Identification of a Mechanism by Which Lens Epithelial Cells Limit Accumulation of Overexpressed Ferritin H-chain*

Malgorzata Goralska‡, Benjamin L. Holley, and M. Christine McGahan
From the Department of Molecular Biomedical Sciences, North Carolina State University, Raleigh, North Carolina 27606

The primary cultures of canine lens epithelial cells were transiently transfected with cDNAs for dog ferritin H- or L-chains in order to study differential expression of these chains. By using chain-specific antibodies, we determined that at 48 h after transfection overexpression of L-chain was much higher (9-fold over control) than that of H-chain (1.7-fold). We discovered that differentially transfected cells secrete overexpressed ferritins as homopolymers in the media. Forty-eight hours after transfection accumulation of H-ferritin in the media was much higher (3-fold) than that of L-ferritin. This resulted in lowering of the concentration of H-chain in the cytosol. Co-transfection of cells with both H- and L-chain cDNAs increased the intracellular levels of H-chain and eliminated secretion of H-ferritin to the media. We concluded that lens epithelial cells differentially regulate concentration of both ferritin chains in the cytosol. The overexpressed L-chain accumulated in the cytosol as predominantly homopolymeric L-ferritin. This is in contrast to H-chain, which is removed to the media unless there is an L-chain available to form heteropolymeric ferritin. These data indicate that the inability of cells to more strictly control cytosolic levels of L-chain may augment its accumulation in lenses of humans with hereditary hyperferritinemia cataract syndrome, which is caused by overexpression of L-chain due to mutation in the regulatory element in the untranslated region of the mRNA of the chain.

Ferritins are the highly conserved iron storage proteins found in species from bacteria to mammals. Mammalian ferritins, located predominantly in cytoplasm, are heteropolymers of 24 subunits of two types, heavy (H); 21 kDa) and light (L; 19 kDa). Despite high (~50%) sequence identity and similar three-dimensional structures, the subunits are genetically different and each has a distinct and complementary role in storing iron. The H subunit has a ferroxidase center responsible for uptake and oxidation of Fe^{2+} into ferric ions. Translocation of these ions into the core of the ferritin shell and their mineralization for long term storage is facilitated by the L subunit. The intracellular level of ferritin correlates with the size of the pool of intracellular iron, which plays the main role in the regulation of ferritin expression. The iron-controlled expression of both chains is primarily translational and involves binding of iron-regulatory proteins IRP1 and IRP2 (iron sensors) to iron-response element (IRE), a stem-loop structure present in the 5'-untranslated region of the mRNA (1). The expression of H-chain gene is also regulated transcriptionally by cytokines (2), hormones, oncogenes (3), and inducers of cell differentiation (4).

The H and L subunit ratio of mammalian ferritins varies and is highly tissue-specific; however, mechanisms by which cells maintain the chain-specific ratio are not fully understood. Although both chains complement each other in the process of sequestering and storing iron, H-chain has a broader range of functions. The overexpression of H-chain reduces cell proliferation (5) and apoptosis (6) and decreases heme and hemo-globin synthesis (7). H-chain efficiently chelates intracellular iron, and if its concentration is increased the free iron pool of the cells can be depleted causing an iron-deficient phenotype (8). However, depletion of the iron pool protects cells from iron-catalyzed oxidative damage caused by free iron (9). The importance of the H-chain for normal cell function was underscored by finding that inactivation of H-chain in knockout mice is lethal during embryogenesis (10).

Contrary to what is known about H-chain, there is little information about the biological role of L-chain other than facilitating deposition of iron. It can be concluded that H-chain and not L-chain is mainly responsible for a wide range of ferritin functions.

The physiological roles of ferritins with different H/L-chain ratios were extensively studied in vitro by using recombinant proteins purified from *Escherichia coli* and transiently or stably transfected cell lines such as primate fibroblastoid (COS) (11), mouse erythroblastic (MEL) (7), or HeLa cell lines (8). The results of studies on cells, which originated from malignant tissues, generated valuable information about the structure and functions of ferritin chains. However, there is concern that physiology and genetics of the cells from the cell lines do not always closely mimic that of the normal cells in vivo. In addition, the regulation of ferritin chain expression is tissue-specific (12), and the molecular mechanisms underlying this regulation are not yet fully understood.

In our previous studies on iron metabolism and resistance to UV irradiation of dog lens epithelial cells, we used a species- and tissue-specific model to change ferritin chain ratio by transiently transfecting primary cell culture with canine H- or L-ferritin cDNA (13). These studies revealed a discrepancy between expression of H- and L-ferritin chains under the...
same conditions. The lens epithelial cells overexpressed L-chain to a much higher degree (7-fold over control) than H-chain (20% over control). However, overexpression of H-chain but not L-chain increased iron incorporation into ferritin and the ability of cells to survive UV irradiation. The large accumulation (10–20-fold) of intracellular L-chain has been also detected in vivo in humans with hereditary hypoferritinaemia cataract syndrome. The accumulation of L-chain in this disease is caused by point mutations in the IRE sequence of L-chain, which results in uncontrollable synthesis of L-chain. There are several known mutations of IRE sequence of L-chain leading to significant overexpression of L-chain (14, 15). However, there are no known cases of significant overexpression of H-chain in vivo, despite the fact that IRE sequences of both chains are almost identical. These findings lead us to the conclusion that the detected discrepancy in expression of H- and L-chains by transfected lens epithelial cells may result from a difference in how the concentration of each ferritin chain type is controlled in order to secure the tissue-specific H/L ratio. The strict control of accumulation of H-chain could be necessary to protect cells from potentially damaging effects of this biologically active protein such as growth impairment or hypoferritinaemia.

The overexpression of L-chain, on the other hand, does not cause any apparent abnormalities in mammalian cells except for lenticular tissue, where formation of light-diffracting L-rich ferritin crystals creates opacities and as a result formation of premature cataracts in humans (16).

The results reported here demonstrate for the first time that transfected lens epithelial cells limit accumulation of overexpressed H-chain but not L-chain, which freely accumulates in the cytosol. We also provide information about the physiological mechanism by which cells prevent the H-chain overaccumulation.

MATERIALS AND METHODS

Cell Culture—Lenses were obtained from mixed breed dogs euthanized at the North Carolina Animal Shelter. The anterior capsules of the lenses, which contain epithelial cells, were removed and placed in a tissue culture dish containing Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Hyclone) and antibiotic-antimycotic (Invitrogen). The cells, which grew out from the capsule, were dispersed by trypsinization and after reaching confluence were plated in 6-well plates at 120,000 cells/well.

Plasmid Construction and Transient Transfection of LEC with Recombinant Plasmid—The coding sequences of dog H- and L-chain ferritin cDNAs were generated from dog lens epithelial cell mRNA by PCR and cloned into pTarget mammalian expression vector (Promega) under the control of the cytomegalovirus promoter. The expression of clones of H- and L-chain was tested using TNT Coupled Reticulocyte Lysate System (Promega), and dyeo sequencing was used to determine the cDNA sequence. Selected clones of the correct sequence, which expressed in vitro, were used for transfection (13). The plated cells were transfected with 2.5 μg of plasmid DNA in 750 μl of DMEM containing 10% serum and 4 μl of FuGENE 6 (Roche Applied Science) for 10–24 h. Transfection was terminated by changing media to serum-free DMEM. Cell lysates and media from transfected cells were collected after an additional 6–40 h of treatment and labeling. The degree of cell lysis after differential transfection was determined based on measurements of lactate dehydrogenase activity in the media and cell lysates with the CytoTox 96 (Promega). Treatment conditions are described in detail under “Results” and in the figure legends.

Designing the Ferritin Chain-specific Antibodies—We designed two peptides corresponding to chain-specific sequences of 21 (78–99) amino acids of canine lens H-chain and to 24 (148–172) amino acids of canine lens L-chain for immunization purposes. Amino acid sequences of dog ferritin chains were obtained from translation of the cloned canine lens ferritin chain cDNA sequences by using Swiss Protein Database (13). We designed the peptides based on known immunological properties of human H- and L-ferritin chains (17, 18). The designed peptides were chemically synthesized, conjugated with keyhole limpet hemocyanin, and used to produce the chain-specific antisera in immunized rabbits by Research Genetics, Inc. The anti-peptide titer determined for ELISA was 1:7,000 for anti-L antibodies and >1:200,000 for anti-H.

Electrophoresis and Immunodetection of Ferritin—Forty-eight hours after transfection, the cell lysates and media from differentially transfected cells were concentrated by filtering through Centricron 10 or 100 (Millipore Corp.). The concentration of proteins in cell lysates was determined by BCA Protein Assay Kit (Pierce). The cell lysate samples containing 70–150 μg of protein and concentrated media samples collected from two wells of transfected cells were analyzed by 10% SDS-PAGE using Tris/Tricine buffer system or on 7% PAGE. The gel-separated proteins were transferred to nitrocellulose membrane (Hybond ECL, Amersham Biosciences) by semi-dry blotting at 20 V for 20 or 80 min (for PAGE separated proteins) in Bjerrum and Shafer-Nielsen transfer buffer. Ferritin bound to nitrocellulose was incubated for 2 h with one of the 1:1500 diluted chain-specific antibodies followed by a 1-h incubation with goat anti-rabbit IgG horseradish peroxidase (Santa Cruz Biotechnology) at a 1:1000 dilution. The immunoreactivity was detected by the ECL Western blotting Analysis System (Amersham Biosciences).

Immunoprecipitation of de Novo Synthesized Ferritin—Differentially transfected cells were labeled with [35S]methionine (50 μCi/750 μl DMEM methionine-free media) for 6–28 h. The total [35S]methionine uptake into control and transfected cells was measured in order to standardize the assay by eliminating variability. The cells were lysed with 0.05 m Tris/HCl buffer, pH 8.0, that contained 0.15 M NaCl, protease inhibitor mixture (Sigma), 0.02% sodium azide, and 1% Triton X-100 and immunoprecipitated with ferritin chain-specific or goat anti-horse ferritin antibodies (ICN Biochemicals). A sample of each cell lysate was precipitated with 20% trichloroacetic acid to quantitate [35S]methionine incorporation into totally, newly synthesized protein. Ferritin was immunoprecipitated with chain-specific antibodies (10–20 μl) from 500 μl of cell lysates at 4 °C for 1 h and subsequently treated overnight with 20 μl of Protein A/G Plus-agarose (Santa Cruz Biotechnology). Ferritin immunoprecipitation with goat anti-horse ferritin antibodies was performed for 1 h at 37 °C followed by treatment with 10% Pansorbin (Calbiochem) for an additional 30 min. Ferritin was released from the antibody complex by boiling in SDS-PAGE loading buffer and analyzed by 10% SDS-PAGE in Tris/Tricine buffer system.

Media from over transfected, [35S]methionine-labeled cells were concentrated using Centricron (Millipore Corp.) and analyzed on 7% PAGE or 10% SDS-PAGE under non-reducing conditions. The gels were autoradiographed, and radioactivity in the bands was quantified by an Instant Imager (Packard-Canberra).

RESULTS

In the present study, we used chain-specific antibodies to determine whether the discrepancy in overexpression of both chains by transfected LEC resulted from a difference in the ability to detect each ferritin chain. We also examined expression of each chain at different times after transfection to find if the distinct pattern of expression was responsible for the observed discrepancy.

Characteristics of Ferritin Chain-specific Antibodies—Results of Western blot analysis of cell lysates of differentially transfected cells are shown on Fig. 1A. The blot developed with anti-L-chain antibodies showed the single L-chain protein band only in cell lysates of L-transfected cells. Anti-L antibodies did not react with overexpressed H-chain protein in H-transfected cells. Anti-H antibodies bound to overexpressed H-chain protein in H-transfected cells and did not detect L-chain overexpressed in L-transfectants. The concentration of L-chain in H-transfectants or H-chain in L transfectants was below the level of detection for both types of antibodies.

The chain-specific antibodies were also used for Western analysis of assembled ferritin in cell lysates from differentially transfected cells separated by PAGE under nonreducing conditions (Fig. 1B). Cell lysates of LEC grown to high confluency were used as a control because the ferritin concentration in subconfluent, plasmid-transfected LEC, was below the level of detection. When tested with anti-L antibodies a single band was detected in control and L-transfected cells; however, only the band detected in the control lysates migrated with horse spleen ferritin standard. The band in L-transfected cells had...
lower mobility than that of the control. This concurs with what has been known about the migration pattern of L-rich ferritin, which is slower than that of H-rich ferritin due to difference in the charge of the chains (19). The difference in the ferritin mobility between control and L-transfected cells indicates that subunit composition of newly synthesized ferritin from L-transfected cells differs from that ofLEC-specific ferritin. The overexpressed L-chain could assemble and create a separate pool of L-rich ferritin, much larger than that of tissue-specific heteropolymeric ferritin, which was undetected by Western analysis of the cytosol of transfected cells.

Anti-H antibodies used for immunodetection did not react with assembled ferritin separated on nonreducing PAGE. It is possible that due to both, lower expression of H- rather than L-chain and low efficiency of semi-dry blotting transfer of large multimeric proteins, the content of H-ferritin was below the detection level.

Subunit Makeup of Ferritin from Differentially Transfected LEC Determined with Chain-specific Antibodies—Both anti-H and anti-L antibodies immunoprecipitated the newly synthesized, assembled ferritin present in cell lysates of differentially transfected cells 48 h after transfection (Fig. 2).

When precipitated with anti-H-chain antibodies, H-transfected showed a significantly increased level of H-chain, 70% over H-chain content of control, plasmid-transfected cells. There was also an increased level of L-chain, about 40% over L-chain of the control. As a result of increased levels of both chains in H-transfectants, the ratio of H/L-ferritin chains in H-transfected remained similar to that of control. L-transfected cells revealed significant increase in L-chain content (2.3-fold) when compared with the corresponding chain of plasmid transfecteds, but this increase was not associated with an increase in the level of H-chain, which remained the same as in control, plasmid-transfected cells. Based on these results, we can conclude that the ferritin of L-transfectants, detected with anti-H antibodies, must be a heteropolymeric protein of lower H/L ratio than that of endogenous LEC ferritin because anti-H antibodies would not react with free L-chain or L-homopolymeric ferritin but only with heteropolymers containing H-chain.

Results of ferritin immunoprecipitation with anti-L antibodies in the most part concurred with data obtained with anti-H antibodies. The overexpression of H-chain in H-transfectants was associated with an increase of L-chain content. The L-transfected cells demonstrated much higher levels of L-chain in L-transfectants, 9-fold over L-chain in control cells, when anti-L antibodies were used, in comparison to that detected with anti-H antibodies (2.3-fold). These results indicated that the majority of overexpressed L-chain remains either as a free chain or is assembled into L-rich or L-homopolymeric ferritin, which would not react with anti-H antibodies.

Distinct Changes in Levels of Overexpressed Ferritin Chains in LEC Over Time—In our previous study we examined ferritin chain overexpression 48 h after a 20-h transfection. The time was selected based on the transfection efficiency determined with pEGFP vector containing green fluorescent protein cDNA and confirmed by dramatic overexpression of L-chain in L-transfectants detected at that time. In the present study we hypothesized that lower expression of H-chain could result from different time courses of L-chain and H-chain expression. To test this possibility, we reduced the time of transfection from 20 to 10 h, and the time at which H- and L-chain overexpression was examined to 10 h post-transfection. The results of ferritin immunoprecipitation with polyclonal, goat anti-horse ferritin antibodies are presented in Fig. 3. Overexpression of H-chain in H-transfected LEC, 20 h after transfection, was 2.4-fold over that expressed in control plasmid-transfected cells. It was significantly more than that detected in both our earlier study at the 68-h time point (20% over control) and at 48 h in our present study with chain-specific antibodies (70% over control). In contrast, overexpression of L-chain, 7-fold over control, plasmid-transfected cells, was comparable with that detected at 68 h (7-fold) (13) and similar to that detected with chain-specific antibodies (9-fold over control). The lower concentration of H-chain in H-transfectants, 68 h after transfection,...
tion, could result not only from lower expression but also from lower accumulation of newly synthesized H-chain when compared with that of L-chain. This would explain the decrease in content of de novo synthesized H-chain as detected between 20 and 68 h after transfection (2.4- to 1.7-fold of control) and steady level of L-chain during the same time.

Ferritin Chains Overexpressed in Transfected LEC Are Released to the Media—The media from LEC transfected for 20 h and labeled with [35S]methionine for an additional 48 h were concentrated by filtration through Centricon-10 filters and analyzed by Tris/Tricine SDS-PAGE under reducing conditions. The results are shown in Fig. 4A. The ferritin chains were detected in the media of both H- and L-transfectants. The media from H-transfectants, 68 h after transfection (labeled for 48 h; Fig. 4A), contained significantly more H-chain (3.4-fold) when compared with L-chain found in the media of L-transfectants. This is despite the fact that the L-chain content of the cytosol of L-transfectants at that time was 7-9-fold higher than that of H-chain in H-chain transfecants. The presence of H-chain in the media from H-transfected cells and L-chain in media from L-transfectants was confirmed by Western blotting with chain-specific antibodies (Fig. 4B). Ferritin subunits were not detected in media from control, plasmid-transfected cells, and media from H- and L-chain-transfected cells did not contain detectable amounts of the opposite subunit.

The media from LEC labeled with [35S]methionine for 20 or 48 h, concentrated on Centricon-100 filters, were also analyzed by nonreducing 7% PAGE (Fig. 5A). The electropherograms show the presence of a single band in media from H- and L-transfectants. The band present in L-transfectant media had much lower mobility (Fig. 5A) than that of H-transfectant media. This documents the similarity in electrophoretic mobility between ferritin found in the media and present in the cytosol of L-chain transfected cells (Fig. 1B). The separation of media ferritin on SDS-PAGE eliminated the difference in media ferritin mobility (Fig. 5B).

The above results indicate that the overexpressed ferritin chains are present in the media as an assembled ferritin, predominantly or exclusively homopolymeric. During 20 h of labeling H- and L-transfectants accumulated a similar amount of ferritin. However, after an additional 28 h (68 h after transfection) concentration of ferritin in the media of H-transfectants was 3-fold over that found in media of L-transfectants.

This indicates a negative correlation between cytosolic level of H-chain, which peaked at 20 h and declined between 20 and 68 h and the accumulation of H-ferritin in the media, which increased significantly during 20–48 h after transfection.

Because there were no differences in the activity of lactate dehydrogenase in the media and cell lysates of differentially transfected cells (data not shown), we concluded that the presence of ferritin in the media resulted from active secretion rather than from leakage.

Co-transfection of LEC with Both H-chain and L-chain Ferritin Clones—Results of immunoprecipitation of newly synthesized ferritin from cell lysates of co-transfected and singly transfected LEC, 20 and 48 h after transfection, are presented in Fig. 6. At 20 h co-transfected cell lysates contained the same level of H-chain as H-transfectants. After an additional 28 h, the H-chain level in H-transfected cells was half that found in co-transfected cells. In contrast, the lower level (50%) of L-chain found at the 20-h time point in co-transfected cells increased after an additional 28 h and reached a concentration not significantly different from that of singly L-transfected cells. The cytosolic H/L-chain ratios calculated for each experimental group showed a 40% decrease (4.50 to 2.75) in singly H-transfected cells between 20 and 48 h. The H/L ratio in L-transfected cells stays the same at both time points indicating that content of L-chain does not change with time. At the 20-h time point in cells co-transfected with both L and H expression vectors, the overexpression of H-chain was associated with lower concentration of L-chain when compared with singly L-transfected cells. However, after an additional 28 h
or 48 h with [35S]methionine. Collected media were concentrated by filtering through Centricon-100, and retentates were analyzed by 7% PAGE (A) or 7% SDS-PAGE under non-reducing conditions (B). The results are the means ± S.E. of three experiments (PL, pTarget; H, ferritin heavy chain cDNA; L, ferritin light chain cDNA).

The concentration of newly synthesized L-chain in co-transfectants reached a level not significantly different from that observed for L-transfectants. The increase in L-chain level at 48 h lowers the H/L ratio in co-transfectants from 1.22 to 0.53 despite the fact that the level of H-chain in these cells was also elevated compared with singly H-chain transfected cells.

Electrophoretic analysis of the ferritin content of the media from co-transfected cells in comparison to single-transfected cells 48 h after transfection is presented in Fig. 7, A and B. The co-transfection with vectors carrying both ferritin genes almost completely abrogates secretion of assembled ferritin into the media (Fig. 7A). When analyzed by SDS-PAGE under reducing conditions, the media from all experimental groups showed the presence of ferritin chains (Fig. 7B). The media from co-transfected cells had only small amounts of L-chain and even less H-chain when compared with single L- and H-transfected cells. These results clearly indicate that there is no accumulation of assembled ferritin and very little accumulation of free chains in the media of LEC if cells are overexpressing both H- and L-ferritin chains simultaneously.

Given that media of both H- and L-transfectants separated by SDS-PAGE under reducing conditions did not contain a significant amount of the opposite subunit (Figs. 4A and 7B), it can be concluded that ferritin secreted by transfected LEC is exclusively or predominantly homopolymeric. The findings that media from non-transfected LEC do not contain ferritin or free ferritin chains (data not shown) corroborates our theory that ferritin secretion is a way of removing overproduced chains that are not assembled into heteropolymeric ferritin of a tissue-specific H/L ratio.

**DISCUSSION**

The high similarity of protein structure between H- and L-ferritin chains creates difficulties in their distinction and quantitation. The antibodies developed against synthetic peptides whose sequences were designed based on determined amino acid sequences of canine ferritin chains (13) proved to be helpful in overcoming these difficulties. The chain-specific antibodies reacted specifically with ferritin chains in free as well as in assembled form. With these antibodies we improved detection of both overexpressed chains by 30–40% in comparison to that detected with polyclonal antibodies, and we confirmed our earlier findings that 48 h after transfection differentially transfected LEC contain 5 times more newly synthesized, overexpressed L-chain than H-chain (13).

We also found that the overexpression of H-chain increases the levels of the endogenous L-chain as it has been detected with both anti-H and anti-L antibodies. This differs from what has been reported in studies on ferritin chain expression in transiently transfected COS (11) and stably transfected MEL (7). In these cell lines overexpression of H-chain caused a decrease in biosynthesis and an accumulation of endogenous L-chain. The discrepancy between these findings may result from lower accumulation of H-chain found in our system but can also indicate that mechanisms by which cells maintain tissue-specific chain ratio may be distinctively different in cells originating from primary culture than those of this particular cell line. We could not determine conclusively whether increased concentration of L-chain affects the H-chain biosynthesis. The association of overexpression of L-chain with the increase of endogenous H-chain was detected only with anti-L but not with anti-H antibodies. It is possible that anti-H antibodies do not recognize ferritin of high content of L-chain as efficiently as anti-L antibodies.

The analysis of the chain ratio of newly synthesized ferritin immunoprecipitated with chain-specific antibodies provides indirect evidence that overexpressed L-chains assemble with newly synthesized endogenous H-chains into heteropolymeric ferritin. The H/L-chain ratio of ferritin from L-transfected LEC was lower than that of endogenous plasmid-transfected cells, even if detected with anti-H antibodies (2.10 versus 0.95). The discrepancy in overexpression of L-chain detected with anti-L- (9-fold over control) and with anti-H antibodies (2.3-fold over control) indicated, however, that the majority of newly synthesized L-chains is assembled into L-homopolymeric ferritin. This was confirmed by Western blot analysis of ferritin present in cell lysates of L-transfectants (Fig. 1B). Based on the above findings we concluded that some of the overexpressed L-chains assemble in the cytosol into heteropolymeric ferritin of lower than the endogenous LEC H/L-chain ratio, but the majority of newly synthesized L-chains create a separate pool of either exclusively L- or L-rich ferritin.

In contrast to L-chain overexpression, the H/L-ferritin chain ratio in H-chain overexpressing LEC was not different from that of control cells. Knowing that the overexpression of H-chain was associated with an increased biosynthesis of endogenous L-chain, we speculated that most of the overexpressed H-chains assembled into ferritin of the chain ratio characteristic for lenticular tissue. Because we could not detect the assembled ferritin on Western blot developed with anti-H antibodies we were not able to confirm this hypothesis.

By having established that the discrepancy in expression of ferritin chains did not result from differences in our ability to detect each chain, we examined the expression of both chains at shorter time, 20 h after transfection, and compared the results with that detected previously, 68 h after transfection (13). The comparison revealed that during the time 20–68 h the ratio of
H- and L-ferritin chains (with pTarget (PL)) or with vector containing cDNA for H- and L-ferritin chains (H and L) and subsequently labeled with [35S]methionine for additional 10 h. B, the cells were transfected for 24 h and labeled with [35S]methionine for an additional 24 h. The cell lysates were analyzed by SDS-PAGE using a Tris/Tricine buffer system. The results are means ± S.E. of four (A) and three (B) experiments; *, p < 0.05, significantly different from corresponding experimental group in A by ANOVA and Tukey’s test.

The simultaneous overexpression of both ferritin chains affects the intracellular concentration of newly synthesized ferritin chains. The increased secretion limits H-chain accumulation in cytosol and may appear as a lower expression of H-chain. The long time (20–48 h) of metabolic labeling used in our study, necessary due to low density of cells used for transient transfection, provided the time frame for newly synthesized chains to be released into the media.

The L-transfected cells released less ferritin to the media when compared with secretion of H-chain by H-transfected LEC. The lower electrophoretic mobility of media ferritin of L-transfected was similar to mobility of the main fraction of cytosolic ferritin of these cells and differed significantly from that of endogenous LEC ferritin. This indicates that overexpressed ferritin assembled in cytosol and released to the media by L-transfectants has a high content of less acidic L-chain.

The Western analysis of subunit makeup of ferritin secreted into the media by H- and L-transfected cells did not show the presence of the matching ferritin chain. We also were not able to detect newly synthesized ferritin in the media of plasmid-transfected cells and in the media from confluent non-transfected LEC. These findings combined with the discovery that ferritin present in the media of transfected cells predominantly if not exclusively consists of the chain overexpressed in the cytosol implied that differential secretion could be a part of physiological mechanisms by which cells maintain the ferritin chain levels in order to secure tissue-specific ferritin H/L-chain ratio. To test this hypothesis we co-transfected LEC with both cDNA coding H- and L-chain and discovered that LEC overexpressing both ferritin chains did not secrete assembled ferritin to the media. These results document for the first time that differential secretion of newly synthesized ferritin chains could play a significant role in regulating the chain makeup of cytosolic ferritin. When enough of both H- and L-chains is made, they can assemble into heteropolymeric ferritin with the correct tissue-specific ratio. However, when either H- or L-chains are overexpressed and there is not enough of the other chain produced endogenously, the excess is assembled into homopolymeric ferritin and secreted to the media. These findings also indicate more strict control of H-chain cytosolic level than that of L-chain in order possibly to prevent H-chain overaccumulation. It is understandable considering that an excess of H-rich ferritin in cytosol could deregulate highly controlled iron homeostasis and affect cell proliferation. The L-transfected LEC accumulate a significant amount of L-ferritin in the cytosol and secrete less L-chain into the media. It has been known that the accumulation of L-homopolymeric ferritin seems not to have a negative effect on any tissue other than lenticular in which the formation of L-rich ferritin crystals causes opacities and premature cataracts in humans (16).

The simultaneous overexpression of both ferritin chains af-
fects their levels. The overexpression of H-chain was associated with lower expression of exogenous L-chain by co-transfected cells particularly at 20 h after transfection. This could not be explained by a decreased cytosolic iron content caused by an increase in H-chain concentration as shown by Picard et al. (22), because L-chain mRNA transcribed from the cDNA cloned into expression vector used in this study did not contain an IRE. The co-transfected LEC maintained the higher level of H-chain expression, in comparison to singly H-transfected cells, during the entire 20–48 h time after transfection. This combined with the finding that media of co-transfected cells do not contain the H-chain strongly indicates that cells retain the overexpressed H-chain in cytosol only if there is enough L-chain to form heteropolymeric ferritin. The observed differences in chain biosynthesis between singly and co-transfected LEC suggest the possibility of post-transcriptional regulation of the expression of one chain by the other.

There are no other studies on overexpression of ferritin chains by transiently transfected cells from primary cell cultures. Transient transfection of primate fibroblastoid COS-7, with cDNA for H- and L-human ferritin chains, resulted in large accumulation of both ferritin chains (200–500-fold when measured by ELISA), which did not cross-hybridize with endogenous chains and did not incorporate iron (11). The stable transfections of both the human (HeLa) (8) and murine (MEL) (7) cell lines with species-specific H-ferritin chain cDNA proved to be more successful. The stably transfected HeLa cells accumulated 14–16-fold more exogenous H-ferritin over the background for 7 days after transfection. The accumulation of H-ferritin in HeLa cells resulted in significantly reduced cell growth, but cells did not show signs of toxicity up to 6 weeks in culture. The very high 10:1 H/L-chain ratio of HeLa ferritin, not found in ferritins of any other normal mammalian cell type, could condition these cells to withstand such a significant concentration of H-chain. The overexpression of H-chain in stably transfected MEL cells was much lower than that in HeLa cells (3–4-fold over control) and therefore is comparable with what we found for transiently transfected LEC (2.4-fold over control) with 30–40% transfection rate.

In conclusion, the data clearly demonstrated that LEC differentially regulate the concentration of both ferritin chains in the cytosol. The overexpression of L-chain results in significant accumulation of L-homopolymeric ferritin in the cytosol and lower secretion of this homopolymer into the media. Contrary to L-chain, regulation of cytosolic concentration of H-chain is very strict. The overexpressed ferritin H-chain does not accumulate in the cytosol to the same extent as the L-chain but is removed into the media unless there is enough L-chain in cytosol to form heteropolymeric ferritin. Lack of such a strong control over L-chain levels could explain why unregulated (due to mutation in IRE) biosynthesis of L-chain leads to its accumulation. This accumulation may result in opacification of lenses in humans with hereditary hyperferritinemia cataract syndrome. The differential secretion may not be the only way the cells control levels of ferritin chains. The selective degradation or deposition of overproduced ferritin chain into insoluble hemosiderin (23) could also contribute significantly to the maintenance of the tissue-specific H/L ratio.

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