Altered Ferritin Subunit Composition: Change in Iron Metabolism in Lens Epithelial Cells and Downstream Effects on Glutathione Levels and VEGF Secretion

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PURPOSE. The iron storage protein ferritin is necessary for the safe storage of iron and for protection against the production of iron-catalyzed oxidative damage. Ferritin is composed of 24 subunits of two types: heavy (H) and light (L). The ratio of these subunits is tissue specific, and alteration of this ratio can have profound effects on iron storage and availability. In the present study, siRNA for each of the chains was used to alter the ferritin H:L chain ratio and to determine the effect of these changes on ferritin synthesis, iron metabolism, and downstream effects on iron-responsive pathways in canine lens epithelial cells.

METHODS. Primary cultures of canine lens epithelial cells were used. The cells were transfected with custom-made siRNA for canine ferritin H- and L-chains. De novo ferritin synthesis was determined by labeling newly synthesized ferritin chains with 35S-methionine, immunoprecipitation, and separation by SDS-PAGE. Iron uptake into cells and incorporation into ferritin was measured by incubating the cells with 59Fe-labeled transferrin. Western blot analysis was used to determine the presence of transferrin receptor, and ELISA was used to determine total ferritin concentration. Ferritin localization in the cells was determined by immunofluorescence labeling. VEGF, glutathione secretion levels, and cystine uptake were measured.

RESULTS. FHsiRNA decreased ferritin H-chain synthesis, but doubled ferritin L-chain synthesis. FLsiRNA decreased both ferritin H- and L-chain synthesis. The degradation of ferritin H-chain was blocked by both siRNAs, whereas only FHsiRNA blocked the degradation of ferritin L-chain, which caused significant accumulation of ferritin L-chain in the cells. This excess ferritin L-chain was found in inclusion bodies, some of which were co-localized with lysosomes. Iron storage in ferritin was greatly reduced by FHsiRNA, resulting in increased iron availability, as noted by a decrease in transferrin receptor levels and iron uptake from transferrin. Increased iron availability also increased cystine uptake and glutathione concentration and decreased nuclear translocation of hypoxia-inducible factor 1-α and vascular endothelial growth factor (VEGF) accumulation in the cell-conditioned medium.

CONCLUSIONS. Most of the effects of altering the ferritin H:L ratio with the specific siRNAs were due to changes in the availability of iron in a labile pool. They caused significant changes in iron uptake and storage, the rate of ferritin synthesis and degradation, the secretion of VEGF, and the levels of glutathione in cultured lens epithelial cells. These profound effects clearly demonstrate that maintenance of a specific H:L ratio is part of a basic cellular homeostatic mechanism. (Invest Ophthalmol Vis Sci. 2010;51:4437–4446) DOI:10.1167/iovs.09-3861

Ferritin is a multimeric iron-storage protein consisting of 24 subunits of two types: heavy (H) and light (L). The ratio of these two chains is tissue specific and controls iron storage and availability. Ferritin H- and L-chain expression is regulated at both the transcriptional and translational levels. In addition, cells can regulate the H:L ratio through differential rates of degradation and secretion for each chain.

The role of ferritin H- and L-chains and the effects of alteration of the H:L ratio on iron storage and physiological and pathologic processes, have been studied by inducing the overexpression and underexpression (siRNA knockdown) of each chain. Overexpression of H-chain ferritin decreases the cytosolic labile iron pool (LIP; considered to be a pool of available iron in the cytosol), increases iron incorporation into ferritin, and increases cell resistance to oxidative stress and UV irradiation in cultured erythroid cells and in lens epithelial cells (LECs).

In addition, altering the H:L ratio with siRNA has significant effects on iron availability in HeLa cells. Ferritin L-chain siRNA (FLsiRNA) decreases L-chain levels by 80%, but has no effect on H-chain levels or any parameters indicative of iron availability. On the other hand, ferritin H-chain siRNA (FHsiRNA) decreases H-chain levels by 75% and increases L-chain levels threefold. FHsiRNA also increases iron availability and decreases resistance to oxidative stress induced by hydrogen peroxide.

We have recently shown that ferritin chains are significantly modified in canine and human lens fiber cells and that these modifications increase with age. Although it is unclear how these modifications affect the assembly of the whole ferritin molecule, they most likely affect the iron storage capability of ferritin. Such changes could contribute to cataractogenesis, either by the aggregation of abnormal chains or by increasing the availability of reactive iron that cannot be safely stored.

Ferritin chains have other physiologic roles beyond safely storing potentially reactive iron. For example, siRNA knockdown showed that H-chain ferritin contributes to malignant mesothelioma cell resistance to apoptosis and that L-chain ferritin plays a role in iron-independent cellular proliferation.

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Indeed, the physiological roles of iron are numerous, and new activities have recently been uncovered (Fig. 1), such as the regulation of the levels of hypoxia inducible factor (HIF)-1α,12,13 a transcription factor that regulates many genes, including vascular endothelial growth factor (VEGF).14,15 which is made by the lens.16 We have recently shown that iron controls VEGF secretion in both LECs (McGahan MC, et al. IOVS 2007; 48:ARVO E-Abstract 2436) and retinal pigmented epithelial (RPE) cells (Ferrell J, Harned J, unpublished data, 2008). Furthermore, iron controls the synthesis and secretion of glutamate in LECs, RPE cells, and neurons.17 The iron-regulated secretion of glutamate had downstream effects on cystine uptake by the cystine/glutamate antiporter and the levels of glutathione (GSH) in these cells.18

The goal of the present study was to alter the ferritin H:L ratio with siRNA for each ferritin chain and to determine how these changes affect iron storage and availability, ferritin levels (including the kinetics of its synthesis and degradation), cystine uptake, GSH levels, HIF-1α translocation to the nucleus, and VEGF production in LECs.

**METHODS**

**Tissue Culture**

Dogs were obtained from the Johnston County Animal Shelter (North Carolina) after they had been euthanatized. The eyes were removed and the anterior lens capsule dissected from the lens and placed on a 100-mm tissue culture plate in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% antibiotic-antimycotic (Mediatech, Manassas, VA). LECs were grown to confluence and subsequently seeded onto six-well plates for the experiments.

**siRNA Transfection**

Custom siRNA for canine ferritin H- and L-chains (SMARTpools, M-010264-00 and M-040576-00, respectively) chains were designed and produced by Dharmacon RNA Technologies (Lafayette, CO), by using canine ferritin sequences determined in this laboratory.7 Dharmacon siRNA design utilizes innovative strategies to optimally reduce off-target effects, including advanced rational design based on bioinformatic analysis, pooling of duplexes, and unique chemical modifications to both the sense and antisense strands to enhance siRNA specificity. Furthermore, the FLsiRNA includes modification reagents (On-TARGET Plus; Dharmacon) to minimize seed region-related mRNA off-target silencing.

Cells were plated at a density of 90,000 to 105,000/well on six-well plates in DMEM+10% fetal bovine serum and incubated overnight at 37°C, typically reaching 40% to 50% confluence. A control nontargeting siRNA pool (Dharmacon) was transfected alongside the ferritin H- and LsiRNA (FH- and FLsiRNA) on each plate to account for any non-sequence-specific cell responses. Lipophilic transfection reagent (0.95 µL/mL, Lipofectamine 2000; Invitrogen) siRNA complexes were formed, according to the manufacturer’s protocol, in serum-free DMEM and added to each well at a final concentration of 100 nM siRNA. After overnight incubation, transfection medium was replaced with the appropriate medium, depending on the specific treatment to follow. De novo ferritin chain synthesis was monitored for each experiment to confirm knockdown.

**Reverse Transcription and Quantitative Real-Time PCR**

Total RNA was extracted from cultured LECs, 24 hours after transfection with CTL, FH, or FLsiRNA (RNaseq kit and Qiashredders; Qiagen, Valencia, CA) according to the manufacturer’s protocol. Cell lysates from four 3.5-cm wells were combined for RNA extraction. The RNA samples were quantitated with a spectrophotometer (NanoDrop Technologies, Wilmington, DE), and 1.0 µg of RNA from each sample was reverse transcribed using oligo dT primers and a transcription system (ImProm-II Reverse Transcription System; Promega, Madison, WI). Real-time PCR was performed with master mix (PowerSYBR Green; Applied Biosystems [ABI], Foster City, CA) in a thermocycler (iCycler iQ; Bio-Rad Laboratories, Hercules, CA). The following primers were prepared in duplicate, 25-µL reactions contained 5 µL of template corresponding to 0.5% or 0.05% of the RT product. Coding sequences..
of ferritin light and heavy cDNAs that we had previously cloned into mammalian expression vector (pTARGET; Promega) were excised (ECoRI, purified with QIAquick Gel Extraction Kit; Qiagen) and used to generate a standard curve. The thermal cycler’s system software was used to analyze the data (ver. 3.0. iCyclerTM iQ Optional System Software; Bio-Rad).

**Ferritin Subunits and Iron Metabolism**

**De Novo Ferritin Synthesis**

The accumulation of newly synthesized ferritin was quantified by incubating transfected cells for 20 hours in serum- and methionine-free DMEM with 60 μCi translabel 35S-methionine (MP Biomedicals, Solon, OH). The cells were lysed on ice in Tris lysis buffer (pH 8.0; 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.02% sodium azide, and 0.006% protease inhibitor cocktail) and the supernatants collected after 12,000g centrifugation. Ferritin was immunoprecipitated with goat anti-horse ferritin (Bethyl Laboratories, Montgomery, TX), and the ferritin subunits were separated by 12% SDS-PAGE in reducing conditions. Radioactivity was quantified with an electronic autoradiography system (Instant Imager; Perkin-Elmer, Waltham, MA) and normalized to radioactivity incorporated into total cell protein precipitated with TCA.

**Iron Incorporation into Ferritin**

Transfected cells were preincubated in 1 hour in serum-free DMEM to remove any transferrin that was still bound to receptors in the membrane, after which the medium was replaced with fresh serum-free DMEM containing 240 μg/mL 59Fe-Tf and incubated for 24 hours. The cells were washed with cold PBS and lysed by adding 200 μL of distilled water to each well, followed by freezing and thawing. Radioactivity in the lysates was measured with a gamma counter (Wallac Wizard; PerkinElmer). Protein content was quantified by bicinchoninic acid (BCA) assay.

**Total Iron Uptake**

Transfected cells were incubated in serum-free DMEM for 1 hour, medium removed and replaced with fresh serum-free medium containing 10 μM Tris-Cl (pH 7.4) containing protease inhibitors and PMSF. Lysates from similar treatments were pooled and concentrated 6- to 10-fold on centrifugal columns (Centricon 10; Millipore). Assay plates (ProBind; BD Biosciences) were coated with affinity-purified goat anti-horse ferritin (4 μg/mL) overnight at 4°C. After the reactions were blocked, samples and standards (dog liver ferritin; NEIA, Inc., Cambridge MA) were applied and incubated for 1 hour at 37°C. After the plates were washed three times with 0.05% Tween-20 in PBS, HRP-labeled goat anti-horse ferritin (Bethyl; 0.5 μg/mL) was added and incubated for 1 hour at room temperature. After further washing, ABTS peroxidase substrate (KPL, Gaithersburg, MD) was applied for 10 to 20 minutes, and absorbance was read at 405 nm on a microplate reader (Sunrise; Tecan, Research Triangle Park, NC).

**Determination of Total Ferritin within the Cells**

Ferritin concentration in cell lysates was measured by sandwich ELISA. Transfected cells were lysed in 10 mM Tris-Cl (pH 7.4) containing protease inhibitors and PMSF. Lysates from similar treatments were pooled and concentrated 6- to 10-fold on centrifugal columns (Centricon 10; Millipore). Assay plates (ProBind; BD Biosciences) were coated with affinity-purified goat anti-horse ferritin (4 μg/mL) overnight at 4°C. After the reactions were blocked, samples and standards (dog liver ferritin; NEIA, Inc., Cambridge MA) were applied and incubated for 1 hour at 37°C. After the plates were washed three times with 0.05% Tween-20 in PBS, HRP-labeled goat anti-horse ferritin (Bethyl; 0.5 μg/mL) was added and incubated for 1 hour at room temperature. After further washing, ABTS peroxidase substrate (KPL, Gaithersburg, MD) was applied for 10 to 20 minutes, and absorbance was read at 405 nm on a microplate reader (Sunrise; Tecan, Research Triangle Park, NC).

**Western Blot Analysis of Transferrin Receptor**

Twenty-four hours after transfection, the cells were rinsed and incubated in serum-free MEM for 24 hours. After lysis in RIPA buffer (Pierce, Rockford, IL) and centrifugation at 14,000g for 15 minutes, the supernatants were concentrated on centrifugal columns (Microcon 30; Millipore, Billerica, MA) and analyzed for protein content by BCA assay (Pierce). Twenty-five micrograms of protein from each sample was loaded onto an 8% denaturing Tris-tricine gel along with 6 μg of purified human placenta Tf receptor (TfR; Alpha Diagnostics, San Antonio, TX) as a positive control. The proteins were transferred for 30 minutes at 20 V to a nitrocellulose membrane (Hybond-ECL; GE Healthcare, Munich, Germany). Blots were blocked in 10 mM Tris buffer (pH 7.5), containing 100 mM NaCl, 0.1% Tween-20, and 5% nonfat dry milk. All incubations were performed for 1 hour at room temperature in blocking buffer. The membranes were incubated in a 1:2000 dilution of monoclonal mouse anti-human TfR (Invitrogen) followed by a series of six washes (6 minutes each) in Tris buffer without milk. The secondary antibody used was HRP-labeled goat anti-mouse (BD Biosciences, Palo Alto, CA) at a dilution of 1:750. After another set of washes and a final wash in Tris-buffered saline, ECL (GE Healthcare) was used for detection. After blocking overnight at 4°C, blots were then reprobed with a 1:500 dilution of HRP-goat anti-human β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), which was used as a loading control.

**Ferritin Chain Degradation**

Twenty-four hours after transfection with the nontargeting control siRNA, FHsiRNA, or FLSiRNA, LECs were incubated for 18 hours with 60 μCi translabel 35S-methionine. After the labeling period, the cells were either harvested (time 0) or rinsed and incubated for an additional 12 or 28 hours in DMEM with 10% FBS containing a large excess of unlabeled methionine (2 mM) and cysteine (0.7 mM) to prevent recycling of the radioactive amino acids. The cells were lysed and the ferritin immunoprecipitated by using the same protocol as for de novo ferritin synthesis, followed by separation of the FH and FL chains by 12% SDS-PAGE. Radioactivity in the ferritin bands was quantified by electronic autoradiography (InstantImager; PerkinElmer) and normalized to radioactivity incorporated into total cell protein precipitated with TCA.

**Determination of HIF-1α Levels in Nuclear Extracts**

LEC were transfected as described with control, FHsiRNA, or FLSiRNA. Ten hours after transfection, nuclear extracts were prepared (NE-PER...
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kit; Pierce) and stored at −80°C. HIF1α levels in the nuclear extracts were measured by sandwich ELISA with an HIF-1α kit (Duoset IC Human/Mouse; R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol. The quantity of HIF-1α was normalized to the amount of protein in the corresponding cytosolic extracts and expressed as a percentage of the HIF-1α found in the control transfected cells.

**Determination of VEGF in Cell-Conditioned Medium (CCM)**

VEGF was determined by ELISA, with antibodies, standard, and reagents from R&D Systems, Inc. LECs were transfected as described, medium replaced with serum-free, glutamine-free MEM and then incubated for 24 hours at 37°C. The plates were plated on ice, CCM collected, and any floating cells removed by centrifugation at 1200g. Samples were stored at −80°C. For the VEGF assay, 96-well assay plates (ProBond; BD Biosciences) were coated with monoclonal anti-human VEGF in PBS (0.8 μg/ml) overnight (all incubations were performed at room temperature). After three washes in PBS+0.05% Tween-20, standards (recombinant human VEGF165) and samples were applied and incubated for 2 hours. PBS+1% BSA was used as diluent throughout the assay. The washes were repeated, followed by incubation with biotinylated anti-human VEGF (25 ng/ml) for another 2 hours. The plate was washed, and a 1:200 dilution of streptavidin-HP was added for 20 minutes. After the final set of washes, the substrate (H2O2/tetramethylbenzidine) was added for 20 minutes followed by 1 M H2SO4 to stop the reaction. Absorbance at 450/540 nm was read within 30 minutes on a microplate reader (Sunrise; Tecan).

**Determination of GSH within the Cells**

Transfected LECs were analyzed for intracellular GSH concentration by using a commercially available kit (Chemicon, Temecula, CA). Four days after transfection, the cells were lysed, centrifuged at 12,000g (at 4°C) and put on ice. The lysates were incubated for 1 hour at room temperature and protected from light in black-walled 96-well plates after addition of monochlorobimane dye dissolved in acetonitrile. A fluorometer (Fluoroskan Ascent; Thermo-Scientific, Waltham, MA) was used to measure fluorescence (390-nm excitation, 460-nm emission). GSH levels were standardized to the amount of protein in the lysates.

**Cystine Uptake**

Transfected cells were labeled with 5 μM 1-35S-cystine in uptake buffer (pH 7.4), consisting of 157 mM NaCl, 10 mM HEPES, 5 mM glucose, 1 mM MgCl2, 1 mM CaCl2, and 0.7 mM KH2PO4 and incubated for 2 minutes at 37°C in 5% CO2. Uptake was terminated by removing the buffer and washing the cells three times with ice-cold PBS. The lysates were prepared by incubating the cells, on ice, in 10 mM Tris buffer containing protease inhibitors and PMSF for 20 minutes, followed by a 5-minute centrifugation (14,600g) at 4°C. Radioactivity was measured in a sample of the lysate using a liquid scintillation counter (1409 Wallac; Perkin Elmer). Incorporation of radioactivity into protein was determined by TCA precipitation.

**Statistics**

The data were analyzed by ANOVA with Tukey’s HSD test for multiple comparisons; differences were significant at P < 0.05.

**Results**

In this study, we altered the ferritin H:L chain ratio by transfecting cultured LECs with siRNA for either ferritin chain (FH- or FLsiRNA) and determining the effects on de novo ferritin H- and L-chain synthesis and degradation rate, iron availability and storage in ferritin, total ferritin concentration, and downstream effects on iron-regulated pathways (see Fig. 1).

**Effect of FH- and FLsiRNA on FH- and FLmRNA and on De Novo Synthesis of Ferritin H- and L-Chains**

FHsiRNA greatly reduced the copy number of FH mRNA while having no effect on FL mRNA. The same specificity of effect on target mRNA was true for FLsiRNA (Fig. 2). FHsiRNA significantly decreased synthesis of ferritin H-chain as early as 2 days after transfection (Fig. 3A). This decrease was maintained at 4 days after transfection. Surprisingly, FHsiRNA caused a very large increase (100%) in de novo ferritin L-chain synthesis by day 2 after transfection. A large increase (50%) was still noted on day 4. Transfection with FLsiRNA did not have similar dramatic effects. De novo synthesis of L-chain was significantly decreased by approximately 40% on day 2 and 26% on day 4 (Fig. 3B). Unexpectedly, H-chain synthesis was also significantly decreased (22%) on posttransfection day 4.

With such a dramatic increase in L-chain ferritin levels in cells transfected with FHsiRNA, we wanted to visualize how ferritin chains were distributed and whether the large increase in L-chain ferritin was localized to any particular subcellular compartment. Cells transfected with a nontargeting control siRNA showed endogenous levels of ferritin H- and L-chain at 4 days after transfection (Figs. 4A, 4D). FHsiRNA transfection resulted in a large decrease in staining for ferritin H-chain (Fig. 4B), whereas ferritin L-chain became even more abundant than in cells transfected with control siRNA (Fig. 4E). Decreases in signal were noted for both H- and L-chain ferritin after FLsiRNA transfection (Figs. 4C, 4F).

**Effect of FH- and FLsiRNA on Ferritin Chain Degradation**

Total ferritin levels are also controlled by the rates of degradation of ferritin H- and L-chains, which are normally quite low. To determine how ferritin siRNAs affect these rates, cellular ferritin in each of the three treatment groups (CTL siRNA, FHsiRNA, and FLsiRNA) was labeled with 35S-methionine for 18 hours. The amount of label present in each chain was then measured 0, 12, and 28 hours later. In control cells, H-chain
steady degraded over 28 hours, whereas L-chain did not significantly decrease over time. FLsiRNA did not alter L-chain ferritin levels at any time compared with the control, but it did inhibit H-chain degradation. In contrast, FHsiRNA significantly blocked degradation of H-chain and caused a large and significant accumulation of labeled L-chain ferritin (Fig. 5).

We were curious as to why L-chain ferritin seemed not to be degraded, and its levels even continued to increase. At higher magnification, cells transfected with control or FLsiRNA generally showed diffuse L-chain ferritin labeling (Figs. 4j, 4l), whereas many cells transfected with FHsiRNA often displayed aggregates of L-chain ferritin accumulation around the nucleus (Fig. 4k). In another study, we had shown that overexpression of L-chain ferritin in LECs results in the accumulation of L-chain ferritin cytoplasmic inclusions.5 In the present study, many of the cells transfected with FHsiRNA contained larger, spherical areas of L-chain ferritin accumulation that were reminiscent of lysosomes (Fig. 4m). To determine whether L-chain ferritin was targeted to lysosomes, as would be expected as part of the normal degradation process, we labeled the transfected cells with red fluorescent dye (LysoTracker Red; Invitrogen) for 1 hour at 4 days after transfection (Fig. 4n). This fluorescent, membrane-permeant dye labels acidic organelles, primarily lysosomes and endosomes. Some structures labeled with the red dye also contained L-chain ferritin (Fig. 4o). However, not all L-chain ferritin colocalized with lysosomes, which would be expected due to the dynamic nature of protein turnover. Of note, the largest ferritin-positive structures colocalized with the largest lysosomes.

**Total Ferritin Concentration in Transfected Cells**

Since ferritin is a long-lived protein, it was important to determine the effects of siRNA-induced changes in de novo ferritin synthesis and degradation on total ferritin concentration in the LECs. As determined by ELISA, transfection with FLsiRNA decreased total ferritin concentration more than 10-fold at 2 days after transfection. This was followed by a rebound to approximately threefold lower than control ferritin at 4 days. In contrast, FHsiRNA increased the concentration of ferritin in LECs almost 200-fold 2 days after transfection. Ferritin concentration continued to increase, reaching levels 500-fold higher than that of the control 4 days after transfection (Table 1). These dramatic increases were most likely due to the very large increase in de novo ferritin L-chain synthesis combined with complete inhibition of L-chain ferritin degradation.

**Iron Incorporation into Assembled Ferritin with Different H:L Ratios**

Other studies have demonstrated that alterations in the ferritin H:L ratio affect the ability of ferritin to store iron.9 Since transfection with FHsiRNA dramatically altered the H:L ratio in LECs, the iron storage capability of ferritin in LECs was determined. The cells were loaded with 55Fe-transferrin after transfec- tion, cytosolic proteins were separated by PAGE, and the radioactivity of the bands determined. The radioactive band corresponded in size to a ferritin standard (450 kDa, not shown). FH siRNA-transfected cells had dramatically reduced iron incorporation into ferritin on day 2 and continued to decrease, reaching as low as 9% of that found in control cells by day 4 (Figs. 6a, 6b). Two ferritin bands of slightly different mobility were detected on the autoradiogram of FHsiRNA-treated cells. FLsiRNA-transfected LECs exhibited a significant increase in iron incorporation into ferritin 2 days after transfection. However, by day 4, the iron incorporation was not different from control levels.

**Changes in Iron Availability in Transfected Cells**

Since synthesis of ferritin is regulated by iron, the FHsiRNA-induced increase in L-chain ferritin may have been due to an increase in iron availability. Since transferrin receptor (TfR) levels decrease when intracellular iron availability increases, TfR levels are often used as an index of cellular iron status. Indeed, TfR levels detected by Western blot analysis were reduced by almost 40% when the cells were treated with FHsiRNA compared with cells treated with nontargeting siRNA controls (Fig. 7a). Furthermore, a functional assay of iron uptake from diferric transferrin by the TfR demonstrated a nearly identical reduction of iron uptake in FHsiRNA-transfected cells (almost 40%, Fig. 7b). These results indicate that FHsiRNA treatment increased iron availability, which ex-

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**Figure 3.** Effect of H- and L-chain siRNA on de novo ferritin subunit synthesis in LECs. (A, B) LECs were seeded in six-well plates, then transfected with nontargeting control or FH- or FLsiRNA. Two and 4 days later, the cells were incubated with [35S]-methionine for 20 hours. A sample for TCA precipitation was removed from the cell lysates, followed by immunoprecipitation of ferritin from the lysates. The solubilized immunoprecipitate was subjected to 12% SDS-PAGE, and radioactivity in the ferritin H- and L-chain bands was normalized to the total amount of radioactivity incorporated into protein (TCA). The data are expressed as a percentage of that of control cells transfected with nontargeting siRNA. The histograms represent the mean ± SEM of at least eight samples. *Significanly different from control (P < 0.05); ANOVA and Tukey’s test. (C) A representative gel showing newly synthesized H- and L-chain ferritin subunits labeled with 35S-methionine after siRNA treatment.
explains why L-chain ferritin synthesis increased in these cells. FLsiRNA had no effect on either TfR levels or iron uptake in the LECs.

Cystine Uptake and GSH Levels in Differentially Transfected LECs

As we recently demonstrated, increased intracellular iron availability increased cystine uptake by the glutamate/cystine antiporter with a concomitant increase in GSH concentration in LECs. In the present study, the amount of available iron in the LIP was increased by FHsiRNA transfection. This increase was confirmed by a decrease in the amount of $^{59}$Fe found in ferritin (Fig. 6), a decrease in the level of TfR in LECs (Fig. 7A), and decreased $^{59}$Fe-transferrin uptake by LECs (Fig. 7B). Levels of TfR are generally considered a reflection of cellular iron status. Indeed, FHsiRNA treatment caused a significant increase in cystine uptake and GSH concentration in LECs, as would be expected in the presence of increased intracellular iron (Fig. 8). In contrast, 4 days after FLsiRNA transfection, there was no significant change in either cystine uptake or GSH levels in LECs.

Concentration of VEGF in Conditioned Medium of Differentially Transfected LECs

Iron is a key regulator of the activity of the transcription factor, HIF-1. HIF-1 regulates production of numerous proteins, including VEGF (Fig. 1). In the present study, excess iron, added in the form of hemin, decreased VEGF, whereas the iron chelator dipyridyl increased VEGF accumulation in the CCM of cultured LECs (Fig. 9). Not surprisingly, FHsiRNA, which increased intracellular iron availability in LECs, significantly decreased the amount of HIF-1α movement into the nucleus by 50%, compared with the control ($P < 0.002, n = 4$). FHsiRNA also decreased VEGF accumulation in the CCM by approximately 60% (Fig. 9). FLsiRNA had no effect on HIF-1α levels in the nucleus, but caused a small decrease in VEGF accumulation in CCM.
DISCUSSION

The multimeric protein, ferritin, is responsible for the safe storage of iron within cells. Ferritin H-chain has ferroxidase activity that facilitates deposition of iron into the ferritin core, whereas ferritin L-chain is thought to facilitate the consolidation of the core.21 The ratio of ferritin H- and L-chains is tissue specific and significantly affects the ability of cells to store and retrieve iron.22

Overexpression of ferritin H- and L-chains was initially used to alter the H:L ratio in several different cell types, allowing for the study of the physiological importance of differences in the composition of this protein. The results of these studies indicate that overexpression of ferritin H-chain increased iron storage in ferritin, decreased the size of the LIP,7,8,23 protected the cells against damage from ultraviolet light or hydrogen peroxide exposure7,8 and was antiapoptotic.24 Overexpression of ferritin L-chain did not significantly affect intracellular iron distribution and was not protective against oxidative stress.7

Overexpression of ferritin L-chain occurs naturally in hereditary hyperferritinemia cataract syndrome (HHCS).25 In this dominantly inherited syndrome, patients have a mutation in the regulatory element of ferritin L-chain mRNA which results in its constitutive synthesis. As a result, high levels of L-chain-rich ferritin accumulate in tissues, including the lens. However, this L-chain-rich ferritin has little, if any, iron sequestered.26 Indeed, iron homeostasis is apparently unaffected in HHCS patients in whom L-chain ferritin is overexpressed. The only

| Table 1. Effect of siRNAs on Ferritin Concentration in LECs |
|---|---|---|
| Treatment | 2 d | 4 d |
| Ctl siRNA | 5.9 ± 4.0 | 4.9 ± 1.8 |
| FHsiRNA | 1066 ± 274* | 2497 ± 1140* |
| FLsiRNA | 0.4 ± 0.3* | 1.5 ± 1.1* |

Data are mean ferritin concentration (ng/mg ± SD). * Significantly different versus control siRNA.
tissue adversely affected by ferritin accumulation is the lens, which becomes cataractous as early as during infancy. This effect is most likely due to aggregation of overexpressed ferritin subunits. Of note, constitutive downregulation of L-chain synthesis in a patient with a mutation in the L-chain ferritin gene results in cataractous as early as during infancy. This finding suggests that L-chain ferritin functions as an iron buffer, controlling the level of potentially redox-active iron.

In our present study, treatment of cultured canine LECs with siRNA custom-made to canine ferritin H- and L-chains, very effectively decreased synthesis of each chain. Quantitation of FH and FL mRNA revealed no off-target effects of either siRNA on the mRNA of the other chain. In an interesting finding, FHsiRNA or FLsiRNA significantly altered the de novo synthesis of the other chain and the iron storage ability/capacity of ferritin. For example, FHsiRNA decreased ferritin H-chain synthesis by 50%, but also caused a dramatic increase in L-chain synthesis by 50%, but also caused a dramatic increase in L-chain synthesis and completely blocked degradation of both chains. As a result, there was a 500-fold increase in the accumulation of ferritin, which had been found free in the cytoplasm or within inclusion bodies with FHsiRNA treatment made cells less resistant to iron supplementation and chelation. It was concluded that H-chain ferritin does not catalyze damaging reactions. One to increase iron storage capacity, since iron stored in ferritin does not catalyze damaging reactions.

We found large amounts of L-chain ferritin accumulated within lysosomes of some LECs in which ferritin L-chain expression was increased (FHsiRNA treatment). However, some cells also were found free in the cytoplasm or within inclusion bodies that did not colocalize with lysosomes. These nonlysosomal, ferritin-containing structures may be similar to those observed when L-chain ferritin was overexpressed in transfected LECs. This accumulation of ferritin, which had a very high L:H ratio, reduced the intracellular storage capacity for iron and likely increased the size of the LIP. Increased availability of iron has been reported to block lysosomal degradation of proteins in RPE cells and ferritin degradation specifically. This mechanism may be an important protective one to increase iron storage capacity, since iron stored in ferritin does not catalyze damaging reactions.

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currying in LECs. However, it did not reveal any information as
to the rate of degradation or whether degradation is blocked
after ferritin enters the lysosome. In some of the cells, the
largest lysosomes contained the largest amounts of ferritin,
which may reflect an attempt to degrade the bulk of excess
ferritin L-chain induced by FHsiRNA transfection. The resulting
increase in intracellular iron availability as well as perturbation of
“normal” iron dynamics in the cell may reduce the efficiency of
ferritin turnover in lysosomes. Since L-chain-specific anti-
body used for immunofluorescence label L-chains in assem-
bled ferritin (“normal” or L-rich) as well as free L-chains, we
cannot determine whether any specific form predominates in
the lysosome compared with the cytoplasm.

The inhibitory effect of FLsiRNA on H-chain synthesis is quite
intriguing, especially since we demonstrated that FLsiRNA had no
effect on FH mRNA levels. FLsiRNA decreased de novo synthe-
sis of both L- and H-chain ferritin, thereby decreasing total
ferritin levels in these cells. This decrease in total ferritin
occurred despite the fact that FLsiRNA blocked the degrada-
tion of H-chain ferritin. In an earlier study of LECs transfected
with H- or L-chain ferritin expression vectors,8 we hypoth-
sized that LECs strictly control H-chain ferritin levels by secret-
ing or degrading this chain when insufficient L-chain ferritin
is available to form heteropolymeric ferritin. The results of the
present study indicate that FLsiRNA reduced L-chain ferritin
availability. The decreased availability would increase H-chain
ferritin secretion, lowering levels of newly synthesized (la-
beled) ferritin, and reduce total ferritin concentration in the
LEC.s. A significant decrease in de novo synthesis of L-chain
ferritin may initially increase the H:L chain ratio and would
favor increased incorporation of iron into ferritin (as seen at
day 2). However, by day 4, due to persistent reduced availability
of newly synthesized H-chain ferritin, the H:L ratio may have
stabilized to the extent that iron incorporation into ferritin was
no longer different from that in the control.

Ferritin synthesis, TIR levels, and iron uptake from TF are
recognized indices of iron availability and the size of the redox-
active LIP. Expression of proteins involved in iron metabolism
such as ferritin and TIR are regulated at the translational level
by iron, since iron dictates the activity of the cytoplasmic iron
regulatory protein (IRP). In iron depleted cells, the IRP can
bind to ferritin mRNA and block its translation. In contrast, IRP
binding to TIR mRNA stabilizes it and increases TIR synthesis.30
Therefore a decrease in TIR expression reflects an increase in
iron availability. IRP contains an iron sulfur cluster. In cells
with a sufficient amount of iron, IRP does not bind to mRNAs,
but functions as a cytosolic aconitase. We have shown that
when IRP functions as an aconitase (i.e., under conditions of
adequate iron availability) it regulates production of gluta-
mate.17 This regulatory system makes sense from a physiolog-
ical perspective, because cells that are iron-replete do not need
more iron. In fact they must decrease iron uptake to avoid the
damaging consequences of excess iron, which include damag-
ing free radical reactions.

The results of our present study indicate that the IRP regu-
ulatory system functions as expected. FHsiRNA-treated LECs
exhibited increased iron availability as measured by decreases in
TIR levels, iron uptake from TF, and iron incorporation into
ferritin in LEC.s. After FHsiRNA treatment, the LECs contained
two pools of iron-labeled ferritin with different mobilities. The
lower mobility (upper) band was mainly composed of L-chain
ferritin, perhaps only L-chain (data not shown). This finding is
not surprising since L-chain ferritin is present in huge excess
and is less acidic than ferritin H-chain. Therefore, it has a lower
electrophoretic mobility. The low level of iron in both bands is
consistent with the low iron storage ability of L-chain rich
ferritin.

De novo ferritin synthesis and ferritin degradation maintain
the level of total ferritin within cells. A 500-fold increase of
total ferritin in FHsiRNA-treated LECs after 4 days is much
larger than the effects on de novo synthesis and degradation
would indicate. However, the very long half-life of this protein
and different ways of measuring ferritin synthesis and total
content makes it difficult or impossible to make quantitative
comparisons. Measurement of de novo synthesis and degrada-
tion require not only interaction of primary antibodies with
ferritin; but also additional steps: isolation of antigen-antibody
complexes, several washes, gel electrophoresis, and autoradi-
ography. Therefore, only newly synthesized, 35S-labeled fer-
ritin is detected and it is possible that not all newly synthesized
ferritin was captured or detected under these conditions. In
contrast, determination of total ferritin concentration by ELISA
would capture most, if not all ferritin in the cell lysate, includ-
ing previously synthesized, nondegraded ferritin. Considering
the long half-life of this protein, as well as the decrease in
degradation rate resulting from siRNA treatment, it would be
expected that the ELISA results would differ greatly from the
de novo synthesis data.

Clearly, the alteration of ferritin subunit synthesis and ulti-
ately the H:L ratio in a complete ferritin molecule significa-
tantly changes intracellular iron dynamics. Our results indicate
that these alterations probably affect the binding activity of the
IRP and its aconitase activity. We have demonstrated in other
work that regulation of aconitase activity by iron has important
downstream effects on glutamate synthesis, cystine uptake,
and GSH levels in both LECs and RPE cells.17,18 As expected,
increased iron availability caused by FHsiRNA significantly in-
creased cystine uptake and GSH levels in LEC.s.

Iron is also important in regulation of VEGF production (Fig.
1) through its effects on HIF-1, which regulates VEGF synthesis
at the transcriptional level. An increase in iron levels increases
HIF-1α degradation and therefore decreases its movement into
the nucleus, where it combines with HIF-1/β to form the het-
erodimer transcription factor HIF-1. HIF-1 controls the synthe-
sis of dozens of proteins, including VEGF. FHsiRNA decreased
HIF-1α levels in the nuclei of LECs as well as VEGF accumu-
lation in the CCM, which is consistent with its ability to increase
intracellular iron availability. FLsiRNA had no effect on HIF-1α
levels in LECs nuclei, but did have a small effect on VEGF
secretion. The reasons for this are unclear; however, they may
be consistent with FLsiRNA’s effect in reducing ferritin H-chain
levels or may be related to the different time points at which
the measurements were made.

The ability of cells to regulate the ferritin H:L chain ratio is
essential for control of iron metabolism as well as the down-
stream regulation of the critical intracellular antioxidant GSH.
Furthermore, secretion of VEGF is also affected by alterations
in the ferritin H:L ratio. Although the role of VEGF in the lens is
unknown, this growth factor plays critical roles in the physi-
ology and pathology of the retina. The results of the current
investigation suggest that there are potentially important rela-
tionships between intracellular iron metabolism and VEGF
and clearly warrant further investigation. It is clear from the
present study that maintenance of cell-type–specific ferritin
H:L ratio is critical for cellular homeostasis, since this ratio
governs cellular iron availability and the multitude of essential
processes dependent on iron, including increasing the level of the
antioxidant GSH, which can protect cells against iron
catalyzed free radical reactions.

References

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