

Expression of the Hemochromatosis (*HFE*) Gene Modulates the Cellular Uptake of ^{67}Ga

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Recent studies have revealed that the wild-type hemochromatosis protein (*HFE*) interacts with the transferrin receptor (*TfR*) and modulates *TfR*-mediated iron uptake by cells. Because of similarities in the transport of gallium and iron and the use of ^{67}Ga scanning in lymphoid malignancies, we examined the effect of *HFE* expression on ^{67}Ga uptake. **Methods:** ^{67}Ga and ^{59}Fe uptakes were measured in HeLa cells transfected with a FLAG-tagged wild-type *HFE* (*fHFE*) gene under control of a tetracycline-repressible promoter. *fHFE* and *TfR* protein levels were measured by Western blotting; cellular transferrin (*Tf*) binding sites were measured by ^{125}I -*Tf* binding assay. **Results:** Induction of *fHFE* expression produced an increase in *TfR* protein that was accompanied by a decrease, rather than an increase, in cellular ^{67}Ga and ^{59}Fe uptake. The difference in ^{67}Ga uptake between *fHFE*-expressing and *fHFE*-nonexpressing cells was markedly increased in the presence of *Tf*. Although *fHFE* expression produced an increase in cellular *TfR* protein, cell surface and intracellular *Tf* binding sites were actually decreased in these cells. **Conclusion:** Our studies suggest that expression of wild-type *HFE* in cells produces a decrease in ^{67}Ga uptake due to a reduction in available *Tf* binding sites for ^{67}Ga -*Tf* on the *TfR*. These results imply that ^{67}Ga uptake by cells with wild-type *HFE* may differ from cells with the *HFE* C282Y mutation.

Key Words: gallium; transferrin receptor; hemochromatosis; iron; tumor imaging

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Scanning with ^{67}Ga is of value in the detection of viable malignant cells in tumor masses that persist after treatment of patients with Hodgkin's and non-Hodgkin's lymphoma (1,2). The mechanisms involved in gallium uptake by cells have been the subject of considerable investigation. Gallium resembles iron in many ways in that it circulates in the blood bound to transferrin (*Tf*), the iron transport protein, and is incorporated into cells by *Tf* receptor (*TfR*)-mediated endocytosis of ^{67}Ga -*Tf* complexes (3-5). In addition, *TfR*-

independent uptake of ^{67}Ga by cells has been described (3-5).

Hemochromatosis, an inherited disorder of iron overload, is one of the most common autosomal recessive diseases in individuals of northern European ancestry, with an occurrence of 1 in 300 and a carrier state of 1 in 10 persons (6). It is characterized by increased iron absorption from the gut and the deposition of excess iron in certain organs, including the liver, heart, and pancreas, resulting in tissue damage, morbidity, and mortality (7). Recent studies of the hemochromatosis (*HFE*) gene have revealed that in the cell, wild-type *HFE* protein binds β_2 -microglobulin and trafficks to the cell surface in association with the *TfR* (8,9). Wild-type *HFE* protein bound to the *TfR* competitively interferes with the binding of *Tf*-*Fe* to the *TfR*, thus decreasing *TfR*-mediated uptake of iron (10,11). The *HFE* C282Y mutation (substitution of cysteine by tyrosine at amino acid 282) is the predominant *HFE* mutation responsible for hemochromatosis (12); it results in the production of an *HFE* protein that fails to bind to the *TfR* (13).

The modulating effect of *HFE* on cellular iron uptake raises important questions regarding the potential impact that this gene may have on the uptake of gallium by cells. To address this issue, we conducted studies to examine ^{67}Ga uptake by HeLa (human cervical cancer) cells under conditions of inducible expression of the wild-type *HFE* gene. Our studies show that changes in the expression of *HFE* significantly affect the uptake of gallium by these cells.

MATERIALS AND METHODS

Materials and Cells

Human *Tf*, anti-FLAG monoclonal antibody M2, puromycin, and doxycycline were purchased from Sigma Chemical Co. Geneticin (G418) was purchased from GIBCO BRL. Anti-*TfR* monoclonal antibody was purchased from Zymed Laboratories. ^{67}Ga citrate was obtained from NycMed-Amersham. ^{59}Fe and Na^{125}I were purchased from Amersham, and ^{59}Fe -*Tf* and ^{125}I -*Tf* were prepared as described (14). The *fwtHFE/tTA* HeLa cell line expressing FLAG epitope-tagged wild-type *HFE* (*fHFE*) under control of the tetracycline-responsive promoter was used in our studies and was generously provided by Dr. Caroline A. Enns (University of Oregon). These cells have been described (8). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 400 $\mu\text{g}/\text{mL}$ geneticin, and 200 ng/mL puromycin.

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fHFE gene expression in cells was turned off by the addition of 1 $\mu\text{g}/\text{mL}$ doxycycline to the culture medium and turned on by the removal of doxycycline. Cells were incubated at 37°C in an atmosphere of 6% CO_2 .

Western Blotting

fHFE and TfR protein levels in cells were determined using an ECL Western blotting detection system (Amersham). Cell lysates were prepared, and total cellular proteins were resolved on a 10% (for HFE) or 7.5% (for TfR) NaDodSO₄-polyacrylamide gel and transferred to nitrocellulose membrane using a Transblot system (BioRad, Richmond, CA). Membranes were incubated in blocking buffer (phosphate-buffered-saline [PBS] with 0.1% Tween-20 containing 10% nonfat dry milk), followed by sequential washes in PBS-Tween. Membranes were then incubated in PBS-Tween containing 2 $\mu\text{g}/\text{mL}$ of monoclonal anti-FLAG antibody or anti-TfR antibody and then washed and incubated for 1 h at room temperature in buffer containing rabbit antimouse immunoglobulin (1:10,000 dilution) conjugated to horseradish peroxidase. For detection of bands, membranes were immersed in ECL detection solution and exposed to XAR-5 film for autoradiography.

Cellular Uptake of ⁶⁷Ga and ⁵⁹Fe and ¹²⁵I-Tf Binding Studies

fwHFE/tTA HeLa cells grown to confluency in flasks with or without 1 $\mu\text{g}/\text{mL}$ doxycycline were harvested and replated at a concentration of 5×10^5 cells/mL in 24-well plates. After cells had been incubated for 24 h in a CO_2 incubator, ⁶⁷Ga citrate (0–74 kBq [0–2.0 μCi]) and various concentrations of Tf (0, 1, or 10 $\mu\text{g}/\text{mL}$) were added to the wells. After 24 h of incubation with ⁶⁷Ga citrate, wells were washed with ice-cold PBS to remove unincorporated ⁶⁷Ga. Cells were removed from the wells, and the total amount of ⁶⁷Ga radioactivity in the cells was counted using a gamma counter.

For studies of ⁵⁹Fe uptake, cells grown in the presence or absence of doxycycline were replated in 24-well plates at a concentration of 5×10^5 cells/mL. ⁵⁹Fe-Tf (final Fe concentration, 8 ng/mL) was added to each well at the start of the incubation. After 24 and 48 h of incubation, cells were harvested and washed by centrifugation with PBS. The total ⁵⁹Fe cpm in the cell pellet was counted to determine the amount of radioactive iron taken up by cells.

Tf binding to cells grown with or without doxycycline was measured using an ¹²⁵I-Tf binding assay. Cells were harvested and washed twice with ice-cold PBS containing 0.1% bovine serum albumin, and binding studies were performed on 5×10^5 cells in 500 μL at 4°C and 37°C as described (14).

RESULTS

Induction of Wild-Type *fHFE* Expression Increases TfR Levels

To confirm that *fHFE* gene expression could be switched on or off in the cells used in our studies, cells were analyzed for the presence of fHFE protein by Western blot analysis after a 96-h incubation with or without doxycycline. As shown in Figure 1A, HFE expression was induced in the absence of doxycycline but was turned off in the presence of 1 μg doxycycline. Since prior studies have demonstrated an important role for the TfR in gallium uptake, cells were also analyzed for TfR expression by Western blotting. As shown

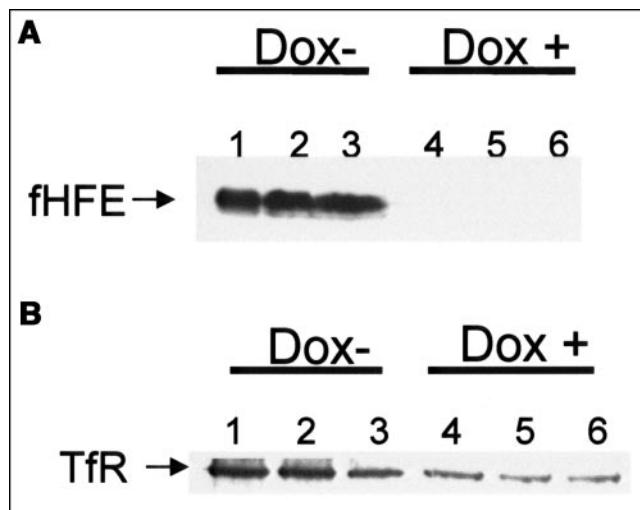


FIGURE 1. Effect of wild-type *fHFE* expression on TfR expression. (A) Western blot shows inducible expression of fHFE protein. Cells were incubated for 96 h in medium in absence (Dox –) or presence (Dox +) of 1 $\mu\text{g}/\text{mL}$ doxycycline. Total cellular protein was loaded on gel as follows: 30 μg in lanes 1 and 4; 60 μg in lanes 2 and 5; and 120 μg in lanes 3 and 6. (B) Western blot shows that induction of *fHFE* expression results in increase in cellular TfR. Dox – (lanes 1–3) represents cells grown in absence of doxycycline; Dox + (lanes 4–6) represents cells grown in presence of doxycycline. All lanes were loaded with 35 μg protein from cell lysates prepared from separate incubations.

in Figure 1B, lanes 1–3, the induction of *fHFE* expression resulted in an increase in cellular TfRs.

Effect of *fHFE* Expression on ⁶⁷Ga Uptake

⁶⁷Ga uptake by cells with or without *fHFE* switched on was measured using a range of ⁶⁷Ga concentrations and exogenously added Tf. As shown in Figure 2A–2C, after 24 h of incubation, cells that expressed fHFE incorporated a significantly lower amount of ⁶⁷Ga than cells in which fHFE was not expressed. In the initial experiment shown in Figure 2A, ⁶⁷Ga uptake was measured in cells incubated in serum-supplemented medium that contained bovine Tf (approximately 0.2 $\mu\text{g}/\text{mL}$). However, bovine Tf is known to have a much lower affinity for the human TfR (present on HeLa cells) than human Tf (15). Therefore, subsequent ⁶⁷Ga uptake experiments (Figs. 2B and 2C) were conducted in the presence of exogenously added human Tf. As shown in these figures, the differences in ⁶⁷Ga uptake between fHFE-expressing and fHFE-nonexpressing cells was more pronounced in the presence of 1 and 10 μg of human Tf, (Fig. 2B and 2C) than in the absence of exogenously added Tf (Fig. 2A).

Effect of *fHFE* Expression on Tf Binding Sites and Iron Uptake

In contrast to the increase in TfR protein seen on immunoblotting with the induction of *fHFE*, ¹²⁵I-Tf binding assay revealed that cells expressing fHFE had a lower number of total cellular and cell surface Tf binding sites (measured at

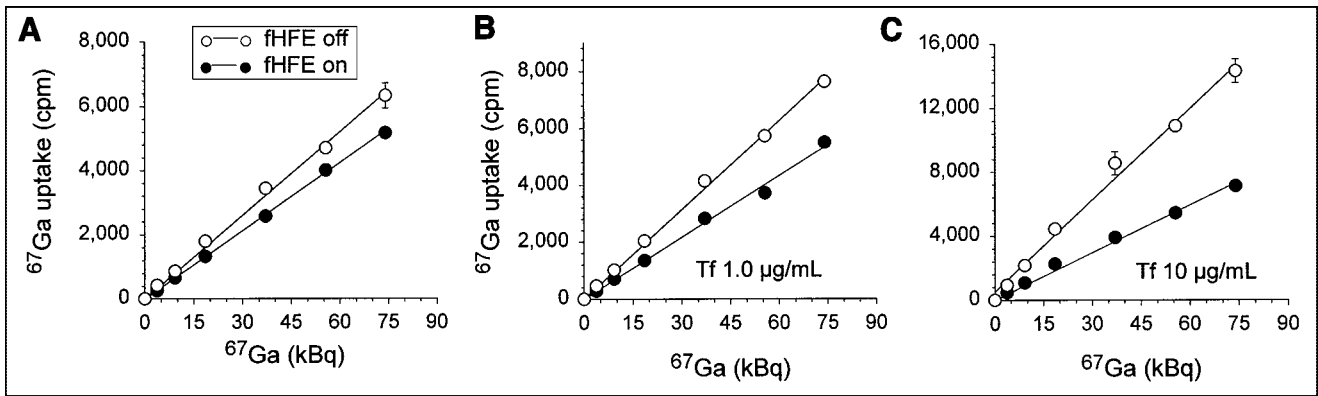


FIGURE 2. Effect of fHFE expression on ^{67}Ga uptake by cells. Cells were incubated for 96 h in absence of doxycycline to induce fHFE gene expression (fHFE on) or in presence of 1 $\mu\text{g}/\text{mL}$ doxycycline to repress fHFE expression (fHFE off). Cells were then replated in medium containing increasing concentrations of ^{67}Ga , and amount of radioactivity taken up by cells was determined after 24-h incubation. (A) ^{67}Ga uptake in complete medium. (B and C) ^{67}Ga uptake in complete medium containing 1 $\mu\text{g}/\text{mL}$ (B) or 10 $\mu\text{g}/\text{mL}$ (C) of exogenously added human Tf. Values shown represent mean \pm SE of representative experiment performed in triplicate. Differences in ^{67}Ga uptake between cells with fHFE turned off and on are significant ($P < 0.01$, by t test) for all data points above 15 kBq ^{67}Ga .

37°C and 4°C, respectively) when compared with cells in which fHFE was not induced (Fig. 3). To confirm that the decrease in Tf binding sites associated with fHFE expression had physiologic significance, iron uptake by cells was measured. As shown in Figure 4, the induction of fHFE expression resulted in a significant reduction in ^{59}Fe accumulation in cells over time.

DISCUSSION

In this study, we show that an increase in the expression of the HFE gene significantly decreases the uptake of ^{67}Ga by cells. Furthermore, our results suggest that the effect of HFE on ^{67}Ga uptake is secondary to a reduction in the binding of Tf with its receptor rather than a reduction in the

number of cellular TfRs. Analysis of TfR expression in fHFE-expressing cells by Western blotting revealed an increase in cellular immunoreactive TfR; however, an increase in TfRs per se would be expected to increase ^{67}Ga -Tf uptake rather than decrease it. In contrast, the decrease in Tf binding sites, as determined by the ^{125}I -Tf binding assay, would be expected to result in a decrease in the Tf-mediated uptake of ^{67}Ga . Therefore, a key finding in our studies that explains the HFE-induced decrease in ^{67}Ga uptake is that the expression of fHFE leads to a decrease in the number of

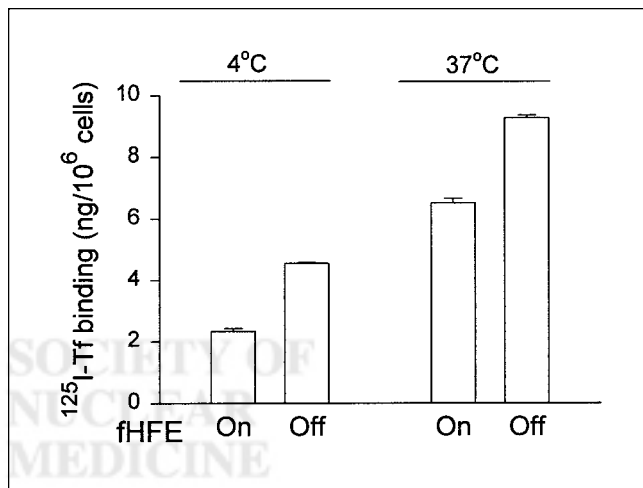


FIGURE 3. fHFE expression produces decrease in cellular Tf binding sites. Cells grown in presence (fHFE off) or absence (fHFE on) of doxycycline were analyzed by ^{125}I -Tf binding assay at 4°C and 37°C as described. $P < 0.003$ for differences in Tf binding between cells with fHFE turned on and off.

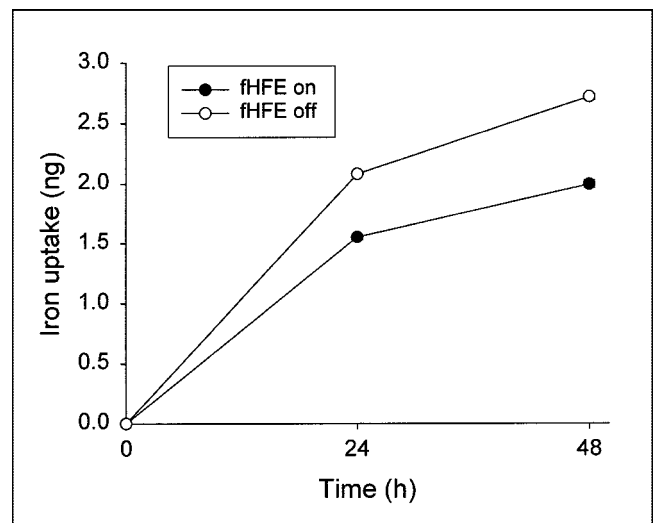


FIGURE 4. Effect of fHFE expression on iron uptake by cells. Cells grown in presence of (fHFE off) or absence (fHFE on) of doxycycline were replated in fresh medium and analyzed for ^{59}Fe uptake as described. Cells were incubated with ^{59}Fe at 8 ng/mL (as ^{59}Fe -Tf), and ^{59}Fe uptake was measured after 24 and 48 h of incubation. Values shown represent means and range of representative experiment performed in duplicate. $P < 0.00001$ for differences in iron uptake between cells with fHFE turned on and off.

Tf binding sites on the TfR in cells, which, in turn, leads to a decrease in the cellular uptake of ^{67}Ga -Tf complexes. The HFE-induced reduction in available Tf binding sites is consistent with recent reports that show that HFE protein competes with Tf for binding to the TfR (16). Our studies also confirm earlier findings demonstrating similarities between ^{67}Ga and iron uptake by cells via the Tf-TfR pathway.

Our study provides information that extends our current understanding of the mechanisms involved in ^{67}Ga uptake and its use as a scanning agent for tumor imaging. First, whereas prior investigations have established a role for the TfR in ^{67}Ga uptake, our results indicate that it is the availability of Tf binding sites rather than the level of the TfR protein per se that is a key factor in determining ^{67}Ga uptake by cells. Hence, studies attempting to correlate TfR with ^{67}Ga uptake may yield equivocal data if TfR levels are measured by immunologic methods alone using anti-TfR antibodies rather than by functional ligand binding assays. Second, the uptake of ^{67}Ga is likely to be decreased in malignant cells that express significant levels of wild-type HFE protein relative to cells that do not express HFE. Although our studies were performed using the HeLa cell line, our results are likely to be applicable to the clinical situation where ^{67}Ga scanning is used. Recently, we showed that immortalized peripheral blood B-lymphocyte cell lines derived from individuals with wild-type HFE or the HFE C282Y mutation express significant levels of wild-type or mutated HFE protein, respectively (17). It is likely, therefore, that HFE may be expressed in certain lymphoid malignancies and that this may impact on the results of ^{67}Ga scanning frequently performed in these disorders.

Our results also support the idea that differences in ^{67}Ga uptake by cells may exist between individuals with wild-type HFE and individuals with the HFE C282Y mutation. Whereas wild-type HFE protein binds to the TfR and interferes with Tf binding, the HFE C282Y protein does not bind to the TfR and thus does not interfere with the binding of Tf to its receptor (13). Accordingly, tumor cells with the HFE C282Y mutation would be expected to have greater ^{67}Ga uptake than those expressing wild-type HFE. Although we did not specifically examine cells with the HFE C282Y mutation, cells in which *fHFE* gene expression was turned off could be considered to be analogous to cells expressing HFE C282Y since an HFE-TfR complex is not formed in either situation.

CONCLUSION

The expression of HFE and its interaction with the TfR may play a role in modifying the uptake of ^{67}Ga by cells.

Further studies are planned to examine the expression of HFE in lymphoma cells since ^{67}Ga scanning is used for tumor imaging in this malignancy. Given the high frequency of the HFE C282Y mutation in the general population, such investigations appear warranted.

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REFERENCES

1. King SC, Reiman RJ, Prosnitz LR. Prognostic importance of restaging gallium scans following induction chemotherapy for advanced Hodgkin's disease. *J Clin Oncol*. 1994;12:306-311.
2. Front D, Israel O. The role of Ga-67 scintigraphy in evaluating the results of therapy of lymphoma patients. *Semin Nucl Med*. 1995;25:60-71.
3. Chitambar CR, Zivkovic Z. Uptake of gallium-67 by human leukemic cells: demonstration of transferrin receptor-dependent and transferrin-independent mechanisms. *Cancer Res*. 1987;47:3929-3934.
4. Luttrupp CA, Jackson JA, Jones BJ, Sohn MH, Lynch RE, Morton KA. Uptake of gallium-67 in transfected cells and tumors absent or enriched in the transferrin receptor. *J Nucl Med*. 1998;39:1405-1411.
5. Weiner RE. The mechanism of ^{67}Ga localization in malignant disease. *Nucl Med Biol*. 1996;23:745-751.
6. Merryweather-Clarke AT, Pointon JJ, Shearman JD, Robson KJ. Global prevalence of putative haemochromatosis mutations. *J Med Genet*. 1997;34:275-278.
7. Powell LW, George DK, McDonnell SM, Kowdley KV. Diagnosis of hemochromatosis. *Ann Intern Med*. 1998;129:925-931.
8. Gross CN, Irrinki A, Feder JN, Enns CA. Co-trafficking of HFE, a nonclassical major histocompatibility complex class I protein, with the transferrin receptor implies a role in intracellular iron regulation. *J Biol Chem*. 1998;273:22068-22074.
9. Parkkila S, Waheed A, Britton RS, et al. Association of the transferrin receptor in human placenta with HFE, the protein defective in hereditary hemochromatosis. *Proc Natl Acad Sci USA*. 1997;94:13198-13202.
10. Roy CN, Penny DM, Feder JN, Enns CA. The hereditary hemochromatosis protein, HFE, specifically regulates transferrin-mediated iron uptake in HeLa cells. *J Biol Chem*. 1999;274:9022-9028.
11. Corsi B, Levi S, Cozzi A, et al. Overexpression of the hereditary hemochromatosis protein, HFE, in HeLa cells induces an iron-deficient phenotype. *FEBS Lett*. 1999;460:149-152.
12. Feder JN, Gnirke A, Thomas W, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet*. 1996;13:399-408.
13. Feder JN, Penny DM, Irrinki A, et al. The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. *Proc Natl Acad Sci USA*. 1998;95:1472-1477.
14. Chitambar CR, Massey EJ, Seligman PA. Regulation of transferrin receptor expression on human leukemic cells during proliferation and induction of differentiation: effects of gallium and dimethylsulfoxide. *J Clin Invest*. 1983;72:1314-1325.
15. Penhallow RC, Brown-Mason A, Woodworth RC. Comparative studies of the binding and growth-supportive ability of mammalian transferrins in human cells. *J Cell Physiol*. 1986;128:251-260.
16. Lebron JA, West AP Jr, Bjorkman PJ. The hemochromatosis protein HFE competes with transferrin for binding to the transferrin receptor. *J Mol Biol*. 1999;294:239-245.
17. Chitambar CR, Wereley JP. Iron transport in a lymphoid cell line with the hemochromatosis C282Y mutation. *Blood*. 2001;97:2734-2740.