Characteristics and Expression of Binding Sites Specific for Ferritin H-Chain on Human Cell Lines


Purified recombinant human ferritin composed solely of H subunit was radiolabeled and incubated with proerythro-leukemic K562 human cells. A specific binding was detected, and it could be displaced only by ferritins, natural or recombinant, containing large proportion of the H subunit. The specific ferritin H-chain binding was saturable, and cells showed 17,000 to 23,000 binding sites per cell. The affinity constant measured at 37°C was of $3 \times 10^4$ M$^{-1}$. Treatment with pronase eliminated the specific binding. The binding sites were expressed in a high number during the cellular exponential phase of growth and progressively decreased to disappear when cells reached the plateau phase. Treatment of the cells with desferrioxamine increased recombinant H-ferritin binding, while iron had little effect. K562 cells induced to differentiate by hemin failed to bind ferritin H. Ferritin H-chain binding capacity is present on various cell lines such as HL60, lung cancer, and hepatoma cells. Analysis of the binding sites by western blotting showed a peptide with apparent mol wt of about 100 Kd.

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MATERIALS AND METHODS

Ferritins. Natural ferritins from horse liver and spleen and from human heart were purified. The recombinant H chain ferritin (rHF) was synthesised by an E coli strain transformed with the expression vector pEMBLex2HFT (p2HFT), which contains the full coding region of human ferritin H chain cDNA. The ferritin was purified by chromatography on Sepharose 6B column, precipitation by centrifugation at 200,000 g for two hours, and preparative polyacrylamide gel electrophoresis (PAGE). The characteristics of the ferritin have been described and are analogous to natural H-chain ferritin. The recombinant L chain ferritin (rLF) was synthesised by a E coli strain transformed with the plasmid pEXHF14, (a generous gift of Dr Seloff, Heidelberg, FRG), which expresses the full coding region of the human ferritin L chain. For easier construction the first two aminoterminal aminoacids were modified from Ser-Ser to Asp-Pro. The characteristics of this protein are described elsewhere. The ferritin was purified with the same method used for rHF. Both proteins appeared pure on electrophoretic analyses. The recombinant H and L and the human ferritins were radiiodinated with $^{125}$I by the chloramidine T method, obtaining a specific radioactivity of 3,000 to 8,000 cpm/ng for rHF and 1,300 to 2,000 cpm/ng for rLF. The radioactivity precipitable with 10% trichloroacetic acid accounted for 95% to 98%.

Cell cultures. Human erythroleukemia K562 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Cells were maintained at densities of 2 to 5 $\times 10^5$ cell/mL, unless specified, and experiments were performed with cells collected from 3 $\times 10^5$ cell/mL. The human acute promyelocytic leukemia cell line HL60, the human small cell lung cancer cell line NCI-H157, and the human hepatoma cell line were grown in RPMI supplemented with 10% FCS. Chang liver cells were grown in BME Eagle supplemented with 10% FCS. All experiments were performed on cells in log phase of growth, unless specified.

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Ferritin binding. For surface binding of $^{125}$I-rHF, $2 \times 10^9$ cells were incubated at 37°C with increasing concentrations of labeled rHF in phosphate-buffered saline (PBS) containing 1 mg/mL of bovine serum albumin. Unless specified the incubation time was 60 minutes. The same experiments were done also at 4°C. Nonspecific binding was determined by the addition of 100-fold excess of unlabeled rHF. Competitive binding assays with ferritins of various origin were performed in the same way.

Pronase treatment. To define whether rHF binding to cell surface was followed by ligand internalization, we examined the release of bound $^{125}$I-rHF from cells exposed to pronase (Calbiochem, La Jolla, CA) for 40 minutes at a final concentration of 100 μg/mL.37

Incubations in presence of hemin. To evaluate whether the expression of rHF binding sites on K562 cells modifies in differentiated cells, the cells were incubated with 40 μmol/L hemin for six days.38 The differentiation was ascertained by the increase of benzidine-positive cells in comparison to control (4% v 65%) assessed by light microscopy.

Incubations in presence of iron or desferrioxamine. The expression of rHF binding site was evaluated after growing K562 cells for 72 hours in presence of 0.5 mmol/L ferric nitroacetate (FeNTA) and 40 μmol/L desferrioxamine.39

Western blotting. It was performed essentially as described previously.40 Washed K562 cells (10^9) were resuspended in 1% sodium dodecyl sulfate (SDS), 0.25 mol/L tris pH 6.8, and incubated for 15 minutes at 37°C. They were then loaded on polyacrylamide gel and run.31 Protein were electrophoretically transferred to nitrocellulose paper, and the filters were overlayed with 2% casein and then with radiolabeled rHF (500,000 cpm/mL). Bound radioactivity was detected by autoradiography.

RESULTS

The radiolabeled recombinant H, and not the recombinant L ferritin, is able to bind specifically to K562 cells, and the binding is inhibited by the presence of excess of unlabeled rHF. The effect of natural and recombinant ferritins on the inhibition of rHF binding to K562 cells is shown in Fig 1. The strongest inhibitory activity is associated with ferritins with high content in H chain, such as rHF (100% H) and heart ferritin (60% H), while liver ferritin (10% H) displays little inhibition, and rLF (0% H) none at all. However, the unlabeled ferritins in the present experiment inhibited about 50% of the binding, the rest being nonspecific binding. In various experiments we have seen that the proportion of nonspecific binding was rather variable, ranging from 35% to 55% of the total. These differences appear to be related to the age of the labeled material, and the lowest nonspecific binding was obtained with ferritin radioiodinated the same day. Therefore all experiments were performed within one day after labeling. Competition assays were also performed with transferrin, lactoferrin, and human normal serum; no inhibition of rHF binding to K562 cells was observed in all cases (not shown). Time-course analysis showed that a steady state is reached after 30 minutes incubation at 37°C, while at 4°C the same level of steady state was reached after 90 minutes (Fig 2). The addition of various amounts of tracer ferritin to cells at 37°C showed a saturable binding, and the number of ferritin-binding sites could be calculated as about 17,000 to 23,000 per cell in various experiments (Fig 3). Scatchard plot analyses performed immediately after labeling rHF gave reproducible values of affinity constant $3 \times 10^6$ mol/L⁻¹ (insert of Fig 3).
Table 1. Effect of Pronase on the 125I-rHF Specific Binding to K562 Cells*

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Before Treatment (Bound cpm)</th>
<th>After Treatment (Bound cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>5.700</td>
<td>710</td>
</tr>
<tr>
<td>4°C</td>
<td>8.130</td>
<td>650</td>
</tr>
</tbody>
</table>

*Cells were incubated with ferritin at the temperature of the experiment, washed, and treated with 100 μg/mL of pronase for 40 minutes. Residual rHF binding was then determined.

Table 2. 125I-rHF Binding to K562 Cells at Various Days of Culture

<table>
<thead>
<tr>
<th>Day of Culture</th>
<th>Cells (10⁵/mL)</th>
<th>rHF Binding (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>350</td>
<td>12,730</td>
</tr>
<tr>
<td>2</td>
<td>820</td>
<td>11,520</td>
</tr>
<tr>
<td>3</td>
<td>910</td>
<td>8,520</td>
</tr>
<tr>
<td>4</td>
<td>1,120</td>
<td>3,280</td>
</tr>
<tr>
<td>5</td>
<td>1,170</td>
<td>1,240</td>
</tr>
</tbody>
</table>

Cells (10⁵/mL) were suspended in 25-mL flasks at day 0. Everyday the cells of two flasks were harvested and rHF binding assayed. The results represent means of assays on two individual flasks.

Table 3. 125I-rHF Binding to Various Cell Lines*

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>rHF Binding (cpm)</th>
<th>Binding Sites (No. per cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>15,050</td>
<td>20,800</td>
</tr>
<tr>
<td>HL60</td>
<td>16,540</td>
<td>23,000</td>
</tr>
<tr>
<td>NCI-N417</td>
<td>11,560</td>
<td>16,000</td>
</tr>
</tbody>
</table>

*Cells were incubated with increasing concentration of the tracer until a binding plateau was reached. Data represent means of triplicate experiments and express saturation binding.

Table 4. rHF Binding to K562 Cells Subjected to Various Treatments*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>rHF Binding (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemin (40 μmol/L)</td>
<td>125†</td>
</tr>
<tr>
<td>FeNTA (0.5 mmol/L)</td>
<td>9,350‡</td>
</tr>
<tr>
<td>Desferrioxamine (40 μmol/L)</td>
<td>27,530‡</td>
</tr>
</tbody>
</table>

*Means of triplicate experiments on 25-mL flasks.
†5 × 10⁴ cells grown for six days in hemin; control cells bound 12,300 cpm.
‡10⁵ cells grown in FeNTA or desferrioxamine for three days; control cells bound 11,800 cpm.

After incubation with labeled ferritin, cells were washed and treated with pronase. The results, shown in Table 1, indicate that all the specific binding was eliminated upon treatment with the proteolytic enzyme, different from control experiments in which radiolabeled transferrin was used. Analysis by trypan blue exclusion showed that cells treated with pronase did not modify their viability.

We studied the cells at different phases of growth by harvesting them after feeding for various days. The capacity to bind specifically the ferritin appeared to be maximum during the period of exponential growth (days 1 and 2) and decreased to disappear after reaching the plateau (Table 2).

Analysis of ferritin-binding sites on other cell lines showed that HL60 (promonocytic) and NCI-N417 (small cell lung cancer) had a similar density of binding sites (Table 3). Preliminary data indicate that cell lines from liver origin have a reduced number of binding sites (not shown).

K562 cells were induced to differentiate by incubation with 40 μmol/L hemin for six days, and the degree of differentiation was monitored by hemoglobin production using specific stain. In the differentiated cells the ferritin-binding sites virtually disappeared (Table 4). Incubation of K562 cells in presence of 0.5 mmol/L iron(III), given as FeNTA, did not affect the expression of ferritin-binding sites, while the iron chelator desferrioxamine caused a large increase of rHF binding sites expression (Table 4).

Preliminary characteristics of the ferritin-binding sites were studied by western blotting after separation of K562 homogenates on SDS electrophoresis. A major peptide of about 100 kd was enlightened after incubation with radiolabeled rHF (Fig 4), and the binding was inhibited by the presence of excess of unlabeled ferritin. The mobility of the peptide was not affected by incubation of the samples with 1% mercaptoethanol before the electrophoresis (not shown).

**DISCUSSION**

In this paper we were able to demonstrate the existence of specific binding sites for rHF on the erythroleukemic cell line K562 by using recombinant ferritin homopolymers of H chain. To the best of our knowledge this is the first report that defines the specificity for H chain of cellular ferritin binding sites. All previous studies on ferritin receptors made use of natural ferritins, which, being mixtures of H and L chains, do not allow definition of which one is the subunit.
that interacts with the cellular binding site. Only Covell et al.\textsuperscript{23} compared ferritins with different subunit compositions and suggested that the binding site may be specific for H subunit, since K562 cells bind preferentially heart (60% H) over liver (10% H) ferritin. It cannot be ruled out that in the studies in which ferritins from liver or spleen were used the cellular binding may depend on the small proportion of H chain present in the ligand. On the other hand, it is possible that H subunit binding sites are unrelated to the previously described liver/spleen ferritin receptors.

K562 cells during exponential growth bind 17,000 to 23,000 molecules of rHF with an average affinity constant of $3 \times 10^{10}$ mol/L$^{-1}$ (Fig 3, Table 3); these findings are in good agreement with the studies on rat hepatocytes in which it was shown that these cells can bind 23,000 to 30,000 rat liver (that is highly homologous to human) ferritin molecules with Ka of $1 \times 10^{10}$ mol/L$^{-1}$.\textsuperscript{19} In both the cell types a peptide is found to be responsible for ferritin binding, but it seems to differ in the two systems, since in rat hepatocytes it has a mol wt of 30 to 50 kd\textsuperscript{19} and in K562 cells of about 100 kd (Fig 4). Moreover, the binding sites in rat hepatocytes do not show specificity for either of the two subunits when using H- or L-enriched rat ferritins.\textsuperscript{20}

The effect of the temperature at which the binding experiments are conducted seems to vary in the different systems used: in rat hepatocytes,\textsuperscript{19,20} in HeLa cells,\textsuperscript{19} in guinea pig reticulocytes,\textsuperscript{29} and in K562 cells (Fig 2) ferritin binding appears to occur at 4°C as well as at 37°C. In contrast it was reported that in guinea pig reticulocytes\textsuperscript{20} ferritin binding does not take place at 4°C.

The variability of the temperature effect on ferritin binding in various systems reflects a further unclear characteristic of ferritin binding: whether it is followed by internalization. Our data suggest that this event does not occur readily, as indicated by the findings that the binding of rHF at 4°C and 37°C reaches the same level (Fig 2) and that all the bound ferritin is available to proteolytic attack of pronase (Table 1). This is in contrast with the electron microscopy analyses showing that liver ferritin binds coated pits in HeLa cells\textsuperscript{19} and undergoes an endocytic cycle in guinea pig reticulocytes\textsuperscript{19} and in human placenta.\textsuperscript{42} Cellular or species differences, most importantly, the different ferritin probes used in our and previous experiments could be responsible for these apparently contrasting results.

If rHF is not internalized, it is unlikely that it is able to donate iron to cell, and the binding site may have other functions not directly related to iron transport or storage. Indeed, it was shown that extracellular ferritin, even of the H type, is iron poor,\textsuperscript{10,43} and to our knowledge it was never shown that H ferritin is able to donate iron to cells.

The expression of rHF binding sites in K562 cells is high in actively proliferating cells and virtually disappears in quiescent cells (Table 2) and in cells that have been induced to differentiate with hemin (Table 4). Treatment with desferrioxamine, which is known to reduce the intracellular iron pool, induces an increase of ferritin-binding capacity (Table 4). These data are insufficient to establish a relationship between cellular iron metabolism and expression of rHF binding (which deserves further attention); however, they are reminiscent of those receptors, including transferrin receptor, whose functions are related to regulation of cellular growth.\textsuperscript{39}

Ferritin H type (and not the L type), either recombinant or natural, is able to inhibit the proliferation of precursors of granulocytes and monocytes in in vitro cultures.\textsuperscript{11,12} Broxmeyer\textsuperscript{44} showed that the interaction between cells and ferritin is related to cell cycle (in S-phase) and that Ia-like antigens may be involved, since no inhibition is apparent on cells missing the Ia. Moreover, H-rich, and not L-rich, ferritins inhibit lymphocytes’ E rosette formation,\textsuperscript{13} lymphocyte migration,\textsuperscript{16} and lymphocyte blastogenesis stimulation by phytohemagglutinin (PHA) and concanavalin A (Con A).\textsuperscript{16} It is expected that these effects on cell proliferation and activities are mediated by a specific binding of ferritin to cell surface. This binding would not require internalization, since it could be aimed at cell–cell surface interactions.

An indirect support to this hypothesis comes from clinical studies that showed that cancer patients have a higher number of ferritin-bearing lymphocytes\textsuperscript{14,15,45} and that the H type of ferritin is mostly increased.\textsuperscript{7} Our findings suggest that ferritin interacts with the cells through the binding site here described. We found rHF binding sites on all the cell lines of different origin we tested, including K562, HL60, lung cancer, hepatoma, and Chang liver cells. Further work is needed to analyze the distribution of the binding site and the function it may have on cellular activities. However, present findings suggest that ferritin H may play a role on the growth and differentiation of various cell types.

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