Liver Endothelium and Not Hepatocytes or Kupffer Cells Have Transferrin Receptors

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Using a visual probe, consisting of latex minibeads covalently linked to transferrin (TF), we found that, in rat liver cell suspensions, transferrin receptors were limited to endothelial cells. Neither hepatocytes nor Kupffer cells contained an appreciable number of TF receptors. Specificity of this reaction was demonstrated by preincubation with nonderivatized TF, which inhibited the binding. This was further confirmed by fractionation of liver cell suspensions on metrizamide gradients. The uptake of either the visual probe or 125I-labeled TF was again limited to the endothelium-rich fraction. Transferrin bound to endothelial membrane was internalized at 37°C, but not at 4°C, via a coated pit system. Again, hepatocytes and Kupffer cells did not internalize the probe. The findings suggest that iron may be first taken up by liver endothelium and then transmitted to parenchymal cells. These results emphasize the generally unappreciated role of endothelium in the transport across the tissue–blood barrier.

MATERIALS AND METHODS

Purified human, rabbit, and rat transferrins were a gift from Drs. U. Muller-Eberhard and H. Leim, Cornell University, New York, NY. Their purity was independently demonstrated in our laboratory by the presence of single bands on polyacrylamide gel electrophoresis. TF was then saturated with iron as described.

Preparation and Fractionation of Liver Cell Suspensions

Liver cells suspensions were prepared from male Sprague-Dawley rats (200–250 g) by a collagenase perfusion method. Briefly, the liver was perfused through the cannulated portal vein with warm calcium-free buffer at the rate of 10 ml/min. The buffer contained 0.123 M NaCl, 2.8 mM MgSO4, 5 mM KCl, 1.2 mM KH2PO4, 25 mM NaHCO3, 10 mM HEPES, 75 μM bovine serum albumin (BSA), 5.5 mM dextrose, and 2,000 U heparin/1,000 ml solution (pH 7.5). Perfusion was subsequently continued with a similar buffer, to which was added 500 mg of collagenase (type IV, Sigma, St. Louis, MO) per 1,000 ml and 2 mM CaCl2 (collagenase activity is Ca2+-dependent). To maintain the optimal temperature of 37°C for collagenase activity, the buffer was kept in a water bath at 42°C.

The liver was then minced in Ca2+-free buffer, and the resulting cell suspension was incubated at 37°C for 30 min, filtered, and centrifuged 3 times at 50 g for 2 min to remove cellular debris. The cell pellet was then resuspended with Dulbecco’s phosphate-buffered saline (PBS) to a final concentration 4 × 106 cells/ml. Viability, as determined by trypan blue exclusion, was 82%–91%.

Fractionation of this cell suspension was done by the method of Seglen. Briefly, the cells were overlayed above the double-layer of metrizamide solutions of 15% (150 g of metrizamide, 10 mM of HEPES, 75 mM of KCl, 1.25 mM CaCl2, and 5.5 ml of 1N NaOH per 1,000 ml; the specific gravity of this solution was 1.08 g/ml, with a pH of 7.6 and osmolarity of 308 mosmole) and 30% (with specific gravity of 1.16 g/ml, pH 7.6, and osmolarity of 330 mosmole). Centrifugation at 1,500 g for 40 min led to the appearance of two cellular fractions: one above the 15% metrizamide solution and the other at the interface of the 15% and 30% solutions. The upper layer contained small cells (5–15 μm) and the lower layer large cells (more than 20 μm). Each fraction was gently collected and washed 3 times with Dulbecco’s PBS. The final cell pellet of the small cell fraction contained 3 × 106 cells, with a viability of 93%–98%. The proportions of different cell types, as determined by EM, in these fractions are given in Table 1.

Enzyme Cytochemistry

Drops of cell suspension were placed on clear glass slides, air dried, and subsequently fixed for cytochemical examination. Alpha-
naphthyl butyrate esterase activity was demonstrated according to Yam et al. Peroxidase activity was demonstrated by incubating cells with 0.05% diaminobenzidine and 0.02% H2O2. Some of the slides were also stained with methyl green-pyronin staining and for acid phosphatase activity and naphthol AS-D chloroacetate esterase activity. The presence of factor VIII antigen was determined, using an indirect method, by incubation of cells with rabbit IgG against factor VIII antigen (Behringer Calbiochem, La Jolla, CA) and subsequent incubation with FITC-labeled anti-rabbit IgG antibody (Polyscience, Warrington, PA).

**Preparation of Latex Minibead Probes**

Amide-modified polystyrene latex minibeads 0.345 μm ± 0.0035 μm SD in diameter; Dow Chemical, Indianapolis, IN) were conjugated with purified transferrin as described. Briefly, 5 mg of TF minibeads were suspended in 1 ml of 0.1 M sodium bicarbonate and 0.15 M NaCl buffer (pH 8.0) was activated by incubation with 8% glutaraldehyde (purified EM grade; Polyscience) at a final concentration of 5% for 48 hr at 37°C in a mechanical shaker. The minibead solution then was dialyzed for 24 hr against 0.1 M NaHCO3, 0.15 M NaCl (pH 8.8) at 4°C. TF was added to the activated minibeads (200 μg/mg beads) and the mixture was gently shaken for 6 hr at 22°C. To saturate the aldehyde group not bound to TF, the mixture was incubated with 1.0 M glycine solution to a final concentration of 0.2 M for 1 hr. Minibead-derivatized TF was then separated from unbound TF by ultracentrifugation at 40,000 g for 20 min at 4°C. Large aggregates were removed by centrifugation at 4,000 g for 10 min. Yeast hexokinase (Sigma, St. Louis, MO) was similarly conjugated to minibeads and used for a control.

TF was labeled with 125I (carrier-free; New England Nuclear, Boston, MA), using the chloramine-T method. The excess 125I was dialyzed against PBS. The specific activity was 9.07 μCi/nmole.

**Incubation**

To 500 μl of the liver cell suspension, and its small or large cell fractions, 500 μl of TF minibead probe was added to a final concentration of 0.4 mg beads/ml. The mixture was incubated at 4°C or 37°C for 60 or 20 min, respectively. To ensure the specificity of the reaction, parallel experiments were carried out in which the liver cell suspension was preincubated with native TF-Fe complex (35 μM) at 4°C for 20 min. After incubation with TF-derivatized minibeads, cells were washed twice with Dulbecco’s PBS containing 0.5% BSA.

To 2 x 10^6 cells (in large and small cell fractions), 20–250 μg/ml of 125I-labeled TF were added. To ensure the specificity of the uptake, cells were preincubated with nonradioactive TF-Fe complex before incubation with the 125I-labeled complex. Incubation was carried out for 1 hr, and the samples were subsequently counted in a gamma counter (Model 1185R, Searle Analytic).

**Electron Microscopy**

For SEM, a drop of cell suspension was mounted on a round coverslip pretreated with poly-1-lysine (50 μg/ml, Sigma) and fixed in a modified Karnovsky’s fixative in cacodylate buffer (0.114 M, pH 7.2) for 30 min at 4°C. The coverslip was then rinsed and postfixed with 2% cold, similarly buffered, OsO4. After dehydration, the cells were critical point dried, sputter-coated with gold-palladium, and examined.

For TEM, cells were fixed and dehydrated as for SEM and embedded in Spurr resin, thin-sectioned, stained, and studied in an electron microscope.

**Statistical Analysis**

Cells were selected randomly at low magnification (in which the beads are not identifiable), and micrographs were obtained at higher magnification. The number of beads on the exposed surface of each cell was counted, and different groups of cells were subjected to nonparametric analysis using the Kruskal-Wallis one-way analysis of variance and the multiple comparison method of Dunn.

**RESULTS**

**Cellular Composition of Liver Cell Suspensions**

Three cell types were recognized in the suspensions. Hepatocytes were larger than 20 μm, displayed short characteristic cytoplasmic organelles in TEM (Fig. 2E). Cytochemically, they were positive for peroxidase, pyronin, acid phosphatase, and esterase. Kupffer cells were 7–15 μm and displayed ridge-like surface foldings on SEM (Figs. 1D and 3C). They were phagocytic and contained lysosomes and phagosomes. Cytochemically, they were positive for acid phosphatase, peroxidase, and esterase. A third cell type was 5–15 μm and displayed a smooth surface with few invaginations in SEM (Fig. 1B). By TEM, it was not phagocytic and could readily be distinguished from other cell types by its sponge-like appearance (Fig. 2A–D and Fig. 3B), which is characteristic of isolated, retracted, endothelial cells. Further evidence for the endothelial nature of this cell type was obtained by staining with FITC-conjugated antibody to factor VIII antigen using the indirect method. In the suspension, only this cell type gave a positive reaction. The antibody also reacted with rat marrow megakaryocytes but not other marrow cells. Table 1 gives the proportions of different cell types in crude liver cell suspensions and the two fractions.

**TF Receptor Distribution**

In both crude and fractionated cell suspensions, the binding of the TF minibead probe was limited to the endothelial cells. Figures 1A, 2A, and 3A demonstrate, respectively, the binding of rat and human TF conjugated to beads as studied by SEM or TEM. Similar results were obtained when rabbit TF was used. Hepatocytes and Kupffer cells did not bind the probe, irrespective of whether rat, human, or rabbit TF was used (Figs. 1–3). This was further documented by semiquantitative studies in which the number of beads was scored on the exposed surfaces of randomly selected cells. Figure 4 shows the data on rat TF probe
and demonstrates that only endothelial cells bind the probe. Similar data using human TF probe are shown in Fig. 5. There were statistically significant differences (<0.05) between the binding for endothelial cells as compared to either hepatocytes or Kupffer cells. There was no difference in either the pattern or the intensity of the binding when TF from various species was used.

The specificity of binding was demonstrated by preincubation with 35 μM native Fe-TF complex, where the binding was 80% inhibited (Figs. 4 and 5). Cross-inhibition was also carried out using human TF to inhibit the binding of rat TF probe. Both homologous and heterologous TF inhibited the binding to the same degree. Moreover, minibeads derivatized with the nontransport protein, hexokinase, did not bind to any cell (Fig. 5). Binding occurred at 4°C as well as 37°C (Figs. 1–3), although internalization of the probe, observed in TEM, occurred only at 37°C (Figs. 2B–D, and 3B). Both rat and human TF probes were internalized at 37°C but not at 4°C. Internalization occurred via coated pits and vesicles (Fig. 2D). No internalization was noted by hepatocytes or Kupffer cells at either 4°C or 37°C (Fig. 2E and F).

**125I-TF Uptake by Liver Cell Fractions**

Incubation of small and large cell fractions with 125I-TF led to the uptake of TF-Fe complex only by the small cell fraction. The large cell fraction did not bind the 125I-TF (Fig. 6). Figure 7 shows the Scatchard plot of the 125I-TF uptake by the small cell fraction. From this plot, the number of receptor sites was calculated to be approximately 5 × 10⁶/cell, with an affinity constant (Kₘ) of 0.9 × 10⁻⁷ M⁻¹.

**DISCUSSION**

These studies indicate that endothelial cells, but not hepatocytes or Kupffer cells, are responsible for the uptake of Fe-TF complex in the liver cell suspensions. The absence of TF receptors on hepatocytes and Kupffer cells suggests the presence of an intercellular transport system for iron, which may be different from that of the TF receptor system.

The uptake of 125I-labeled TF by crude liver suspen-
Fig. 2. Binding and internalization of rat TF-derivatized minibeads by various cell types in the liver cell suspension, studied by TEM. (A) An endothelial cell, recognizable by its sponge-like appearance, incubated with the probe at 4°C. Binding is present (arrow), but internalization is not seen at this temperature (×9,500). (B) An endothelial cell incubated with the probe at 37°C. Numerous beads have been internalized or are in the process of internalization (arrows). Note again the sponge-like appearance of the cell (×11,000). (C) Incubation was again carried out at 37°C. An endothelial cell shows numerous beads bound to the cell, in process of internalization, or completely internalized (×11,000). (D) Higher magnification of a part of an endothelial cell incubated with the probe at 37°C. Internalization occurs via coated pits and vesicles (arrows) (×20,000). (E) A hepatocyte incubated with the probe at 37°C. Neither binding nor internalization is seen (×3,500). (F) A Kupffer cell incubated with the probe at 37°C. No binding is seen (×9,500).
Fig. 4. Binding capacity of various cell types in the liver cell suspension for rat TF-derivatized minibeads. Each point represents the number of minibeads bound by an individual cell. Hepatocytes (H) and Kupffer cells (K) did not bind the probe, but endothelial cells (E) did. The binding was inhibited by preincubation with nonderivatized Fe-saturated TF (E-pre), indicating the specificity of the probe. All data are based on 4°C experiments.

Fig. 5. Binding capacity of various cell types in the liver cell suspension for human TF-derivatized and control minibeads. Each circle represents the number of minibeads bound by an individual cell. Hepatocytes (H) and Kupffer (K) did not bind the minibead probe significantly, but endothelial cells (E) did. The binding was inhibited by preincubation with native Fe-saturated TF (E-PRE). Control beads derivatized with hexokinase (E-HK) did not bind to the endothelial cells, indicating that the binding is not a nonspecific characteristic of the probe with respect to the endothelial cell. Experiments were carried out at 4°C.
sions has led to the conclusion that hepatocytes are the initial site of the uptake of Fe-TF complex. Such a conclusion would be inevitable if liver cell suspensions were homogeneous in their cellular compositions. However, the data presented here indicate that these suspensions are not homogeneous. They have a considerable contamination with the smaller endothelial and Kupffer cells. The uptake of $^{125I}$-TF does not permit the differentiation of the cell type responsible for the uptake. The minibead method, although not quantitatively as accurate as the $^{125I}$-method, has the merit of permitting observations on the binding of the probe to individual cells and the topography of the binding. By using this method, the uptake of Fe-TF complex was found to occur on endothelial cells rather than hepatocytes. The uptake was comprised of two steps: The first step was the binding of Fe-TF complex to the cell surface. This binding occurred at both 4°C and 37°C and was inhibited by the presence of excess nonconjugated Fe-TF complex, indicating the specificity of the binding. The second step consisted of the internalization of the probe. This step occurred at 37°C, but not at 4°C. Moreover, the internalization occurred via the system of coated pits and vesicles, indicating receptor-mediated endocytosis. No internalization occurred by either hepatocytes or Kupffer cells at 37°C, again supporting the contention that the internalization was the result of receptor-mediated endocytosis. The absence of internalization at 37°C by Kupffer cells, a cell known to be phagocytic, may be a time phenomenon. Phagocytosis of particles such as latex requires more time exposure than was provided in these experiments.

Our conclusion, derived from the experiments with visual probe, was further substantiated by fractionation of liver cell suspensions and the uptake of $^{125I}$-labeled TF by individual fractions. Again, it was the endothelial-rich fraction and not hepatocytes that took up the label.

Using these methods, we have obtained a similar pattern of receptor distribution in liver cell suspensions for at least 3 other transport proteins (unpublished data), as well as insulin, suggesting that TF may not be unique in this regard.

The endothelium is a critical element of the tissue-blood barrier. Its function in controlling the traffic of cells and particulate substances has recently come into focus. It is also the first cellular element to come into contact with circulating Fe-TF and other circulating proteins. Although some of these proteins may bypass the endothelial barrier by diffusion, it is probable that many are initially taken up by the endothelium, which has appropriate surface receptors. The path through which these complexes are then transported to the tissue parenchyma is not clear, but in the case of iron, it does not appear to involve TF receptor. It is possible that the iron may be first processed by the endothelial cell and is then transported to other tissue cells, such as hepatocytes or Kupffer cells. The role of endothelium in this regard may therefore be comparable to the role of intestinal epithelium in the regulation of iron absorption. The endothelium may thereby have a function in controlling the magnitude of iron uptake by hepatocytes. In regulating the intestinal iron absorption, a function has recently been proposed for the TF. It is possible that the TF-endothelial interaction may also play a function in the regulation of iron uptake by the liver.

Of further interest is the magnitude of uptake of Fe-TF complex by the endothelium. Whereas in crude liver cell suspensions, the number of receptor sites has been calculated to be $367 \times 10^2$/cell, in the small cell...
fraction, we calculated the number of receptor sites to be approximately $5 \times 10^6$/cell. Considering that this fraction contains only 37% endothelial cells, the number of receptors per endothelial cell can then be extrapolated at $14 \times 10^6$/cell. This is more than 2 orders of magnitude larger than in crude liver cell suspensions, and even 1 order of magnitude more than those reported for such other cell systems as L-1210 leukemia cells and reticulocytes. This large number of receptor sites may reflect the fact that the endothelium is involved mostly in channeling the traffic of Fe-TF rather than in the metabolism of iron per se, as is the case with hepatocytes and other cell systems. The affinity constant of the TF binding to the small cell fraction, obtained in this study, is in agreement with the figures reported for crude liver cell suspensions and such other cell systems as reticulocytes.

We also found no difference in the pattern and intensity of TF binding to endothelial cells using a homologous (rat) and two heterologous (human, rabbit) TF. This was further substantiated by cross-inhibition of the binding using heterologous TF species. Both the homologous and heterologous systems similarly inhibited the uptake.

REFERENCES

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