Accumulation of Iron by Primary Rat Hepatocytes in Long-Term Culture

Changes in Nuclear Shape Mediated by Non-Transferrin-Bound Forms of Iron

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We have previously shown that hepatocytes in long-term dimethylsulfoxide (DMSO) culture, fed a chemically defined medium, are highly differentiated and an excellent *in vitro* model of adult liver. Hepatocytes in long-term DMSO culture can be iron loaded by exposure to non-transferrin-bound iron (NTBI) in the form of ferrous sulfate (FeSO$_4$), ferric nitrilotriacetate, or trimethylhexanoyl (TMH)-ferrocene. Holotransferrin, at equivalent times and concentrations, was unable to load hepatocytes. Of the iron compounds tested, TMH-ferrocene most accurately simulated the morphological features of iron-loaded hepatocytes *in vivo*. When exposed to 25 μmol/L TMH-ferrocene, hepatocytes loaded increasing amounts of iron for 2 months before the cells died. When exposed to lower concentrations of TMH-ferrocene (as low as 2.5 μmol/L), hepatocytes continuously loaded iron and remained viable for more than 2 months. The cellular deposition of iron was different in hepatocytes exposed to TMH-ferrocene compared with those exposed to FeSO$_4$; exposure to TMH-ferrocene resulted in the presence of more ferritin cores within lysosomes than were seen with FeSO$_4$. When the concentration of TMH-ferrocene was increased, a greater number of ferritin cores were observed within the lysosome, and total cellular ferritin, as assessed by Western blot, increased. The formation of hemosiderin was also observed. Furthermore, nuclear shape was distorted in iron-loaded hepatocytes. The extent of deviation from circularity in the nucleus correlated with increasing concentrations of TMH-ferrocene and was greater in hepatocytes exposed to FeSO$_4$ than an equivalent concentration of TMH-ferrocene. The deviation from circularity was smallest in hepatocytes that contained well formed ferritin cores and increased in hepatocytes that contained greater amounts of hemosiderin. Furthermore, in hepatocytes treated with FeSO$_4$, a large amount of cell-associated iron was detected but without a significant increase in the total amount of ferritin. The deviation from circularity was the largest in FeSO$_4$-treated hepatocytes, indicating that iron not properly incorporated into ferritin caused more cellular damage. We conclude that iron-loaded hepatocytes in long-term DMSO culture represent a flexible system for studying the effects of chronic iron loading on hepatocytes. *(Am J Pathol 1998, 152:781–792)*

Iron is essential for life, and yet, inorganic unbound iron is toxic for all living cells. Iron transport and storage are regulated by well defined mechanisms.[1] Dietary iron,[2] including iron from the degradation of heme,[3] is normally bound to transferrin.[4,5] Normal systemic transport of dietary iron starts with the binding of iron by transferrin at the luminal surface of the upper gastrointestinal tract.[5,6] Transferrin can bind two molecules of ferric iron; however, only approximately 30% of the total binding capacity of transferrin is used under normal conditions.[4,5] Holotransferrin and, to a lesser degree, monoferric transferrin is bound by the transferrin receptor and released in the lysosomes after receptor-mediated endocytosis.[4] Dietary 3,5,5-trimethylhexanoyl (TMH)-ferrocene does not use the transferrin/transferrin receptor system and gains access to the liver without prior metabolism. However, once inside the hepatocyte, the iron is freed from the cyclopentadiene rings of TMH-ferrocene and incorporated into ferritin.[7] The precise mechanism by which TMH-ferrocene and other non-transferrin-bound iron (NTBI) enters mammalian cells is not fully understood.

Iron loading of whole animals is generally accomplished by means of a dietary iron supplement such as iron carbonyl[8,9] or TMH-ferrocene.[7,10,11] In humans with hemochromatosis, iron overload of the liver and

other organs occurs with a concomitant increase in transferrin saturation.[12] In experimental models of iron overload and in humans with hemochromatosis, the ability of transferrin to accommodate the available iron becomes saturated and NTBI appears in the serum.[13] Because the transferrin/transferrin receptor system is so well controlled via the iron-responsive element (IRE; for reviews see Refs. 5 and 14), it is generally thought that the iron that is the proximate catalyst of cellular damage is NTBI.[14,15] NTBI, which can be transported by specific cellular processes,[16–18] has a strong association with hepatocellular damage in both animal models and humans.[14,19]

Primary rat hepatocytes plated on rat tail collagen-coated plates and maintained in serum-free, chemically defined medium (RPCD) supplemented with 2% dimethylsulfoxide (DMSO), survive, retain hepatocyte morphology, and continue to secrete albumin at high levels for over a year.[20,21] They do not synthesize DNA or proliferate under routine culture conditions. This long-term DMSO-treated hepatocyte culture system has been used to study molecular mechanisms of albumin expression,[20,22] immortalization and transformation of hepatocytes,[23–25] and DNA synthesis.[26] The cells are heterogeneous in size, and both mononucleated and binucleated cells are present in the cultures. Examination at the ultrastructural level showed that the hepatocytes contain large numbers of mitochondria and extensive rough and smooth endoplasmic reticulum, indicating that the cells are intact and metabolically active.[21] Cell-cell junctions with desmosomes are apparent as are numerous bile canaliculi with microvilli. The cells contain regular circular-shaped nuclei with prominent nucleoli and have a low nucleus-to-cytoplasm ratio. In a recent study, we showed that exposure of hepatocytes, in the presence of 2% DMSO, to copper, iron, and zinc induced DNA synthesis and a limited number of rounds of cell replication during the first 40 days in culture without loss of hepatic differentiation.[27] Furthermore, when either 0.75 μmol/L holo-transferrin or 0.75 μmol/L apo-transferrin and $FeSO_4$ were used as the iron source in these studies, the cultures remained free from iron loading as assessed by Perls' Prussian blue staining.[27]

In this study, long-term primary hepatocytes in 2% DMSO-supplemented cultures were used to assess the efficacy of iron loading from various forms of iron; holo-transferrin, TMH-ferrocene, $FeSO_4$, and ferric nitrilotriacetate. This particular culture system allows for the addition of specific forms and concentrations of iron for long periods of time, a feature not available in any other experimental *in vitro* system used for studying iron overload. The immediate goals of this study were 1) to determine which form(s) of iron can be used to most efficiently load hepatocytes, 2) to assess the time and concentration dependence of iron loading, 3) to assess cellular damage of the hepatocytes at the ultrastructural level after long-term exposure to iron, and 4) to assess the physiological relevance of this model in light of previously established models.

Materials and Methods

Materials

Epon 812 was from Tousimis Research Corp. (Rockville, MD). TMH-ferrocene was a gift from Peter Nielsen, Universitätkrankenhaus Eppendorf, Hamburg, Germany. All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Methods

Primary hepatocytes were isolated with collagenase perfusion of adult male Fischer 344 rats as described.[26,28–30] The primary hepatocytes were plated in 60-mm dishes coated with rat tail collagen[21,31] at a density of 1×10^6 cells per 60-mm dish.[20,26] DMSO (2%) and epidermal growth factor (25 ng/ml) were added to the cultures in M6 medium 24 hours after plating when the medium with 3% fetal calf serum was removed.[27]

Histology

Perls' Prussian blue staining was done using a 1:1 (v/v) mixture of 10% potassium ferrocyanide/20% HCl for 15 minutes.[32] The cells were counterstained with 0.1% nuclear fast red for 1 minute. A light counterstain was used so that any faintly positive cells could be visualized more easily.

Electron Microscopy

Primary cultures of rat liver hepatocytes were fixed directly in the tissue culture dish with 1% paraformaldehyde and 0.5% gluteraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.3, for 1 hour at 4°C. The cells were post-fixed in 1% osmium tetroxide plus 1.5% potassium ferrocyanide overnight at 4°C. The cells were then dehydrated in graded ethanols and embedded in Epon 812. Ultrathin (~70 nm) sections were cut from the embedded cells and stained with uranyl acetate and lead citrate before visualization on a transmission electron microscope (Phillips EM400, Eindhoven, The Netherlands).

Determination of Cellular Iron Content

Total cellular iron content was assessed using a Ferrochem II Analyzer (ESA, Chelmsford, MA). Cells were removed from the dishes by scraping in PBS, transferred to an Eppendorf tube, and lysed by sonication. Protein content was assessed on a portion of the cellular sonicate using the bicinchoninic acid assay with bovine serum albumin as the protein standard.[33] Samples containing only PBS and handled in a similar fashion were tested in parallel along with the cellular samples to ensure that no iron contamination was introduced during the harvesting and preparation procedures.

Morphometric Analysis of Nuclear Shape

Electron micrographs (EMs) were digitized by a color flat-bed scanner at 100 dpi and 8 bits (256 shades of gray). Increasing the amount of digitized information by either increasing the resolution and or the color depth did not increase the power of the morphometric data (data not shown). The digitized images were imported into NIH Image (v1.61) software. When possible, only complete nuclei within the EMs were analyzed; however, to maximize the number of measurements from the sections, the perimeter was extrapolated as the most circular path when less than 5% of perimeter was missing. The outer perimeter of the nucleus was traced by hand and bounded by a polygonal region of interest. In this analysis, internal holes were excluded. The circularity (C) of the nucleus was calculated using the following formula:

$$C = \frac{Area_{meas}}{\Pi\left(\frac{Per_{meas}}{2\Pi}\right)^2}$$

where Per_{meas} is the outer perimeter of the region of interest and $Area_{meas}$ is the area of the region of interest. The nuclear roundness factor (NRF) was calculated using the described method[34,35]:

$$NRF = \frac{R_{meas}}{r_{area}} = \frac{\frac{Per_{meas}}{2\Pi}}{\sqrt{\frac{Area_{meas}}{\Pi}}}$$

At least nine nuclei were analyzed for each separate treatment. No attempt was made to convert the sizes to microns, as the absolute size of a nucleus in an EM is dependent on the section analyzed and could be subject to overinterpretation.

Western Blotting

Protein samples were total cellular sonicates harvested as described for the iron assay. The samples were placed immediately into Laemmli sample buffer[36] (4X) and stored at −20°C until separation by SDS-polyacrylamide gel electrophoresis. Total protein (25 μg) was loaded into each lane and separated on a 15% acrylamide gel using Tris/glycine as a buffer system. Prestained broad-range molecular weight standards (BioRad, Hercules, CA) were included to assess specific protein sizes and the efficiency of electrophoretic transfer. The samples were electrophoretically transferred to a supported nitrocellulose membrane (Micron Separations, Westboro, MA), and ferritin was detected using an anti-human ferritin antibody (Dako, Carpinteria, CA) at a 1:500 dilution. The blot was developed using a peroxidase-conjugated anti-rabbit IgG as a secondary antibody and enhanced chemiluminescence development kit (NEN, Boston, MA).

Statistical Analysis

Statistical analysis was done on a Macintosh computer using JMP 3.02 software (Cary, NC). Analysis of variance and Dunnett's test *versus* control were done as appropriate. The null hypothesis was rejected for $P > 0.05$.

Results

Histochemical Detection of Hemosiderin Formation in Hepatocytes Exposed to Various Forms of Iron

We were interested in determining whether hepatocytes in long-term DMSO culture could be iron loaded and which form of iron most efficiently loads these cells. The basal medium (designated M6 medium) contains 0.75 μmol/L holo-transferrin (>98% Fe saturation). As there are two molar equivalents of iron per mole of transferrin, hepatocytes fed medium supplemented with 0.75 μmol/L holo-transferrin are exposed to 1.5 μmol/L iron. We have recently determined that the properties of hepatocytes in long-term DMSO culture do not change when the cells are fed medium supplemented with holo-transferrin.[27] We tested four forms of iron for their ability to iron load hepatocytes in long-term DMSO culture: holo-transferrin (>98% Fe saturation, hTf), $FeSO_4$ (FS), TMH-ferrocene (TMHF), and ferric nitrilotriacetate (FeNTA). Primary rat hepatocytes were maintained in culture for 8 weeks in M6 medium and exposed to a total of 6.5 μmol/L iron in one of four different forms. Under all four different culture conditions used to attempt to achieve iron loading, 1.5 μmol/L iron was in the form of holo-transferrin. The other 5 μmol/L iron was in the form indicated. At the end of the 8-week period, the cells were fixed with ethanol/acetic acid and stained for iron using the Perls' Prussian blue protocol. No stainable iron was detected in hepatocytes maintained in M6 medium (1.5 μmol/L iron in the form of holo-transferrin) even after 2 months in culture.[27] Similarly, no positively stained cells were detected in hepatocytes maintained for 8 weeks when the holo-transferrin concentration was raised to 6.5 μmol/L (Figure 1, hTf). These findings were confirmed by an independent experiment showing that hepatocytes positive for Perls' staining were not observed when hepatocytes were treated with holo-transferrin for 10 weeks (data not shown). In cultures treated with $FeSO_4$, Perls'-positive cells were detected, but insoluble iron, presumably ferric hydroxide, was observed as evidenced by the blue staining on the unoccupied plating surfaces (Figure 1, FS). TMH-ferrocene was the most efficient compound at loading hepatocytes with iron and remained in solution for the duration of the experiments (Figure 1, TMHF). The pattern of Perls'-positive cells was not the same in cultures treated with TMH-ferrocene compared with those treated with $FeSO_4$. Hepatocytes exposed to TMH-ferrocene that became iron loaded were evenly distributed throughout the culture dish whereas the cells exposed to $FeSO_4$ were more positive around the borders of cell islands. In cells treated with ferric nitrilotriacetate, a more soluble form of
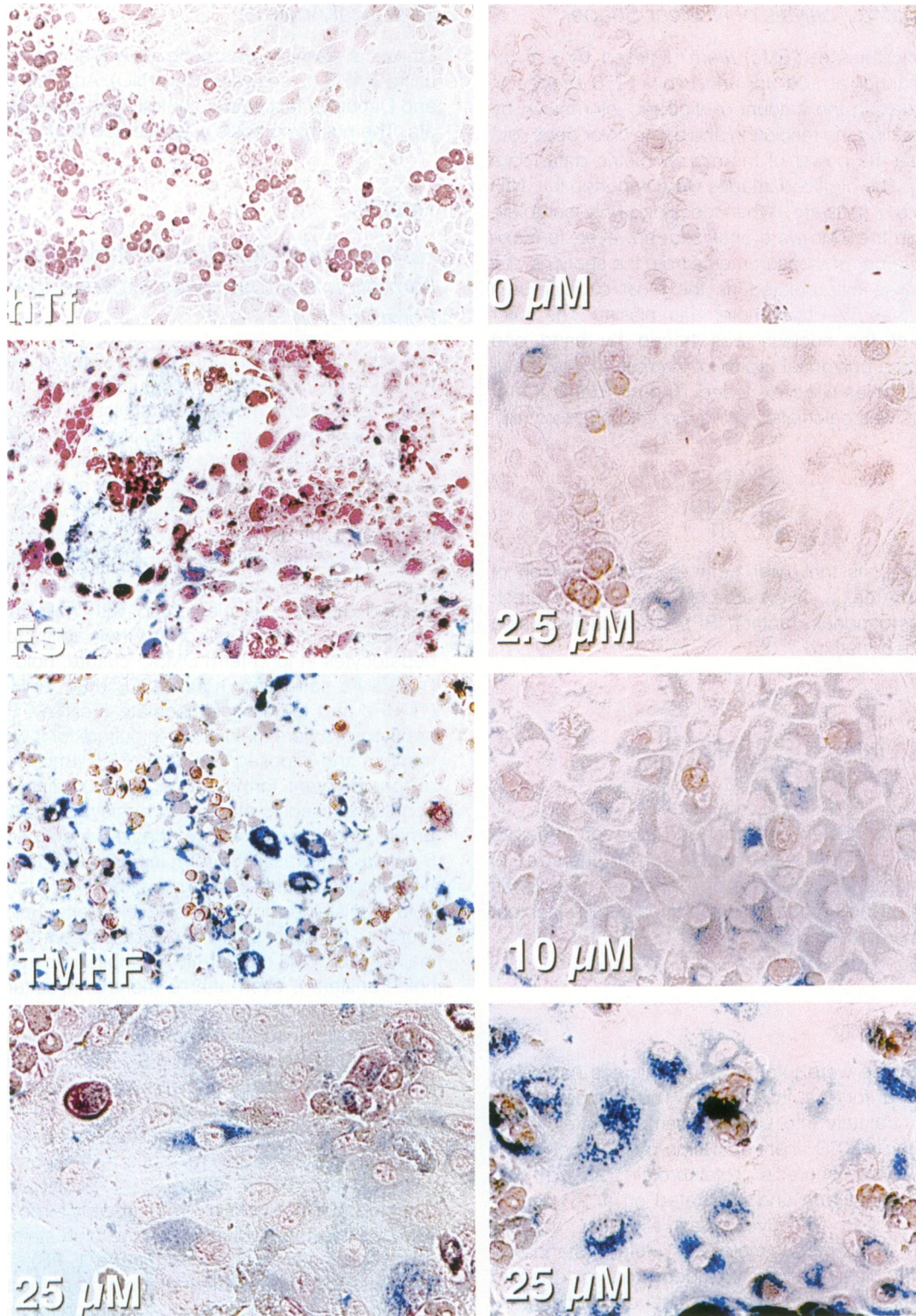
Figure 1. Histochemical detection of hemosiderin formation in hepatocytes exposed to various forms of iron. In the three photomicrographs labeled hTf, FS, and TMHF, the primary rat hepatocytes were in culture for 8 weeks and exposed to a total of 6.5 μmol/L iron. As it has been previously demonstrated that some transferrin is required to maintain hepatocytes in long-term DMSO culture, 1.5 μmol/L iron was in the form of holo-transferrin. The other 5 μmol/L was in the form indicated: holo-transferrin (hTf), FeSO$_4$ (FS), or TMH-ferrocene (TMHF). Representative photomicrographs shown are at magnification ×120. In other experiments, the concentration of TMH-ferrocene was increased, the rat hepatocytes were in culture for either 10 or 21 days, and 1.5 μmol/L iron was in the form of holo-transferrin. The photomicrograph on the bottom left shows hepatocytes exposed to 25 μmol/L TMH-ferrocene for 10 days. The four photomicrographs on the right show hepatocytes exposed to the indicated amount of TMH-ferrocene (in μmol/L) for 21 days. The photomicrographs shown are at magnification ×240.

iron than $FeSO_4$, iron loading occurred but was not as efficient as that observed with TMH-ferrocene (data not shown). The pattern of iron loading as measured by Perls' staining with ferric nitrilotriacetate was analogous to that for TMH-ferrocene, and no iron precipitate was observed on the unoccupied plating surfaces (data not shown).

Effects of Increasing Concentrations of TMH-Ferrocene on Iron Loading of Primary Rat Hepatocytes

Primary rat hepatocytes in long-term DMSO culture were treated with various concentrations of TMH-ferrocene (0, 2.5, 5.0, 7.5, 10, 20, 25, 37.5, or 50 μmol/L) for either 10 or 21 days before Perls' Prussian blue staining. In cells treated for 10 days, Perls'-positive cells were not observed in the cultures exposed to 0, 2.5, or 5 μmol/L TMH-ferrocene. Iron-loaded hepatocytes were detectable in cultures treated with 7.5 to 25 μmol/L TMH-ferrocene (Figure 1, bottom left, 25 μmol/L), and the number of hepatocytes that contained iron increased in a concentration-dependent fashion (data not shown). Treatment with 37.5 or 50 μmol/L TMH-ferrocene was toxic to the hepatocytes.

After 21 days of treatment with TMH-ferrocene, Perls'-positive cells were detected (Figure 1, right side) even at the lowest concentration of 2.5 μmol/L TMH-ferrocene. At higher concentrations of TMH-ferrocene, the number of Perls'-positive hepatocytes was greater than had been observed at the same concentration when treatment was for only 10 days. This was observed for all concentrations of TMH-ferrocene, but only the cells treated with 25 μmol/L are directly compared in Figure 1 (bottom left panel; bottom right panel, 21 days). By 21 days, essentially all of the cells in the hepatocyte cultures treated with 25 μmol/L TMH-ferrocene were positive by Perls' staining, and a punctate nature of the blue staining in the cytoplasm of the hepatocytes was readily apparent.

Ultrastructural Features of Hepatocytes Exposed to Various Forms of Iron

Primary rat hepatocytes in long-term DMSO culture were treated with 6.5 μmol/L iron in the form of holo-transferrin (>98% Fe saturation), $FeSO_4$, or TMH-ferrocene for 8 weeks, at which time they were fixed and embedded for electron microscopy. Hepatocytes treated with 6.5 μmol/L iron in the form of holo-transferrin showed no deviation in the ultrastructural features that were observed in hepatocytes exposed to the normal amount of iron, 1.5 μmol/L iron in the form of holo-transferrin.[27] These features include a normal round nucleus, Golgi bodies, lysosomes, rough endoplasmic reticulum, smooth endoplasmic reticulum, mitochondria, and filaments, which appear to be tonofilaments (Figure 2, hTf). There was no evidence of ferritin formation within the lysosomes (Figure 2, hTf). In hepatocytes treated with $FeSO_4$, the nuclei were invaginated and irregular, and

condensation of heterochromatin to the inner nuclear envelope was apparent (Figure 2, FS1). Ferritin cores were evident within the lysosomes (Figure 2, FS2). The ferritin cores did not form a paracrystalline array and were mostly, if not completely, in the form of hemosiderin (Figure 2, FS2). There were areas within the cells that contained high amounts of filamentous material, and these were in close proximity to bile canaliculi (Figure 2, FS3). In addition, hepatocytes that had undergone necrosis were present (Figure 2, FS1).

In hepatocytes treated with TMH-ferrocene, the shape of the nucleus deviated from circularity but was not as invaginated as in cells treated with $FeSO_4$ (Figure 2, TMHF1). The amount of ferritin within the lysosomes was greater in hepatocytes treated with TMH-ferrocene than was observed in cells exposed to $FeSO_4$. Furthermore, the ferritin cores in cells treated with TMH-ferrocene were frequently found in paracrystalline arrays and associated with the membranes within the lysosome (Figure 2, TMHF2 and TMHF3). As the lysosomes in hepatocytes in long-term DMSO culture normally contain multilayered vesicles it was not unexpected that the ferritin cores aligned with these multilayered, intralysosomal membranes (Figure 2, TMHF2 and TMHF3). Hepatocytes treated with TMH-ferrocene also contained hemosiderin (Figure 2, TMHF2 and TMHF3), ferritin cores not associated with lysosomes (Figure 2, TMHF3), and a greater number of filaments than were present in hepatocytes treated with 6.5 μmol/L holo-transferrin (Figure 2, hTf) or untreated hepatocytes (data not shown).

Quantitation of Changes in Nuclear Shape Associated with Iron Overload

To quantitate the differences in nuclear shape, two parameters of nuclear shape were analyzed: circularity and the NRF (Figure 3).[35,37] In cells exposed to 6.5 μmol/L iron as holo-transferrin, no changes in nuclear shape were observed compared with hepatocytes fed M6 medium. When cells were exposed to 6.5 μmol/L iron in the form of TMH-ferrocene or $FeSO_4$, the circularity of the nucleus was significantly less than that observed in cells fed M6 medium (Figure 3). In hepatocytes exposed to 6.5 μmol/L $FeSO_4$ or TMH-ferrocene, the NRF was greater than for hepatocytes fed M6 medium or medium supplemented with 6.5 μmol/L holo-transferrin. However, a statistically significant increase was observed only in hepatocytes treated with $FeSO_4$ compared with hepatocytes fed M6 medium.

Effects of Increasing Concentrations of TMH-Ferrocene on Nuclear Shape Parameters

To test whether TMH-ferrocene could cause cellular damage, the shape of nuclei in hepatocytes exposed to increasing concentrations of TMH-ferrocene was calculated (Figure 4). There was a significant relationship between the increase in TMH-ferrocene concentration
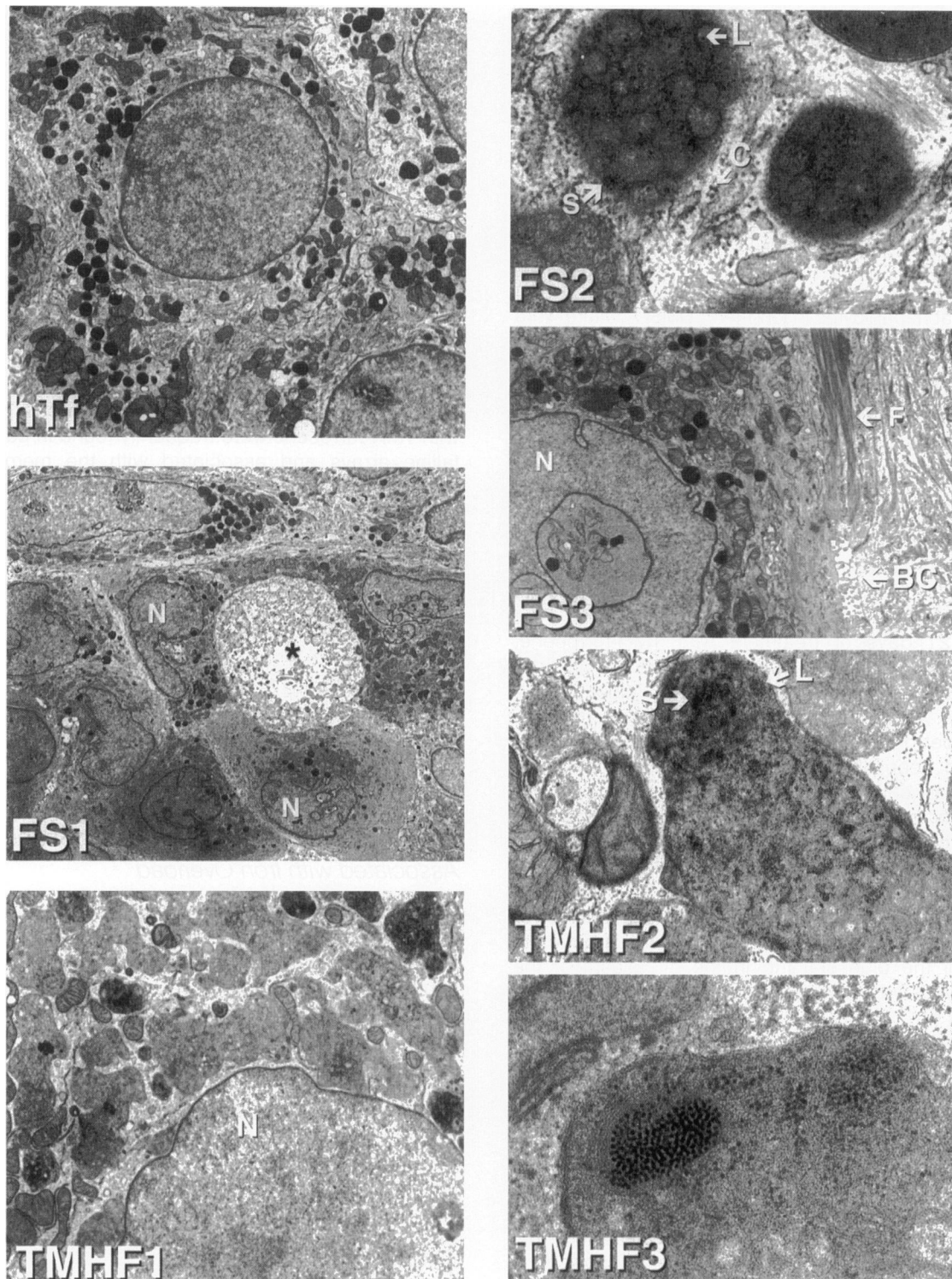
Figure 2. Ultrastructural features of hepatocytes exposed to various forms of iron. Primary rat hepatocytes were in culture for 8 weeks and exposed to a total of 6.5 μmol/L iron. The cultures were exposed to 1.5 μmol/L iron in the form of holo-transferrin and 5 μmol/L iron in the form indicated. The cells were in primary culture for 8 weeks and were fixed and embedded for electron microscopy as described in Materials and Methods. The cells exposed to 6.5 μmol/L *hTf* (magnification, ×3550) displayed no ultrastructural differences from cells exposed to only 1.5 μmol/L hTf (data not shown). The cells treated with FeSO$_4$ (FS) displayed invaginated nuclei (N), occasional cell death (*), hemosiderin formation (S; *FS-1*; magnification, ×1650), increased number of filaments (F; *FS-3*; magnification, ×4600) associated with bile canaliculi (BC), and both cytosolic (C) and lysosomal (L) ferritin (*FS-2*; magnification, ×35,500). The cells exposed to TMH-ferrocene (TMHF) had nuclei that were slightly distorted (*TMHF-1*; magnification, ×7700) and lysosomal (L) ferritin and (S) hemosiderin (*TMHF-2*; magnification, ×27,500), with the lysosomal ferritin forming a paracrystalline structure (*TMHF-3*; magnification, ×100,000).
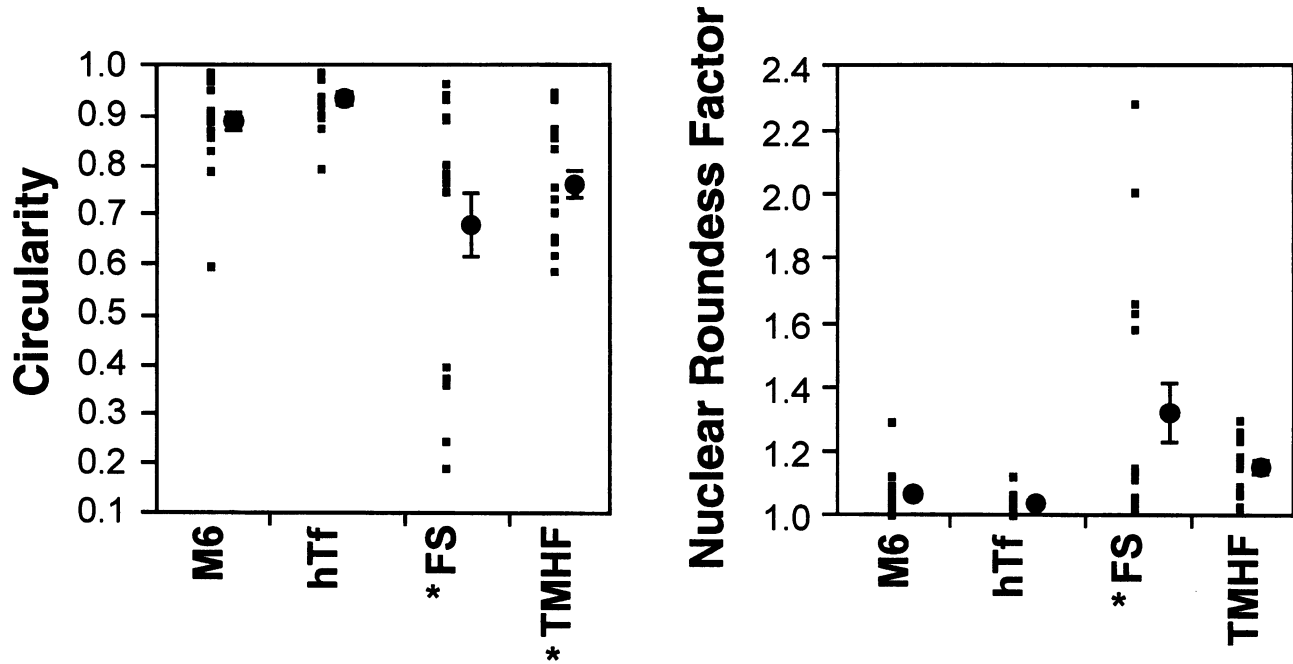
Figure 3. Morphometric analysis of nuclear shape. The numbers of nuclei analyzed for each separate treatment were as follows: 19 for M6, 14 for hTf, 16 for FS, and 15 for TMHF. The graphs represent the circularity (C) as defined in the text and the nuclear roundness factor (NRF). The data show means ± SEM. Where no error bar is shown, the error falls within the size of the symbol. The individual data points are shown as well. The analysis of variance was $P = 0.0001$ for C and $P = 0.0008$ for the NRF. Using the Dunnett's test *versus* control (M6 a significant decrease *versus* M6 for C and a significant increase *versus* M6 for NRF are indicated by asterisks in the appropriate graphs.

and the decrease in the circularity of the nuclei ($P = 0.0064$). A correlation between the concentration of TMH-ferrocene and an increase in NRF was not significant (data not shown). This finding reflects the relative lack of sensitivity of NRF to small deviations in shape (discussed below).

Effects of Increasing Concentrations of TMH-Ferrocene on Lysosomal Iron Content

To assess the subcellular localization and to estimate the amount of cellular iron contained in cells that had been exposed to increasing concentrations of TMH-ferrocene,
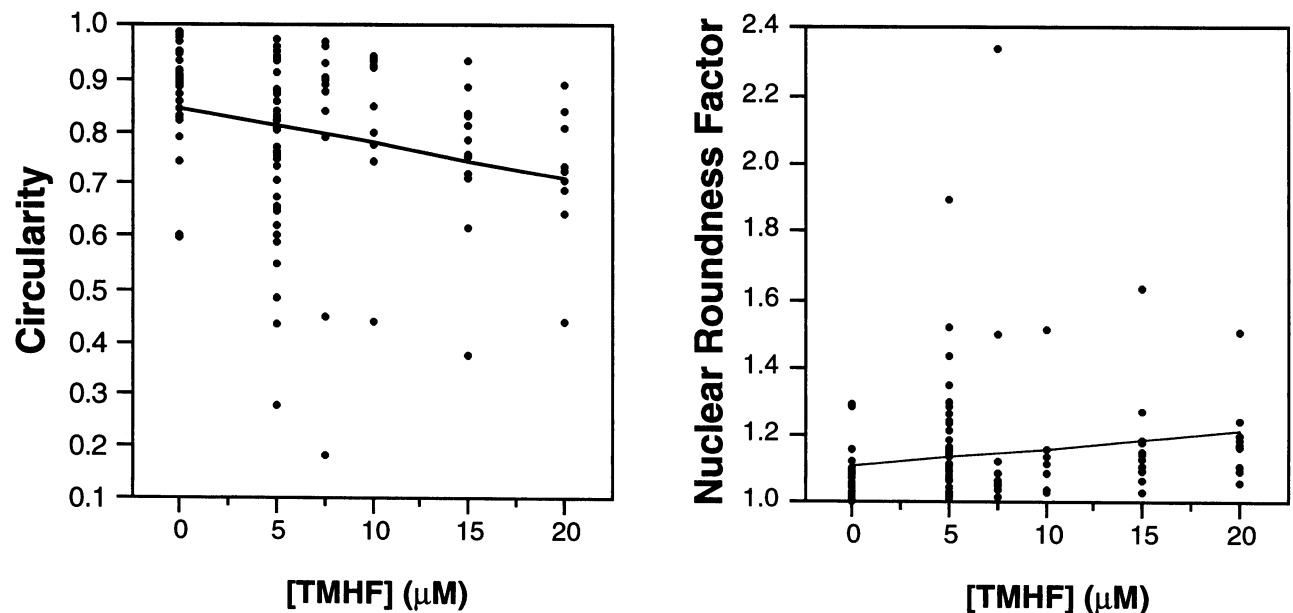

Figure 4. Aberrations in nuclear shape associated with increasing concentrations of TMH-ferrocene. Primary rat hepatocytes were in culture for 12 weeks and exposed to the indicated amount of TMH-ferrocene. The cultures were also exposed to 1.5 μmol/L iron as holo-transferrin. The graphs represent the circularity (C) as defined in Materials and Methods and the nuclear roundness factor (NRF). The individual data points are shown for each concentration of TMH-ferrocene. The P values for the regression analysis were significant for C ($P = 0.0064$) but were not significant for NRF ($P = 0.053$).
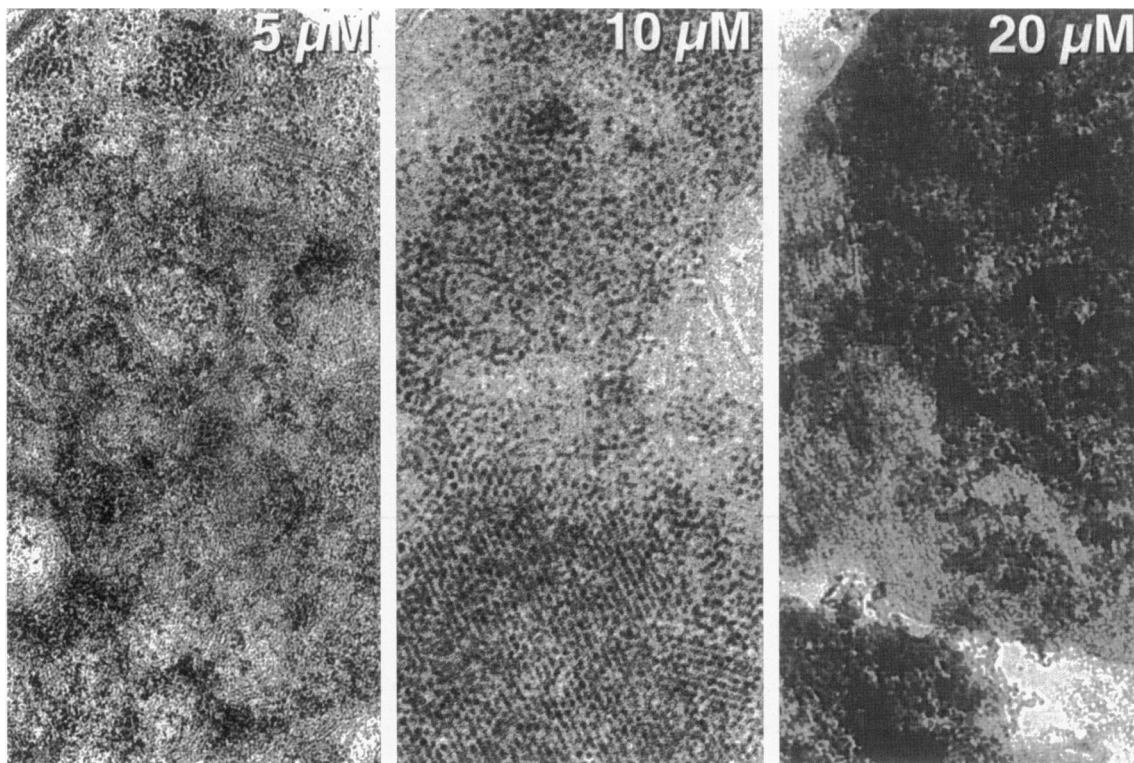
Figure 5. Effects of increasing amounts of TMH-ferrocene on iron loading of lysosomes in primary rat hepatocytes. Primary rat hepatocytes were in culture for 8 weeks. The cultures were fixed and embedded as described in Materials and Methods. Representative electron micrographs were taken of the lysosomes showing the increasing concentrations of hemosiderin and ferritin cores contained within these vesicles. The concentration of TMH-ferrocene is indicated: 5 μmol/L (magnification, $\times 95,500$), 10 μmol/L (magnification, $\times 123,750$), and 20 μmol/L (magnification, $\times 123,750$).

hepatocytes exposed to 5, 10, and 20 μmol/L TMH-ferrocene for 8 weeks were fixed for electron microscopy (Figure 5). As expected, almost all of the ferritin cores were contained within the lysosomes. No significant concentration of ferritin cores was observed in any other subcellular organelle. The concentration of iron within the lysosomes increased as the concentration of TMH-ferrocene within the medium was increased. The form of iron present within the lysosomes also varied depending on the concentration of TMH-ferrocene. In lysosomes of cells treated with 5 μmol/L TMH-ferrocene, ferritin in paracrystalline arrays was present in some lysosomes, and hemosiderin could also be detected. At 10 μmol/L TMH-ferrocene, the number of ferritin cores increased to essentially fill some of the lysosomes. At 20 μmol/L, all of the lysosomes contained large amounts of ferritin cores, and there was a marked increase in hemosiderin, an electron-dense conglomeration of ferritin cores.

Effects of Different Iron Compounds on Total Cell-Associated Iron in Primary Rat Hepatocytes

To assess whether the increasing amount of iron observed in the lysosomes of iron-loaded hepatocytes was associated with an increase in the total iron content of the cells we used a Ferrochem II analyzer to measure the total iron content of the hepatocytes in long-term DMSO culture after exposure to various forms and concentrations of iron (Figure 6). We tested cells fed RPCD me-

dium, a medium that does not contain any iron and contains apo-transferrin; M6, a medium that contains holo-transferrin; and two concentrations each of TMH-ferrocene and FeSO$_4$. As predicted, the lowest amount of total iron was observed in the cells fed the iron-deficient medium, RPCD. Cells fed M6 medium demonstrated an increase in total iron content when compared with cells fed RPCD. TMH-ferrocene treatments statistically increased the cell-associated iron. The largest increase in total iron content was observed in cultures containing cells treated with FeSO$_4$.

Effects of Iron Compounds on Total Ferritin Content of Primary Rat Hepatocytes

Evaluation of EMs (Figure 2) suggested that hepatocytes exposed to TMH-ferrocene were able to properly incorporate iron from TMH-ferrocene into ferritin, whereas hepatocytes exposed to FeSO$_4$ incorporated much less iron into ferritin. To obtain a quantitative comparison of the amount of ferritin within the cells, we analyzed total ferritin by Western blotting (Figure 7). The amount of ferritin increased in proportion to the amount of iron associated with the hepatocytes in hepatocytes fed M6 medium or medium supplemented with TMH-ferrocene (Figure 7 compared with Figure 6). However, in hepatocytes fed medium supplemented with FeSO$_4$, which contained the highest levels of total cell-associated iron, the amount of ferritin did not increase in proportion to total
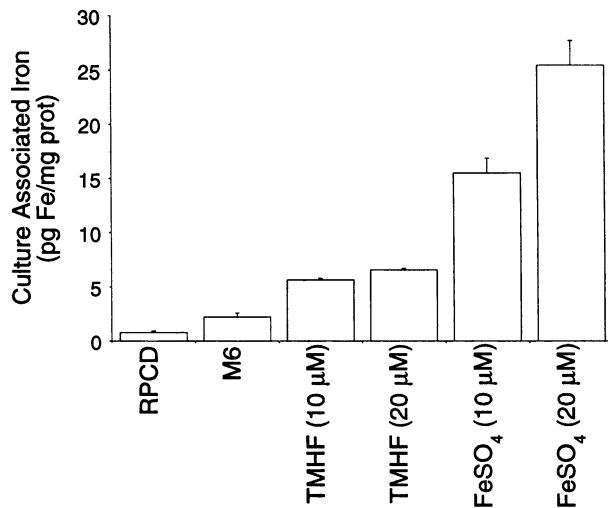
Figure 6. Effects of various forms on iron on total iron content in culture dishes. Primary rat hepatocytes were in culture for 21 days and exposed to the indicated amount of iron. Hepatocytes were washed with PBS, harvested by scraping, and sonicated, and total iron content was assessed using a Ferrochem II analyzer (ESA, Chelmsford, MA). The total iron content apparent in the $FeSO_4$-treated dishes included iron associated with the tissue culture dishes as an insoluble precipitate (see Figure 1, FS). The data represent means + SEM; n = 3.

iron content and was similar to the levels in hepatocytes fed M6 medium. These findings suggest that the smaller changes in circularity of the nuclei in the presence of TMH-ferrocene, when compared with cells treated with $FeSO_4$, may be attributed to increased ferritin levels.

Discussion

Primary hepatocytes in long-term culture retain the ability to accumulate iron from exogenous iron sources in a fashion analogous to that observed in whole animals. Specifically, we concluded the following from these studies. 1) Primary rat hepatocytes in long-term DMSO culture can be iron loaded by exposure to NTBI in the form of $FeSO_4$ or TMH-ferrocene but not with holo-transferrin at the concentrations tested. 2) Because iron loading can be carried out over long time periods (months) in hepatocytes in DMSO, it is possible to obtain iron loading using concentrations as low as 2.5 μmol/L TMH-ferrocene. When exposed to 25 μmol/L TMH-ferrocene, hepa-
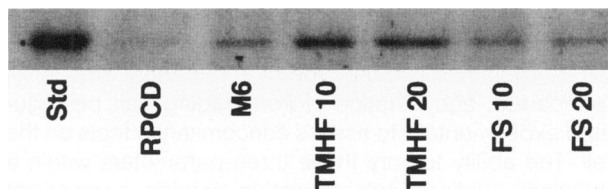

Figure 7. Ferritin protein levels in primary rat hepatocytes treated with various forms of iron. Primary rat hepatocytes were in culture for 21 days and exposed to the indicated amounts and forms of iron. Cells were washed and harvested by scraping, sonicated, and immediately placed into SDS-PAGE sample buffer. Total protein (25 μg) was loaded into each lane and separated by electrophoresis through a 15% gel. Purified rat liver ferritin (from Sigma) was included as a positive control (lane 1) and migrated to a position just below the 21.5-kd molecular weight marker (data not shown).

tocytes continued to load increasing amounts of iron for 2 months before the cells died; when exposed to lower concentrations, such as 2.5 or 5.0 μmol/L TMH-ferrocene, hepatocytes were able to continuously load iron and remain viable for more than 2 months. 3) Hepatocytes do not appear to down-regulate TMH-ferrocene uptake as the cells continued to sequester iron even after there was an appreciable amount of iron already in the cell. 4) The cellular deposition of iron is different in hepatocytes exposed to TMH-ferrocene compared with those exposed to $FeSO_4$; exposure to TMH-ferrocene resulted in the presence of more ferritin cores within lysosomes. 5) Iron loading distorted nuclear shape in hepatocytes, the amount of nuclear distortion was greater in hepatocytes exposed to $FeSO_4$ than in those exposed to TMH-ferrocene, and TMH-ferrocene caused a decrease in the circularity of the nuclei of hepatocytes that correlated with an increase in concentration of the iron compound. 6) TMH-ferrocene was capable of delivering iron to hepatocytes in culture so that it can undergo normal physiological processing as evidenced by the larger amount of ferritin detected by electron microscopy or Western blot. However, the iron from $FeSO_4$, which is quite insoluble, cannot participate in the normal physiological processing. These results demonstrate that primary hepatocytes in long-term culture can accumulate iron from NTBI, and this accumulation is associated with changes in morphology and physiology.

Little success has been achieved to date with iron loading of liver cell lines or primary hepatocytes. Studies using hepatocyte-derived cell lines are limited because the cells continue to grow and divide, and therefore, a high level of iron loading is difficult to achieve. It is also difficult to load primary hepatocytes in short-term culture, not because they divide but rather because the cells do not remain viable and differentiated for the time needed for successful loading. Studies on cultures of primary hepatocytes have been limited to short-term studies, ranging from 1 to 48 hours. Treatment of hepatocyte-derived cell lines and primary hepatocytes in short-term culture with exogenous iron has been used to study the effects of iron on iron metabolism, the transport of NTBI, and iron-induced cellular damage. To our knowledge, the ability of primary hepatocytes to load iron as demonstrated (using electron microscopy) by hemosiderin formation and the presence of ferritin iron cores has not been previously reported.

NTBI has long been suspected to directly cause hepatocellular damage.[13,14] Elevations in levels of NTBI in patients with either homozygous hemochromatosis[13,38] or other types of iron overload[39-41] or in rats treated with TMH-ferrocene[19] have been correlated with liver iron overload, suggesting that NTBI is the source of the iron that is loaded into the liver. The proteins responsible for iron transport and storage, transferrin and ferritin, respectively, are known to sequester iron in a form that is not available to catalyze oxidation reactions. Once the transferrin transport system has been overwhelmed, there is an increase in NTBI. NTBI can be taken up by hepatocytes using specific transport mechanisms[16,42-46] and incorporated into ferritin, thereby protecting the cell from

damage caused by iron-catalyzed oxidation reactions. However, when the NTBI is not sequestered into ferritin, for example, in $FeSO_4$-treated hepatocytes, or when the ferritin system is overloaded, for example, in hepatocytes treated with higher concentrations of TMH-ferrocene or in hemochromatosis patients with high hepatic iron concentrations, the iron is available to catalyze oxidation reactions, and cellular damage occurs. These data are consistent with the concept that an intracellular pool of low molecular weight iron is more catalytically active and capable of causing cellular damage than is ferritin-bound iron.[47-49]

Although TMH-ferrocene is a nonphysiological source of iron, it has been previously reported that a highly useful model for iron overload can be achieved by feeding rats TMH-ferrocene.[7] It was apparent from these animal studies that iron from TMH-ferrocene can be successfully delivered to the liver and incorporated into ferritin. Although TMH-ferrocene obviously cannot be used to study how hepatocytes take up NTBI, this nonphysiological form of NTBI can be successfully used to iron load hepatocytes. The ability of three different forms of NTBI to load long-term primary rat hepatocytes maintained in a chemically defined medium, without the cells being loaded by an equivalent concentration of transferrin-bound iron, is the first direct evidence that hepatocytes can be specifically loaded by NTBI. The forms of iron tested were $FeSO_4$, TMH-ferrocene, ferric nitrilotriacetate (data not shown), and holo-transferrin. The data showing inability of holo-transferrin to load hepatocytes is not surprising in light of the highly regulated transferrin/transferrin receptor iron uptake system[50,51] that regulates the uptake of transferrin-bound iron into hepatocytes. The ability of both TMH-ferrocene and ferric nitrilotriacetate to produce iron loading in cells randomly distributed throughout the culture dish indicate that the mechanisms responsible for NTBI uptake continue to function in most, if not all, hepatocytes in this culture system even though the hepatocytes are no longer present in an intact liver. One possible explanation as to why hepatocytes in long-term DMSO culture do not load iron from holo-transferrin could be that the transferrin/transferrin receptor system has ceased to function in this system. However, this explanation is highly unlikely as 1) it has been previously reported that primary rat hepatocytes can be used to study regulation of the transferrin receptor,[52,53] and 2) we recently demonstrated that iron in the form of holo-transferrin in combination with increased levels of copper and zinc can be used to induce DNA synthesis in hepatocytes in the presence of 2% DMSO.[27] The inability of $FeSO_4$ to uniformly load hepatocytes in long-term DMSO culture is most likely due to its insolubility in basic, aqueous solvents than an inherent property of the hepatocytes within the culture system.

Cellular damage, as assessed by both electron microscopy and the analysis of nuclear shape, is different in cells exposed to $FeSO_4$ *versus* cells exposed to TMH-ferrocene. In this system, the extent of cellular damage, as assessed by changes in nuclear shape parameters, is inversely correlated with the amount of iron found contained within ferritin. That is, the amount of ferritin iron

observed in hepatocytes treated with $FeSO_4$ was considerably less than the amount observed in hepatocytes treated with TMH-ferrocene. This observation is in agreement with previous findings that incorporation of intracellular iron into ferritin minimizes cellular damage. As there was an appreciable amount of iron precipitated on the surface of the hepatocytes in cultures exposed to $FeSO_4$, the larger deviations in nuclear shape are consistent with the concept that perturbations in the cytoskeleton can lead to changes in nuclear shape.[54-57] Exposing hepatocytes to increasing concentrations of TMH-ferrocene leads to an increase in the amounts of ferritin and hemosiderin in cells and a decrease in circularity of the nuclei. Indeed, the amount of cellular damage can be manipulated by increasing the concentration of TMH-ferrocene in the media, the time of the cells in culture, or both.

The use of circularity to measure the shape of the nucleus is more sensitive to changes in shape when the starting shape of the nuclei are almost circular. The NRF is more sensitive to large changes when the starting nuclear shape is already significantly out of round. This is because the relationship between the NRF and circularity (C) is described by the following equation:

$$NRF = \sqrt{\frac{1}{C}}$$

The use of circularity in this study is therefore preferable to using the NRF because the nuclei in normal hepatocytes are almost perfect circles. Changes in circularity in a population of hepatocyte nuclei in response to exogenous iron, or other damaging agents, can be more easily quantified than changes in the NRF, as was the case with the changes in shape associated with increasing concentrations of TMH-ferrocene (Figure 4). Whether circularity can be used to test for hepatocellular damage in patients remains to be determined.

We have previously shown that hepatocytes in long-term DMSO culture are highly differentiated and an excellent *in vitro* model for adult liver for studying gene expression, growth control, and pathological changes in adult hepatocytes.[21,22,26,58,59] In this study, we have demonstrated that it is possible to superimpose on the long-term DMSO culture system the ability to iron load these cells. The results from this study show that NTBI is more effective at loading iron into hepatocytes than holo-transferrin. The appearance and ultrastructure of the ferritin cores within the lysosomes appears to be identical to that observed in both patients and in whole-animal models of iron overload. One major strength of this *in vitro* culture system is that the type of iron loaded, amount of iron loaded, and duration of iron loading can be regulated experimentally to assess concomitant effects on the cell. The ability to vary these three parameters within a chemically defined culture system provide a variety of combinations of conditions that can be used to study the effect of iron on specific cellular events, including lipid peroxidation, formation of covalent protein adducts, and the effects of specific antioxidants and cytokines on cellular processes of interest. Furthermore, the ability to load the cells with large amounts of iron provides a system to

study the effects of increasing concentrations of intralysosomal iron in hepatocytes. In summary, we have demonstrated that hepatocytes in long-term DMSO culture can be iron loaded and represent a flexible system for studying the effects of chronic iron loading on hepatocytes.

Acknowledgments

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