Changes in the characteristics and distribution of ferritin in iron-loaded cell cultures

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(Received 6 May 1980/Accepted 19 August 1980)

When Chang liver cells are grown in an iron-rich medium for up to 20 weeks, iron loading up to 50 times the normal cellular iron content may be obtained, although ferritin increases only to about 10 times normal. Ferritin has been isolated from such cells, and the isoferritin pattern found on elution from DEAE-Sephadex A-50 by increasing chloride concentrations has been used as a basis for studying changes in the properties of ferritin under conditions of cellular loading. A consistent shift of peak ferritin-elution position to higher chloride concentrations (lower pI) occurs when cells are loaded with ferric nitrilotriacetate for increasing lengths of time. A change in immunoreactivity also takes place on loading, the ratio of ferritin reacting with heart and spleen ferritin antibodies increasing at any particular value of pI. Cells were pulse-labelled with [^{59}Fe]ferric nitrilotriacetate and [^3H]leucine followed by non-radioactive iron in the same form. During the 72 h after the synthesis of new protein and its incorporation of iron, there is a slight acid shift in its isoelectric point. This effect is seen in both normal and loaded cells, with the whole spectrum being shifted towards lower pI in the loaded state. These findings suggest that the shift to more acidic ferritins on iron loading and the associated changes in antigenicity may be unrelated to subunit composition.

Cellular iron metabolism in cultured Chang cells derived from normal human hepatocytes was investigated previously (White *et al.*, 1976). These cells take up iron from transferrin and most of this is later found in the cytosol, partly incorporated in ferritin and partly in an easily chelatable non-haem non-ferritin complex. Iron uptake is about 30 times as great when ferric nitrilotriacetate is used as a donor as when transferrin iron is used (White & Jacobs, 1978). Progressive iron overload can be induced by incubation with ferric nitrilotriacetate for 4–6 weeks, after which time a state of equilibrium is reached (Jacobs *et al.*, 1978). There is a rapid rise in cytosolic ferritin over a 3-day period, followed by a slower accumulation. After 5 days there is a rapid increase in membrane-associated iron, the eventual degree of iron overload being related to the iron content of the medium. Electron microscopy shows the presence of single ferritin cores during the early stages of loading, followed by membrane-bound accumulations of cores from 4 to 8 weeks. After this time, amorphous iron-rich deposits are found and the appearances are similar to those found in human biopsy material (Jacobs *et al.*, 1978). Initial exposure of cells to iron concentrations above 450 $\mu\text{mol/litre}$ causes rapid death, but similar exposure after

initial stimulation of ferritin synthesis and siderosome formation by low iron concentrations is well tolerated, suggesting that these processes are protective. Freely proliferating Chang cells with up to 50 times the normal iron content can be maintained in culture with no morphological or functional signs of toxicity.

The stimulation of ferritin synthesis is similar to that seen in other studies of cellular iron uptake (Harrison *et al.*, 1974) and the ferritin concentration in Chang cells may reach 10 times the normal value. Previous studies of isoferritins from human tissues (Wagstaff *et al.*, 1978; Powell *et al.*, 1975a), horse spleen (Russell & Harrison, 1978) and rat ferritin (Hoy & Harrison, 1976), have presented differing views regarding the effect of iron loading on the isoferritin distribution in liver. In the present study an attempt has been made to compare the isoferritin patterns in normal and iron-loaded cells in culture and to gain some insight into their evolution. Iron-loaded cells produced previously and that had been stored at -20°C for 12 months were used for the isolation and characterization of ferritin. In addition, pulse doses of [^{59}Fe]ferric nitrilotriacetate and [^3H]leucine were given to cell cultures simultaneously in order to

study the process of ferritin synthesis in greater detail.

Methods

Normal and iron-loaded deep-frozen Chang cells collected during a previous 26-week experiment (Jacobs *et al.*, 1978) were used for the extraction and characterization of ferritin. For 'chase' experiments, Chang cells (Flow Laboratories, Irvine, Scotland, U.K.) were cultured in minimum essential medium containing 10% (v/v) foetal-calf serum. The iron concentration of the basal medium was $6\text{ }\mu\text{mol/litre}$ which, for the purpose of iron loading, was supplemented with $161\text{ }\mu\text{mol}$ of iron/litre in the form of ferric nitrilotriacetate as prepared previously (Jacobs *et al.*, 1978). Cells were subcultured every 7 days and pulse-labelled 3 days after subculturing by adding [^{59}Fe]ferric nitrilotriacetate ($25\text{ }\mu\text{Ci}$ /subculture) to give a concentration of $161\text{ }\mu\text{mol}$ of Fe^{3+} /litre and [^3H]leucine (The Radiochemical Centre, Amersham, Bucks., U.K.) [$25\text{ }\mu\text{Ci}$ (0.5 nmol)/subculture] for a period of 90 min, after which the label was removed by washing and reincubating with non-radioactive medium containing the appropriate concentration of iron. The cells were harvested at intervals into 10 ml of fresh medium, centrifuged at $200g$ for 5 min and washed three times in 10 ml of NaCl (154 mmol/litre). Cells were sonicated for 30 s to disrupt membranes and a portion of the sonicate was taken for analysis. The remainder was centrifuged at $20000g$ for 30 min to form a pellet from which the supernatant was removed. Purified human spleen ferritin ($100\text{ }\mu\text{g}$) and anti-(human ferritin) serum were added to 7 ml of the supernatant, incubated for 1 h at 37°C and left at 4°C overnight. The precipitate was washed three times with NaCl (154 mmol/litre) and dissolved in 1 ml of 0.01 M-HCl . Portions (1 ml) of whole cell homogenate, supernatant and ferritin were counted for radioactivity in an Intertechnique liquid-scintillation counter in 10 ml of scintillation cocktail {700 ml of toluene, 350 ml of Triton X-100, 3.5 g of PPO (2,5-diphenyloxazole) and 70 mg of POPOP[1,4-bis-(5-phenyloxazol-2-yl)benzene]}.

Ferritin was prepared from either the sonicated or the frozen cells by heating rapidly to 70°C and maintaining this temperature for 10 min, followed by cooling on ice for 1 h. The $20000g$ supernatant after centrifugation for 30 min was adjusted to pH 4.8 with 1.0 M-acetic acid and cooled on ice for 2 h. After further centrifugation, concentration of the supernatant was carried out if necessary by using an Amicon UM 10 filtration membrane (mol.wt. cut-off 10000), followed by chromatography on Sepharose 6B, further concentration of ferritin-containing fractions (previously determined by calibration with human spleen ferritin) by UM 10 filter and finally

anion-exchange chromatography with DEAE-Sephadex A-50 (Worwood *et al.*, 1976). ^{59}Fe and ^3H were measured in the fractions eluted from the ion-exchange resin by the method described above. Ferritin concentrations were determined by immunoradiometric assays for heart and spleen ferritin (Jones & Worwood, 1978). Estimations were carried out in triplicate on each sample, with each assay system.

Results

Anion-exchange chromatography of Chang-cell ferritin

The concentration of ferritin eluted from a Sephadex A-50 column as detected by the 'spleen' assay is shown in Fig. 1 as a function of increasing chloride concentration. Ferritin from cells grown on either 81, 161 or $312\text{ }\mu\text{g}$ of Fe^{3+} /litre for 1–10 weeks is eluted at a higher concentration than ferritin from normal cells. Cells grown at the same iron concentrations for 11–25 weeks produce ferritin that has even greater affinity for the column. The amounts of ferritin have been normalized to the same peak height for direct comparison of the peak elution positions. A progressive shift of the peak elution position from 0.255 M-Cl^- to 0.288 M-Cl^- is seen with increasing periods of iron loading. The concentration of iron in the medium and consequently the iron and ferritin concentration in the

Fig. 1. Profiles of ferritin prepared from Chang cells, eluted from Sephadex A-50 by increasing chloride concentrations and detected by antibodies to spleen ferritin

●, Normal cells; ▲, cells grown for 11–26 weeks in media enriched with up to $312\text{ }\mu\text{g}$ of iron/litre as ferric nitrilotriacetate. The curves have been normalized to the same peak height and concentrations are expressed in arbitrary units.

cells has no effect on the elution position of the ferritin peaks. The shift of the ferritin elution peak to a higher chloride concentration when cells are iron-loaded for increasing lengths of time has been consistently observed on several occasions, though the starting and finishing points do vary to a small extent from experiment to experiment.

Immunoreactivity of ferritin

The fractions eluted from Sephadex A-50 were assayed by using antibodies to spleen or heart ferritin and the ratio of their immunoreactivities was obtained. Heart-reacting ferritin has a greater affinity for the column, and the protein from normal cells shows the peak of heart-reacting ferritin at 0.245 M. The heart/spleen ratio rises sharply in the fractions eluting at chloride concentrations above 0.24 M (Fig. 2). When the same procedures are carried out with cells that had been grown for 10 weeks in medium supplemented with 161 µmol of Fe³⁺/litre the peak concentrations of spleen and heart reacting ferritin are found at 0.258 and 0.274 M-Cl⁻ respectively, both peaks having an increased affinity compared with the corresponding peaks derived from normal cell ferritin. The heart/spleen ratio is higher than normal in all fractions and shows a continuous rise throughout the gradient (Table 1).

Pulse experiments with ⁵⁹Fe and ³H

Cells were incubated for 90 min with [⁵⁹Fe]ferric

nitrilotriacetate and [³H]leucine, followed by non-radioactive medium as described above. They were harvested at intervals and the distribution of both ⁵⁹Fe and ³H were determined after simple cell fractionation and antibody precipitation after addition of carrier ferritin. In normal cells, maximum incorporation of ³H into cytosol ferritin is at 24 h and from this time onwards there is an exponential decay, indicating an apparent ferritin protein half-life of about 48 h (Fig. 3). After the initial pulse, most of the cytosolic ⁵⁹Fe is in a non-ferritin form corresponding to the labile intermediate described pre-

Table 1. Ratio of results for heart and spleen assays for ferritin eluting at selected chloride concentrations from anion-exchange chromatography for normal and iron-loaded cells

[Cl ⁻] (M)	Cells	Assay result ratio (heart/spleen)	
		Normal	Iron-loaded
0.219		0.67	1.63
0.235		1.27	1.73
0.261		2.27	3.79
0.285		4.70	7.01
0.310		4.61	7.61
0.335		3.45	9.42
0.360		3.26	11.22

Fig. 2. Comparison of ferritins detected by heart and spleen assays in a preparation from normal cells eluted from Sephadex A-50

(a) Ratio of heart to spleen assay results; (b) ●, heart assay; ▲, spleen assay, (arbitrary units).

Fig. 3. Distribution of radioactive labels with time after an initial 90 min pulse of [⁵⁹Fe]ferric nitrilotriacetate (162 µmol/litre) and [³H]leucine [25 µCi (0.5 nmol)]/sub-culture

●, ⁵⁹Fe in ferritin; ▲, ⁵⁹Fe in non-ferritin components of the cell supernatant; ♦, ³H in ferritin.

viously (White *et al.*, 1976). ^{59}Fe in ferritin rises to a peak only after 72 h. Similar changes are seen when iron-loaded cells are exposed to the same pulse of ^3H and ^{59}Fe , though in this case the half-life of $[^3\text{H}]$ ferritin appears to be in excess of 72 h and ^{59}Fe incorporation into ferritin continues to rise for 96 h. Similar results were obtained when ferritin was isolated from the cell samples by heating, centrifugation and gel chromatography.

^3H incorporation into ferritin represents about 1% of the incorporation into total protein at all times, though after 72 h values of 0.5–0.8% are found. Immediately after the initial pulse when most of the cytosolic ^{59}Fe is in a non-ferritin form (Fig. 5) the proportion of cytosol iron in the form of ferritin is

Fig. 4. ^3H incorporated into ferritin prepared from normal cells and eluted from anion exchange at various times after an initial pulse as in Fig. 3
♦, 1½ h; ●, 24 h; ▲, 72 h.

Fig. 5. ^{59}Fe incorporation into ferritin after pulse-labelling as in Fig. 3

Peak ^{59}Fe positions are given as $[\text{Cl}^-]$ (M): 1½ h, 0.247; 24 h, 0.256; 72 h, 0.273; 168 h, 0.270.

the same in both normal and iron-loaded cells. However, the cytosolic radioactive iron represents 24% of the total cell uptake in normal cells, but 67% of the total cell uptake in iron-loaded cells, suggesting an expanded labile iron pool in the latter case. After 24 h there is no difference in distribution between normal and loaded cells, about 25% of the total cell iron being in the cytosol and about 60% of the cytosol iron being in the form of ferritin in both cases.

Anion-exchange chromatography of cytosol ferritin after the initial pulse shows a changing distribution of the isotopes with time. In normal cells the peak incorporation of ^3H immediately after the pulse is at 0.255 M Cl^- . This remains unchanged after 24 h, but after 72 h the peak incorporation is found to elute at 0.275 M (Fig. 4). Similarly ^{59}Fe incorporation after the pulse is in a peak at 0.247 M and this shifts progressively to 0.256 M at 24 h and 0.273 M at 72 h, remaining unchanged at 168 h (Fig. 5). There is a steady change in the position of both isotope peaks from a more basic fraction to a more acidic fraction between 1½ and 72 h after a pulse dose. The final positions of both isotopes correspond well with each other.

Fig. 6. (a) ^3H and (b) ^{59}Fe incorporated into ferritin of cells grown in medium supplemented with $161\text{ }\mu\text{mol}$ of ferric nitrilotriacetate/litre after pulse-labelling

For iron-loaded cells all the peaks appear at a somewhat higher NaCl molarity than in normal cells, though a similar progression with time is noted (Fig. 6). Immediately after the pulse ^3H appears in a peak at 0.268 M NaCl, shifting to 0.285 M after 96 h (Fig. 6a). ^{59}Fe shifts from an initial peak at 0.270 M to one at 0.284 M after 96 h (Fig. 6b).

Discussion

Previous studies have shown that Chang cells take up iron both from transferrin (White *et al.*, 1976) and from the nitrilotriacetate (White & Jacobs, 1978). In both cases ferritin synthesis results. Long-term incubation in media supplemented with iron results in loading of the cells proportionately to the concentration of iron in the medium (Jacobs *et al.*, 1978), though the intracellular ferritin concentration does not exceed 6–10 times the basal value. Iron loading is associated with morphological changes, including lysosomal accumulations of amorphous iron, which do not appear to be harmful to the cells.

It has been suggested that the isoferritins found in all tissues result from the formation of molecules with different proportions of two types of subunits (Drysdale, 1977), and there is some evidence to support this. Adelman *et al.* (1975) have demonstrated two subunit types (H and L), and Arosio *et al.* (1978) have shown these to be produced in a cell-free wheat-germ system where postsynthetic modification is not likely. In iron-loaded human liver obtained *post mortem*, different isoferritin patterns have been observed by different workers. Powell *et al.* (1975b) compared three cases of iron overload with one normal subject and were impressed by the increase of basic isoferritins. Wagstaff *et al.* (1978) found no significant deviation from normal in four cases but Bomford *et al.* (1978) compared six control subjects with five patients having idiopathic haemochromatosis and showed a predominance of acidic isoferritins with increasing amounts of iron storage. They suggested that this might be due to preferential synthesis or retarded degradation of acidic isoferritins in iron-loaded states. Previous studies have demonstrated a direct relationship between the pI of human heart and liver ferritin and the salt concentration at which it is eluted from an anion-exchange column (Wagstaff *et al.*, 1978).

During the investigations described here, a shift in the spectrum of ferritin elution from anion-exchange columns towards the acidic isoferritins has been noted on several occasions when Chang cells have been grown in an iron-supplemented medium. However, the phenomenon is not produced rapidly, but progresses to a maximum shift over a period of up to 20 weeks. In our previous study the amounts of iron and ferritin in the cells reached equilibrium

after only 4 weeks (Jacobs *et al.*, 1978), though morphological changes continued to develop over a period of 8 weeks before fully developed iron-loaded cells with dense siderosomes were observed by electron microscopy. The intralysosomal aggregation of discrete iron cores to form large amorphous masses does not occur for 12 weeks, and it might be inferred that acid isoferritins appearing late in iron loading are more likely to form the grossly irregular membrane-bound bodies seen at this stage and that the more basic isoferritins tend to form membrane-bound accumulations of iron cores that have a more discrete nature resembling intact ferritin molecules, the latter being observed earlier in the iron-loading process. Although the shift in isoferritin pattern on iron loading shown in Fig. 1 is related only to the duration of loading and not to the concentration of iron used, it should be noted that the lowest concentration of iron used in these experiments was 13 times that of the basal medium.

On iron loading, in addition to the appearance of more acidic isoferritins, the isoferritins isolated in a particular fraction change their immunoreactivity, as shown by an increase in the ratio of heart-reacting to spleen-reacting ferritin. This is consistent with the view that in tissues with a high capacity for producing H-subunits, the synthesis of these may be stimulated by iron (Wagstaff *et al.*, 1978). At present the formation of apoferritin by the assembly of two different subunit types synthesized as separate gene products has not been conclusively demonstrated, and the contrary view, that there is postsynthetic modification of some subunits either before or after they are assembled into ferritin, has to be considered. The present investigation does not provide any information as to whether there are two gene products or whether a postsynthetic modification occurs, but it does suggest that the surface properties of an isoferritin are not solely dependent on its 'subunit' composition. If it is assumed that ferritin of a fixed pI has a fixed subunit composition, then an increase in the net proportion of H-subunits after iron loading would be expected to shift the whole spectrum of pI. However, as well as causing a shift in the pI spectrum, iron loading also increases the proportion of ferritin reacting to heart antibody at a given pI (and decreases the proportion reacting to spleen antibody), suggesting that changes in immunoreactivity induced by iron do not depend entirely on subunit composition.

^3H and ^{59}Fe are both incorporated into ferritin eluting at approximately the same chloride concentration (pI), and with time the two isotopes progress in parallel to more acidic ferritins. Apoferritin corresponding to a chloride elution concentration of 0.255 M is synthesized in normal cells and to 0.27 M-chloride in iron-loaded cells, these ferritins having apparent half-lives of 48 and 72 h

respectively. In normal Chang cells stimulated with ferric nitrilotriacetate, the newly synthesized ferritin appears to become progressively more acidic as it accumulates iron, though we cannot exclude the possibility of preferential iron uptake into ferritin molecules of low pI after 24 h. Iron loading over long periods of time leads to a shift of the whole process, including synthesis, towards more acidic ferritins. The change in peak position is maximal at the time of maximal iron incorporation after the initial pulse, and this suggests that iron loading of the ferritin molecule could itself be a factor causing changes in surface charge. The change in immunoreactivity could result from a rearrangement of subunits, with the exposure of new antigenic sites rather than a change in subunit composition.

The short-term studies indicate a rapid effect of iron loading on the surface properties of the ferritin molecule. The long-term effects of iron loading also lead to changes in the surface of the molecule, but these take many weeks to appear. The relationship between these two phenomena is not clear. Two intriguing possibilities arise from these observations. Firstly, a consideration of the short-term experiments in conjunction with the data of Wagstaff *et al.* (1978) and Russell & Harrison (1978), showing a relationship between ferritin charge and functional activity, suggests that the changes induced by iron accumulation might themselves result in a parallel change in the avidity of the molecule for iron. Secondly, the electron-micrographic studies of Iancu *et al.* (1977) on iron-loaded human liver shows that ferritin cores within lysosomes are larger and denser than those in the cytosol, suggesting preferential uptake of iron-rich molecules. A change in protein

configuration on iron loading might supply the signal triggering lysosomal uptake.

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