Heavy Chain Ferritin siRNA Delivered by Cationic Liposomes Increases Sensitivity of Cancer Cells to Chemotherapeutic Agents

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Abstract

Approximately half of all gliomas are resistant to chemotherapy, and new therapeutic strategies are urgently needed to treat this cancer. We hypothesized that disrupting iron homeostasis in glioma cells could block tumor growth, based on an acute requirement for high levels of iron to meet energy requirements associated with their rapid growth. Ferritin is best known as an intracellular iron storage protein, but it also localizes to tumor cell nuclei where it seems to protect DNA from oxidative damage and to promote transcription. In this study, we hypothesize that silencing the H-ferritin (heavy chain ferritin) gene could increase tumor sensitivity to chemotoxins. To test this hypothesis, H-ferritin siRNA was delivered to several human cancer cell lines by using cationic liposomes (C-liposome). H-ferritin siRNA decreased protein expression by 80% within 48 hours, and this decrease was associated with more than 50% decrease in the LD50 for DNA-alkylating agent carmustine (BCNU), which is commonly used to treat glioma in clinic. In a subcutaneous mouse model of human glioma, intratumoral injections of liposomes containing H-ferritin siRNA reduced the effective dose of BCNU needed for tumor suppression by more than 50%. A plasmid supercoil relaxation assay showed that H-ferritin specifically and directly protected DNA from BCNU treatment. H-ferritin siRNA additionally seemed to increase apoptosis in glioma cells in vitro upon H-ferritin knockdown. Overall, our results illustrate how silencing H-ferritin can effectively sensitize tumors to chemotherapy and also show the ability of C-liposomes to serve as a novel in vivo delivery tool for siRNAs. Cancer Res; 71(6); 1–10. ©2011 AACR.

Introduction

Malignant glioblastoma multiforme is a deadly brain tumor for which there is no effective treatment. The limitations associated with treatment strategies include drug-resistant characteristics of the tumors, inability of chemotoxic agents to traverse the blood–brain barrier in effective amounts, radiation toxicity to normal brain tissue, and potential risk of significant neurologic damage associated with tumor resection in elegant brain regions. Administration of higher dosages of chemotherapeutic agents is generally not an option, because they are often accompanied by stronger adverse side effects (1); the same is true of increasing radiation toxicity (2). One effective way to increase efficacy of therapeutic agents is to increase the sensitivity of cancer cells to these agents.

Herein, we propose to increase the efficacy of chemotherapeutics by using cationic liposomes (C-liposome) that are capable of delivering therapeutically effective concentrations of siRNA to tumors cells.

The use of siRNAs for treating cancer is an area of considerable interest, and many promising siRNAs have been previously identified. More than 28 genes have been evaluated as siRNA targets in different types of cancer. VEGF siRNA is implicated for treating fibrosarcoma, Ewing’s sarcoma, and prostate cancer, and EGFR (epidermal growth factor receptor) siRNA is implicated for the treatment of glioma (3). The major focus of our efforts to find a suitable target gene for siRNA has been ferritin, which is a key protein in maintaining cellular iron homeostasis (4). Ferritin tightly regulates the intracellular availability of iron in aerobic or anaerobic organisms (5) and is thus essential to cell viability. It functions as an iron-storage and -regulating protein in the cytosol, mitochondria, and nucleus (6). Iron is an essential trace element for cell growth and proliferation, as it is involved in a variety of redox reactions (7), which makes it essential for maintaining cellular homeostasis (8). We identified ferritin as a potential target for siRNA therapy after it was discovered that one of the ferritin subunits, H-ferritin (heavy chain ferritin), was present in the nuclei of glioma cells (6, 9). Nuclear ferritin is distributed nonrandomly, with preferential association with heterochromatin, which suggests that it might be involved in facilitating
DNA synthesis (9, 10). Ferritin in the nucleus has a protective function, as shown by its ability to protect DNA of cornel epithelial cells from UV damage and oxidative stress (11–13). Ferritin may also play a role in drug resistance mechanisms, as repeated exposure of cells to hydroxyurea results in an increased resistance to this toxic agent and an increase in ferritin mRNA (14). Ferritin overexpression is associated with the development of various cancers (15–19). On the basis of these reports, we hypothesized that downregulation of H-ferritin by siRNA in cancer cells could provide a mechanism to increase the chemotherapeutic sensitivity of tumors by eliminating the protective functions of H-ferritin. To test our hypothesis, C-liposomes carrying H-ferritin siRNA were prepared and characterized and the vulnerability of tumor cells treated with H-ferritin siRNA to chemotherapy was verified in vitro and in vivo.

BCNU [1,3-bis(2-chloroethyl)-1-nitrosurea] is one of the carmustines which is primarily used in brain tumor therapy, either as single agent or in combination with other drugs, due to its ability to cross blood–brain barrier. It is one of the few drugs approved by the Food and Drug Administration for treating malignant glioma, with a reported response rate of up to 30%. It generally acts by alkylation of DNA and RNA and unlike other alkylating agents, it is not cross-resistant to other alkylators. It also acts by inhibiting several key enzymatic processes by carbamoylation of amino acids in the proteins. Although BCNU has been reported to have superior activity, its clinical use is limited because of serious side effects. We chose BCNU in this study to determine whether silencing H-ferritin expression in cell and xenograft in vivo models could significantly decrease the effective therapeutic dose.

Materials and Methods

DC-cholesterol/DOPE lipids (30:70 w/w) and rhodamine-labeled phospholipids [l-α-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)] were purchased from Avanti Polar Lipids. BCNU (carmustine) was purchased from Sigma. Fluorescent siRNA and early endosomal antigen antibody (EEA1) were obtained from Santa Cruz Biotechnology. The nonspecific (NS) siRNA was from Qiagen, Milan, Italy. Female adult athymic nude mice were purchased from Charles Rivers Laboratories, Inc. Chamber slides were obtained from Nalgene Nunc International, Inc. MTS/PEMS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium/phenazine methosulfate assay] was purchased from Promega, Inc.

Cell culture

U251, U87, and malignant peripheral nerve sheath tumor cell line sNF6.2 were purchased from American Type Culture Collection, which were cultured according to the company’s instruction sheet and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% FBS (v/v), 0.1% (v/v) Gentamicin Reagent Solution (Gibco), and 1% (v/v) Non-essential Amino Acid Solution (Sigma). MCF-7 cell was a gift cell line from Dr. Andrea Mamm, Penn State University, Hershey, PA. These cells were maintained, according to the author’s instructions, in MEM (Gibco) supplemented with 10% FBS (v/v) and 0.1% (w/v) insulin from bovine pancreas (Gibco). All cells were maintained in a humidified atmosphere of 5% CO2/95% air at 37°C. The cumulative culture length of all the cell lines was less than 6 months. All the cells were originally tested and certified by the provider or the company.

Preparation of C-liposomes

To formulate the C-liposomes, lipids were dissolved in methanol/chloroform (1:1 v/v) and subsequently rotary evaporated to obtain a lipid film, which was dried over N2 and further dried at 4°C in a desiccator overnight. The lipid film was hydrated in PBS (2.8 mg/mL w/v) and sonicated. A polycarbonate membrane of gradually decreasing pore size was used to produce small unilamellar vesicles by extrusion through a 2-stacked, 0.1-μm polycarbonate membrane and subsequently with a 0.05-μm membrane, using a nitrogen pressure–operated extruder (LIPEX Extruder; Northern Lipids, Inc.). All the extrusions were carried out 10 times at an operating pressure of 800 psi (5,440 kPa). To study cellular uptake, the C-liposomes were labeled with rhodamine by adding a 1% (molar ratio) of rhodamine-labeled phospholipids [l-α-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)] to the liposome formulation.

Characterization of C-liposomes

The size distribution of C-liposomes was determined by light scattering analysis, using an ALV/DLS/SLS-5022F compact goniometer system (ALV). The zeta potential and polydispersity index (PDI) of C-liposomes were measured by the PALS Zeta Potential Analyzer (Ver. 3.16; Brookhaven Instruments Corp.). The PDI represents the distribution of particle size of the liposomes in a given medium or buffer in which they are suspended.

Cytotoxicity of C-liposomes

To evaluate the potential cytotoxicity of the C-liposomes, U251 cells (4,000 per well) were seeded in 96-well plates and allowed to attach overnight. The cells were transfected with 0, 2, 4, or 6 μL of the C-liposome stock in DMEM serum-free media. After 3 hours, the medium was replaced with complete medium. An MTS assay was conducted at 24, 48, and 72 hours posttransfection. The cytotoxicity of C-liposomes was also evaluated in mice. Athymic nude mice were divided into 3 groups, with 3 mice in each group. Control mice were injected with 50 μL of PBS. For the treatment groups, 6 μL of C-liposomes and 6 μL of 1.5 μg of C-liposomes/siRNA complex was injected intraperitoneally once per week for a total of 6 weeks. After 6 weeks, 300 μL of the blood was collected through tail vein. Liver and kidney toxicity, total bilirubin, blood urea nitrogen (BUN), creatine, aspartate amino transferase (AST, also called SGOT), alanine aminotransferase (ALT, also called SGPT), and alkaline phosphatase were determined using an automated chemistry analyzer and kits from Thermo Electron Inc., according to the manufacturer’s instruction.
Formation of C-liposomes and siRNA complexes

To determine the best ratio of C-liposomes and siRNA, C-liposomes and siRNA-FITC (fluorescein isothiocyanate) were mixed on ice under the following 6 conditions: ratio of C-liposomes and siRNA-FITC (fluorescein isothiocyanate) were prepared by mixing C-liposomes and siRNA-FITC (fluorescein isothiocyanate) at a ratio of (v/w) 0:1, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1. The mixtures were equilibrated at room temperature for 5 minutes and then DNA loading buffer was added. The samples were then loaded onto a 1% agarose gel and electrophoresed at 100 V for 18 minutes in TAE buffer. The image was taken by an image reader (FujiFilm LAS-3000).

Uptake of C-lipomesssiRNA

To visualize the uptake of the C-liposomes:siRNA complex by cells, U251 cells (10,000 per well) and U87 cells (20,000 cells per well) were seeded in 8-well chamber slides and allowed to attach overnight. After exposing the cells to serum-free media for 1 hour, cultures were exposed to siRNA-FITC:DMEM (1 μg/mL) or C-liposomes:rhodamine siRNA:FITC:DMEM (4 μg/mL) for 1 hour. The cells were then quenched with 4% paraformaldehyde and then stained with 1% (w/v) of DAPI (4,6-diamidino-2-phenylindole) in DMEM, mounted, and dried overnight. The cells were analyzed on a confocal microscope (Leica TCS SP2; Leica Microsystems) and subject to Z-plane sectioning (0.6-μm steps), followed by averaging by the method of SDs to obtain the final image.

Internalization of C-liposomes by the endosomal pathway

To determine whether the C-liposomes were internalized into cells via the endosomal pathway, we investigated the colocalization of C-liposomes with the early endosomal marker EEA1. The cells were treated as in the preceding paragraph, with the exception that following fixation, immunocytochemistry was conducted using a primary antibody for the endosomal marker EEA1 (1:15 dilution), subsequently followed by a secondary FITC-conjugated anti-goat IgG antibody (1:500 dilution). Images were captured through a confocal microscope.

Western blot analysis

The level of H-ferritin expression in each of the cell lines under investigation was determined by Western blot analysis. Briefly, U251 cells were allowed to attach overnight and were subsequently transfected with C-liposomes:siRNA (4 μL/1 μg) in 1 mL of DMEM for 3 hours before the media was replaced with complete media in which the DMEM was supplemented with 10% FBS. Lysates were harvested using radioimmunoprecipitation assay (RIPA) buffer (Sigma) at 24, 48, and 72 hours posttransfection and Western blotting for H-ferritin was done (6). Bands were scanned by a densitometer (GS-800 Calibrated: Bio-Rad) and quantified using Quantity One-4.5.0 software (Bio-Rad). The data quantified are expressed as a percentage of the controls.

Chemotherapeutic sensitivity

To test the hypothesis that H-ferritin siRNA will increase chemotherapeutic sensitivity, U251 and sNF96.2 cells (2,000 cells per well) and MCF-7 cells (4,000 per well) were seeded into 96-well plates and allowed to attach overnight. Cultures were transfected with C-liposomes:siRNA (4 μL/1 μg) in 1 mL of DMEM for 3 hours before the media was replaced with complete media. At 48 hours posttransfection, cells were exposed to BCNU for 48 hours at concentrations of 0, 20, 40, 60, 80, and 100 μmol/L. An SRB (sulforhodamine B) assay was done 48 hours after BCNU exposure. Data were expressed as a percentage of the control with respect to the untreated cells.

H-ferritin expression in vivo

To show that the H-ferritin siRNA delivered to tumors in vivo decreased H-ferritin expression, subcutaneous tumors were prepared by implanting 15 × 106 human U251 glioma cells in the flank of adult female athymic nude mice. When the tumor volume reached 10 to 14 mm3 in 2 weeks, the mice were randomly assigned to receive (a) 0.1 mol/L PBS, (b) C-liposomes:siRNA, or (c) C-liposomes:H-ferritin siRNA. Tumors were excised 48 hours postinjection and homogenized with RIPA buffer. Total protein was isolated and analyzed for H-ferritin expression by Western blot analysis.

Chemotherapeutic efficacy in vivo

To test the chemotherapeutic sensitivity in vivo, subcutaneous tumors were prepared as described earlier. When tumors reached 10 to 14 mm3, mice were randomly assigned to receive (a) C-liposomes:NS siRNA or (b) C-liposomes:H-ferritin siRNA (6 μL/1.5 μg). In both cases, the C-liposomes were injected intratumorally on a weekly basis. The day after C-liposomes were injected, all of the animals received intraperitoneal injections of BCNU. The BCNU was delivered on the opposite side of the tumor at a dosage of 12.5 mg/kg of body weight. This regimen was conducted for a total of 7 weeks. Tumor volume was recorded weekly by an individual blinded to the treatment strategy. A group receiving only BCNU was not included in this study for humane reasons, as the effect of BCNU alone on tumors is well established in the literature (20). Moreover, the aim of this study was to compare mice receiving H-ferritin siRNA with mice receiving the NS siRNA in order to determine whether there is a difference in efficacy between these 2 groups.

Supercoil relaxation assays

To determine whether the protection provided by H-ferritin could be directly on DNA, DNA backbone cleavage was determined by a supercoil relaxation assay. Covalently closed circular pUC19 DNA plasmid (BioLabs) was utilized, as has been in our previous experiments (6). For the experiments herein, 1 μg of plasmid was dissolved in 10 mmol/L HEPES (pH 7.5), 50 mmol/L NaCl, 2.5 mmol/L MgCl2, and 2.5 mmol/L DTT (total volume 30 μL), which allows the resolution of topoisomers under standard electrophoresis without ethidium bromide. The plasmid and reaction mixture was treated with BCNU in the presence or absence of H- or L-ferritin for 1 hour at 37°C and terminated with 50% (v/v) glycerol, 50 mmol/L EDTA, and 0.1% bromphenol blue. The sample was loaded on a 1.5% agarose gel and run under 40 mA for 6 or 8 hours at room temperature. After the electrophoresis,
the gel was immersed in 100 mL of TAE buffer containing 5 µL of SYBR Safe DNA gel stain (Invitrogen) for 10 minutes. The conversion of relaxation, linear, and supercoiled forms of DNA was examined using a LAS-3000 Fuji image reader.

Apoptotic effects
To determine whether H-ferritin siRNA induced apoptotic pathways, U251 cells were seeded into 6-well plates at a density of 166,000 cells per well and MCF-7 cells at a density of 333,000 cells per well and allowed to attach overnight. Cultures were transfected using C-liposomes:siRNA:DMEM (4 µL/1 µg/1 mL) for 3 hours before media was replaced with complete media. Cell lysate was collected using the lysis buffer from the Caspase-3 Fluorometric Assay Kit (R&D catalogue no BF1100) at 48 hours posttransfection, and the assay was conducted following the manufacturer’s instruction.

Statistical analysis
Student’s t tests were conducted as appropriate to determine whether significant differences existed between groups. A value of $P < 0.05$ was regarded as significant.

Results
Uniformly sized C-liposomes
The average particle size for 3 different batches of C-liposomes was 95 nm and ranged from 80 to 180 nm (Fig. 1A). The PDI of C-liposomes fell within the range of 0.1 to 0.2 (X PDI = 0.168, SD = 0.0065). The mean zeta potential for different batches of the C-liposomes equaled 35 mV ($\pm$0.25) before complexation and 5 mV ($\pm$0.25) after complexation with siRNA. The maximal optimal ratio of C-liposomes:siRNA 4 µL/1 µg (8 µL) in 1 mL of DMEM was 4:1 (v/w) C-liposomes:siRNA (Fig. 1B). Increasing the concentration of C-liposomes beyond this ratio did not result in an increased siRNA load. No cytotoxicity, based on cell viability assays (Fig. 1C), was observed with increasing concentrations of C-liposomes (2, 4, and 6 µL of C-liposome stock per mL of media).

Cellular uptake of C-liposomes:siRNA
No uptake was detected in U251 cells (Fig. 2A; top panels, c) or in U87 cells (Fig. 2B; top panels, k), when cells were exposed to FITC-labeled siRNA without C-liposomes. Uptake was observed in both U251 (Fig. 2A; bottom panels, g) and U87 cells (Fig. 2B; bottom panels, o) when the cells were exposed to the FITC-labeled siRNA with C-liposomes:rhodamine. Following the exposure of the cells to C-liposomes:rhodamine, the cells were immunostained for the early endosomal marker EEA1. Colocalization of C-liposomes and early endosomal marker EEA1 evidently indicated that uptake of the C-liposomes was through the endosomal pathway, which was consistent with our previous report for nanovesicle uptake (21).

H-ferritin siRNA downregulates H-ferritin expression in U251 cells
C-liposomes efficiently delivered H-ferritin siRNA into U251 cells and released a functional siRNA that successfully down-

regulated 70% to 90% of H-ferritin expression at 24, 48, and 72 hours, respectively (Fig. 3A). Delivery of NS siRNA as control had almost no effect on the H-ferritin expression levels (Fig. 3A).

H-ferritin siRNA enhanced chemotherapeutic sensitivity in vitro
The presence of H-ferritin siRNA decreased the LD$_{50}$ for BCNU from more than 100 to 40 µmol/L in U251 cells (Fig. 3B). The presence of H-ferritin siRNA also significantly increased
chemotherapeutic efficacy in MCF-7 (Fig. 3C) and sNF96.2 cells (Fig. 3D).

C-liposomes were not toxic tested through in vivo toxicology

There was no increase in the levels of liver and kidney enzymes AST, ALT, alkaline phosphatase, and total bilirubin (Fig. 4A and B) in the serum of the treated mice when compared with control mice.

H-ferritin siRNA downregulated H-ferritin expression in a tumor xenograft

Direct intratumoral injection of C-liposomes:H-ferritin siRNA resulted in greater than 50% decrease in H-ferritin expression in subcutaneous tumor xenografts 48 hours post-injection compared with mice receiving C-liposomes or C-liposomes:NS siRNA (Fig. 4C).

H-ferritin siRNA enhanced chemotherapeutic sensitivity in vivo

The chemotherapeutic efficacy of BCNU was enhanced by the presence of C-liposomes:H-ferritin siRNA. Tumor growth was effectively suppressed by 50% at week 5 and 100% at week 7 in the group receiving C-liposomes: H-ferritin siRNA compared with mice receiving C-liposomes:NS siRNA (Fig. 4D). Throughout the course of the C-liposomes:siRNA administration, both groups received 12.5 mg/kg of BCNU weekly through intraperitoneal

Figure 2. Internalization of C-liposome:siRNA complexes in U251 and U87 cells. A and B, C-liposome:siRNA complexes were internalized. a–d (U251 cells) and e–h (U87 cells), representative confocal images of the cells exposed to siRNA-FITC alone as control. FITC signal is not observed, indicating lack of siRNA uptake. e–h (U251 cells) and m–p (U87 cells), representative confocal images of the cells exposed to C-liposomes-rhodamine:siRNA-FITC complexes. Rhodamine and FITC colocalization around DAPI-labeled nuclei indicative of cellular uptake of liposomes:siRNA complexes is observed. Channels: blue, DAPI; red, C-liposomes-rhodamine; green, siRNA-FITC. C, colocalization of C-liposomes with endosomal marker EEA1. q–t (U251 cells) and u–x (MCF-7 cells), representative confocal images of the cells exposed to C-liposomes-rhodamine (red) and stained for EEA1 (with FITC secondary antibody, green); DAPI, blue. Rhodamine and FITC localization indicative of endosomal internalization pathway for C-liposomes. Lip, C-liposomes.

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injection. This concentration of BCNU is less than 50% of the reported effective dose (20) and was chosen on the basis of the decreased LD50 observed in the human cell culture. The study was terminated at week 7 because the tumor growth was too large to continue in the control group.

The action of BCNU on DNA coiling was inhibited by rH-ferritin

A supercoil relaxation assay using DNA plasmid pUC19 revealed that BCNU exposure converts DNA to the supercoiled form with a concomitant decrease in the linear form of DNA (Fig. 5A). This effect was attenuated by rH-ferritin (Fig. 5A and B) but not by horse spleen ferritin, which is composed of 95% L-ferritin (Fig. 5B).

H-ferritin siRNA increased caspase-3 activity in U251 and MCF-7 cells

Caspase-3 activity was evaluated 48 hours posttransfection of C-liposomes:siRNA. The presence of siRNA for H-ferritin in U251 cells increased caspase-3 activity by 100% (**P < 0.0005; Fig. 6A) and 20% in MCF-7 cells (*P < 0.05; Fig. 6B) compared with cells transfected with NS siRNA.

Discussion

The following in vitro and in vivo results of this study were obtained: (i) C-liposomes composed of DC-cholesterol/DOPE lipids were nontoxic in vitro and in vivo. (ii) C-liposomes could complex with siRNA to form C-liposomes:siRNA, which was internalized into the cells through the endosomal pathway.
(iii) H-ferritin siRNA downregulated H-ferritin expression in cancer cells both in vitro and in vivo and resulted in increased vulnerability of glioma U251, sNF96.2, and breast cancer MCF-7 cells to chemotherapy. (iv) An antitumor effect of H-ferritin siRNA may be associated with its ability to alter the ability of BCNU, a DNA-alkylating agent to convert DNA to a supercoiled form, and maintain a significant portion of DNA in the relaxed and linear form which is associated with increased transcription. This function would be consistent with the presence of ferritin in the nucleus of cancer cells and our previous results suggesting H-ferritin promotes DNA relaxation (6, 10). (v) Downregulation of H-ferritin protein expression increased activation of caspase-3. Ferritin is typically considered a stable cytosolic iron-storage protein with slow turnover, thus targeting this protein with siRNA may seem counterintuitive. However, it has been reported that the half-life of cytosolic ferritin in K562 cells (22), human fibroblast cells (23), and HeLa cells (24) is approximately 12 hours. These data are consistent with the dramatic downregulation of H-ferritin seen within 24 hours in the human astrocytoma cell line. The results from this project are a promising initial step toward the development of siRNA gene therapy involving H-ferritin for the treatment of multiple tumor types. A crucial component of siRNA gene therapy is an effective delivery mechanism. C-liposomes were selected as a gene delivery vehicle for this study as an extension of the nanotechnology platform currently in use in our laboratory (21, 25). The C-liposomes in this study and the neutralized liposomes used in our previous studies share some common physical properties. Both were simple to prepare at a nanometer scale with similar methods and are nontoxic. Both liposomes are internalized by glioma cells through endosomal pathways; however, the C-liposomes were synthesized with positively charged lipids, so they were capable of forming a complex with siRNA or DNA by electrostatic interactions (26). Cytotoxicity and transfection efficiency of C-liposomes depend on the scale and uniformity of their size. During the preparation process, multiple
extrusions with different membrane filter sizes resulted in uniform size, which contributed to a transfection efficiency of more than 90%. The homogeneous size distribution of the C-liposomes diminished the chance of aggregation, and therefore a lack of observed toxicity, which corresponds with previous studies (26, 27). Efficacy and toxicity were examined both in vitro and in vivo. In the past, we have shown that siRNA delivered through nuclear transfection without C-liposomes downregulated H-ferritin expression approximately 5-fold in glioma cells compared with controls (10). This same siRNA delivered by C-liposomes downregulated H-ferritin expression by more than 9-fold both in vitro and in vivo. Thus, our present results further indicate that a nanoliposomal delivery mechanism can increase the efficacy of siRNA and optimize the amount of siRNA delivered.

In addition to showing that H-ferritin siRNA sensitizes cancer cells to chemotoxins, we addressed the potential mechanisms by which H-ferritin siRNA may be involved. As reported previously, H-ferritin was concentrated in the nuclear fraction containing heterochromatin in SW1088 astrocytoma cells (10). H-ferritin binds to DNA in SW1088 glioma cells and promotes DNA relaxation, thus functionally it should be associated with increased transcription (28). We have previously shown that ferritin releases iron in a controlled manner to promote DNA relaxation, although, in the higher concentration range, there is an increase in the linear form of DNA, which is associated with DNA damage (9, 10). These previous reports are consistent with the observations in this study that H-ferritin maintained DNA in a relaxed form even in the presence of high concentrations of an alkylating agent and blocking the formation of supercoiled DNA. Thus, the data presented herein provide a compelling argument for a protective and trophic role of H-ferritin in tumor growth through a direct effect on DNA.

In contrast, downregulation of H-ferritin was associated with an increased activation of caspase-3 in U251 and MCF-7

Figure 5. Influence of H-ferritin on DNA relaxation as evidenced by a supercoil relaxation assay. A, the effect of BCNU with H-ferritin on DNA. The DNA plasmid pUC19 was treated with incremental concentrations of BCNU in the presence (left) or absence (right) of 25 μmol/L rH-ferritin. The conversion to the supercoiled form of DNA was induced by BCNU (right). This effect was inhibited by H-ferritin (left). B, comparison of the effect of H-ferritin and L-ferritin on DNA. The DNA plasmid pUC19 was treated with 5 μmol/L of BCNU and then exposed to incremental concentrations of H-ferritin (left) or L-ferritin (right). The conversion to relaxed and linear forms was observed in the presence of H-ferritin (left) but not in the presence of L-ferritin (right). The presence of H-ferritin was associated with increased relaxed and linear forms (middle). L-ferritin had a minimal effect. rHF, rH-ferritin; LF, L-ferritin. R, relaxed form; L, linear form; SC, supercoiled form.
H-Ferritin siRNA Sensitizes Cancer Cells to Chemotoxin

Figure 6. The role of H-ferritin in apoptotic pathways. A, caspase-3 activity in U251 cells. B, caspase-3 activity in MCF-7 cells. The exposure of astrocytoma U251 cells (A) and MCF-7 cells (B) to C-liposomes:H-ferritin siRNA is accompanied by an increase in caspase-3 activity. No effects on caspase-3 activity were noted in MCF-7 cells receiving C-liposomes:NS siRNA. Values were normalized to controls without treatment. The differences between the NS and HF siRNA are significant at *P < 0.05.

References


14. McClarty GA, Chan AK, Choy BK, Wright JA. Increased ferritin gene expression is associated with increased ribonucleotide reductase cells. This observation is consistent with reports that downregulation of H-ferritin resulted in an increase in apoptosis of malignant mesothelioma cells (29) and that overexpression of H-ferritin inhibited TNF-α–induced apoptosis (30). The induction of caspase-3 upon silencing the H-ferritin in MCF-7 cells is similar to reports using other anticancer treatments (31, 32). These observations in vitro supported our hypothesis for the potential therapeutic value of silencing H-ferritin in these cells. In addition to the intracellular effects, H-ferritin is secreted by numerous types of cancer cells (15–19). The effect of secreted ferritin in cancer may be limited immunosuppressive responses (33). Thus, targeted siRNA therapy for H-ferritin in vivo may have an additive impact on tumor suppression and is an area warranting future exploration.

In conclusion, our study has shown that the vulnerability of cancer cells to chemotherapy can be increased by limiting H-ferritin expression. The importance of ferritin in cancer cell biology suggests that efficient delivery of siRNA for H-ferritin could be a viable therapeutic option for sensitizing tumors to chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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