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Patterns of iron distribution in liver cells in β-thalassemia studied by X-ray microanalysis

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Background and Objectives. β-thalassemia is an important public health problem in the countries bordering the Mediterranean sea. One of the major consequences of this disorder, primarily (due to an ineffective erythropoiesis) or secondarily to blood transfusions (which are necessary for the patient's survival), is iron storage. Applying X-ray microanalysis we wanted to demonstrate the different sites of iron storage in subcellular compartments (mitochondria, cytosol, nucleus, rough endoplasmic reticulum and lipid droplets) and whether there were any other trace elements stored in liver cell.

Design and Methods. X-ray microanalysis was performed (at 100 kV in the STEM mode of a Hitachi H7000) on thin sections from specimens of liver biopsies from 6 patients affected by β-thalassemia, during follow-up after bone marrow transplantation.

Results. Spectra showed no correlation between iron peaks of lysosomes and hepatic iron concentration (HIC) or serum ferritin levels. Iron peaks were also detected in other subcellular compartments such as cisternae of rough endoplasmic reticulum, mitochondria and cytosol. No iron peaks were detected in lipid droplets and no significant iron peaks were found in the nuclei. Traces of copper were almost constantly found in lysosomes and cytosol.

Interpretation and Conclusions. These results demonstrated iron storage within subcellular organelles other than lysosomes and highlighted a non-correlation between lysosomal iron peaks and HIC or serum ferritin levels. The presence of traces of copper in the lysosomes and in the cytosol may be correlated with the stronger hypothesis of links in the metabolism of the two elements (iron and copper), as ceruloplasmin is a ferroxidase copper-dependent protein. X-ray microanalysis may become a relevant tool in the localization of iron storage within hepatocytes in the evaluation of the effectiveness of bone marrow transplantation and iron chelation therapy. It also may provide some interesting information about iron metabolism in hepatocytes.

Key words: transmission electron microscopy; X-ray microanalysis; β thalassemia; iron; liver metals.

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β-thalassemia major, an autosomal recessive disease characterized by decreased or absent synthesis of the β globin chain,[1] is one of the most common diseases in regions of the world where malaria has long been present. It constitutes an important public health problem in countries bordering the Mediterranean sea,[2] in India and in Southeast Asia.[3] This inherited disorder of hemoglobin synthesis causes severe anemia which is transfusion-dependent. Since an ideal iron chelator is not available yet in clinical practice,[4,5] the major consequence of chronic transfusion therapy is a progressive accumulation of iron, which may be increased by iron absorbed from the diet as a result of the ineffective erythropoiesis.[6] The liver, as a major storage depot for iron, constantly shows hemosiderosis, often shows fibrosis and ultimately, in some cases, cirrhosis.[7] Previous ultrastructural studies on livers of thalassemic patients[8,9] highlighted the presence of hemosiderin-loaded lysosomes in the cytoplasm of hepatocytes and Küpffer cells as the most peculiar feature of thalassemia-associated liver disease. In a scanning electron microscopy study after osmium maceration, we observed splitting in the membrane of siderosomes with diffusion of hemosiderin into the cytosol.[10]

The aim of this study was to correlate morphologic findings with X-ray microanalysis and clinical data in order to observe whether:

1. iron is only stored in lysosomes (siderosomes) or whether it is stored in other cell compartments;
2. other trace elements are stored in liver cells.

Design and Methods

Six needle liver biopsies were obtained from subjects affected by β-thalassemia after marrow transplantation in order to determine the degree of hemosiderosis.[11] These patients had received a marrow transplant between 1.5 and 10 years before the biopsies were taken. Clinical data are

summarized in Table 1. As a control group, we analyzed liver biopsies from 3 patients affected by HCV-related chronic hepatitis. The control subjects were selected from our case records of patients with chronic hepatitis C with the following criteria: at histology, all showed mild chronic hepatitis, without fibrosis and with negative histochemistry for iron.

In all liver biopsies, the determination of hepatic iron concentration (HIC) was performed by atomic absorption spectroscopy according to previously reported procedures.[12] For transmission electron microscopy studies, liver samples were immediately fixed in a fixative consisting of 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, post-fixed with osmium tetroxide and routinely embedded in epoxy resin (Agar 100, Agar Scientific, Stansted, UK), sectioned and stained with uranyl acetate and lead citrate.

X-ray microanalysis was performed at 100 kV in the STEM mode of a Hitachi H7100, with a Link ISIS energy-dispersive X-ray microanalysis system. Spectra showing the presence of electron excitement were acquired over periods of 50 seconds. For each biopsy, at least 30 spectra were obtained. X-ray microanalysis was performed targeting the following hepatocellular organelles: siderosomes, mitochondria, endoplasmic reticulum, cytosol, lipid droplets, nucleus, and biliary canaliculi.

Siderosomes and mitochondria were also analyzed in Küpffer cells.

Control spectra were obtained by X-ray microanalysis on the empty grid and from the resin.

Results

There was a great variation in HIC (measured by atomic absorption spectroscopy) among thalassemic patients, ranging from 720 μg per gram of dry tissue (μg/g dt) up to 21,080 mg/g dt (Table 1). In the control group, liver iron content was in the normal range.[13]

Control spectra (obtained with X-ray microanalysis) using empty grids and resin did not show any iron peaks. A 0.8 cps peak at 8 KeV was present in both spectra, indicating the presence of copper in the electron microscope frame. It should be stressed that *cps* is not a form of quantitative measurement as it only indicates the presence of excited electrons.

Siderosomes

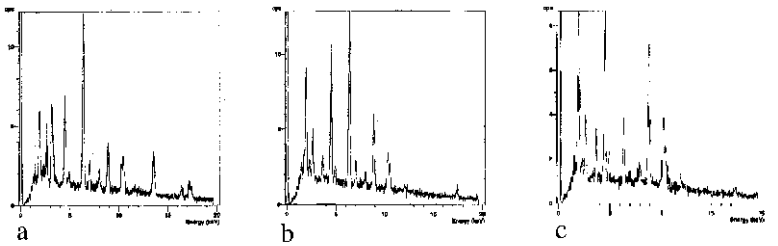
X-ray microanalysis showed, in all 6 subjects affected by beta-thalassemia major, iron accumulation in hemosiderin-loaded lysosomes (siderosomes). Interestingly, we observed a marked inter-individual variability in iron peaks seen in siderosomes. The highest peaks were found in cases #1 and #6 which showed peaks of 23 and 28 cps respectively (Figures 1a and b). Lower values were observed in cases #2, 4 and 5, with iron peaks around 7 cps, while intermediate values were present in case #3 (18 cps). In particular, in cases #2 and #4, with the lowest value of HIC, X-ray spectra from lysosomes constantly showed iron peaks below 7 cps (Figure 1c). The level of lysosomal iron peaks in spectra obtained by X-ray microanalysis did not always correlate with HIC. Cases with very similar HIC (case #5 and 6) showed marked differences in the levels of iron peaks; on the other hand, similar iron peaks were observed in cases with different HIC (see case #2 and case #4). An intra individual variability in siderosome iron content was also observed: secondary, large, multivacuolated siderosomes showed higher peaks (Figure 1d) than small round siderosomes (Figure 1e).

While the majority of siderosomes analyzed showed a specific increase in iron content, some of

Table 1. Clinical and laboratory data and maximum level of iron peaks (cps-counts per second) in different cell structures, detected at 6.41 keV.

	Sex	Age	Years after BMT	Serum ferritin ng/mL	HCV RNA	HCV Ab	GPT	HIC μg/g dt	Hepatocytes [cps - count per second] SID	MIT	ER	CYT	HAEM	NUC	Küpffer SID
Case #1	M	13	8	1.095	-	-	93	7,460	23	1.8	1.4	2.7	2.6	1.5	1.4
Case #2	M	17	10	40	+	+	58	720	6.8	0.8	1.0	1.8	MV	2.0	MV
Case #3	F	18	1.5	2.605	-	-	85	21,080	18	1.3	1.7	1.7	12	MV	16.0
Case #4	M	15	4	259	+	+	35	3,080	6.8	2.2	2.2	2.6	3.0	1.8	MV
Case #5	F	21	6	1.177	-	+	17	19,880	11.0	1.4	0.8	1.8	MV	1.4	21.0
Case #6	M	19	2	3.864	-	+	225	19,770	28.0	2.6	2.6	3.6	7.0	MV	MV

BMT: bone marrow transplantation; HIC: hepatic iron concentration; HCV: hepatitis C virus; HCV Ab: Ab against HCV; HIC: hepatic iron concentration (expressed in μg per g of dry tissue); SID: siderosomes; MIT: mitochondria; ER: endoplasmic reticulum; CYT: Cytosol; HAEM: "free" hemosiderin; NUC: nuclei; SID: siderosomes in Küpffer cells; MV: missing value.



Figures 1. a and b) Cases #1 and 6: lysosomes, iron peak at 6.41 keV; c) case #4: lysosome; d) case #1: lysosome; e) case #1: large multivacuolated lysosome; f) control liver): no iron peak.

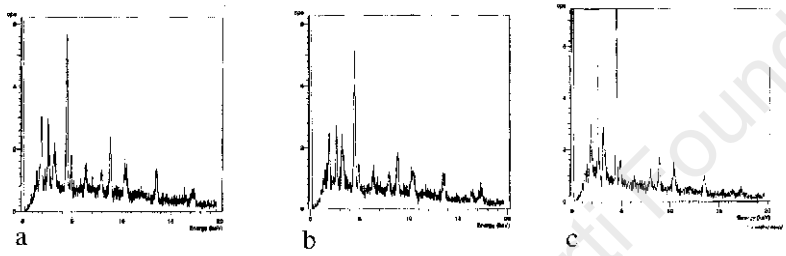


Figure 2. a) case #1: smooth endoplasmic reticulum; b) case #1: rough endoplasmic reticulum; c) case #3: dilated endoplasmic reticulum containing granular material.

them also showed an increase in copper content, whose peaks reached 4 cps (Figure 1d). No iron peak was detected in control livers (Figure 1f).

Hemosiderin free in the cytoplasm

In some cases (cases #1, 3, 4, and 6), hemosiderin granules were also found freely diffused in the cytoplasm, not bound by membranes. X-ray microanalysis of free hemosiderin showed marked differences in their iron content: the highest peaks (12 cps) were found in case #3; the lowest peaks were observed in cases #1 and 4 (2.6 and 3 cps, respectively); case #6 showed a 7 cps iron peak.

Endoplasmic reticulum

Amounts of iron comparable to those found in mitochondria (*see below*), were present in spectra from smooth (Figure 2a) and rough endoplasmic reticulum (Figure 2b). A higher iron peak was found in dilated cisternae of endoplasmic reticulum containing mildly electron-dense granular material in case #3 (Figure 2c). Even for iron peaks detected in endoplasmic reticulum, no strict correlation was found between their level and HIC. Iron peaks were absent in spectra from endoplasmic reticulum in control livers.

Cytosol

Spectra obtained from the cytosol of hepatocytes in areas devoid of cell organelles constantly showed the presence of stored iron: iron peaks were particularly high in areas of glycogen deposition around mitochondria, where they reached the level of 2.7 cps in case #1 (Figure 3a) and 3.6 cps in case #6. A cytosolic iron peak was also present in the case with the lowest HIC (case #2), reaching a maximum of 1.8 cps (Figure 3b). No strict correlation was found between the level of cytosolic iron peaks and HIC. Very similar peaks (2.7-2.6) were found in cases #1 and 4, with markedly different HIC.

Lipid droplets

No iron peak was found in the lipid droplets present in the cytoplasm of hepatocytes (Figure 3c).

Mitochondria

X-ray microanalysis of hepatocytic mitochondria showed a constant but mild iron storage: the iron peaks only occasionally reached the level of 2 cps (Figure 4a). The highest peaks were found in cases #6 and 4, which showed peaks of 2.6 and 2.2, respectively. Iron content was particularly low in case #2,

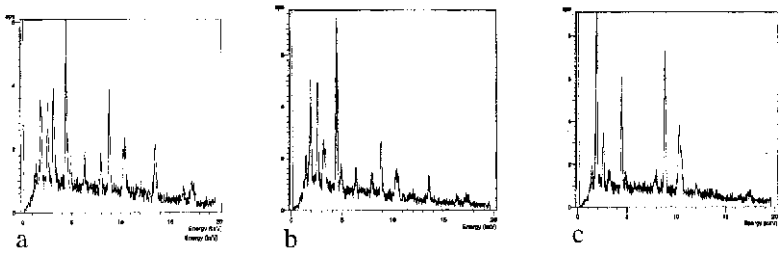


Figure 3. a) case #1: cytosol. b) case #2: cytosol. c) case #3: lipid droplets.

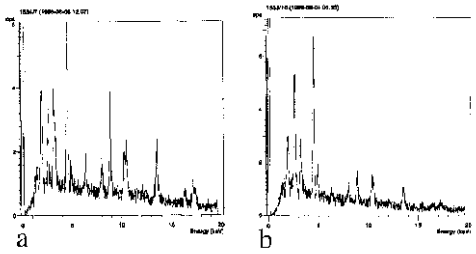


Figure 4. a) case #1: mitochondria; b) case #2: mitochondria.

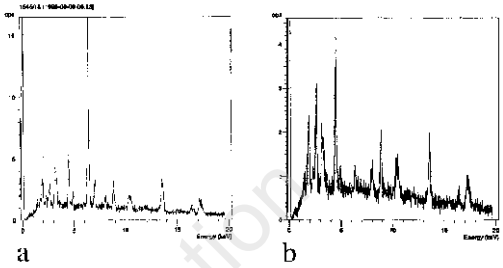


Figure 5. a) case #3: Küpffer cell, lysosome. b) case #3: Kupffer cells, mitochondria.

in which the mitochondrial iron peak never exceeded the level of 1 cps (Figure 4b). The iron peaks did not correlate with levels of HIC. Case #4, with a very low HIC, showed iron peaks with a ten-fold higher HIC. Spectra from mitochondria in the control group did not show the presence of iron peaks.

Nuclei

When nuclei were analyzed, a small iron peak was occasionally detected in the nuclear membrane and in the chromatin. A higher peak was often observed in nucleoli, with a maximum level of 2 cps. No iron was present in nuclear structures from control subjects.

Küpffer cells

In Küpffer cells, we observed a very high iron peak in all siderosomes analyzed (Figure 5a), reaching levels comparable to those found in siderosomes of hepatocytes. A small iron peak was also observed in spectra from secondary lysosomes in Küpffer cells from control livers: the observed peaks for iron never exceeded 1.5 cps. Low iron storage was also detected in Küpffer cell mitochondria from thalassemic patients (Figure 5b), the iron peaks never exceeding 1.5 cps.

Discussion

The clinical and pathologic picture of patients affected by β-thalassemia is mainly characterized by systemic hemosiderosis. Target organs for iron deposition are liver and, in adolescence and adulthood, heart.[10] The intimate mechanism which regulates the movements of iron through cytoplasmic organelles inside the cells is not well known yet in either physiologic conditions or pathologic states of iron overload.

Conflicting results have been reported on the site of iron deposition inside cells in thalassemia. Previous ultrastructural studies of liver in β-thalassemia showed the presence of siderosomes as the most peculiar feature of the disease, suggesting that iron overload was mainly confined to lysosomes. A recent electron microscopic study on one kidney biopsy in a patient who had received a BMT, applying X-ray microanalysis, showed the presence of high iron peaks confined to hemosiderin deposits accumulated in the tubular basal membrane. X-ray spectra from siderosomes within tubular cells and from other cell structures showed only small iron peaks.[14] The recent report of changes in liver mitochondria in thalassemia patients[15] raised the question of whether iron may accumulate in hepatocytic organelles other than lysosomes. As a result of this study, we propose X-ray microanalysis as a useful tool to reveal iron storage inside hepatocytes in thalassemia. Our preliminary results show that iron overload is present not only in lysosomes, but also in other cell structures; by X-ray microanalysis iron was detected even in mitochondria, inside cisternae of endoplasmic reticulum, in the nuclei and in the cytosol, mainly in areas of glycogen deposition.

These findings support the hypothesis that iron overload in thalassemia is not restricted to the lysosomal compartment and that the whole hepatocyte is affected by excess iron. This is in accordance with the modern views on the process of iron uptake, distribution and release in the hepatocyte, mainly based on iron-kinetic studies.[16-18] Iron delivery to the hepatocyte begins with the binding of plasma transferrin to a transferrin receptor (TR), which clusters with other TR complexes and forms an internal vesicle, an *endosome*. The *endosome* fuses with an acidic vesicle and, in the acidic environment, iron is released from the TR. After release, iron is transported across the endosomal membrane into the cytosol.[19] Some endosomes, containing apotransferrin and TR, are shunted to the Golgi apparatus for re-sialation and repair before returning to the cell membrane.[20] The exact form and fate of iron delivered from the endosome are still unknown. The newly released iron is presumably available to mitochondria for heme synthesis. Another target of iron released in the cytosol is ferritin for storage. Only few data are available to indicate whether incorporation into ferritin is an obligatory step during the passage of all iron through hepatocytes or whether only the iron fraction destined to be stored is incorporated into ferritin.[16,17]

To be released from ferritin, iron must be oxidated from ferrous iron (Fe^{2+}) to the ferric form (Fe^{3+}), since apotransferrin only binds Fe^{3+}. The most important ferroxidase in the hepatocyte is now considered to be ceruloplasmin, which is probably localized in the endoplasmic reticulum.[21] The relevance of this step in iron metabolism has only been recently clarified by the report of systemic hemosiderosis in patients affected by congenital deficiency of ceruloplasmin[22,23] and by the finding of severe hemosiderosis in a patient affected by Wilson's disease.[24] All these data, based on iron-kinetic studies, evidence that all the compartments of the hepatocyte are involved in iron metabolism. Our data confirm this hypothesis and suggest that even in thalassemia, iron overload is a general problem for the hepatocyte and not only a *lysosomal* problem.

The observation of iron peaks in spectra of hepatocytic mitochondria is new. Conflicting results have been reported regarding iron targeting to mitochondria in iron overload. In hemoglobin-synthesizing erythroid cells, iron is considered to be specifically targeted toward mitochondria, in a form (which is not ferritin) available for heme synthesis.[18] On the other hand, mitochondria in non-erythroid cells have been reported not to accumulate non-heme iron, even in severely overloaded individuals.[18] Only one transmission electron microscopic study evidenced the presence of ferritin deposits inside mitochondria from thalassemia patients.[9] Our analysis by X-ray microanalysis shows that hepatocytic mitochondria are involved by iron overload in thalassemia; this observation may explain the mitochondrial alteration previously described in a SEM study.[15] Iron peaks were observed in spectra from the mitochondrial matrix and from the mitochondrial membrane, in the absence of hemosiderin or ferritin deposits. The observation of iron peaks in spectra obtained in the cytosol, in areas with no hemosiderin accumulation, poses new problems in the interpretation of liver cell necrosis due to iron overload. In fact, while hemosiderin-bound iron sequestered inside lysosomes is completely neutralized by ferritin, the molecules which iron is bound to in the non-ferritin bound pool are not able to neutralize iron, which promotes the formation of free radicals and tissue damage.[25] X-ray microanalysis evidenced iron peaks in the cytosol of all 6 cases analyzed. It is interesting to note that, even in cases with low HIC, iron peaks in the cytosol were present and comparable to peaks observed in cases with much higher HIC. This finding could demonstrate that iron stored in the cytoplasm remains even when iron metabolism is effective (after bone marrow transplantation), causing cell death and tissue damage even in these patients. This observation may, therefore, be important even in the evaluation of the effects of iron chelators, which should be targeted mainly toward this *free* cytosolic iron pool, in the follow-up of non-transplanted patients. Following this study we have been unable to clearly explain the significance of the *non-correlation* between iron deposits and clinical data (ferritin value). A possible, immediate explanation for this disagreement could be the previously reported uneven distribution of iron in β-thalassemia,[26] which could be at the basis of sampling variability in iron content determination in a small needle liver biopsy. Finally, our study shows that X-ray microanalysis may become a relevant tool in the discovery of the sites of iron accumulation and of iron flux inside the hepatocyte in β-thalassemia major, as well as in other disorders of iron metabolism with iron overload. The unique ability of X-ray microanalysis to localize iron stores at a subcellular level may provide new data on the sites of iron deposition inside the hepatocyte and on the different iron pools targeted by iron chelators used in clinical practice.

Contributions and Acknowledgments

Authors are listed according to a criterion of decreasing individual contribution to the work, with the following exceptions: the first and the last authors contributed equally to this article. This work was carried out at the Laboratory of Electron Microscopy of the Department of Anatomy, University of Uppsala, Box 571, S-751 23 Uppsala, Sweden, thanks to the generosity of Professor Godfried M. Roomans.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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What is already known on this topic

Iron overload is a major complication in transfusion-dependent thalassemia. The liver is a major target for iron overload: iron seems to be deposited in lysosomes both in hepatoocytes and Küpffer cells.

What this study adds

X-ray microanalysis studies have identified significant accumulation of iron outside of lysosomes, in cellular compartments such as cisternae of rough endoplasmic reticulum, mitochondria and cytosol. No iron accumulation was found in lipid droplets and nuclei.

Potential implications for clinical practice

X-ray microanalysis may provide a tool for the precise intracellular localization of liver iron accumulation and assess efficacy of existing and novel iron chelators, as well as follow-up of changes in iron accumulation after a bone marrow transplant.

Carlo Brugnara, Deputy Editor