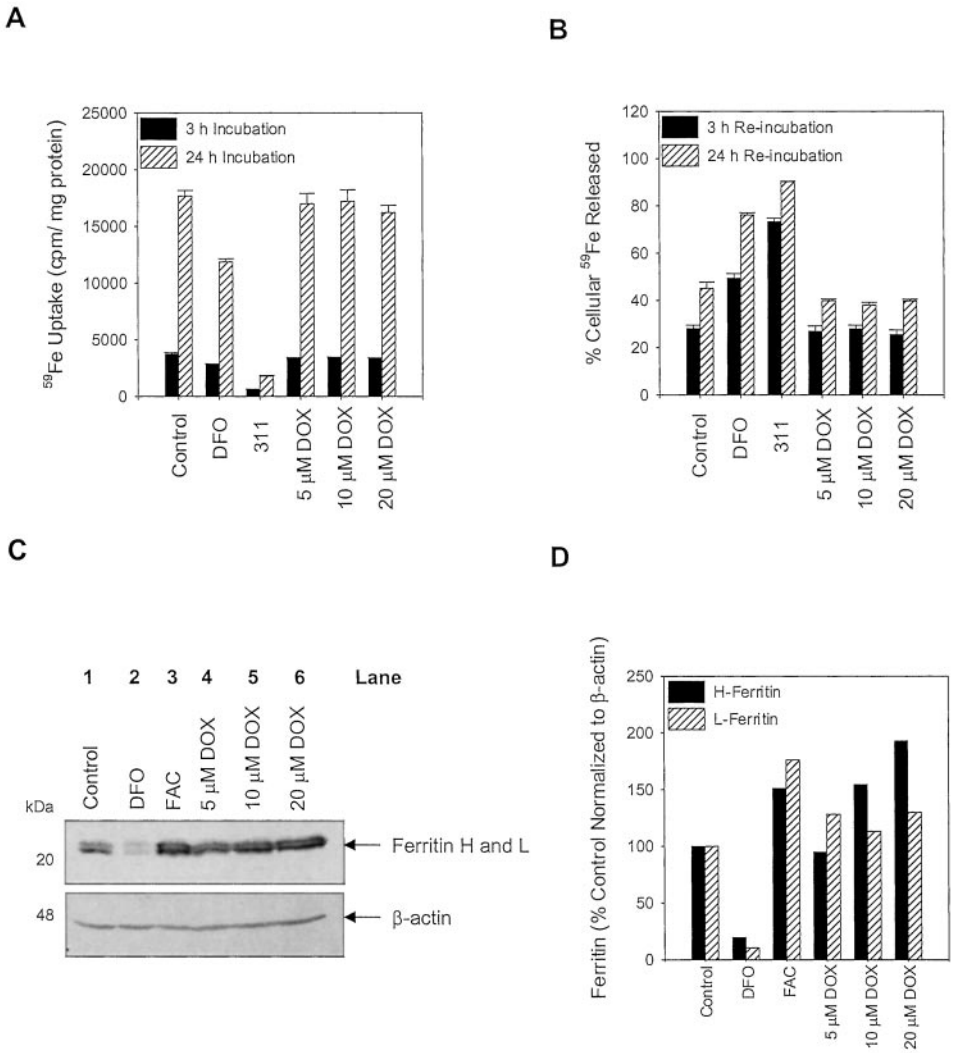


Fig. 3. DOX affects neither total cellular Fe content nor total cellular ferritin protein levels. A, SK-Mel-28 cells were incubated with ^{59}Fe-Tf (0.75 μM) in the presence or absence of DFO (0.5 mM), 311 (50 μM), or DOX (5–20 μM) for 3 h or 24 h at 37°C. Cells were washed and total cellular ^{59}Fe content was assessed (see *Materials and Methods* for details). Results are mean ± S.D. (three determinations) in a typical experiment from three experiments performed. B, SK-Mel-28 cells were incubated with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C. Cells were then washed and re-incubated with control media, DFO (0.5 mM), 311 (50 μM), or DOX (5–20 μM) for 3 or 24 h at 37°C and the release of cellular ^{59}Fe assessed (see *Materials and Methods* for details). Results are mean ± S.D. (three determinations) in a typical experiment from three experiments performed. C, SK-Mel-28 cells were incubated with control media, DFO (100 μM), FAC (100 μg/ml), or DOX (5–20 μM) for 18 h at 37°C. Western blot analyses were performed as described under *Materials and Methods*. Results shown are a typical experiment from three performed. D, densitometry of the results in C, normalized to β-actin.

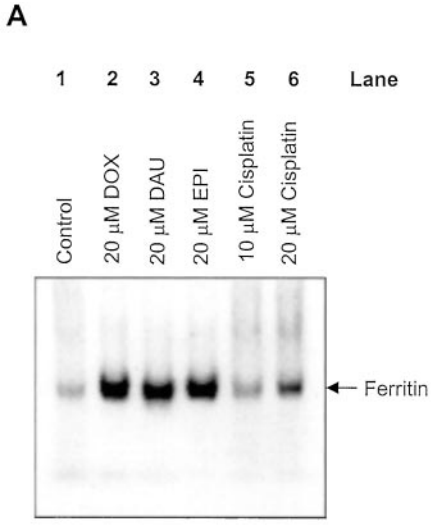
dium azide (5 mM) was used as a positive control and markedly reduced ATP to 8% of control levels. However, DOX, at all concentrations examined, had no effect on ATP levels. This latter result, together with the data using the cytotoxic agent cisplatin, suggested that the lack of ^{59}Fe mobilization from ferritin was not caused simply by the ability of DOX to depress cellular metabolism or deplete ATP.

Other Redox-Cycling Drugs also Caused a Marked Accumulation of ^{59}Fe in Ferritin. A major mechanism of anthracycline toxicity is believed to involve redox cycling and the production of free radicals (Gianni and Myers, 1992). To determine whether other redox cycling agents exerted the same effect as anthracyclines on Fe accumulation in ferritin, experiments were performed using the classic redox cycling agent menadione (Gutteridge and Halliwell, 1989). The SK-Mel-28 cells were incubated with ^{59}Fe-Tf (0.75 μM) for 18 h in the presence or absence of menadione (1 to 10 μM) or DOX (5 μM) and ferritin-^{59}Fe accumulation was assessed (Fig. 5, A and B).

Incubation of cells with menadione resulted in a concentration-dependent increase in ferritin-^{59}Fe accumulation (Fig. 5A, compare lane 1 with lanes 3 to 5). At a menadione concentration of 10 μM, ferritin-^{59}Fe accumulation was 4-fold higher than that observed in control cells and was comparable with the 5-fold increase observed with 5 μM DOX (Fig. 5A, compare lanes 1, 2 and 5; Fig. 5B). Similar results were also observed with another redox-cycling agent, namely paraquat, although much higher concentrations (200 μM) were required to cause an increase in ferritin-^{59}Fe accumulation (data not shown).

Considering the data above showing that menadione and paraquat had a similar effect on ferritin-^{59}Fe accumulation as DOX, it could be suggested that redox cycling may play a role in this process. To determine whether free radicals were involved, experiments were designed to examine the effects of a range of free radical scavengers on ferritin-^{59}Fe accumulation in the presence or absence of DOX or menadione (Fig. 6, A and B). Free radical scavengers examined included the membrane-impermeable agents SOD (1000 U/ml) and catalase (1000 U/ml), the cell-permeable SOD mimetic MnTBAP (200 μM), and the cell-permeable glutathione peroxidase mimetic ebselen (15 μM) (Konorev et al., 1999). The concentrations of free-radical scavengers examined were within the effective range reported in the literature (Minotti et al., 1998; Konorev et al., 1999).

In control cells incubated with free radical scavengers in the absence of redox-cycling agents (Fig. 6A, lanes 1–6), only MnTBAP could slightly reduce ferritin-^{59}Fe accumulation compared with untreated control cells (Fig. 6, A, compare lanes 1 and 5, and B). The other free-radical scavengers had little effect on ferritin-^{59}Fe accumulation compared with untreated cells (Fig. 6A, compare lane 1 with lanes 2–4 and 6). As demonstrated previously, after incubation of cells with DOX (5 μM), accumulation of ^{59}Fe in ferritin was observed compared with the untreated control incubated with medium alone (Fig. 6, A, compare lanes 1 and 7, and B). Again, simultaneous incubation of DOX with MnTBAP or the combination of DOX with ebselen and MnTBAP reduced the accumulation of ferritin-^{59}Fe to 67 and 77%, respectively, compared with cells treated with DOX alone (Fig. 6, A, compare lanes 7 with lanes 11 and 12, and B). However, other scavengers had little effect on ferritin-^{59}Fe accumulation compared with cells treated with DOX alone (Fig. 6, A, compare lane 7 with lanes 8–10, and B). As shown in Fig. 5A, menadione alone increased ferritin-^{59}Fe levels compared with the untreated control (Fig. 6, A, compare lanes 1 and 13, and B) and coincubation in the presence of SOD or catalase had little effect on ^{59}Fe-ferritin accumulation (Fig. 6, A, compare lanes 13 with 14 and 15, and B). Interestingly, ebselen reduced ^{59}Fe-ferritin accumulation to 66% of that observed for cells treated with menadione alone (Fig. 6, A, compare lanes 13 and 16, and B). Incubation of cells with menadione in the presence of MnTBAP or the combination of menadione, MnTBAP, and ebselen decreased ^{59}Fe-ferritin levels to 27 and 30%, respectively, compared with cells incu-



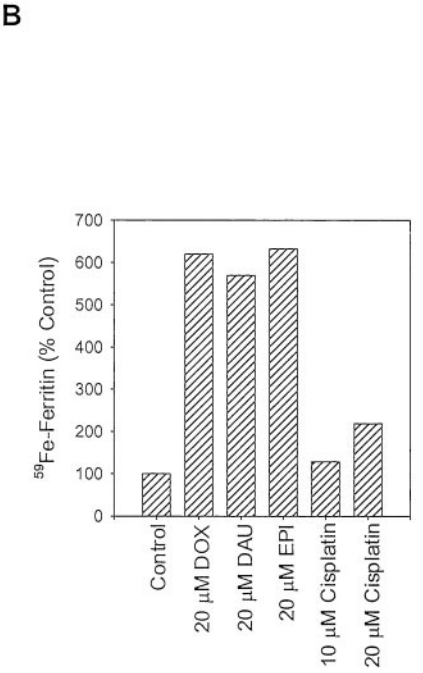


Fig. 4. Other cytotoxic agents are far less efficient than anthracyclines at inducing ferritin-^{59}Fe accumulation. A, SK-Mel-28 cells were incubated with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C. Cells were then re-incubated with control media, DOX (20 μM), DAU (20 μM), EPI (20 μM), or cisplatin (10 or 20 μM) for 18 h at 37°C and ^{59}Fe-PAGE autoradiography was performed (see *Materials and Methods* for details). The results are a typical experiment from two performed. B, densitometric analysis of the autoradiograph shown in A.

bated with menadione alone (Fig. 6, A, compare lanes 13 with 17 and 18, and B).

Considering that MnTBAP is a permeable superoxide scavenger, these results suggest that this free radical could play a role in the ^{59}Fe-ferritin accumulation induced by the redox-cycling activity of DOX or menadione. The ability of anthracyclines to interact with and decrease certain cellular reductants, such as GSH (Gianni and Myers, 1992), as well as its ability to alter the redox status of cells (Gianni and Myers, 1992) may be a possible mechanism by which these drugs inhibit ferritin-^{59}Fe mobilization and/or favor Fe uptake and incorporation into ferritin. Indeed, incubation of SK-Mel-28 cells with 20 μM DOX for 6 and 24 h decreased cellular GSH to 72 and 47% of control levels, respectively. Experiments were performed using the GSH-depleting agent BSO (Griffith and Meister, 1979) and NAC, which reconstitutes cellular GSH levels (Lomonosova et al., 1998). Incubation of cells with BSO (0.01 mM) for 18 h at 37°C reduced GSH levels to 30% of the control but had no effect on the ability of DOX to induce ferritin-^{59}Fe accumulation (data not shown). In addition, incubation with NAC (1 mM) also had no effect on the ability of DOX to increase ferritin-^{59}Fe levels (data not shown). These results indicate that although DOX has a marked effect on GSH levels, this does not seem to alter ^{59}Fe incorporation into ferritin.

The Effects of Chelators on Preventing Doxorubicin-Mediated Ferritin-^{59}Fe Accumulation by Cells during Fe Uptake. The DOX-induced accumulation of ferritin-^{59}Fe and the inhibition of ferritin-^{59}Fe mobilization to other vital Fe-containing proteins (e.g., ribonucleotide reductase) could potentially result in their decreased enzymatic activity and lead to cytostasis or cytotoxicity via Fe deprivation. To determine whether these effects of DOX could be prevented, we examined the effect of a number of Fe chelators (Fig. 7, A and B). In these studies, we assessed the clinically used Fe chelators DFO (50 μM) and ICRF-187 (500 μM) and also the active membrane-permeable aroylhydrazone chelator PCIH (50 μM), which has been demonstrated to have potential for the treatment of Fe-overload diseases (Becker and Richardson, 1999) (Fig. 7, A and B).

Cardiomyocytes were incubated with ^{59}Fe-Tf (0.75 μM) and the chelators for 18 h at 37°C in the presence or absence of DOX (5 μM). Accumulation of ^{59}Fe-ferritin was prevented almost completely by DFO (Fig. 7, A, compare lanes 1 and 2, and B), whereas PCIH reduced ferritin-^{59}Fe to 7% of the control (Fig. 7, A, compare lanes 1 and 3, and B). In contrast, ICRF-187 was less effective, reducing ^{59}Fe-ferritin to 63% of the control value (Fig. 7, A, compare lanes 1 and 4, and B). Again, treatment of cells with DOX (5 μM) markedly increased ^{59}Fe-ferritin levels to 360% of the control (Fig. 7, A, compare lanes 1 and 5, and B). However, simultaneous incubation of cells with DOX and DFO almost totally inhibited ferritin-^{59}Fe accumulation compared with DOX alone (Fig. 7, A, compare lanes 5 and 6, and B). The chelator PCIH was also moderately effective at inhibiting ferritin-^{59}Fe accumulation in the presence of DOX, reducing levels to 48% of that found with DOX alone (Fig. 7, A, compare lanes 5 and 7, and B). It is of interest to note that when cells were coincubated with PCIH and DOX, there was a change in ^{59}Fe distribution from the lower to the upper sections of the ferritin band compared with DOX-treated cells (Fig. 7A, compare lanes 5 and 7). In cells simultaneously treated with ICRF-187 and DOX, the total amount of ferritin-^{59}Fe was comparable with DOX alone (Fig. 7, A, compare lanes 5 and 8, and B). However, the incubation of ICRF-187 with DOX caused a slight change in ^{59}Fe distribution from the lower to the upper band of ferritin compared with DOX alone (Fig. 7A, compare lanes 5 and 8). The significance of the redistribution of Fe in the two ferritin bands remains unknown at present. Collectively, these data suggest that DFO, and to a lesser extent, PCIH, are effective at inhibiting the accumulation of ^{59}Fe-ferritin in control and DOX-treated cells during the Fe uptake process.

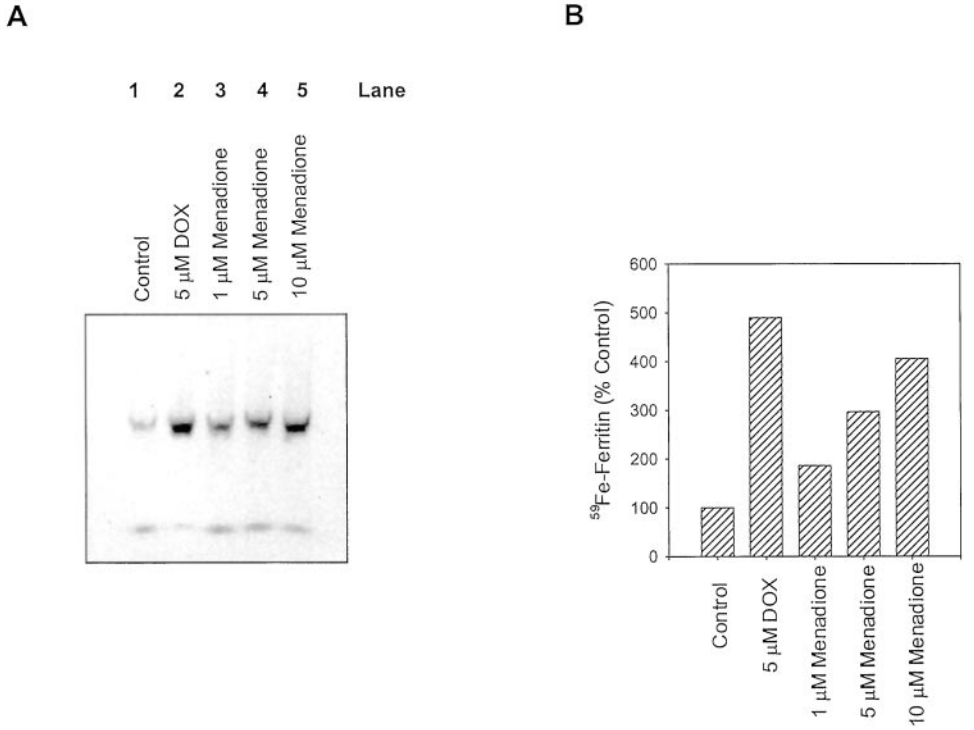


Fig. 5. The redox-cycling agents DOX and menadione induced ferritin-^{59}Fe accumulation. A, SK-Mel-28 cells were incubated with ^{59}Fe-Tf (0.75 μM) for 18 h at 37°C in the presence or absence of DOX (5 μM) or menadione (1–10 μM). ^{59}Fe-PAGE autoradiography was then performed (see *Materials and Methods* for details). The results shown are a typical experiment from 3 performed. B, densitometric analysis of the gel is shown in A.

Iron Chelators Only Partially Mobilize ^{59}Fe from Ferritin after its Accumulation in DOX-Treated Cells. Although DFO and, to a lesser extent, PCIH, seemed to be able to prevent DOX-mediated ferritin-^{59}Fe accumulation during the ^{59}Fe uptake process (see Fig. 7 above), it was important to examine whether these chelators were effective at mobilizing ^{59}Fe already bound within ferritin as a result of DOX treatment (Fig. 8).

Cardiomyocytes were prelabeled for 18 h at 37°C with ^{59}Fe-Tf (0.75 μM) in the presence or absence of DOX (5 μM; Fig. 8, A and B). The efficacy of DFO (50 μM), PCIH (50 μM), and ICRF-187 (500 μM) at mobilizing ^{59}Fe from ferritin was examined after a 24-h reincubation at 37°C. In cells reincubated in control media for 24 h, no discreet ^{59}Fe-ferritin band was observed (Fig. 8A, lanes 1–4). Rather, there was a broad distribution of ^{59}Fe in very diffuse bands within the lane of the gel. As shown in Figs. 1, A and B, and 2, A and B, ^{59}Fe redistributes from ferritin to other cellular compartments during long periods of reincubation and uptake. Because of the length of both the labeling and reincubation periods (18 and 24 h, respectively) used in Fig. 8, the effect was more marked. Reincubation of ^{59}Fe-labeled control cells with the chelators had little effect compared with cells reincubated in control media alone (Fig. 8, A, compare lane 1 with 2–4, and B).

In prelabeled DOX-treated cells (Fig. 8A, lanes 5–8), despite a 24-h reincubation period in control media, a marked accumulation of ferritin-^{59}Fe was still observed (Fig. 8, A, compare lanes 1 and 5, and B). Reincubation of prelabeled DOX-treated cells with medium containing DFO, PCIH, or ICRF-187 reduced ferritin-^{59}Fe accumulation to 58 to 74% of that found for control media alone (Fig. 8A, compare lanes 5 with lanes 6–8). It is of interest to note that DFO or PCIH reduced ferritin-^{59}Fe accumulation in both the upper and lower ferritin bands; this was slightly more pronounced for the upper band (Fig. 8A, compare lane 5 with lanes 6–7). Further experiments demonstrated that both DFO and PCIH significantly mobilized ^{59}Fe from control and DOX-treated cells (Fig. 8C), suggesting that although these chelators are effective in cellular Fe mobilization, ferritin-Fe does not seem to be the main source of intracellular Fe targeted by these chelators. Considering these results, although DFO and PCIH are capable of inhibiting the process of DOX-induced ^{59}Fe accumulation into ferritin (Fig. 7A), they are far less

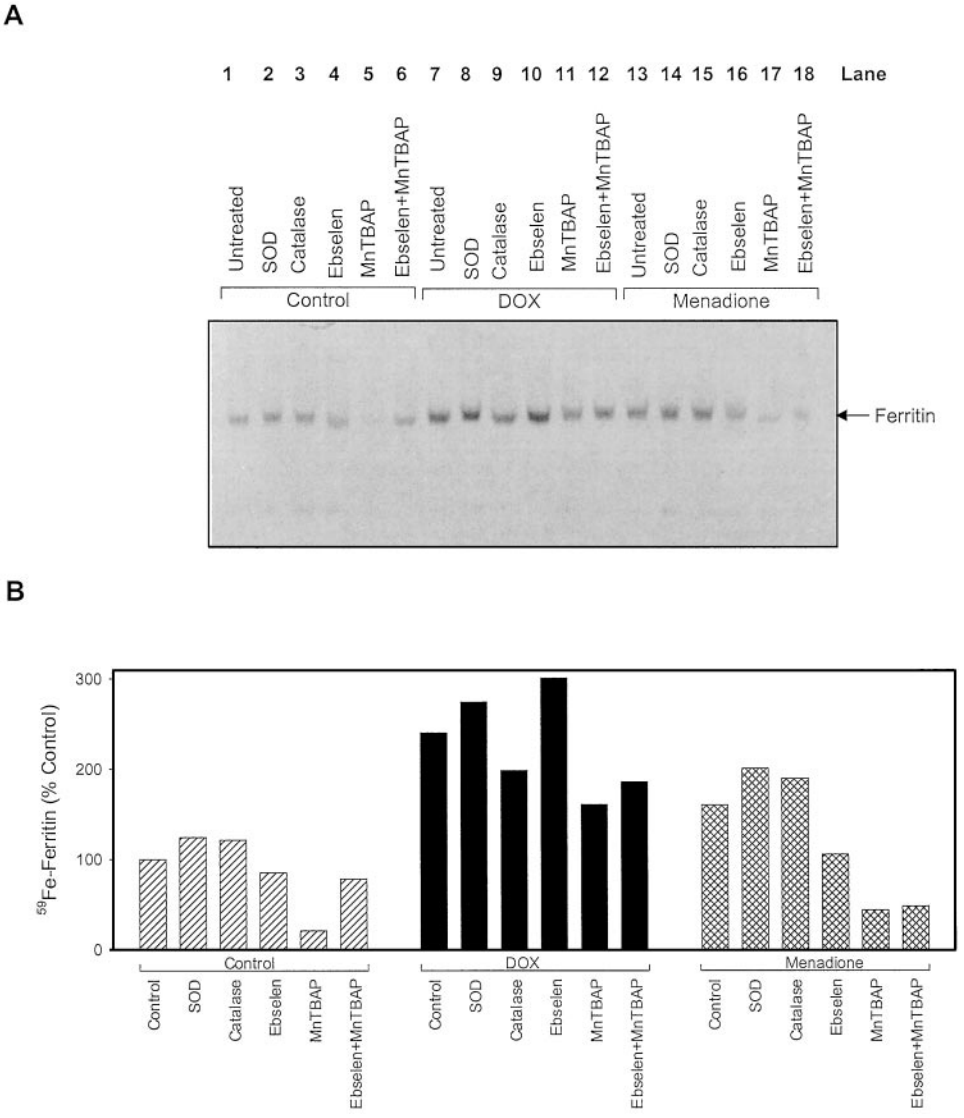


Fig. 6. The ferritin-^{59}Fe accumulation induced by the redox-cycling agents DOX and menadione can be partially inhibited by the cell-permeable superoxide scavenger, MnTBAP. A, SK-Mel-28 cells were incubated with ^{59}Fe-Tf (0.75 μM) for 18 h at 37°C with either control media, DOX (5 μM), or menadione (10 μM) in the presence or absence of the free radical scavengers, catalase (1000 U/ml), SOD (1000 U/ml), ebselen (15 μM), MnTBAP (200 μM), or the combination of ebselen (15 μM) and MnTBAP (200 μM) for 18 h at 37°C. ^{59}Fe-PAGE autoradiography was then performed (see *Materials and Methods* for details). The results shown are a typical experiment from three performed. B, densitometric analysis of the autoradiograph shown in A.

effective at mobilizing ^{59}Fe after it has been incorporated into this molecule in the presence of DOX (Fig. 8A).

Discussion

Anthracyclines are potent antineoplastic agents that avidly bind Fe; this property is thought to play a role in their cardiotoxic effects (Gianni and Myers, 1992). Despite this, there have been very few studies on the effects of these drugs on Fe metabolism in neoplastic cells or cardiomyocytes. The most comprehensive experiments to date have examined the IRPs that are crucial for cellular Fe homeostasis (Minotti et al., 1998; Minotti et al., 2001; Kotamraju et al., 2002; Kwok and Richardson, 2002). In addition, very little is understood concerning the actual mechanisms involved in intracellular Fe trafficking and ferritin-Fe release and the effect of anthracyclines on these processes.

The current study is the first to investigate the effects of anthracyclines on intracellular Fe trafficking and distribution in both tumor cells and differentiated cardiomyocytes. We demonstrated that during and after cellular ^{59}Fe uptake from ^{59}Fe-Tf in control cells, ^{59}Fe was mainly incorporated into ferritin during the first 6 h (Figs. 1 and 2). Thereafter, ferritin-^{59}Fe levels dramatically decreased, the ^{59}Fe being redistributed to other cellular compartments (Figs. 1 and 2). However, the presence of anthracyclines inhibited the ^{59}Fe mobilization process from ferritin, resulting in a marked accumulation of ^{59}Fe within this protein (Figs. 1 and 2). Cellular ^{59}Fe uptake and efflux experiments demonstrated that the total cellular Fe content was not affected by anthracyclines (Fig. 3, A and B). These observations indicated that the changes in ferritin-^{59}Fe content were not caused by altered ^{59}Fe uptake or release from cells. The inhibition of ^{59}Fe redistribution to other cellular compartments by anthracyclines may have detrimental effects on vital Fe-dependent processes and may, at least in part, be responsible for its cytotoxicity.

Our observations are the first to demonstrate the effect of anthracyclines on inhibiting ferritin-Fe redistribution in an intact cellular system. In contrast to our data, previous studies using isolated and purified ferritin have demonstrated that anthracyclines induce Fe release from this protein (Demant, 1984; Thomas and Aust, 1986). However, it is likely that the in vivo mechanisms involved in mobilizing Fe from ferritin are quite different from that observed with the purified protein. Once isolated from its cellular environment, the structure and function of the molecule could be altered. In addition, Fe may only be mobilized within certain cellular compartments such as the lysosome (Radisky and Kaplan, 1998), and it is probable that interaction of ferritin with other molecules may be essential for Fe release. Furthermore, in studies using purified ferritin, it was difficult to determine whether Fe was being mobilized from the ferritin core or from Fe nonspecifically bound to the outer surface of the protein (Ponka and Richardson, 1997).

Although many mechanisms have been proposed for in vitro ferritin-Fe mobilization (Dognin and Crichton, 1975; Harrison and Arosio, 1996), currently there are few data describing the process of Fe release from ferritin within cells. Studies using isolated ferritins in vitro have shown that when conserved residues in the 3-fold axes are mutated, ferritin-Fe release is markedly enhanced (Takagi et al., 1998; Jin et al., 2001). Similarly, it can be speculated that residues could be altered to inhibit Fe release. Considering this, a major mechanism of anthracycline toxicity is through their ability to redox cycle, generating free radicals that result in lipid peroxidation and protein oxidation (Gianni and Myers, 1992). Oxidation of the ferritin protein could theoretically alter the Fe release process. In our current investigation, other redox-cycling drugs, including paraquat and menadione, also resulted in ferritin-Fe accumulation (Figs. 5 and 6). In addition, experiments with the intracellular superoxide scavenger, MnTBAP, suggested that superoxide may play a role in the accumulation of ferritin-Fe induced by these redox-cycling drugs (Fig. 6, A and B). Further studies are underway to determine the precise molecular mechanism involved.

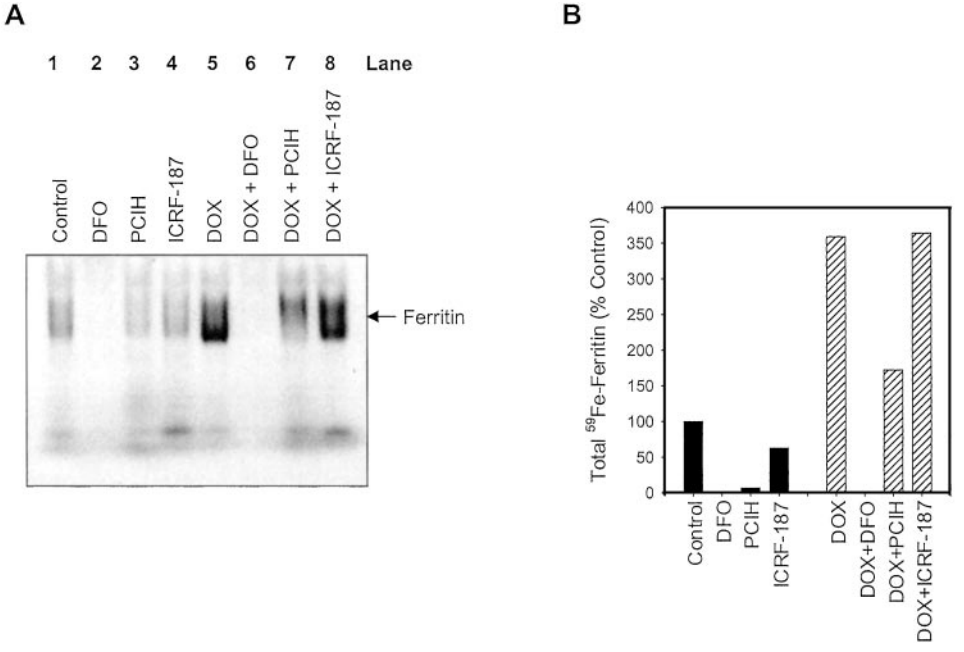


Fig. 7. Some iron chelators are effective at inhibiting DOX-mediated ferritin-^{59}Fe accumulation during cellular ^{59}Fe uptake from ^{59}Fe-transferrin (^{59}Fe-Tf). A, cardiomyocyte cultures were incubated with ^{59}Fe-Tf (0.75 μM) for 18 h at 37°C with control media, DFO (50 μM), PCIH (50 μM), or ICRF-187 (500 μM) in the presence or absence of DOX (5 μM) and ^{59}Fe-PAGE autoradiography performed (see *Materials and Methods* for details). The results shown are a typical experiment from 3 performed. B, densitometric analysis of the results in shown in A.

At present, it is unclear whether the accumulation of ferritin-Fe after treatment with DOX is involved in its cardiotoxic or antineoplastic effects. However, the release of stored Fe from ferritin is probably critical for important physiological processes (e.g., ribonucleotide reductase activity that is necessary for DNA synthesis) and the ability of anthracyclines to prevent this may be a mechanism of cytotoxicity. Alternatively, it could also be argued that the increased storage of Fe within ferritin may be a protective cellular response that helps to prevent the interaction of Fe with anthracyclines, inhibiting its ability to redox-cycle and generate toxic free radicals.

We demonstrated in our studies that Fe chelators such as DFO and PCIH could inhibit the DOX-mediated accumulation of ferritin Fe (Fig. 7) and slightly mobilize Fe once it had been incorporated into ferritin after incubation with DOX (Fig. 8). It is important to note that DOX has been shown to be more cytotoxic in Fe-overloaded cardiomyocytes compared with controls (Hershko et al., 1993), and the ability of DOX to accumulate Fe in ferritin (Figs. 1 and 2) and prevent its redistribution may potentiate its cardiotoxicity. In addition, DFO can reduce toxicity in Fe-overloaded cardiomyocytes (Hershko et al., 1993). However, it remains to be established whether the ability of chelators to prevent ferritin-Fe accumulation (Figs. 7 and 8) is beneficial in terms of reducing DOX cardiotoxicity. It is interesting that some Fe chelators are potent antitumor agents (Richardson and Milnes, 1997) and the combination of DOX and DFO is more cytotoxic than either alone (Blatt and Huntley, 1989). Therefore, suitable chelators may both prevent DOX-mediated cardiotoxicity and potentiate its antitumor activity.

The uptake of ^{59}Fe into ferritin of the differentiated cardiomyocytes resulted in a diffuse band that seemed to consist of two major components (e.g., see Figs. 1C, 2E, 7A, and 8A), whereas ^{59}Fe uptake into ferritin by all the tumor cell lines resulted in a single well-defined band (e.g., Figs. 1A and 2, A, C, and E). This observation could be indicative of a difference in the metabolism of ferritin in the cardiomyocytes compared with the neoplastic cells (Andrews et al., 1987; Campbell et al., 1993). Although the role of ferritin as an Fe-storage molecule is well defined, there have been few studies in living cells examining the Fe uptake and release process from this protein. Further investigation is required to fully characterize the significance of the difference observed in ferritin-^{59}Fe incorporation between cardiomyocytes and tumor cell types.

In our previous study, we examined the effect of anthracyclines on IRP-RNA-binding activity in the same cell types assessed in this investigation, namely, SK-Mel-28 melanoma cells and cardiomyocytes (Kwok and Richardson, 2002). These experiments were important because IRPs are major

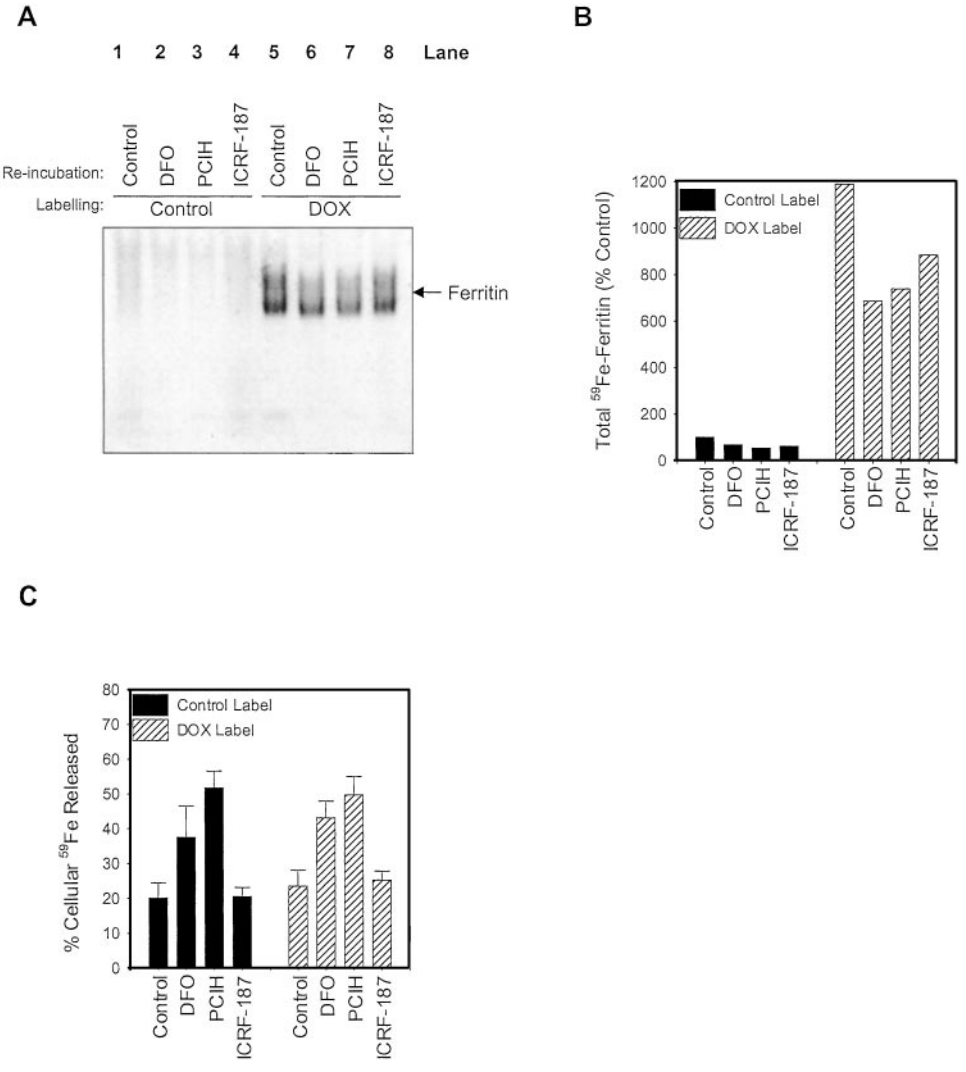


Fig. 8. Iron chelators can partially mobilize ^{59}Fe from ferritin in DOX-treated cells. A, cardiomyocyte cultures were prelabeled for 18 h at 37°C with ^{59}Fe-Tf (0.75 μM) in the presence or absence of DOX (5 μM). Cells were then washed and reincubated for 24 h at 37°C in control media or media containing the chelators DFO (50 μM), PCIH (50 μM), and ICRF-187 (500 μM), and ^{59}Fe-PAGE was autoradiography performed (see *Materials and Methods* for details). The results shown are a typical experiment from three performed. B, densitometric analysis of the results in A. C, cardiomyocyte cultures were treated as described in A, and cellular ^{59}Fe released into the overlying media was assessed after the 24 h reincubation in the presence or absence of chelators. Results are mean ± S.D. (three experiments).

regulators of intracellular Fe metabolism (Hentze and Kühn, 1996). The ferritin mRNA contains a hairpin loop structure in its 5′-untranslated region called an iron-responsive element (Hentze and Kühn, 1996). In ferritin mRNA, binding of IRP to the iron-responsive element inhibits translation, thereby decreasing Fe storage (Hentze and Kühn, 1996).

We showed in a prior investigation that the effect of anthracyclines on IRP-RNA-binding was complex, with an initial and delayed response being found (Kwok and Richardson, 2002). The initial response to DOX resulted in a decrease in IRP-RNA-binding activity observed after a 6-h incubation (Kwok and Richardson, 2002), which should theoretically increase ferritin protein synthesis. Surprisingly, this was not observed in the current study examining ferritin protein levels as a function of time. The reason for this remains unclear at present and is a subject for further investigation. The delayed effect of DOX on IRP-RNA-binding was observed after an 18- to 24-h incubation in which the initial decrease was followed by restoration of IRP-RNA-binding activity to control levels (Kwok and Richardson, 2002). This was in agreement with the observations of the present work, in which ferritin protein levels in the presence of DOX were comparable with the untreated controls (Fig. 3C). However, ^{59}Fe incorporation into ferritin continued to increase (Fig. 1, A and B). Again, these results suggest that the elevated ^{59}Fe levels in ferritin after incubation with DOX was independent of de novo ferritin synthesis.

In conclusion, this is the first study to demonstrate that anthracyclines induce ferritin-Fe accumulation by inhibiting Fe mobilization from the protein. To date, very little is understood concerning the mechanism of ferritin Fe-release, and this investigation represents the first examination of this process using intact cells and the physiological Fe donor Tf. Our experiments also showed that ferritin is a dynamic molecule that can take up and release Fe depending on cellular requirements. The inhibition of Fe redistribution to other cellular compartments by anthracyclines may have consequences in terms of the cytotoxic effects of these drugs.

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