Role of Melanotransferrin (p97) in Non-transferrin Iron Uptake by HeLa and K562 Cells

(melanotransferrin / p97 / non-transferrin iron uptake / HeLa cells / K562 cells)

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Abstract. We tested whether melanotransferrin (p97), an iron-binding protein of the plasma membrane, is involved in the transport of non-transferrin iron into human HeLa and K562 cells. The expression of melanotransferrin was detected in HeLa but not in K562 cells. PI-PLC treatment dramatically decreased (to 20% of the original value) p97 expression by HeLa cells. However, the rate of iron uptake from ^{55}Fe-ferric citrate by PI-PLC-treated HeLa cells was comparable or only slightly lower (80–100%) than the rate of iron uptake by untreated cells. PI-PLC treatment had no effect on iron uptake by K562 cells. These findings strongly support the suggestion that melanotransferrin does not play a substantial role in non-transferrin iron uptake by either HeLa or K562 cells.

Iron is essential for nearly all organisms. This indispensable trace element is acquired by mammalian cells either via the classical transferrin-transferrin receptor pathway (Dautry-Varsat et al., 1983; Klausner et al., 1983) or via poorly defined transferrin-independent pathway(s) (Goldenberg and Scheiber, 1995). Transport of non-transferrin iron in various cell types has been demonstrated by many authors (Brissot et al., 1985; Basset et al., 1986; Morgan, 1988; Wright et al., 1988; Sturrock et al., 1990; Kaplan et al., 1991; Richardson and Baker, 1991; Seligman et al., 1991; Hamazaki and Glass, 1992; Núñez et al., 1992; Inman and Wessling-Resnick, 1993; Kriegerbecková et al., 1995; Parkes et al., 1997; Olakanmi et al., 1997; Baker et al., 1998; Musílková et al., 1998) and the ability of non-transferrin iron to substitute fully for transferrin as a source of iron in defined cell culture media was also demonstrated (Kovář and Franěk, 1987). Studies concerning non-transferrin iron uptake support the suggestion that there are several pathways of the uptake (Goldenberg and Scheiber, 1995), while transporters per se remain poorly defined.

Recently, some progress in understanding the nature of non-transferrin iron transporters has been made. One experimental approach was based on the identification of iron-binding proteins in cellular membranes. Such proteins were found in the intestine (Teichmann and Stremmel, 1990; Conrad et al., 1993), K562 cells (Conrad and Umbreit, 1994), reticulocytes (Umbreit et al., 1997), reticulocyte endocytic vesicles (Núñez et al., 1989), melanoma and other tumor cells (Brown et al., 1981a; Brown et al., 1982; Richardson and Baker, 1990). Focusing on the molecular background of defects in iron metabolism represents another approach. It is suggested that the *HFE* gene responsible for hereditary hemochromatosis regulates intestinal iron absorption. The function of the gene is known to be related to the function of the *Nramp2* gene. Nramp2 itself is a member of the natural resistance-associated macrophage protein (Nramp) family. Proteins of this small family are supposed to be iron transporters. Nramp2 was found in all tissues, including the intestine and hematopoietic cells, while Nramp1 is expressed only in reticuloendothelial cells (Atkinson et al., 1997; Fleming et al., 1997). Divalent cation transporter (DCT1) from rat duodenum is a transporter for Fe^{2+} and other divalent cations. It also belongs to the Nramp family (Gunshin et al., 1997). Stimulator of Fe transport (SFT) from K562 cells represents another membrane protein involved in non-transferrin iron transport (Gutierrez et al., 1997). SFT is unrelated to the Nramp family.

Melanotransferrin, known as p97, was identified as an iron-binding protein of the plasma membrane (Brown et al., 1981a; Brown et al., 1982). Melanotransferrin is structurally similar to the serum iron-transporting protein transferrin (Baker et al., 1992). It is localized as glycosylphosphatidylinositol (GPI)-anchored protein (Food et al., 1994) in the membranes of melanoma or other tumor cells. Only trace amounts are present in normal tissues (Brown et al., 1981a; Brown et al., 1982). Melanotransferrin was an obvious candidate for a specific iron-binding site in the plasma membrane (Richardson and Baker, 1990), and thus it was supposed to be involved in non-transferrin iron uptake (Kennard et al., 1995). Previously, we have described non-transferrin iron uptake by HeLa and K562 cells (Kovář et al., 1997; Musílková et al.,

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Abbreviations: BSA – bovine serum albumin, FITC – fluorescein isothiocyanate, GPI – glycosylphosphatidylinositol, PBS – phosphate-buffered saline, PI-PLC – phosphatidylinositol-specific phospholipase C.

1998). In the present study we tested whether p97 is involved in non-transferrin iron uptake by these cells.

Material and Methods

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), except the following ones: fetal bovine serum (J. Kysilka, Brno, Czech Republic) and $^{55}FeCl_3$ (DuPont NEN, Boston, MA) for ^{55}Fe-ferric citrate preparation.

Human tumor cell lines HeLa (cervical carcinoma) and K562 (erythroleukemia) were obtained from J. Kaplan (University of Utah School of Medicine, Salt Lake City, UT) and from M. Wessling-Resnick (Harvard School of Public Health, Boston, MA), respectively. Human cell line HPB-ALL (T lymphoblastic leukemia) was from V. Hořejší (Institute of Molecular Genetics, Prague, Czech Republic). RPMI 1640 medium containing extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), HEPES (15 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) was used as a basic medium (Kovář and Franěk, 1987). Cells were maintained in the basic medium supplemented with 10% fetal bovine serum (FBS medium) at 37°C in a humidified atmosphere of 5% CO_2 in air. In the experiments, we employed defined iron-free medium. Iron-free medium is a defined protein-free (PFH) medium described previously (Kovář and Franěk, 1987) without addition of any iron source. PFH medium represents the basic medium described above plus other supplements described previously (Kovář and Franěk, 1987).

To determine the expression of cell-surface p97, indirect immunofluorescence and flow cytometry analysis were used. The cells were collected by low-speed centrifugation (8 min at 500 *g*), washed twice with phosphate-buffered saline (PBS) and incubated for 30 min in 0.3% bovine serum albumin (BSA)/PBS on ice (1 × 10^6 cells/200 μl). After two washes with 0.3% BSA/PBS, the cells were incubated for 30 min on ice with 200 μl of mouse monoclonal IgG antibody 96.5 (10 μg/ml of 0.3% BSA/PBS) specific to p97 or with 200 μl of nonspecific mouse IgG (Sigma Chemical Co., St. Louis, MO) (10 μg/ml of 0.3% BSA/PBS) as a negative control. The monoclonal antibody 96.5 (Brown et al., 1981b) was a gift of J. Kemp (University of Iowa, Iowa City, IA). Cells were washed twice with 0.3% BSA/PBS and incubated with 200 μl of anti-mouse IgG (Fab-specific) fluorescein isothiocyanate (FITC) conjugate (20 μg/ml of 0.3% BSA/PBS) for another 30 min on ice. After incubation the cells were washed twice with PBS and resuspended in 300 μl of PBS. After addition of propidium iodide (1 μg/ml), the cells were analyzed on a FACScan Analyzer (Becton Dickinson).

For the measurement of iron uptake from ferric citrate (Brunner-Döpper et al., 1998) we employed a method derived from the method used by Olakanmi et al. (1997). Cells grown in FBS medium were harvested by low-speed centrifugation and washed twice with the basic medium. The washed cells were diluted with iron-free medium to a concentration of 10 × 10^6 cells/ml and transferred to wells (50 μl per well) of 96-well U plates (Corning Costar, Cambridge, MA). The plates were warmed at 37°C for 15 min in the incubator. The uptake was started by addition of 50 μl of ^{55}Fe-ferric citrate in iron-free medium (final concentration 1 μM ^{55}Fe, 0.11 μCi/well) to the cell suspension. After the required incu-

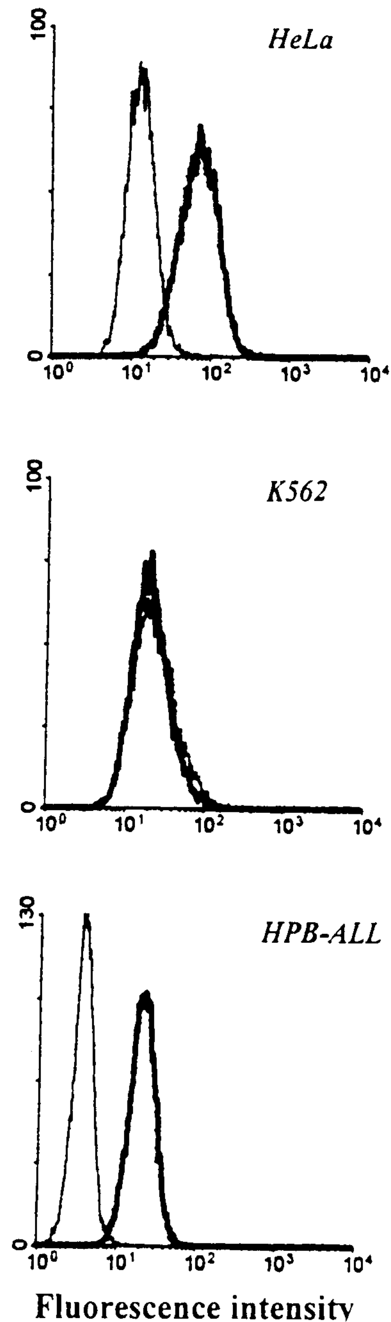

Fig. 1. Expression of p97 by HeLa (a), K562 (b) and HPB-ALL (c) cells. Indirect immunofluorescence subsequent to staining with mouse monoclonal antibody 96.5 (IgG) specific for human p97 (bold line) was used. Control staining was carried out with nonspecific mouse IgG (fine line). HPB-ALL cells represent the positive control for p97 expression.

bation period at 37°C, iron uptake was stopped by transferring the plates to ice and simultaneously adding 100 μl of ice-cold 500 μM nonradioactive ferric citrate in iron-free medium. Cells were washed three times with 200 μl of 500 μM nonradioactive ferric citrate in iron-free medium. After washing, the cells in the individual wells were resuspended in 100 μl of distilled water and transferred to scintillation vials. Radioactivity incorporated by the cells was measured by liquid scintillation in a Beckman LS 7800 counter (Beckman Instruments).

To remove GPI-bound p97 from the cell surface before starting the indirect immunofluorescence or the iron uptake experiments, harvested cells were treated with phosphatidylinositol-specific phospholipase C (PI-PLC). Cells washed with the basic medium were incubated in iron-free medium (4×10^6 cells/ml) containing PI-PLC (300 mU/ml) for 1 h at 37°C in the incubator. After the incubation period the cells were washed twice with PBS and used in experiments.

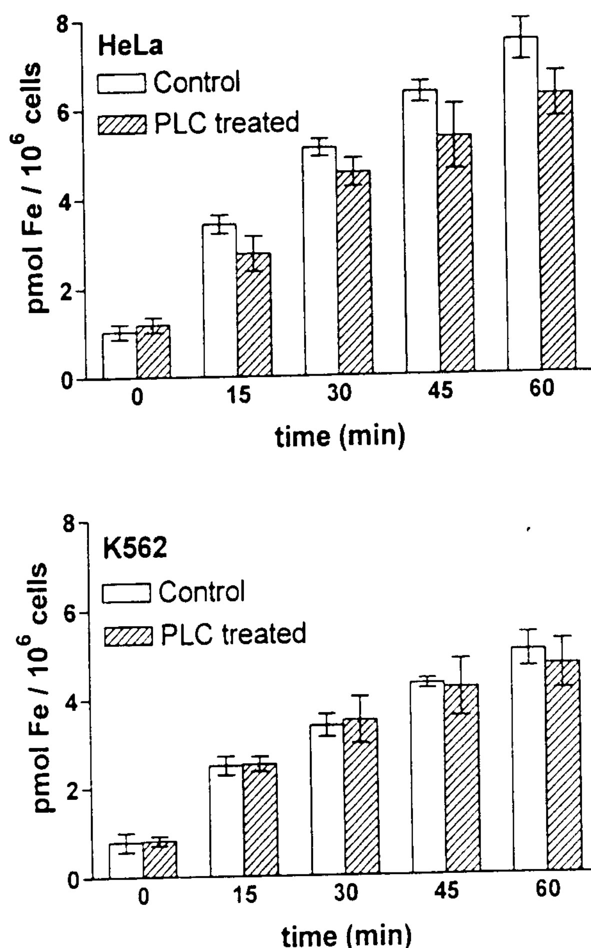

Fig. 2. The effect of phosphatidylinositol-specific phospholipase C (PI-PLC) treatment on Fe uptake from ^{55}Fe-ferric citrate (1 μM) by HeLa and K562 cells. Treated cells (4×10^6 cells/ml) were incubated with phospholipase C (300 mU/ml) for 1 h at 37°C. Control cells were incubated without phospholipase C. Data presented here and data presented in Table 1 were obtained with the same group of treated cells in one representative experiment. Each column represents the mean ± standard error of the mean (SEM) of four determinations.

Results

As shown in Fig. 1, the expression of p97 was detected on HeLa cells as well as on HPB-ALL cells used as a positive control. In contrast, no expression of p97 was detected on K562 cells.

It was shown previously (Kennard et al., 1993) that p97 attached to mammalian cell-surface membranes by a GPI anchor can be released from the cell surface by treatment with PI-PLC. We found that after 1-hour treatment with PI-PLC (300 mU/ml), the p97 on the cell surface of HeLa cells decreased dramatically. The mean fluorescence intensity due to specific binding decreased to about 20% of the original value. Similar data were also obtained in repeated independent experiments. The same experiment with PI-PLC treatment was also carried out with p97-negative K562 cells. As expected, no effect on fluorescence intensity was found (Table 1).

Non-transferrin iron uptake from ^{55}Fe-ferric citrate (1 μM) by PI-PLC-treated and untreated cells was compared for HeLa as well as for K562 cells (Fig. 2). The rate of iron uptake by treated HeLa cells (85 ± 8 fmol Fe/min & 10^6 cells), calculated from the data presented in Fig. 2, was slightly lower than the rate of iron uptake by untreated HeLa cells (106 ± 9 fmol Fe/min & 10^6 cells). Statistical analysis showed that the rates of iron uptake were not significantly different at the 5% level of probability. However, in another independent experiment the rate of iron uptake by PI-PLC-treated HeLa cells (158 ± 8 fmol Fe/min & 10^6 cells) was fully comparable with the rate of iron uptake by untreated HeLa cells (150 ± 8 fmol Fe/min & 10^6 cells). In this case, the rates of iron uptake were not significantly different at the 1% level of probability. On the basis of data from several independent experiments, we can say that the slight decrease, if any, in iron uptake by PI-PLC-treated HeLa cells did not

Table 1. The effect of PI-PLC treatment on p97 expression by HeLa and K562 cells

PI-PLC treatment[a]	Mean fluorescence intensity[b]			
	HeLa		K562	
	IgG[c]	96.5[d]	IgG[c]	96.5[d]
–	15.7	87.0	30.0	29.6
+	16.4	31.0	29.7	30.3

[a]Cells (4×10^6/ml) were incubated with phospholipase C (300 mU/ml) for 1 h at 37° C.
[b]Data presented here and data presented in Fig. 2 were obtained with the same group of treated cells in one representative experiment.
[c]Control staining with nonspecific mouse IgG.
[d]Staining with mouse monoclonal antibody 96.5 (IgG) specific for human p97.

correspond with the dramatic and reproducible decrease in p97 expression by these cells (see Table 1). As expected, the rate of iron uptake by PI-PLC-treated K562 cells (68 ± 7 fmol Fe/min & 10^6 cells) was also nearly the same as the rate of iron uptake by untreated K562 cells (70 ± 7 fmol Fe/min & 10^6 cells). These rates were again calculated from the data presented in Figure 2.

Discussion

Melanotransferrin (p97) has at least one functional iron-binding site and it is expressed particularly on melanoma cells. However, it has been found also on other cell types of tumor and normal tissue origin (Brown et al., 1981b; Brown et al., 1982). As the only known plasma-membrane protein binding iron specifically, it has been considered to be involved in non-transferrin iron uptake (Kennard et al., 1995). Because the mechanism(s) of non-transferrin iron transport still remains obscure, the possible role of melanotransferrin in the transport is of special interest (Musílková et al., 1998).

In order to assess the role of melanotransferrin in non-transferrin iron uptake by mammalian cells, we employed human cell lines HeLa and K562, sharing similar characteristics of non-transferrin iron uptake (Kovář et al., 1997). We decided (i) to compare p97 expression by these two cell lines and (ii) to study the effect of p97 release from the cell surface by treatment with PI-PLC on non-transferrin iron uptake. Kennard et al. (1995) demonstrated that PI-PLC treatment decreased non-transferrin iron uptake in CHO cells transfected with the human *p97* gene.

We found that HeLa cells expressed p97 and, in contrast, that K562 did not express any detectable amount of p97. On the other hand, according to our previous data both cell lines have similar characterictics of non-transferrin iron uptake (Kovář et al., 1997). These two findings do not correspond with the possibility of a significant role for p97 in non-transferrin iron uptake by HeLa and K562 cells. The treatment of HeLa cells with PI-PLC led to a dramatic decrease in cell surface p97 (to about 20% of the original value). However, treatment with PI-PLC resulted in similar or only slightly decreased non-transferrin iron uptake by HeLa cells. If there was any decrease in the uptake, it resulted rather from an indirect effect of the PI-PLC treatment than from the removal of p97. As expected, there was no effect of PI-PLC treatment on iron uptake by K562 cells. Taken together, all the findings strongly support the suggestion that melanotransferrin does not play any substantial role in non-transferrin iron uptake by HeLa as well as K562 cells.

Our conclusion concerning human HeLa and K562 cells does not correspond with the finding of Kennard et al. (1995) that p97 is involved, together with other unknown mechanism(s), in non-transferrin iron uptake by Chinese hamster CHO cells. However, various cell types can employ or prefer differing mechanisms of non-transferrin iron uptake. It is generally accepted that there are multiple pathways of the uptake (Goldenberg and Scheiber, 1995). Furthermore, Kennard et al. (1995) employed cells transfected with the *p97* gene, while we employed cells naturally expressing p97. Differing amounts of expressed p97 could also play a role.

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