Cell surface expression of HFE protein in epithelial cells, macrophages, and monocytes

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ABSTRACT

Background and Objectives. Most patients with hereditary hemochromatosis are homozygous for a Cys282 →Tyr mutation in the HFE gene. This mutation has been shown to impair the association of the HFE gene product with β2-microglobulin and to prevent its cell surface presentation in transfected COS-7 and 293 cells. This study was performed to examine the expression of HFE protein in epithelial cells, macrophages, and circulating leukocytes obtained from normal subjects and patients with hereditary hemochromatosis.

Design and Methods. Antisera against two different peptides of the HFE protein were used to immunostain tissue sections and isolate granulocytes, lymphocytes and monocytes.

Results. Immunocytochemical staining showed that the HFE protein is expressed in gastric epithelial cells, tissue macrophages, and circulating monocytes and granulocytes. The cell surface associated signal, which was seen in normal gastric epithelial cells, monocytes and macrophages, was also present in C282Y mutant cells from patients with hereditary hemochromatosis, although at apparently reduced amounts in these cells.

Interpretation and Conclusions. From these studies, it is clear that the C282Y mutation reduces but does not completely prevent presentation of the HFE protein on the cell surface of human monocytes, tissue macrophages, and gastric epithelial cells.

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Key words: hereditary hemochromatosis, HFE, iron, leukocytes, plasma membrane

Hereditary hemochromatosis (HH) is the most common autosomal recessive disorder in populations of Northern European origin, affecting approximately 1:250 individuals. Its pathogenesis involves a defective regulation of intestinal iron absorption, which leads to iron overload of parenchymal cells in many organs. Clinical consequences of iron accumulation include cirrhosis of the liver, hepatocellular carcinoma, diabetes, heart failure, arthritis, and hypogonadism. One characteristic feature of iron overload in HH is the relative paucity of iron in Kupffer cells and macrophages. This contrasts with transfusional iron overload and other forms of secondary iron overload, which are characterized by progressive iron accumulation in these cells. The paradox of modest reticuloendothelial iron stores in HH has led some investigators to suggest that pathogenic mechanisms in HH involve a defective function of reticuloendothelial cells.

A novel candidate gene (HFE), which is responsible for most cases of HH, was cloned by Feder et al. Eighty-three percent of 178 HH patients were found to be homozygous for the same missense mutation (Cys282 →Tyr) in this gene. The high frequency of the C282Y mutation in HH patients has been confirmed by other investigators. The human HFE protein predicted from the cDNA sequence is an integral membrane protein which shares structural homology with major histocompatibility complex class I molecules. The C282Y mutation has been suggested to disrupt a critical disulfide bond in the α3 domain of the HFE protein and abrogate binding of the mutant HFE protein to β2-microglobulin (β2M). Feder et al. and Waheed et al. showed that the C282Y mutant HFE protein does not associate with β2M in human embryonic kidney cells (293 cells) or in COS-7 cells transfected with the mutant cDNA. Waheed et al. also demonstrated that much of the C282Y mutant protein remains in high molecular weight aggregates, fails to undergo late Golgi processing, and is rapidly degraded. While these studies provided a mechanism whereby the C282Y mutation impairs cell surface expression and Golgi processing of the HFE protein, they did not reveal how the HFE protein is involved in iron homeostasis or how the C282Y mutation might produce HH. Definite proof that HFE is the major gene that causes HH was pro-

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vided by recent observations in HFE gene knockout mice. These mice have abnormally high transferrin saturation and increased iron storage in hepatocytes, indicating that disruption of the HFE gene results in excessive accumulation of iron. It is also notable that these mice showed a similar pattern of hepatic iron storage to that seen in HH patients with relatively little iron in Kupffer cells, suggesting that functionally abnormal HFE protein is most likely involved in this defect in HH.

Previous immunohistochemical studies have demonstrated the localization of the HFE protein in the normal human gastrointestinal tract and placenta. The HFE protein in placenta is expressed on the apical surface of the syncytiotrophoblast cells, where transferrin-bound iron is normally transported to the fetus via transferrin receptor (TfR)-mediated endocytosis. In fact, we found placental HFE protein to be physically associated with TfR as well as with β2M. These studies together with recent observations of Feder et al. and Lebrón et al. showing the association of HFE protein with TfR, suggest that the HFE protein may regulate TfR-mediated iron uptake. We previously reported that surface epithelial cells of the gastric mucosa and some subepithelial leukocytes express the HFE protein. In the present study, we compare the staining of the HFE protein in gastric epithelium from HH patients and controls. We also compare HFE protein expression in tissue macrophages, circulating monocytes, and granulocytes from HH patients and controls. The difference in the expression pattern of the HFE protein between normal and C282Y mutant subjects may be linked to the paucity of iron deposition observed in reticuloendothelial cells of HH patients express the HFE protein. In the present study, we compare the staining of the HFE protein in gastric epithelium from HH patients and controls. We also compare HFE protein expression in tissue macrophages, circulating monocytes, and granulocytes from HH patients and controls. The difference in the expression pattern of the HFE protein between normal and C282Y mutant subjects may be linked to the paucity of iron deposition observed in reticuloendothelial cells of HH patients.

**Design and Methods**

**Antibodies**

Based on the structure of the HFE protein derived from cDNA sequencing, antisera against two different peptides of the HFE protein were produced in rabbits. The production and characterization of the CT16 antibody raised against a polypeptide of 16 C-terminal amino acids have previously been described. The second polyclonal antibody against a polypeptide predicted for the amino acids 76 to 91 was raised similarly in rabbits as described elsewhere, and we refer to this antibody as ECTO16. Using corresponding peptide-Affigel 10 affinity resins, affinity-pure and peptide-specific IgGs were isolated and stored in 50% glycerol at –20°C. Mouse anti-human macrophage antibody (Dakopatts, Glostrup, Denmark) was used to identify macrophages in paraffin-embedded tissue sections. Mouse anti-human CD14 antibody (Zymed Laboratories, South San Francisco, CA, USA) was used to identify the circulating monocytes. The following secondary antibodies were used for the immunocytochemistry studies: biotinylated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO, USA), biotinylated swine anti-rabbit IgG (Dakopatts), tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Sigma Chemical Co.), and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma Chemical Co.).

**Isolation of leukocytes**

Granulocytes were isolated by Ficoll-Paque density gradient centrifugation (Pharmacia Biotech, Piscataway, NJ, USA), after which the cell pellet was hemolyzed with a lysis solution containing 154 mmol/L NH4Cl, 10 mmol/L KHC03, and 0.1 mmol/L EDTA. The cells were washed twice with a balanced salt solution containing 0.01% D-glucose, 5 mmol/L CaCl2, 98 μmol/L MgCl2, 0.34 mmol/L KCl, 14.5 mmol/L Tris base, and 126 mmol/L NaCl, supplemented with a mixture of protease inhibitors (1 mmol/L pheynylmethylsulfonyl fluoride, 1 mmol/L benzamidine, and 1 mmol/L L-cycloheximide). After washing, the cells were frozen in liquid nitrogen. Giemsa staining indicated that >95% of these cells were granulocytes.

Control mononuclear cells were isolated from blood of a genotyped normal (wild type) individual by Ficoll-Paque density gradient centrifugation according to the protocol of Pharmacia Biotech. Viability on isolation was >95% by the trypan blue exclusion test. Monocytes were enriched by culturing them as described previously. Morphologically, more than 95% of non-adherent cells were lymphocytes as identified by Giemsa staining. The adherent cells were predominantly (>80%) monocytes.

Peripheral blood cells were also purified using a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson Immunocytometry Systems, San José, CA, USA) on the basis of their light scattering properties. Based on the size and granularity of the cells, leukocytes from a genotyped normal subject were separated into three distinct morphologic types: granulocytes, lymphocytes, and monocytes. For each of the groups 60,000 cells were separated, which were then centrifuged 100 x g for 10 min at room temperature. Cell preparations were placed on microscope slides, fixed in methanol, and stained with Giemsa stain. The purity of each cell fraction was >80%.

**Immunocytochemistry**

The specimens from pyloric antrum (three normal controls and three HH patients) were obtained after informed consent, together with routine histopathologic specimens taken during a surgical operation or endoscopy. All HH patients were C282Y homozygotes, and all had their body iron stores reduced to normal by phlebotomy before the endoscopy. They were all Caucasian males, and their ages at endoscopy were 49, 49, and 53 years. The samples were processed and immunostained as described elsewhere.

Isolated leukocytes were spread onto the microscope slides and fixed in 4% formaldehyde in PBS for 20 min. Saponin (0.05%) was used to permeabilize the cells. The steps in the immunostaining were essentially the same as described by Waheed et al. All reagents for SDS-PAGE are from Bio-Rad Laboratories (Richmond, CA, USA) or Sigma Chemical Co. SDS-PAGE was performed using a Mini-PROTEAN electrophoresis unit (Bio-Rad Laboratories) under reducing conditions according to Laemmli.
Western blotting was carried out using the CT16 antibody as described earlier.14

Results

HFE protein in circulating leukocytes

FACS-purification of normal leukocytes allowed us to immunostain enriched fractions of granulocytes, monocytes, and lymphocytes. Figure 1a-c shows typical immunostaining patterns of the HFE protein in granulocyte (a), monocyte (b), and lymphocyte (c) fractions. The granulocytes showed a granular, intracellular reaction. The monocytes showed predominantly a plasma membrane-associated immunoreaction. No positive signal for the HFE protein was detected in lymphocytes. Figure 1d shows that the cultured monocytes also expressed the HFE protein, but the signal was weaker than in fresh cell preparations, suggesting that the expression level of the HFE protein on the surface of monocytes depends on the culture conditions and/or cell differentiation.

Western blotting

The specificity of the HFE immunostaining in circulating monocytes and granulocytes was confirmed by Western blotting. Figure 2 shows that under reducing conditions, the major polypeptide identified by this antibody in each cell preparation was the 48-kDa monomeric HFE protein. The 48-kDa signal was prominent in granulocytes and monocytes, but was absent from lymphocytes.

Comparison of the protein expression in normal and C282Y mutant leukocytes

The staining patterns of normal and C282Y mutant HFE proteins in leukocytes were compared using double-immunostaining of cells from fresh peripheral blood. Monocytes were identified using a monoclonal anti-CD14 antibody and the expression of HFE protein was examined using the polyclonal CT16 antibody. Figure 3a shows that much of the wild-type HFE protein was expressed on the cell surface of a monocyte. Figure 3b shows the immunolocalization of the HFE protein in a monocyte from a genotyped C282Y homozygous HH patient. The cell surface-associated signal was lower. There was also a slight decrease in the intensity of the intracellular signal in the monocytes from C282Y homozygous patients when compared to the intensity of signal from normal monocytes. This difference might be due to faster intracellular degradation of the C282Y-mutant HFE protein that has been described by Waheed et al.14 The granulocytes from C282Y homozygous HH patients showed similar immunostaining to that seen in normal granulocytes (data not shown).

Comparison of the HFE protein expression in normal and C282Y mutant gastric epithelial cells and tissue macrophages

Normal human gastric mucosa (Figure 4a) showed a positive signal for the HFE protein on the basolateral surface of the epithelial cells. It also showed a positive immunoreaction in the tissue macrophages, where the staining was predominantly in the plasma membrane. A similar distribution pattern was
Expression of HFE protein obtained using the ECTO16 antibody (data not shown). Positive staining for the HFE protein was also seen in the gastric mucosa from C282Y homozygous HH patients (Figure 4b), but the intensity of the reaction was reduced, both in the surface epithelium and in the tissue macrophages. The positive immunostaining of the HFE protein was blocked in the presence of added CT16 peptide, and no staining was seen with non-immune serum (data not shown).

Discussion

Kupffer cells and macrophages have been suggested to be important in iron metabolism. Macrophages are numerous in the cellular lamina propria of intestinal villi, where absorbed iron is transported from epithelial cells to the circulation.24 Kupffer cells and splenic macrophages also play important roles in the destruction of aged, abnormal, or damaged erythrocytes. The iron from hemoglobin is normally freed and then released by the Kupffer cells and macrophages. In transfusional iron overload, iron is stored as ferritin and hemosiderin in these cells. In contrast, the excess iron in HH accumulates mainly in hepatocytes with relative sparing of reticuloendothelial cells until late in the disease.2,3

The paradox of modest reticuloendothelial iron stores in the face of extensive iron overload in parenchymal cells in HH has even led some investigators to suggest that the primary pathogenic mechanism in HH involves a defect in iron metabolism in Kupffer cells, monocytes, and macrophages. It has been observed that in HH, the lamina propria macrophages of the gut are located in the lower portions of the villi, whereas in secondary iron overload they are confined to the tips of the villi.24,25 The reason for this difference is not clear. Björn-Rasmussen et al.26 demonstrated that it cannot be explained by defective chemotactic properties of monocytes. Other approaches to search for the basic defect in HH in reticuloendothelial cells included studies of the uptake of transferrin-bound iron, of expression of TFR, and of metabolism of ferritin in monocytes of HH patients.27-32 Despite these studies, conclusive evidence for a specific defect has not been found. Recently, Cairo et al.33 showed that the binding activity of iron regulatory protein (IRP) was inappropriately high in monocytes of HH patients, which would be consistent with impaired reticuloendothelial iron storage of ferritin in macrophages. The mechanism of the reduced iron storage in C282Y mutant reticuloendothelial cells is most likely linked to defective function of the mutant HFE protein in these cells.

Recent studies have demonstrated an association between HFE protein and TFR, suggesting that the HFE protein may play a role in TFR-mediated iron transport.19-22 Comparisons of previous immunohistochemical results on the distribution of the HFE protein23 and TFR24,25 reveal that these proteins may be co-expressed in many tissues including esophageal, gastric and intestinal mucosa, and the placenta. So far, the physical association between these proteins has been demonstrated only in placental syncytiotrophoblasts,24 duodenal crypt cells24 and mammalian cell lines.19,20 Since the HFE protein and TFR21,22,23 are...
both expressed in monocytes and macrophages, the physical association of the normal HFE protein and TfR may exist in these cells as well.

The abundance of HFE protein in granulocytes and its localization predominantly in intracellular granules or vesicles are provocative observations. Cytochemical iron staining has shown that neutrophils contain large amounts of intragranular iron, which participates in bacterial killing.46 This is similar to the localization of β7M, which is present as an intragranular protein in neutrophils.47 It seems likely that HFE protein and β7M are associated in these cells as they are in transfected cells13,14,19,20 and placentomal syncytiotrophoblasts.48 An iron-binding protein, lactoferrin, has also been demonstrated in the cytoplasmic granules of neutrophils with a staining pattern that resembles the localization of the HFE protein.47 It will be of interest to determine whether the HFE protein in neutrophils associates with lactoferrin or lactoferrin receptor.

Although immunocytochemistry is, by nature, a semiquantitative method, the present data suggest that cell surface expression of HFE protein is reduced in the epithelial cells, macrophages, and circulating monocytes of HH patients. Nonetheless, these results indicate that some C282Y mutant HFE protein is expressed on the cell surface of these cells. This contrasts with earlier results in human Kupffer cells and transfected 293 and COS-7 cells in which cell surface expression of C282Y mutant HFE was not evident.13,14,42 Some studies have suggested that normal transportation of major histocompatibility complex class I proteins to the cell surface requires association with β7M.13,14,43,44 This association is clearly impaired between the C282Y mutant HFE protein and β7M.13,14 Nonetheless, there is good evidence that limited expression of human leukocyte antigens can occur on the cell surface even in the absence of β7M.46,47 This evidence is in agreement with our results showing a weak but definite expression of the C282Y mutant HFE protein on the cell surface of epithelial cells and macrophages. An interesting question is whether the C282Y mutation produces the HFE phenotype by reducing the amount of functional HFE protein at the cell surface, or by producing a dysfunctional protein that disrupts the regulation of iron absorption. The recently developed knockout mouse models of HFE13,19,15,16 may be helpful in addressing this and other important questions related to the mechanism of iron overload in HH.

Contributions and Acknowledgments

SP designed and performed the experiments. SP, RSB and WSS wrote the manuscript. AKP helped with cell cultures and immunocytochemistry. AW prepared antibodies. RSB and BRB were responsible for patient referral to our laboratory. XYZ, REF and ST contributed to the review of the paper. All authors were equally responsible for conception and design of the study and approved the final version of the paper.

The order of names takes into account the time, work and scientific contribution given by all authors. BRB and WSS, as senior authors, are cited last. We thank Dr. Lynn Dusin for the help with FAC5 purification and acknowledge editorial assistance of Elizabeth Torno.

S. Parkkila et al.

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Disclosures

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Potential Implications for clinical practice

- The reduced cell-surface expression of HFE protein in C282Y-homozygous HH patients may contribute to the paucity of iron deposition observed in reticuloendothelial cells and mononuclear phagocytes in these patients.

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345


