Regulation, mechanisms and proposed function of ferritin translocation to cell nuclei

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Summary

Ferritin is traditionally considered a cytoplasmic iron-storage protein, but recent reports indicate that it is also found in cell nuclei. Nuclear ferritin has been proposed to be involved in both the protection of DNA and the exacerbation of iron-induced oxidative damage to DNA. We demonstrate that H-rich ferritin is present in the nucleus of human astrocytoma tumor cells. To study the mechanism and regulation of ferritin translocation to the nucleus, we developed a cell culture model using SW1088 human astrocytoma cells. Changes in cellular iron levels, cytokine treatments and hydrogen peroxide exposure affected the distribution of ferritin between the cytosol and the nucleus. Ferritin enters the nucleus via active transport through the nuclear pore and does not require NLS-bearing cytosolic factors for transport. Furthermore, H-rich ferritin is preferred over L-rich ferritin for uptake into the nucleus. Whole cell crosslinking studies revealed that ferritin is associated with DNA. Ferritin protected DNA from iron-induced oxidative damage in both in vitro and in cell culture models. These results strongly suggest a novel role for ferritin in nuclear protection. This work should lead to novel characterization of ferritin functions in the context of genomic stability and may have unparalleled biological significance in terms of the accessibility of metals to DNA. The knowledge generated as a result of these studies will also improve our understanding of iron-induced damage of nuclear constituents.

Key words: Iron, Oxidative damage, DNA protection, Nuclear translocation

Introduction

Although iron is required for oxidative metabolism, it can be toxic because of its ability to catalyze the generation of free radicals (Yagi et al., 1992). Iron-induced oxidative damage can result in changes in membrane fluidity and permeability, enzyme inactivation and accelerated proteolysis, mutation, sister chromatid exchange, and chromosomal aberrations (Gardiner, 1989; Halliwell, 1992; Lesnefsky, 1994; Loeb et al., 1988; Stadtman, 1990). Specific roles for iron-dependent radical formation of DNA mutations in aging and cancer have been clearly established (Toyokuni and Sagripanti, 1992). For example, individuals who suffer from hemochromatosis, an iron-overload disorder, have a 20% higher incidence of cancer than do unaffected subjects (Witte et al., 1996). Protection against oxidative and free-radical damage takes two forms. Antioxidant enzymes, such as superoxide dismutase, catalase, peroxidase and glutathione peroxidase, convert superoxides and peroxides into less reactive species (Basaga, 1990; Floyd, 1990; Sies, 1993). In addition, most organisms possess proteins that can sequester transition metals, reducing the availability of transition metals that catalyze free-radical formation. The iron-storage protein ferritin is one such protein, and is considered an important cytoprotectant (Crichton, 1990).

Most vertebrate ferritins occur as hollow, spherical assemblies of 24 protein subunits (aggregate M_r ~450,000). As many as 4500 iron atoms can be accommodated within a ferritin assembly (Aisen and Listowsky, 1980). Ferritin assemblies comprise two functionally and genetically distinct subunit types: H (heavy) and L (light), which are present in varying ratios in different tissues. Subunits of type L contribute to the nucleation of the iron core, but lack the ferroxidase activity necessary for uptake of ferrous (Fe^{2+}) iron. Subunits of type H possess ferroxidase activity and promote rapid uptake and oxidation of ferrous iron (Lawson et al., 1989).

Ferritin has been observed in the nucleus of rat hepatocytes (Smith et al., 1990), chicken corneal epithelial cells (Cai et al., 1997; Cai et al., 1998), the human K562 cell line (Pountney et al., 1999) and rodent neurons during development and after hypoxic ischemic insult (Cheepsunthorn et al., 1998; Cheepsunthorn et al., 2001). There is little agreement between these studies either about the mechanism of action of ferritin in the nucleus or the mechanism by which it enters the nucleus. In corneal epithelial cells, the nuclear expression pattern of ferritin is developmentally regulated (Cai et al., 1997), whereas in hepatocytes the ferritin in the nucleus is thought to follow iron passively across a concentration gradient after iron overload (Smith et al., 1990). Recently, Cai and Linsenmayer (Cai and Linsenmayer, 2001) suggested that nuclear localization of ferritin involved tissue-specific mechanisms. Functionally, the presence of ferritin in the nucleus in corneal

epithelial cells protects DNA from UV damage (Cai et al., 1998), but ferritin in the nuclei of hepatocytes is hypothesized to be a catalyst for hydroxyl radical formation during toxic, carcinogenic and aging processes (Smith et al., 1990).

In this study, we demonstrate that ferritin is present in the nucleus of human astrocytoma cells in vivo and a human SW1088 astrocytoma cell line. Normally astrocytes do not contain ferritin in either their nuclei or cytoplasm. Some cytoplasmic staining for L-ferritin has been observed in diseased states (Connor, 1994; Connor and Menzies, 1995). We established a cell culture model using the SW1088 cells to elucidate the mechanism for ferritin uptake into the nucleus, in order to begin to identify factors that regulate the concentration of ferritin in the nucleus. Subsequently, we used a supercoil strand break assay and the cell culture model to test directly the hypothesis that ferritin protects DNA from iron-induced oxidative damage.

Materials and Methods

Astrocytoma tumor tissue preparation

Human astrocytomas obtained by biopsy were immersed in 4% paraformaldehyde and embedded in paraffin was for sectioning. The sections were immunoreacted for H-ferritin (HS-59 antibody; 1:100). To control for nonspecific antibody immunoreactions, sections were incubated without the primary antibody, or with mouse IgG (1:50), glial fibrillary protein (GFAP; 1:500) or neurofilament (1:20) antibodies. The avidin biding complex (ABC) protocol was followed for immunodetection and the immunoreaction visualized using 3-amino-9-ethylcarbazole (AEC; Scy-tek). Sections were counterstained with Blue Counterstain (TACS) to visualize cell nuclei.

SW1088 cell culture

Human astrocytoma SW1088 cells (HTB-12; ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (BioCell), 4 mM L-glutamine (Sigma) and antibiotics [100 U/ml penicillin and 1 ng/ml streptomycin (Gibco)]. Cultures were maintained in 75 mm and 150 mm culture flasks and passaged every 5 days. For immunohistochemical analyses, astrocytoma cells were plated on 12 mm poly-L-lysine-coated glass coverslips at a density of 1.5×10^4 cells. Cells were fixed with 4% paraformaldehyde and nonspecific antibody reactions were blocked by incubation for 30 minutes in 5% non-fat dry milk. The cells were incubated with the monoclonal anti-human rH-ferritin antibody (HO2; 1:250) followed by a Texas Red-conjugated IgG secondary (Sigma) at a dilution 1:100. Control immunoreactions were performed without the primary antibody. Nuclei were visualized using DAPI (100 ng/ml; Molecular Probes). DNA synthesis was detected using the 5-bromo-2′-deoxyuridine (BrdU) Labeling and Detection Kit (Boehringer Mannheim). Results were visualized by fluorescence microscopy.

Myc-ferritin constructs and cellular localization of Myc tagged H-ferritin

The human H-ferritin cDNA was inserted 5′ of the Myc segment of the Myc containing vector pcDNA3 (Invitrogen). The H-ferritin Myc construct was a generous gift from Dwight Stambolian (University of Pennsylvania). SW1088 cells at 50-60% confluence were transfected with the construct using the Lipofectamine transfection reagent (Boehringer Mannheim). Cells were grown in standard culture conditions for 12 hours, fixed with 4% paraformaldehyde and the Myc epitope visualized with the Ab-1 anti-Myc antibody (Calbiochem; 1:250) and FITC-conjugated IgG secondary (Sigma; 1:100). Nuclear localization of staining was verified using DAPI.

Iron chelation in SW1088 cells

The iron chelator deferoxamine (DFO; Sigma) was used to examine the effect of iron chelation on the presence of ferritin in nuclei of astrocytoma cells. Cells were plated in the presence of 100 μM DFO for 6, 12, 24, 48 and 72 hours. Immunohistochemical and uptake studies were routinely performed on cells treated with DFO for 72 hours. The Live/Dead Stain (Molecular Probes) was used to demonstrate that the DFO-treated cells were still viable.

Cell stressors

Astrocytoma cells were treated with 100 μM DFO for 72 hours to minimize ferritin expression in the cells. After this treatment, the cultures were rinsed with Hanks balanced salts solution and incubated for 12 hours in standard medium alone (control) or medium containing ferric ammonium citrate (FAC; 0, 50, 100, 200 μM), hydrogen peroxide (H_2O_2; 0, 50, 100, 300 μM), tumor necrosis factor α (TNFα; 1, 10, 50 ng/ml) or interleukin 1β (IL-1β; 50, 100, 200 U/ml). Treatments were performed in triplicate and nuclear and cytosolic fractions were obtained separately for each trial.

Isolation of nuclear and cytosolic fractions

Nuclear and cytosolic fractions of harvested astrocytoma cells were isolated according to standard methods (Abmayr and Workman, 1997). The cultures were rinsed with Hanks Balanced Salts Solution, trypsinized and the cells collected by centrifugation. The pelleted cells were rinsed with 5 ml 0.1 M phosphate-buffered saline (PBS) and collected by centrifugation at 1850 ***g*** for 5 minutes. The cell pellets were resuspended in five pelleted cell volumes (p.c.v.) of hypotonic buffer [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT]. The resuspended cells were collected by centrifugation for 5 minutes at 1850 ***g***. These cells were lysed by resuspension in five p.c.v. of hypotonic solution for 10 minutes on ice and homogenized using a Dounce homogenizer. The cells were lysed with 20 up-and-down pestle strokes of a type B pestle and cell lysis was verified by light microscopy. The nuclei were collected by centrifugation for 15 minutes at 3300 ***g***. The supernatant was collected for cytosolic analysis.

Relative amounts of ferritin in nuclear and cytosolic extracts

The relative amounts of H-ferritin in the cytosolic and nuclear extracts were determined by western immunoblot analysis. Equal amounts of nuclear (5 μg/50 μl) or cytosolic (10 μg/50 μl) proteins from control and experimental groups were examined by 15% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. H- and L-rich ferritins (0.5 ng/50 μl) were added as standards and also served as positive and negative controls for the antibodies. Membranes were blocked with 5% non-fat dry milk, washed in TBS-Tween (Tris-buffered saline/0.2% Tween-20), and probed with mouse anti-human rH-ferritin (HS-59) at a dilution of 1:1500, overnight at 4°C. The blots were washed and incubated with goat anti-mouse IgG conjugated with peroxidase for the SW1088 cell extracts, at a dilution of 1:5000 for 60 minutes at room temperature. The immunoreaction was visualized using chemiluminescent detection (ECL+Plus, Amersham). Autoradiography films were scanned and the band intensities for each experiment were assessed using the image analysis software program Collage (Fotodyne). The threshold value for each analysis was set to zero. S/N values [area intensity–(pixel intensity×background)/background pixel intensity] for each band were obtained. The S/N values were converted to percent of control (12 hour media replete samples) to compare experiments between the

three trials for each experimental manipulation. Each experiment was repeated three times and a separate immunoblot analysis performed each time.

Proteins and antibodies

The human recombinant ferritins used in this study (rH, rL and 222) and ferritin subunit-specific monoclonal antibodies were generously supplied by Paolo Arosio (Milan, Italy). The specificity of the HS-59 and HO2 monoclonal antibodies to the H-ferritin subunit has been well characterized (Cavanna et al., 1983; Luzzago et al., 1986; Ruggeri et al., 1992). The HS-59 antibody was used to detect the denatured form of H-ferritin present in the western blot and paraffin wax-embedded tumor sections as a result of sample processing. The 222 ferritin is an H-ferritin with amino acid substitutions E62K and H65G that diminish the ferroxidase activity (Levi et al., 1988). The recombinant proteins have proper assembly, folding and functional properties (Lawson et al., 1989; Levi et al., 1987; Levi et al., 1989). The monoclonal GFAP antibody was obtained from Boehringer Mannheim, the Mouse IgG from Santa Cruz Biotechnologies, (500 μg/ml) and the monoclonal neurofilament antibody from AMAC (#0168). Crystalline bovine serum albumin, chymotrypsin, horse spleen apoferritin, transferrin and ovalbumin were obtained from Sigma. CAP protein was purified from *E. coli* strain pp47 containing the plasmid pHA5 according to the method of (Fried and Crothers, 1983). The ferritins (5 mg/ml) and BSA (10 mg/ml) were labeled with fluorescein-5-EX-succinimidyl ester (Molecular Probes) according to the manufacturer's protocol.

Characterization of ferritin nuclear import

Nuclear import of ferritin was measured using digitonin (Aldrich)-permeabilized cells (Adam et al., 1990; Cserpan and Udvardy, 1995). To demonstrate ferritin uptake, cells were permeabilized with digitonin (40 μg/ml) for 5 minutes in transport buffer (20 mM Hepes, pH 7.3, 100 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT and 1 μg/ml each aprotinin, leupeptin and pepstatin) while on ice. Excess digitonin was removed with three rinses in transport buffer. Fluorescein (FITC)-labeled recombinant H-ferritin was added to control and DFO-treated astrocytoma cells for 30 or 60 minutes in standard media. Cells were exposed to a range of concentrations of FITC-ferritin (10 nM, 100 nM, 1 μM, 5 μM, 10 μM) and 5 μM was selected as the optimal concentration, based on detectability and cell viability. To demonstrate that the permeabilization of the cells with digitonin did not affect nuclear membrane integrity, we performed the following control experiments in parallel cultures: cultures were treated with TRITC-dextran (70 kDa; Molecular Probes 5 μM final concentration), 222 mutant ferritin, L-rich ferritin, BSA (Sigma) or transferrin (Sigma). The latter were added at the same concentration as that used for ferritin.

Once conditions in which a fluorescently tagged ferritin could enter the nucleus were established, the effect of cellular iron status on ferritin nuclear transport was investigated. Cells were exposed for 72 hours to either standard media or media containing the iron chelator deferoxamine (100 μM). To control for a nonspecific deferoxamine effect, DFO and FAC were combined in a 1:1 molar ratio to form ferroxiamine B, which blocks the iron chelating effect of deferoxamine (Bergamini et al., 1999; Gutteridge et al., 1994). After 72 hours of treatment, FITC-rH ferritin (5 μM) was added to the media for 1 hour. The cells were then rinsed briefly in PBS, and viewed with a confocal microscope. These experiments were performed three times each.

To identify the mechanism for rH-ferritin nuclear translocation ferritin nuclear uptake was monitored under the following conditions: temperature variation (4°C and 37°C), nuclear pore receptor inhibition [wheat germ agglutinin (WGA); 200 μg/ml], ATP depletion (25 U/ml apyrase; Sigma), or ATP repletion (apyrase followed by addition of ATP) (Adam et al., 1990; Adam and Adam, 1994; Duverger et al., 1995). For each of these conditions, cells were first depleted of iron by exposure to DFO (100 μM) for 72 hours and then permeabilized with digitonin (40 μg/ml). Fluorescein-labeled rH-ferritin (5 μM in transport buffer) was added to the cells after each treatment for 60 minutes at 37°C. To study the effect of nuclear pore inhibition on ferritin translocation to the nucleus, WGA was added to the culture medium (200 μg/ml) for 10 minutes on ice in transport buffer. Excess WGA was removed by three washes in transport buffer. To determine if ATP is required for ferritin nuclear translocation, ATP was depleted by apyrase treatment (25 U/ml) in transport buffer for 15 minutes at 37°C. As a control for the ATP depletion experiments, after apyrase treatment, ATP was reintroduced in a parallel set of cells by incubating them in an ATP regeneration system (9 mM ATP, 20 mM phosphocreatine and 20-100 U/ml creatine kinase) in the presence of FITC-rH ferritin (Newmeyer et al., 1986). In addition, because nuclear transport is temperature dependent, an additional set of uptake studies was performed at 4°C. To determine if cytosolic factors were required for translocation of ferritin to the nucleus, cells were exposed to N-ethylamleimide (NEM) (Adam et al., 1990). Permeabilized cells were treated with 2 mM NEM (Aldrich) for 10 minutes at 4°C in transport buffer lacking DTT (Adam et al., 1990; Duverger et al., 1995). At the end of the incubation in FITC-rH-ferritin, cells were rinsed three times in transport buffer containing DTT, fixed in 4% paraformaldehyde and examined by fluorescence and confocal microscopy. Each experiment was repeated three times.

Ferritin/DNA crosslinking studies

Human astrocytoma cells (SW1088) were grown to 80% confluence in 75 cm^2 flasks in the presence or absence of 100 μM deferoxamine for 72 hours. The cells were rinsed and permeabilized with 40 μg/ml digitonin for 5 minutes on ice. The permeabilization step is necessary for exogenous ferritin to enter the cell. ^{125}I-rH-ferritin was added to the medium for 30 minutes at 30°C. The media was replaced with media containing 1% formaldehyde or standard media alone and the flasks placed at 4°C for 4 days (Solomon et al., 1988). After incubation at 4°C the cells were harvested mechanically and DNA-protein complexes isolated. Samples (50 μl) were analyzed by slot blot analysis and autoradiography. Intensity of each band was measured using the image analysis program Collage and normalized for DNA concentrations.

Supercoil relaxation assay

The DNA used for the supercoil assays was the plasmid pUC19 (Yanisch-Perron et al., 1985). Plasmid pUC19 was propagated in DH5a cells, and the covalently closed circular fraction purified by two cycles of centrifugation in CsCl density gradients. Reaction mixtures (50 μl) contained supercoiled pUC19 DNA (10 nM) dissolved in 10 mM Tris, 100 mM KCl, (pH 7.4), 50 μM $FeCl_3$, and 10 mM H_2O_2, plus varying amounts of proteins (both ferritins and non-ferritins were tested). Reaction mixtures were assembled as follows. The DNA was dissolved in 10 mM Tris, 100 mM KCl, (pH 7.4). The protein of interest was added, in amounts sufficient to give the desired final concentration, and the sample was incubated at room temperature for 15 minutes. $FeCl_3$ was added to the sample and, following a 15-minute incubation, H_2O_2 were added to the mixture. The assembled samples were incubated for 1 hour at 37°C. Reactions were terminated by the addition of 25 μl of 4 M urea, 50% sucrose, 50 mM EDTA and 0.1% Bromophenol Blue. Aliquots were subjected to electrophoresis on 1.5% agarose gels, and the mole fractions of superhelical and relaxed forms measured by densitometry of photographic negatives of the gels after staining with Ethidium Bromide (0.5 μg/ml).

Whole cell damage assay

To examine the potential DNA protective effects of ferritin in live cells, comparisons were made between DFO-treated cells (control) and DFO-treated cells incubated with WGA. DFO treatment for 72 hours resulted in a loss of detectable ferritin in the nucleus. After DFO treatment, WGA was added to the cells at 200 μg/ml for 10 minutes on ice in transport buffer. WGA blocks the relocalization of actively transported proteins to the nucleus. Cells were incubated for 30 or 60 minutes in 100 μM hydrogen peroxide or ferric ammonium citrate in standard culture conditions (standard conditions contain 5 μM iron). Cells were then fixed with 4% paraformaldehyde and ssDNA strand-breaks were detected using the TUNEL assay (Cell Death Kit, Boehringer Mannheim). The TUNEL assay uses terminal deoxynucleotidyl transferase to introduce fluorescein-dUTP into partially degraded DNA that can result from oxidative damaging reagents. Nuclei were counterstained with DAPI (100 ng/ml). Results were ranked according to labeling intensity and scored by double-blind analysis.

Results

H-ferritin immunostaining in human astrocytoma sections

A total of six tumors were examined. In all cases, cells within the tumor were H-ferritin positive. Ferritin was present in both cytoplasmic and nuclear compartments, although not all cells contained ferritin in the nucleus (Fig. 1A,B). Staining was not detected in control sections stained with mouse IgG (Fig. 1C,D) or cells processed through the immunoreaction without the primary antibody (data not shown). Two additional human monoclonal antibodies were examined as controls. Sections exposed to a monoclonal antibody for GFAP stained only intermediate filaments in the cytoplasm (Fig. 1E). A monoclonal antibody to neurofilament was used as an irrelevant control and neither cytoplasmic nor nuclear staining was detected (Fig. 1F).

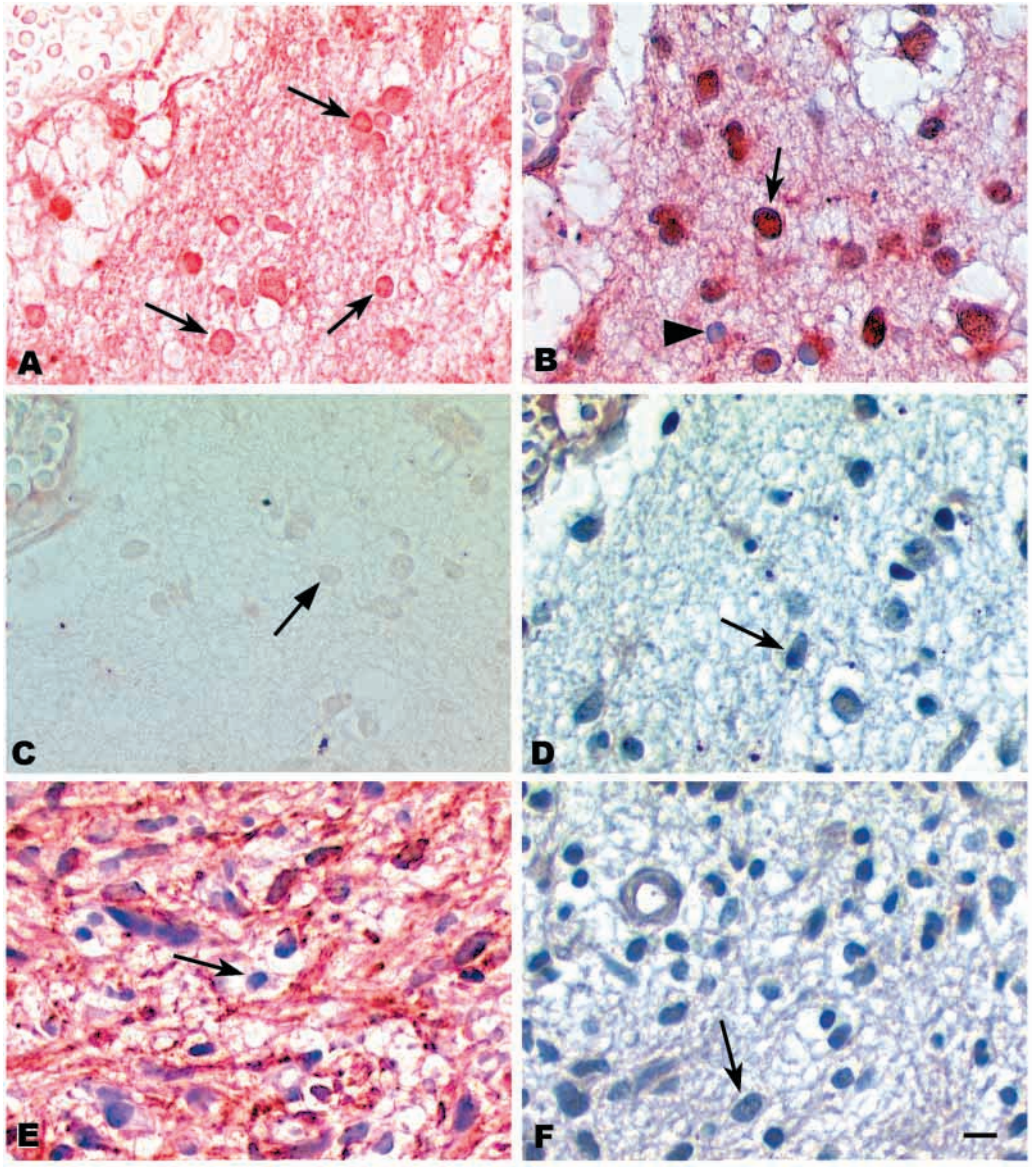

Fig. 1. H-ferritin immunostaining in a human astrocytoma tumors. A total of six different tumors were analyzed. The sections in A-D represent contiguous sections from the same tumor. Note the blood vessel containing red blood cells in the upper left-hand corner for orientation. (A) H-ferritin was detected with the HS-59 monoclonal mouse anti-human rH-ferritin antibody (red immunoreaction product). The arrows indicate examples of positive cell nuclei. (B) A contiguous section was reacted for H-ferritin (red) and a blue counterstain was applied to show the presence of cell nuclei. An example of a ferritin positive nucleus is indicated by the arrow. A non-ferritin positive nucleus is indicated by the arrowhead and appears blue. (C) This section was stained for Mouse IgG as a nonspecific control for the secondary antibody. No staining is visible, although nuclei can be seen in the field (arrow). (D) This section was counterstained to reveal the cell nuclei (blue, arrow) after incubation with mouse IgG. (E) This section represents GFAP staining in the human astrocytoma. Intermediate (GFAP-positive) filaments are seen coursing throughout the cytoplasm (red). The section was counterstained to visualize the nuclei that appear blue (arrow). The staining with GFAP represents a control for nuclear ferritin staining with a different human monoclonal antibody that would stain cellular features of the astrocytoma. (F) An immunoreaction for neurofilament was performed on the astrocytoma as an irrelevant control monoclonal antibody. There is no red reaction product for neurofilaments. The section was counterstained blue to reveal nuclei (arrow). Bar, 10.8 μm.

Immunohistochemical analysis of ferritin nuclear localization in cell culture

A cell culture model using the human astrocytoma SW1088 cell line was established to manipulate ferritin experimentally in the nucleus. To establish the fundamental conditions under which ferritin is present in the nucleus, the cells were first examined immunohistochemically. Human astrocytoma cells (SW1088) stained with anti-human rH-ferritin monoclonal antibody display intense immunohistochemical nuclear staining and light cytoplasmic staining (Fig. 2A). Staining was not detected in control sections (data not shown). The cultures were triple stained with rH-ferritin, DAPI and BrdU. The triple stain confirmed the presence of ferritin in the cell nuclei (Fig. 2A,B) and also indicated that ferritin was present in the nucleus of both BrdU-positive and BrdU-negative cells (compare Fig. 2C,D), but is not associated with condensed chromatin of actively mitotic cells (Fig. 2E,F). Almost all

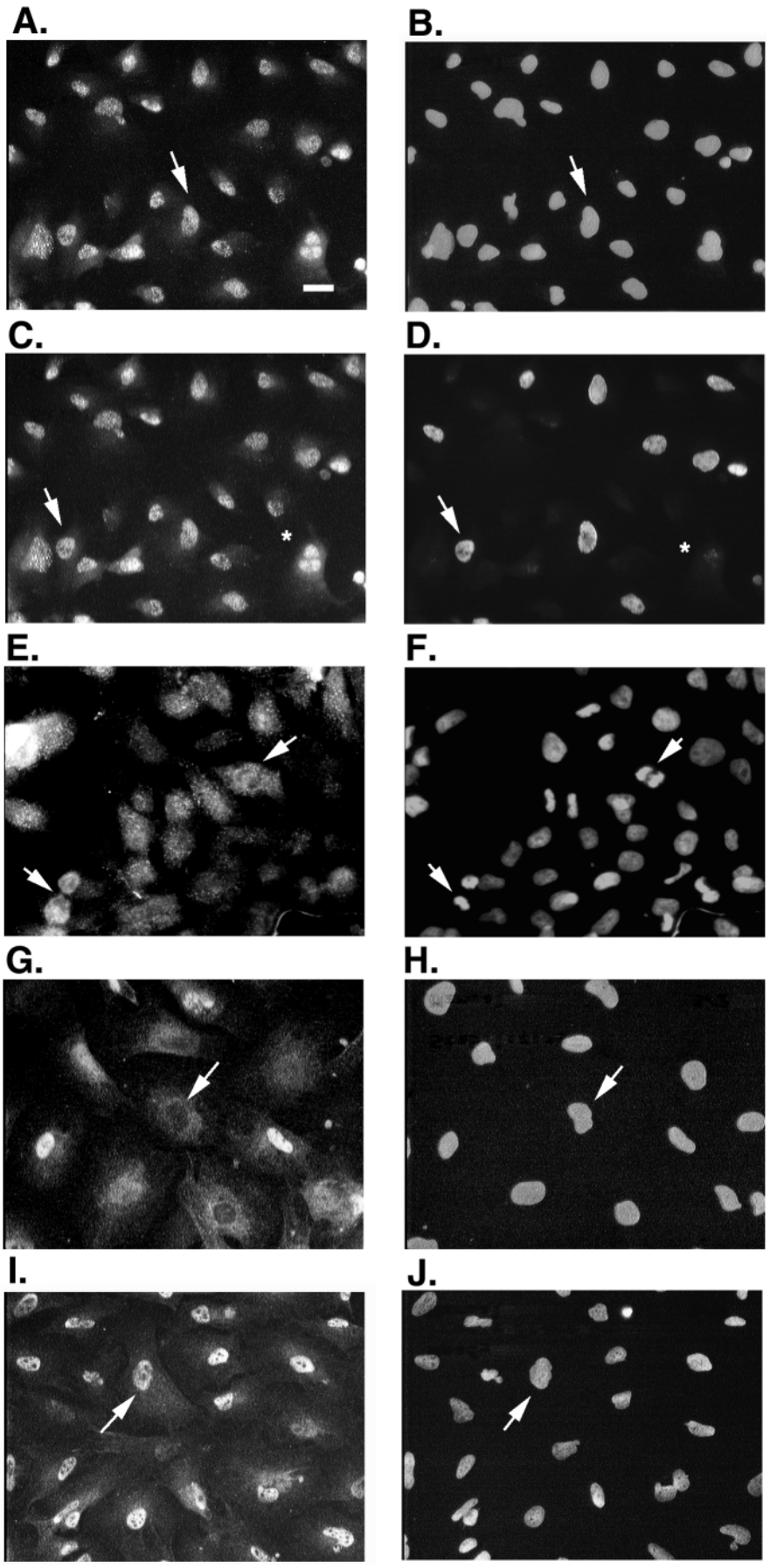

Fig. 2. H-ferritin immunostaining in SW1088 human astrocytoma cells is not regulated by DNA synthesis but does change in response to iron chelation. H-ferritin was detected with the HO2 monoclonal mouse anti-human rH-ferritin antibody (A,C,E,G,I). The sections were co-localized with DAPI in order to visualize the nucleus (B,F,H,J) or BrdU for DNA synthesis (D). (A-D) Cells that have been triple-stained for H-ferritin (A,C), DAPI (B) and BrdU incorporation (D). (A,B) H-ferritin is detected in the nucleus of astrocytoma cells grown in standard media. The arrow indicates an example of a single cell nucleus. The cell nuclei from A are shown in B using DAPI. The arrow indicates the same cell in A and B. (C,D) A is reproduced as C in order to compare H-ferritin staining with that of BrdU (D). H-ferritin is found in the nuclei of both BrdU and non-BrdU positive cells. (D) The arrows in C,D indicate the nucleus of the same cell that is both H-ferritin and BrdU positive. The asterisk near the cell in C indicates a cell that has H-ferritin in the nucleus but is not BrdU positive. (E,F) H-ferritin is detected in the nucleus and cytoplasm of astrocytoma cells (E) but is not localized to the nucleus in dividing cells (F). The DAPI stain in F reveals two cells (arrows) that are dividing and the area containing the chromosomes is not visible in those cells in E. (G,H) H-ferritin is only cytoplasmic in most (>85%) DFO-treated cells. The nuclei of the cells in G are demonstrated using DAPI (H) and the arrows depict a cell in which nuclear ferritin is not detected. (I,J) H-ferritin is detected in the nucleus after being returned to standard media after DFO treatment (I). The arrows indicate the nucleus of a cell that is stained for H-ferritin (I) and DAPI (J). Bar, 10 μm.

of the cells observed in these cultures were H-ferritin nuclear positive and 50% of these cells are BrdU positive. Treatment with 100 μM DFO routinely results in loss of detectable nuclear staining of ferritin in at least 80% of the cells (Fig. 2G,H). The ferritin nuclear immunostaining reappears when normal medium is reapplied (Fig. 2I,J). The Live/Dead cell death assay (Molecular Probes) demonstrated that 95% of the cells were viable after 72 hours of DFO treatment (data not shown).

Fluctuations in nuclear ferritin concentrations in response to iron, H_2O_2 and cytokines

The results reported in Fig. 2 identified a treatment paradigm wherein ferritin levels in cell nuclei appear to increase or decrease based on a non-quantitative immunohistochemical detection method. With this basic model established, the next set of experiments were designed to test the hypothesis that the amount of ferritin found in the nucleus could be altered by changing culture conditions. To test this hypothesis, astrocytoma cells were plated in DFO to remove ferritin from the nucleus. The cells were then exposed to standard medium (control) or media supplemented with FAC, TNFα, IL-1β or H_2O_2. Addition of FAC in the media resulted in a significant increase in ferritin protein levels over control in both the cytosolic and nuclear extracts at each FAC concentration (Fig. 3A). The cytokines TNFα and IL-1β are known to increase ferritin levels in cells (Fahmy and Young, 1993; Kwak et al., 1995; Miller et al., 1991; Torti and Torti, 1994) and were used to determine how increases in ferritin not directly related to iron would alter nuclear ferritin levels. Treatment with either 10 or 50 ng/ml TNFα resulted in a significant increase in nuclear but a decrease in cytosolic ferritin levels (Fig. 3B). IL-1β treatment, in general had less of an effect than treatment with TNFα, but did result in a slight (but statistically significant) increase in nuclear ferritin at 50 U/ml (Fig. 3C). However, IL-1β treatment at greater concentrations resulted in slightly but significantly decreased nuclear ferritin levels compared with control, without changing the cytosolic ferritin levels (Fig. 3C). Astrocytoma cells exposed to hydrogen peroxide (Fig. 3D) had significantly higher ferritin protein levels in the nucleus at 100 μM H_2O_2 when compared with control nuclear levels. Cytosolic levels did not vary from control levels at any H_2O_2 concentration.

Demonstration of ferritin nuclear translocation

To demonstrate directly that ferritin enters the nucleus, FITC-conjugated ferritin was added to digitonin permeabilized cells. A TRITC-dextran complex (70 kDa) was one approach used to demonstrate that digitonin permeabilization did not affect the integrity of the nuclear membrane (Fig. 4A). FITC-rH ferritin did not enter the nuclei of cells in standard culture conditions but, rather, remained in the cytoplasm even after 60 minutes of exposure (Fig. 4B). If cells were first treated with deferoxamine, FITC-rH-ferritin was found in their nuclei within 60 minutes of exposure (Fig. 4C). Translocation of ferritin to the nucleus did not occur in the presence of iron saturated DFO (Fig. 4D). BSA-FITC (Fig. 4E) and Tf-FITC

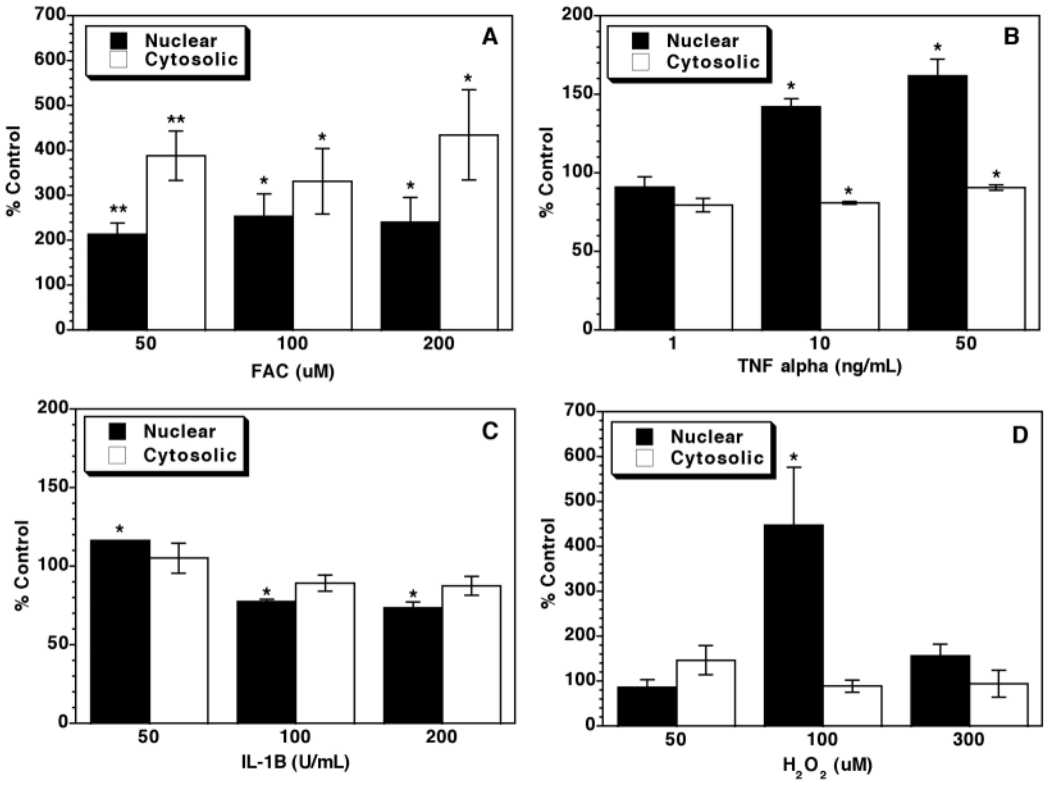

Fig. 3. Ferric ammonium citrate, cytokines and hydrogen peroxide affect ferritin nuclear concentrations in SW1088 human astrocytoma cells. SW1088 astrocytoma cells were treated with deferoxamine and then placed in standard media (control) or standard media, including varying concentrations of ferric ammonium citrate (FAC), tumor necrosis factor α (TNFα), interleukin 1β (IL-1β) or hydrogen peroxide (H_2O_2). Nuclear and cytosolic extracts were isolated and ferritin levels were determined using the HO2 monoclonal mouse anti-human rH-ferritin antibody. Ferritin levels are expressed as a percentage of control values. The concentration of the experimental factors is shown at the bottom of each graph. (A) Nuclear and cytosolic ferritin protein levels both increase in response to FAC exposure, but the increase is not concentration dependent. (B) Nuclear ferritin levels are increased in response to TNFα, but cytosolic levels are decreased when compared with control at each concentration. (C) Nuclear ferritin levels increase at the lowest dose of IL1β, but decrease at the higher doses. The cytosolic levels of ferritin are not different from control. (D) Nuclear ferritin levels increase in response to H_2O_2 only at 100 μM H_2O_2, while cytosolic levels remain constant. The asterisks indicate a statistically significant difference from control values. $^{**}P<0.001$, $^{*}P<0.05$.

(data not shown) conjugates were also used as controls in this study. Both of these proteins enter the digitonin permeabilized cells but both remained within the cytoplasm. These proteins provide additional evidence that the digitonin treatment did not affect the integrity of the nuclear membrane and further suggest specificity of ferritin translocation. The specificity of ferritin uptake was further examined using rL-ferritin and the ferroxidase mutant rH-ferritin 222, respectively, under the same conditions as in Fig. 4C. The FITC-222 ferroxidase mutant entered cell nuclei (Fig. 4F), suggesting the iron-binding status of ferritin may not be a factor in ferritin uptake because the ability of the 222 mutant to store iron is compromised (Levi et al., 1988). FITC-rL ferritin entered the cell but not the nucleus (Fig. 4G). The failure of the rL-ferritin conjugate to enter the nucleus indicates subunit preference for uptake and is a further control that demonstrates the integrity of the nuclear membrane. As an additional control for the uptake studies that have to this point used permeabilized cells, we transfected astrocytoma cells with Myc epitope-tagged rH-ferritin and detected Myc-tagged H-ferritin in the cell nuclei and cytoplasm (Fig. 4H).

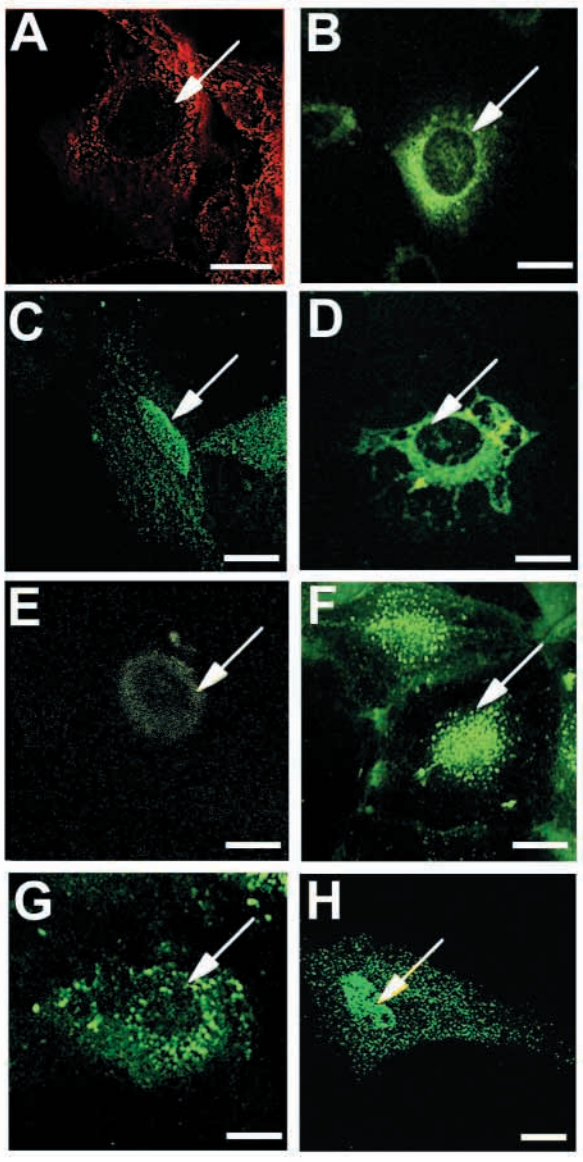

Fig. 4. Ferritin nuclear translocation in SW1088 human astrocytoma cells responds to iron chelation treatment and is ferritin-subunit specific. These are a series of confocal images that are shown for each condition as a representative of the entire cell population. Each experiment was repeated three times and random cells chosen. The arrow in each panel points to the cell nucleus. Fluorophore-labeled proteins (5 μM) were added to permeabilized cells for 60 minutes prior to visualization. (A) TRITC-dextran (70,000 kDa) enters the cells but does not translocate to the nucleus, indicating that the nuclear membrane remained intact following digitonin treatment. (B) FITC-rH ferritin enters the cells but is not imported into the nucleus of cells plated in standard culture conditions. (C) If cells are pretreated with deferoxamine and then returned to standard medium containing FITC rH-ferritin, the labeled ferritin translocates to the nucleus. (D) The combination of DFO and ferric ammonium citrate (FAC) results in the formation of ferrioxamine B that limits the chelating ability of DFO. Under this condition, FITC rH-ferritin does not translocate to the nucleus but remains cytoplasmic. (E) FITC-labeled BSA was added to the standard media following DFO pretreatment (same condition as in B) as a control for the selectivity of the uptake mechanism and to show the nuclear membrane is still intact following permeabilization. FITC-BSA enters the cell but does not enter the nucleus. (F) The 222 mutant, an H-ferritin that lacks a ferroxidase center enters the cell nuclei (two cells are shown in this micrograph, one is indicated by the arrow) of DFO-treated cells indicating the iron status of ferritin is not a factor in the nuclear uptake. (G) To determine if there is a preferred subunit type of ferritin that is translocated to the nucleus, FITC rL-ferritin was added to the medium after pretreatment with DFO. This subunit of ferritin does not translocate to the nucleus under these conditions, indicating there is a preference for H-rich ferritin and also demonstrating the integrity of the nuclear membrane after digitonin permeabilization. (A-G) Exogenously applied ferritin. In H, an example of a cell transfected with Myc-tagged ferritin is shown. The epitope tagged ferritin is found in the nucleus of SW1088 cells. These data illustrate that endogenously expressed tagged H-ferritin can translocate to the nucleus. Bars, 7 μm.

Mechanisms controlling ferritin nuclear import

Having demonstrated ferritin translocation to the nucleus, the next set of experiments were designed to test the hypothesis that ferritin uptake into the nucleus is active rather than passive. The combination of DFO pretreatment and digitonin was used in these experiments. FITC-rH ferritin was imported to the nucleus in DFO-treated permeabilized cells (Fig. 4C, Fig. 5A). In the presence of WGA, which blocks import through nuclear pores, FITC-rH ferritin accumulates in the cytoplasm, near the nuclear envelope, but does not translocate to the nucleus (Fig. 5B). To determine if nuclear import of FITC-ferritin is energy dependent, uptake studies were performed at 4°C on DFO-treated, digitonin permeabilized cells (already described). At this temperature, nuclear transport of ferritin was markedly reduced compared with controls (37°C) (Fig. 5C). Nuclear uptake of ferritin was also decreased in the presence of apyrase, an ATP-hydrolyzing enzyme (Fig. 5D). In the presence of an ATP regeneration system, nuclear uptake of ferritin was re-established (Fig. 5E).

These experiments indicated that the uptake of ferritin was active and occurred via the nuclear pore. However, those experiments did not address whether cytosolic factors were

required for ferritin nuclear translocation. Consequently, cell cultures were exposed to NEM which inactivates nuclear import of NLS-bearing proteins (Adam et al., 1990; Duverger et al., 1995). NEM did not inhibit translocation of ferritin into the nucleus (Fig. 5F).

Ferritin/DNA interactions in cultured SW108 cells

Having established ferritin translocation to the nucleus is regulated and characterized the import mechanism, we designed experiments to test the hypothesis that ferritin in the nucleus interacts with DNA. Formaldehyde crosslinking techniques were used to test the hypothesis that ferritin binds DNA in cell culture (Solomon et al., 1988). Astrocytoma cell cultures were DFO treated and the cells were permeabilized with digitonin before adding ^{125}I-rH ferritin to the culture medium. Control cultures were not treated with DFO. Protein-DNA complexes were isolated by precipitation of DNA. ^{125}I-labeled ferritin crosslinked to DNA. DFO pretreatment increased the amount of ferritin that was crosslinked (Fig. 6A,B). A group of control and DFO-treated cells subjected to ^{125}I-rH-ferritin but not exposed to formaldehyde showed very low to no detectable ferritin associated with the DNA (Fig. 6C).

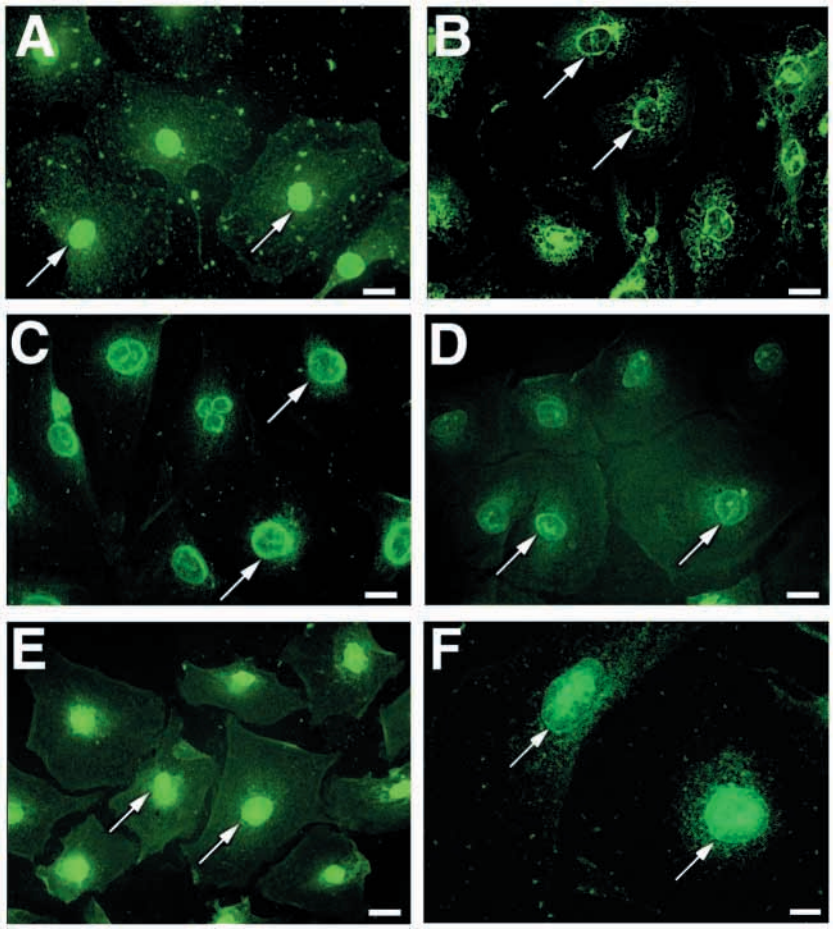

Fig. 5. Mechanism of nuclear translocation for FITC labeled rH-ferritin. SW1088 cells were pretreated with deferoxamine for 72 hours followed by digitonin treatment. The cells were then exposed to treatments that affect nuclear translocation of proteins in the presence of FITC-conjugated H-ferritin. The cells in the micrographs are representative of the general population. The arrows indicate the nuclei of individual cells. Each experiment was performed in triplicate. (A) As previously established, FITC-labeled rH-ferritin translocates to the nucleus. (B) Treatment of the cells with wheat germ agglutinin (WGA), to block the nuclear pore complex, blocks nuclear translocation of FITC-labeled recombinant H-ferritin, which collects at the perinuclear membrane. (C) Incubation of cells with FITC-labeled recombinant H-ferritin at 4°C decreased the appearance of the ferritin in cell nuclei. FITC-labeled rH-ferritin collects at the perinuclear membrane. (D) Depletion of ATP in the cells with apyrase results in a decreased appearance of FITC-labeled recombinant H-ferritin in cell nuclei. FITC-labeled rH-ferritin collects at the perinuclear membrane. (E) Regeneration of ATP following apyrase treatment results in FITC-labeled rH-ferritin nuclear translocation. (F) Exposure of the cells to NEM does not inhibit ferritin nuclear translocation. Two cells are shown in this micrograph with immunolabeled nuclei (arrows). These studies demonstrate that ferritin uptake into the nucleus is via the nuclear pore complex; it requires energy but does not require an NLS bearing cytosolic protein. Bars, 7 μm (A-E); 4 μm (F).

Ferritin protects DNA from iron-induced oxidative damage

The close proximity of nuclear H-ferritin to DNA (Fig. 6), suggests that one possible function of this protein may be a DNA protectant. Many soluble iron species catalyze the formation of hydroxyl radicals, which react readily with nucleic acids, giving a spectrum of products (Henle et al., 1996; Imlay and Linn, 1988). Breakage of the DNA backbone as a consequence of hydroxyl radical attack on deoxyribose moieties (Floyd, 1990; Tachon, 1989) is particularly easy to detect using a superhelical DNA relaxation assay (Tachon, 1989). This assay was used to determine whether ferritin is capable of protecting DNA from iron-catalyzed damage (Fig. 7A). Exposure of a sample of DNA to 10 mM H_2O_2 and 50 μM $FeCl_3$ for 60 minutes at 37°C resulted in the conversion of the form I monomer to form II (relaxed circular DNA); in some cases a small amount of linear (form III) DNA was also produced. DNA damage was only observed when both ferric chloride and hydrogen peroxide were present. Neither reagent alone damaged the DNA to a detectable extent (data not shown). Addition of increasing amounts of recombinant H-ferritin to the reaction mixture, prior to the sequential addition of $FeCl_3$ and H_2O_2, resulted in the preservation of an increasing fraction of supercoiled DNA. The incubation of ferritin and hydrogen peroxide with DNA (in the absence of added $FeCl_3$) resulted in no detectable damage to the DNA (data not shown). A qualitatively similar DNA relaxation was obtained when 200 μM $FeSO_4$ was substituted for the $FeCl_3/H_2O_2$ solution and this relaxation was also prevented by the inclusion of recombinant H-ferritin in the reaction mixture (data not shown). An additional observation in these studies is that the electrophoretic mobilities of the protected DNAs were reduced

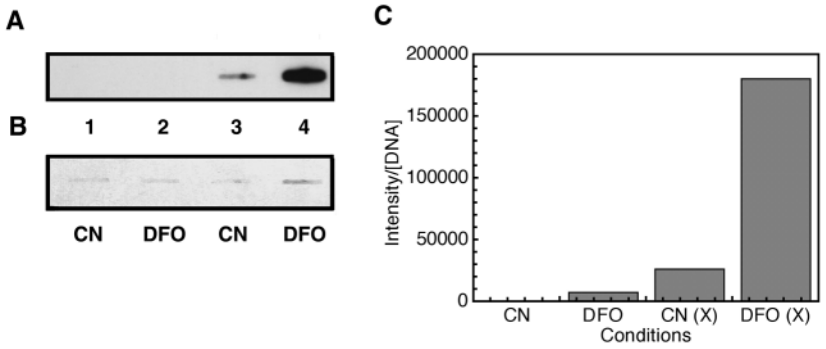

Fig. 6. ^{125}I-rH-ferritin crosslinks to DNA in whole cells. ^{125}I-rH ferritin was incubated with SW1088 astrocytoma cells grown in standard, untreated medium as a control (CN) or in the presence of 100 μM deferoxamine (DFO). (A) One group of cells from both CN and DFO conditions were placed in 1% formaldehyde to crosslink DNA-protein complexes (lanes 3 and 4). The other group was not subjected to crosslinking (lanes 1 and 2). (B) The membranes containing the crosslinked complexes were incubated with a rH-ferritin-specific antibody to demonstrate that the crosslinked protein was ferritin. Lane 1, CN; lane 2, DFO; lane 3, CN crosslinked; lane 4, DFO crosslinked. (C) The numerical values obtained from the densitometric analysis of each lane in A are shown. The data shown in the figure are representative of three different experiments. ^{125}I-rH ferritin-DNA complexes were identified in CN and DFO crosslinked conditions with the greatest level found in the DFO group.

in a ferritin concentration-dependent manner (Fig. 7A, lane 7). Such mobility shifts are consistent with the formation of protein-DNA complexes (Fried and Crothers, 1981; Fried and Garner, 1997).

To determine whether the ability of ferritin to protect DNA from iron-induced oxidative damage is related to its ability to bind iron, supercoil relaxation experiments were carried out with recombinant H-ferritin, recombinant 222 ferritin, horse spleen apoferritin (Sigma; ~84% L-subunit, iron poor) and several non-ferritin proteins (Fig. 7B). All ferritins protected DNA to some degree, although ferritins with reduced ferroxidase activity (the L-subunit-rich spleen apoferritin and the mutant 222 ferritin) were significantly less effective. Transferrin was also tested in this assay at concentrations (10-90 μM) that were more than adequate to bind the 50 μM iron present in the reaction (Fig. 7B). Transferrin did not offer DNA protection at any concentration tested. In addition, bovine serum albumin, chymotrypsin and ovalbumin did not significantly inhibit iron/H_2O_2-induced DNA damage. The addition of non-ferritin proteins in this assay demonstrated that not only was the iron-binding capacity of ferritin required for DNA protection, but protein mass was not responsible for the DNA protection that was observed. Because the non-ferritin proteins used as controls do not bind DNA, we also used a DNA-binding protein, *E. coli* cAMP receptor protein (CAP), as a control. No more than 30% of the DNA was protected by CAP at concentrations sufficient to coat the DNA (>2 μM), whereas a similar concentration of ferritin afforded nearly 100% protection (Fig. 7B). Finally, DNA protection requires incubation of ferritin with $FeCl_3$ for at least 15 minutes, prior to addition of H_2O_2. If the two reagents are added simultaneously, no reduction in DNA damage is observed (data not shown). Taken together, these results support the notion that ferritin protects DNA and that the degree of protection is at least qualitatively related to the iron-binding activity of the ferritin.

Ferritin protects DNA in live SW1088 cells

We attempted to develop a cell culture model to test the ability of ferritin to protect DNA by taking advantage of our observation that ferritin can be removed from the cell nucleus with deferoxamine treatment and that translocation of ferritin into the nucleus can be blocked by treatment with wheat germ

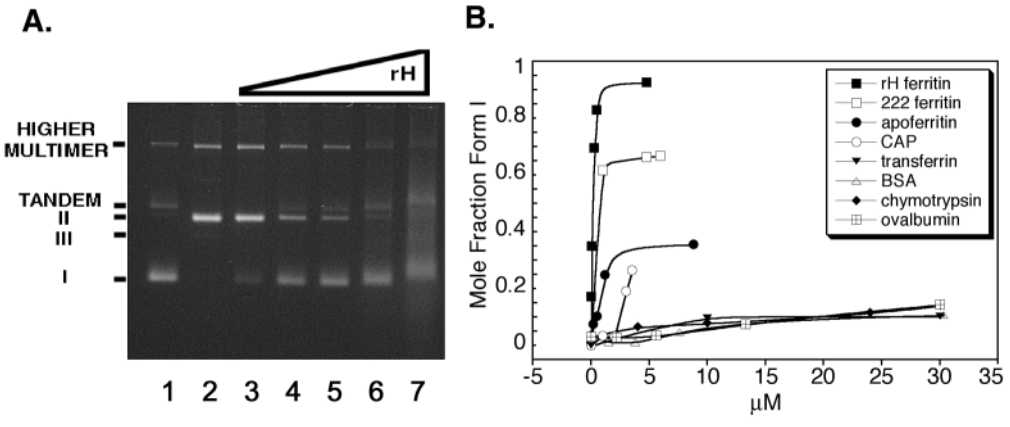

Fig. 7. Ferritin protects DNA from iron-induced oxidative damage, in vitro. (A) Electrophoretic supercoil relaxation assay. The positions to which supercoiled (I), relaxed circular (II) and linear (III) monomeric DNAs migrated are indicated in the on the left. Reaction mixtures contained supercoiled pUC19 DNA in the following conditions: lane 1, DNA alone; lanes 2-7, DNA plus 50 μM $FeCl_3$ and 10 mM H_2O_2; lanes 3-7, recombinant H-ferritin at increasing concentrations (95 nM, 240 nM, 475 nM, 5 μM and 14.9 μM) in addition to the 50 μM $FeCl_3$ and 10 mM H_2O_2. (B) Graph of the mole fraction of form I DNA remaining at the end of the reaction as a function of the concentrations of ferritin and non-ferritin proteins. Reactions were performed as described in A, with the following ferritin samples: rH-ferritin (black square); apoferritin ferritin (black circle); recombinant 222 mutant ferritin (white square); and transferrin (black triangle). The CAP protein (white circle) was used in place of ferritin as a control for DNA-binding proteins. As controls for nonspecific protein effects, ferritin was replaced by ovalbumin (crossed white square), bovine serum albumin (white triangle) or chymotrypsin (black diamond) in duplicate reactions. All experiments were repeated three times. These data demonstrate that ferritin will protect DNA from iron-induced oxidative damage.

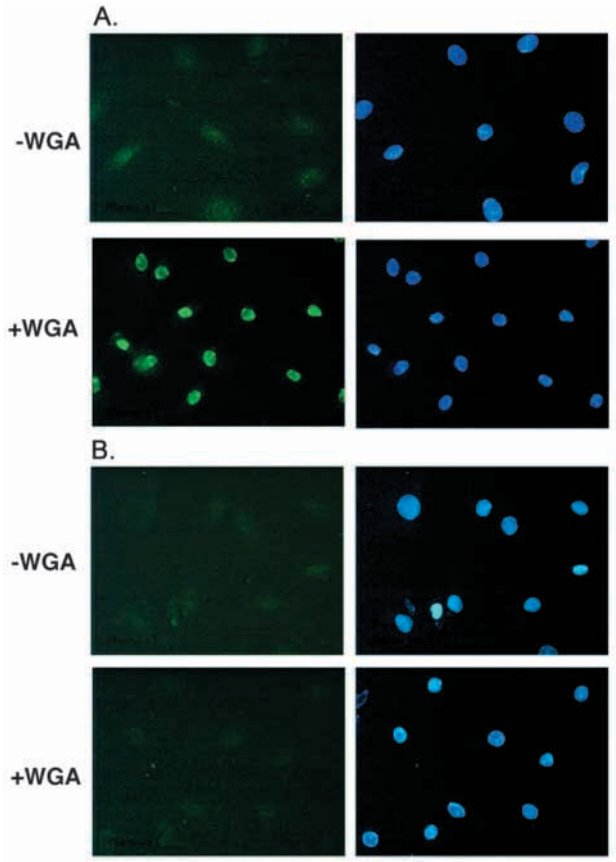

Fig. 8. Cell culture model to study ferritin and DNA damage. To demonstrate that SW1088 astrocytoma cells in culture are susceptible to DNA damage when nuclear ferritin is not present, we used the following model. Intracellular ferritin was depleted by deferoxamine and then the cells were reincubated with standard media, which results in a reappearance of ferritin in the nucleus. However, one group of cells was exposed to wheat germ agglutinin (WGA), to block the translocation of ferritin to the nucleus, and the other group was not exposed to WGA. Both sets of SW1088 cells were treated with digitonin, which is necessary to allow WGA to enter the cells. DNA strand breaks are detected using the TUNEL assay (green; Boehringer Mannheim). DAPI staining (blue) was used to locate cell nuclei. The same microscopic field is represented in both right and left panels. (A) In this experiment, 100 μM H_2O_2 was added to the reincubation media after DFO pretreatment. If WGA was included (+WGA) in the reincubation media (to block ferritin translocation to the nucleus), most of the cells became TUNEL positive (green). If WGA was excluded from the reincubation media (–WGA) (permitting ferritin translocation to the nucleus), no TUNEL-positive cells were present. (B) In this experiment, ferric ammonium citrate (100 μM) was added to the reincubation media. There were no TUNEL-positive cells regardless of whether or not WGA was absent (–WGA) or present (+WGA) in the reincubation media. The micrographs have been chosen as representative. Experiments were repeated three times each.

agglutinin (WGA). In this model, astrocytoma cells were treated with DFO followed by digitonin treatment to decrease ferritin expression in the cells, as described earlier. The DFO-containing medium was then replaced with media containing WGA, followed by either 100 μM H_2O_2 or 100 μM FAC. FAC and H_2O_2 were used as cell stressors and these concentrations were chosen because they resulted in increased nuclear ferritin concentrations (Fig. 3A,D). The control for this experiment was media that did not contain WGA. The results indicate that when WGA is present in the media (blocking the translocation of nuclear ferritin), there is an increase in the number of TUNEL-positive cells compared with the number found in the non-WGA-treated cultures when the cells are exposed to H_2O_2 (Fig. 8A). The addition of iron (FAC) to the media did not affect the number of TUNEL-positive cells regardless of the presence or absence of WGA (Fig. 8B). Although WGA will block the movement of all actively transported proteins into the nucleus, the data still indicate that those cells that do not have nuclear ferritin are susceptible to DNA damage after hydrogen peroxide treatment.

Discussion

The presence of ferritin in cell nuclei was demonstrated in this study with immunohistochemistry, fluorescent tagging and epitope tagging. NEM, which inhibits translocation of NLS-containing proteins, did not affect ferritin nuclear translocation. Thus, the entry of ferritin into the nucleus is not dependent upon NLS-containing proteins (Duverger et al., 1995). This latter observation is also consistent with sequence data for ferritin (Boyd et al., 1985) and a report that no specific region in ferritin functions as an NLS (Cai and Linsenmayer, 2001). The nuclear import studies indicate a regulated nuclear uptake mechanism that prefers H-rich ferritin, is energy dependent and occurs through the nuclear pore. Once inside the nucleus, crosslinking demonstrates that nuclear ferritin is associated with DNA. In vitro experiments indicate that ferritin protects DNA from iron-induced oxidative damage. Finally, our cell culture model reveals that the amount of ferritin in the nucleus is responsive to iron, H_2O_2 and cytokine exposure.

Because there are specific iron-binding sites on DNA (Henle et al., 1999), a mechanism for iron transport into the nucleus, presumably involving a protein (because ‘free iron’ is not thought to be present in biological systems), must exist. A mechanism for iron transport into rat liver nuclei has been identified (Gurgueira and Meneghini, 1996) and recently the divalent metal transport protein (DMT1) has been demonstrated in the nucleus of some neural cells (Roth et al., 2000). Lactoferrin in the only other iron-binding protein besides ferritin that has been demonstrated in cell nuclei (Garré et al., 1992; He and Furmanski, 1995). Ferritin is unique because it can play a role in both iron delivery and iron sequestration. In this study we demonstrate that ferritin is present in nuclei of human astrocytoma cells both in tissue and in cell culture. These observations extend the original observations of cells that contain ferritin in the nucleus and expand the notion that ferritin is present in the nucleus to protect cells from u.v. damage (Cai et al., 1998) to the more general concept of protection from iron-induced oxidative damage. Our DNA protection assays are the first to test directly the hypothesis that DNA is protected by ferritin and our studies may suggest a potential role for nuclear ferritin in the growth of tumor cells.

Modulation of relative amounts of ferritin in the nucleus and cytoplasm

The cell culture system was developed to characterize the

conditions under which ferritin was present in the nucleus. Ferritin was present in the astrocytoma cells from the time that they were initially plated. The appearance of ferritin in the nucleus could be demonstrated immunohistochemically, with uptake of exogenously applied fluorescently tagged ferritin and epitope-tagged endogenously synthesized ferritin. The presence of ferritin in the nucleus was not dependent on whether the cells were synthesizing DNA because it is found in the nucleus of both BrdU-positive and -negative cells. However, ferritin is not present in cells that were actively dividing. Iron chelation with DFO resulted in loss of ferritin from the nucleus to below immunodetectable levels. The crosslinking studies indicate that ferritin is associated with DNA. The amount of ferritin associated with DNA can be increased if the cells are first treated with DFO and then exposed to control media, which is consistent with the quantitative and fluorescently labeled ferritin uptake studies. These studies show that the presence of ferritin in the nucleus is not dependent on the state of the cell's growth with respect to cell division and proliferation, but is dependent upon iron availability

To determine if iron is the sole modulator of the amount of ferritin in the nucleus, we exposed cells to peroxide and cytokines as forms of biological stress. Our results show that stress-induced changes in the relative concentrations of ferritin are most evident in nuclei, and less evident in cytoplasm. The increase in nuclear ferritin with 100 μM H_2O_2 correlates well with the finding of maximal DNA nicking at this concentration of peroxide (Luo et al., 1994). These results support our hypothesis that the increase of ferritin in the nucleus is an attempt to sequester iron and prevent DNA-Fe^{2+} interactions with H_2O_2 that can result in double strand breaks and cell death (Stevens and Kalkwarf, 1990). At concentrations of H_2O_2 higher than 100 μM (Luo et al., 1994), there was decreased DNA damage and we found no significant increase in ferritin (nuclear or cytoplasmic) over control levels. These latter results suggest that cell damage at this concentration of peroxide is not occurring within the nucleus and thus the lack of DNA damage and lack of nuclear ferritin response are consistent.

Cytokine exposure can also be a form of stress to a cell and TNFα and IL-1β have been shown to increase H-ferritin biosynthesis selectively relative to L-ferritin (Fahmy and Young, 1993; Kwak et al., 1995; Miller et al., 1991; Torti and Torti, 1994). These cytokines induced significant changes in nuclear levels of ferritin. The lowest concentration of IL-1β resulted in a small, but significant increase in nuclear ferritin, whereas the two higher concentrations decreased nuclear ferritin levels. The two higher concentrations of TNFα resulted in a significant increase in nuclear ferritin and a decrease in cytosolic ferritin. These data indicate that the amount of ferritin translocated to the nucleus depends on the nature of the stressor. Even small alterations in ferritin levels may have a large physiological impact, as one ferritin molecule has the capacity to take up 4500 atoms of iron.

Ferritin nuclear translocation

Having established that the concentration of ferritin in the nucleus could be manipulated, studies were undertaken to examine the mechanism by which ferritin entered the nucleus. Permeabililzation of the cells with digitonin was necessary for exogenous ferritin to enter the cell. The permeabilization step was also necessary for WGA, the nuclear pore inhibitor, to enter the cell. Several controls, such as dextran, transferrin, BSA and even the L-rich subunit of ferritin were included to demonstrate that permeabilization with digitonin did not alter the integrity of the nuclear membrane. The inclusion of L-rich ferritin not only supported the data that the nuclear membrane was still intact in our studies, but also revealed that there is selective ferritin subunit transport into the nucleus. The H-ferritin mutant 222, which is structurally similar to H-chain ferritin but lacks a ferroxidase center (Levi et al., 1988), is also translocated. This latter result suggests that selected ferritin uptake may be due to protein sequence or structure and not iron uptake efficiency of the ferritin. In addition to the exogenously applied labeled proteins, a Myc epitope-tagged ferritin was also translocated to the cell nucleus, indicating that translocation of ferritin to the nucleus is a normal process of viable cells.

The nuclear pore complex (NPC) provides the sole avenue for macromolecular transport between the nucleus and cytoplasm (Davis, 1995). The movement of ferritin from the cytoplasm to the nucleus could occur either by passive diffusion or active transport through the NPC. The preference for H-ferritin subunit for transport suggests that ferritin transport into the nucleus is selective. Such selectivity is a hallmark of a facilitated process, as opposed to passive diffusion. The mechanism of pore-mediated protein import requires two steps: docking and translocation. The docking of the import complex [nuclear-targeted proteins and NPC receptors (importins)] can be blocked by the addition of wheat germ agglutinin (WGA), while the translocation process can be inhibited by ATP depletion and temperature changes (Adam and Adam, 1994; Newmeyer et al., 1986; Richardson et al., 1988). H-ferritin nuclear entry was blocked by WGA and translocation of H-ferritin into the nucleus was blocked at 4°C and by depletion of ATP. Combined, these results are consistent with models in which H-ferritin is actively transported into the nucleus through the NPC. The inability of NEM to inhibit ferritin translocation to the nucleus indicates that a NLS bearing cytosolic factor is not involved in translocating ferritin into the nucleus (Duverger et al., 1995).

DNA protection by ferritin

The ability of ferritin to sequester iron may allow it to protect DNA from iron-induced oxidative damage. The supercoil assay results show that ferritin can act on iron to reduce its ability to catalyze oxidative damage, even when DNA is present as a competing ligand for iron. Addition of other proteins, including CAP (at concentrations that coat DNA) and transferrin (at concentrations to bind the iron in the system), did not protect DNA to the same extent as ferritin. Thus, coating the DNA, as a large protein like ferritin might do to some extent, is not an effective protection mechanism. The conclusions from the transferrin results suggest that the iron bound to transferrin may still be available to peroxide for redox reactions and/or that some affinity of the protein for DNA is necessary for protection. Our crosslinking studies show that ferritin interacts with DNA. A report exists that nuclear ferritin binds a region of the β-globin promoter (Broyles et al., 2001; Pountney et al., 1999). The order of addition experiments indicate that the

ferritin-iron interactions require time to complete, as DNA protection requires pre-incubation of ferritin with $FeCl_3$ prior to the addition of H_2O_2. Several previous studies have implicated ferritin as a source of hydroxyl radical-production, either through the oxidation of iron after sequestration, or after iron release from the molecule (Reif, 1992; Reif et al., 1988; Samokyszyn et al., 1988). However, the absence of DNA cleavage in reaction mixtures containing ferritin, DNA and H_2O_2, but not $FeCl_3$, described above, is most consistent with the notion that on interaction with ferritin, the iron becomes unavailable to participate in H_2O_2-dependent DNA cleavage. In addition, the absence of DNA cleavage in reaction mixtures containing ferritin, DNA, $FeCl_3$ but not H_2O_2, suggest that the reactions associated with iron uptake by ferritin do not damage the DNA. The increased DNA damage seen in the WGA-treated cells in response to hydrogen peroxide (Fig. 8A) further supports the concept that ferritin protects DNA, and extends our in vitro findings to a cell culture system. That induction of DNA damage requires peroxide is to be expected, as significant damage to the cell would not occur solely in the presence of an iron source without an oxidative stressor. In a previous report, we have shown that iron loading of astrocytes in culture does not induce damage in the absence of peroxide (Robb and Connor, 1998). These results are consistent with the idea that ferritin in the nucleus is associated with protection of DNA. A caveat in the cell culture model is that translocation of all nuclear-targeted factors that require active transport are inhibited by WGA. However, the deferoxamine pre-treatment that was required to decrease nuclear ferritin to below detectable levels so that the protection studies could be initiated should affect primarily only proteins transcriptionally and translationally regulated by iron. Thus, while the cell culture experiments do not provide direct evidence of DNA protection by ferritin, the experiment was designed so that ferritin is absent at times when DNA damage is induced. Taken together, these data strongly suggest that nuclear ferritin acts as a DNA protectant.

Conclusions

The presence of ferritin in cell nuclei after elevated iron or biological stress is consistent with the concept that ferritin is an acute phase response protein and extends this notion to the nucleus (Theil, 1987). In this study, ferritin was present in nuclei of both human astrocytomas and a human astrocytoma cell line, suggesting a relationship between nuclear ferritin, iron and tumorigenesis. Nuclear ferritin could serve dual functions: both protecting DNA and serving as iron source for growth. Iron is thought to be a carcinogenic factor because of its ability to initiate free-radical activation and promote cell growth (Deugnier et al., 1998). Iron has been shown to enhance human hepatoma proliferation (Hann et al., 1990) and iron chelation will block G_1 phase in human neuroblastoma cells (Brodie et al., 1993). Iron depletion has been tested clinically as a means to treat neuroblastomas (Donfrancesco et al., 1990), neonatal acute leukemia (Estrov et al., 1987) and Hodgkins disease (Vriesendorp et al., 1991), and specific intracellular iron chelators are being developed as potential cancer therapies (Torti et al., 1998). Perhaps a better understanding of the role of ferritin in the nucleus may be useful in elucidating the role of iron in tumorigenesis.

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