Characterization and Quantitation of the Circulating Forms of Serum Transferrin Receptor Using Domain-Specific Antibodies

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To characterize the nature of the immunoreactive transferrin receptor in human serum, antisera were developed to peptide sequences of the extracellular domain of human transferrin receptor between amino acids 107 and 120 and the intracellular domain between amino acids 40 and 54. Antisera against the extracellular domain exhibited reactivity against both purified intact receptor and immunopurified circulating receptor, whereas antisera against the intracellular domain reacted only with intact receptor. Using competitive binding enzyme-linked immunosorbent assays, transferrin receptor in ultracentrifuged sera from normal subjects and patients with sickle cell anemia could be detected with antisera against the extracellular but not the intracellular domain. When the pellet obtained by ultracentrifugation of these sera was assayed after solubilization in 1% teric (polyoxyethylene-9-lauryl ether), only 0.6% of total serum receptor was detected in normal subjects and 3.8% in subjects with sickle cell disease. Roughly equal amounts of this pelleted immunoactivity were detected with antibodies against the extracellular and intracellular domains. These results indicate that less than 1% of transferrin receptor in normal human sera is intact receptor consistent with an exosomal origin and that virtually all circulating transferrin receptor is in the form of a truncated extracellular domain.

PUBLICATIONS from several laboratories indicate that transferrin receptor can be detected invariably in human sera using sensitive immunologic methods. Recent studies from our laboratory have shown that the serum transferrin receptor is a truncated soluble form resulting from a cleavage in the extracellular domain between arginine, at amino acid position 100, and leucine, at amino acid position 101. On the other hand, in vitro studies with maturing sheep reticulocytes have indicated that transferrin receptor is released intact in small vesicles termed exosomes in which the extracellular domain of the transferrin receptor is positioned on the external surface. It has been suggested in several reports that this particular form accounts for the immunoreactivity detected in human serum. In our recent study demonstrating that serum receptor consists predominantly of a truncated soluble form, the material used for amino acid sequencing was isolated by affinity purification, and it is conceivable that a particulate form of circulating receptor would not be extracted by this purification approach. Moreover, the monoclonal enzyme-linked immunosorbent assay (ELISA) used in our laboratory for routine measurements of serum transferrin receptor does not distinguish between the intact and soluble forms of transferrin receptor. The current investigation was undertaken to further characterize the nature of the serum transferrin receptor in humans by extending our search for a vesicular or intact form of transferrin receptor in serum. To distinguish and quantify the intact and soluble forms of the circulating receptor, we developed polyclonal antibodies to peptide sequences in the extracellular and intracellular domains for use in competitive binding ELISAs.

MATERIALS AND METHODS

Keyhole limpet hemocyanin (KLH), glutaraldehyde, complete and incomplete Freund’s adjuvant, ammonium sulfate, glycine, tris-[hydroxymethyl]aminomethane (TRIS), polyoxyethylene-9-lauryl ether (teric), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co (St Louis, MO). o-Phenylenediamine dihydrochloride was from Eastman Kodak (Rochester, NY). Peroxidase-conjugated swine IgG to rabbit IgG was obtained from Dako (Carpinteria, CA). Affi-gel protein A gel, 4% to 20% polyacrylamide gradient gel and nitrocellulose membrane were from Bio-Rad Laboratories (Richmond, CA). Intact transferrin receptor was purified from human placenta using a transferrin affinity column by the method of Turkewitz et al. Immunoaffinity chromatography using monoclonal antitransferrin receptor antibody was employed to purify soluble receptor from human serum.

Selection of peptide sequences. The two peptide sequences selected from the deduced amino acid sequence for the preparation of antisera were positioned on either side of the truncation site of the soluble transferrin receptor. The specific sequences were chosen to maximize regional hydrophilicity while maintaining an optimal position of lysine residues for complexing the KLH carrier protein. The peptide in the extracellular domain was located six residues distal to the truncation site of the intact receptor between amino acid residues 107 and 120 (Fig 1). The chosen sequence PVREEEPGEDFPAR possessed high hydrophilicity but required a lysine residue to be appended at the C-terminus to facilitate coupling to the carrier protein KLH. The intracellular peptide sequence LAVDEENADNNTKA was located in the cytoplasmic domain of the molecule between amino acid residues 40 and 54 and possessed one lysine residue at the second position from the C-terminus.

Peptide synthesis, purification, and conjugation. The two peptides were synthesized commercially by the solid-phase method at Multiple Peptide Systems (San Diego, CA). Purification of the desired peptide from the unpurified mixture of peptide sequences was accomplished by high-performance liquid chromatography (HPLC) using a Varian Liquid Chromatograph, model Vista 5500 (Varian Instruments, Sunnyvale, CA) and a Vydac C18 reverse phase column (4.6 mm x 25 cm) (Alltech Associates Inc, Deerfield, IL). The sample was eluted with a flow rate of 1 mL/min using a linear gradient of 5% to 65% acetonitrile/0.1% trifluoroacetic acid for 30 minutes. Peptide conjugation to the carrier protein KLH was performed as described previously.

Preparation of antisera. Polyclonal antisera against the purified peptides were prepared by injecting female New Zealand white rabbits subcutaneously with 0.5 mg of peptide conjugate emulsified in 500 µL complete Freund’s adjuvant and 500 µL normal saline. The an-

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The arrow between residues in the cytoplasmic domain and gel electrophoresis (SDS-PAGE) under nonreducing conditions by the method of Laemmli and transblotted indicates the site of soluble receptor truncation. Cysteine residues at the receptor antibody (Mab).

Fig 1. Predicted amino acid sequence of the amino-terminal end of the transferrin receptor. The transmembrane sequence (amino acids 62 to 89) is underlined. The selected domain-specific sequences are doubly underlined and consist of residues 40 to 54 in the cytoplasmic domain and 107 to 120 in the extracellular domain. The arrow between residues 100 (arginine) and 101 (leucine) indicates the site of soluble receptor truncation. Cysteine residues at position 89 and 98 are involved in disulfide linkage to form the receptor dimer.

Animals were immunized a further two times at 2-week intervals by the same technique and bled 10 days after the final injection.

A portion of the antiserum to synthetic peptides was precipitated with ammonium sulfate and purified using protein A chromatography as follows. Ammonium sulfate was slowly added to the antiserum at 40% saturation and allowed to stand for 2 hours at 4°C. The supernatant, obtained by centrifugation at 3,800g for 20 minutes, was brought to 50% saturation with ammonium sulfate, allowed to stand for 2 hours, and recentrifuged. Both pellets were pooled and loaded onto an affinity-capture protein A gel column and washed with phosphate-buffered saline (PBS) until the A260 approached zero. Antibody was then eluted with 0.1 mol/L glycine, pH 2.5, immediately neutralized with 1 mol/L TRIS, pH 8.3, and dialyzed against PBS.

Electrophoresis and Western blotting. Purified intact and serum receptor were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions by the method of Laemmli and transblotted to nitrocellulose membrane as described previously. After blocking with 1% nonfat dry milk in PBS for 1 hour, the nitrocellulose membrane strips were incubated for 2 hours with 10 mL cytoplasmic or extracellular domain peptide antibody that had been purified using protein A chromatography or monoclonal antibody (MoAb) to receptor at concentrations of 45, 50, and 40 μg/mL, respectively, in PBS containing 0.1% nonfat dry milk. After washing with PBS, the nitrocellulose membrane strips were incubated with 1:1,000 swine antirabbit IgG antibody conjugated to horseradish peroxidase (HRP), and those in MoAb to receptor were incubated with 1:1,000 rabbit antimouse IgG antibody–HRP for 2 hours. The immunoreactive proteins were detected by adding the chromogenic substrate 4-chloro-l-naphthol after extensive washing with PBS.

Competitive binding ELISA. The protocol for the competitive binding ELISA was modified from that described by Torffvit et al.²⁰ Flat-bottomed 96-well microtiter plates (NUNC-Intermed; NUNC Inc, Naperville, IL) were coated overnight at 4°C with 200 ng/mL purified intact receptor diluted in 0.05 mol/L carbonate buffer, pH 9.6, blocked for 30 minutes with 0.5% BSA in carbonate buffer, and washed three times with PBS and 0.05% Tween (PBST). In the competition mixture, 80 μL of standard or sample in PBST was added to each well followed by 20 μL of diluted peptide antiserum and incubated for 2 hours at room temperature. After washing three times with PBST, swine antirabbit IgG conjugated with HRP diluted 1:1,000 with 1% BSA/PBST (100 μL) was added to each well and incubated at room temperature for 2 hours. After thorough washing, 100 μL of o-phenylenediamine dihydrochloride (1.9 mmol/L) in 0.15 mol/L citrate-phosphate buffer, pH 5.0, containing 0.01% hydrogen peroxide, was added and after a 30-minute incubation in the dark, the reaction was stopped by adding 25 μL of 25% sulfuric acid. A492 was determined on a microplate reader (Tietter Multiskan, Flow Laboratories, McLean, VA). The standard curve was obtained by plotting the concentration of affinity-purified transferrin receptor added in the assay against the difference in A492 from measurements performed with PBST only.

Preparation of human sera. Plasma samples were obtained from three normal subjects and five patients with sickle cell anemia. Samples were diluted 1:1 with saline and ultracentrifuged at 100,000 g for 90 minutes at 4°C. The resulting supernatants were stored, and the pellets were solubilized in Hank’s buffered salt solution containing 1 mmol/L TRIS (HBS-TRIS) and 1% tenc, pH 7.4, while rotating continuously overnight at 4°C. The solubilized materials were then centrifuged at 15,000 g for 15 minutes and supernatants collected. The serum transferrin receptor concentration was measured in the native serum before and after ultracentrifugation and in the solubilized pellets. Both the competitive binding ELISA using polyclonal peptide antibodies outlined previously and an ELISA employing monoclonal antibodies to the intact receptor as described previously were used.

RESULTS

The immunoreactivity and specificity of the peptide antibodies for both purified intact and serum transferrin receptors was first examined by Western blotting (Fig 2). MoAb against the intact receptor detected placental receptor at M,
= 190,000 and serum receptor at 85,000 under nonreducing electrophoretic conditions. An identical reactivity pattern was obtained with the antibody raised against the extracellular peptide domain. However, the peptide antibody against the intracellular domain recognized only the intact receptor.

These reactivities were confirmed when competitive binding ELISAs were established using peptide antibodies against the extracellular and intracellular domain. When increasing amounts of intact placental receptor were added, a parallel dose response was obtained with the intracellular and extracellular domain antibody (Fig 3). On the other hand, when increasing amounts of affinity-purified serum receptor were added, only the antisera against the extracellular domain gave a dose-response curve (Fig 4).

To assess the ability of these assays to provide a quantitative measure of intact and soluble receptor when contained in the same mixture, simultaneous measurements were performed with assays using the two domain-specific antisera (Table 1). Samples containing only intact receptor could be assayed using either the extracellular or intracellular assay system, whereas the latter assay was unable to detect the soluble form of transferrin receptor. Thus, in samples containing a mixture of the intact and soluble form of transferrin receptor, subtraction of the assay value obtained with the intracellular peptide antibody from the extracellular assay provided a reliable measure of the concentration of the truncated soluble form.

These competitive binding ELISAs were next used to determine the quantities of soluble and intact transferrin receptor in human sera. Because of greater sensitivity and qualitatively identical reactivity, the monoclonal ELISA was used rather than the competitive ELISA established with peptide antibodies against the extracellular domain. In postultracentrifugation supernatant of both normal sera and sera from patients with sickle cell anemia, negligible amounts of transferrin receptor were detected with the assay against the intracellular domain that measures only intact receptor (Table 2), whereas predictable amounts were measured with the monoclonal ELISA. In normal serum, both ELISAs also failed to detect significant quantities of transferrin receptor in the solubilized postcentrifugation pellet. On the other hand, detectable quantities of transferrin receptor could be measured in this serum fraction in patients with sickle cell anemia using the monoclonal ELISA. The amount varied between 1.7% and 6.7% of the total quantity present in the serum with a mean of 3.8%. In the sera from patients with sickle cell anemia, the two assay systems detected comparable amounts of receptor in the solubilized pellet. In four of the five sera examined, greater amounts of transferrin receptor were detected with the assay developed for the cytoplasmic portion of the receptor, suggesting that there was a small amount of intracellular remnant of the transferrin receptor in addition to intact receptor in the pelleted material.

**DISCUSSION**

Prior work suggesting that the exosome is the major pathway of transferrin receptor loss from cells has been
Table 2. The Assayed Receptor Content of Ultracentrifuged-Serum Supernatant and Solubilized Ultracentrifugation Pellets From Normal Subjects and Patients With Sickle Cell Anemia Using MoAb ELISA (MoAb) That Reacts With the Extracellular Receptor Domain and Cytoplasmic Domain Peptide Antibody Competitive Binding ELISA (intra) That Reacts With the Cytoplasmic Receptor Domain

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>Ultracentrifuged Serum Supernatant</th>
<th>Solubilized Postcentrifugation Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MoAb* (ug/100 mL plasma)</td>
<td>intra† (ug/100 mL plasma)</td>
</tr>
<tr>
<td>Normal</td>
<td>435</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>543</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>570</td>
<td>11</td>
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<td>Sickle cell anemia</td>
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<tr>
<td></td>
<td>4,172</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2,599</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1,770</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1,829</td>
<td>0</td>
</tr>
</tbody>
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* Detects both intact and serum receptor.
† Detects only intact receptor.

Conducted primarily in animals. In rat studies performed by Beguin et al., the electrophoretic pattern of the immunoreactive plasma transferrin receptor from normal and iron-deficient rats was considered to be the same as intact placental receptor. Using gel filtration, this group later concluded that transferrin receptor in human serum was also in the same molecular form as intact placental receptor. In a more recent study, the release of transferrin receptor during the maturation of rat reticulocytes in vitro was studied. These workers reported that two thirds of the receptor shed during a 44-hour incubation was present in vesicles and the remainder in a soluble form. The latter was found to be similar in size to the vesicular and cellular receptor, leading these investigators to conclude that the soluble form of the transferrin receptor is released as an intact molecule from the vesicles. These observations are in sharp contrast to recent studies with K562 erythroleukemic cells that released both an intact exosomal form and a quantitatively dominant truncated monomeric form identical to that recently described in human sera.

In the present study, antisera were developed against peptide sequences in the extracellular and intracellular domains of human transferrin receptor to further characterize the nature of circulating receptor in human sera. These antisera exhibited the expected reactivities with intact placental and truncated serum receptor when examined by Western blotting (Fig 2). The establishment of quantitative assays with these immunologic reagents proved to be the major obstacle in this investigation. We were unsuccessful in our efforts to establish a sandwich-type ELISA using varying combinations of polyclonal antibodies and MoAbs against intact receptor or synthetic peptides. The results were not improved by purifying antibodies with protein A or peptide affinity chromatography. All combinations failed to give a dose-response curve with purified intact receptor. Because free peptide bound to the microtiter plate could readily bind the peptide antibody, it must be assumed that these difficulties were caused by diminished affinity of the antisera for the linear peptide sequences when present as the native protein.

These problems were circumvented by the use of a competitive binding ELISA in which various fractions of human sera were incubated with an appropriate dilution of the peptide antisera. When assayed in microtiter plates coated with intact human receptor, a progressive decrease in the binding of the peptide antisera to this receptor occurred when increasing quantities of transferrin receptor were added. Because the sensitivity of the sandwich ELISA with MoAb was greater than the competitive binding ELISA using extracellular domain peptide antibodies, the former was used in conjunction with the intracellular domain peptide antibody competitive binding ELISA when examining the nature of circulating transferrin receptor. It should be noted that the use of these two assays in tandem permits the quantitative detection of mixtures of either intact and soluble receptor or intact and the cytoplasmic remnant, but it does not provide measurements of mixtures of all three forms of the receptor.

In normal subjects, negligible amounts of transferrin receptor were detected in the solubilized pellet following ultracentrifugation; the fraction in which vesicular or exosomal receptor would be contained. The pelletable receptor accounted for only 0.6% of the total serum receptor (range 0.5% to 0.7%) and was thus virtually undetectable. When the concentration of circulating receptor was substantially increased in patients with sickle cell anemia, the soluble truncated form in the ultracentrifuged supernatant of serum again was the predominant form of the circulating receptor, although more receptor was detectable in the pelleted fraction. The latter accounted for an average of 3.8% of the total serum receptor with a range of 1.7% to 6.7% based on measurements using the monoclonal ELISA. The monoclonal assay performed in conjunction with the competitive ELISA using the intracellular domain antibody indicated a slightly higher concentration of transferrin receptor containing the intracellular domain, except in the patient with the highest concentration of circulating receptor. These findings are consistent with an exosome containing intact receptor and a small amount of additional cytoplasmic remnant.

The present investigation provides additional evidence that the predominant form of the circulating transferrin receptor in human serum is a truncated monomeric form of transferrin receptor. The majority of investigations which have led to evidence that transferrin receptor is released from cells in intact vesicles have used maturing reticulocytes from animals with marked hemolysis. Therefore, it is not surprising that small amounts of receptor can be detected in this form in patients with severe hemolytic anemia. In addition to the patients with sickle cell anemia examined in the present study, we have detected receptor in pelleted form averaging 5.9% in patients with autoimmune hemolytic anemia and 3.9% in patients with thrombotic thrombocytopenic purpura. Nevertheless, the predominant, if not only, form in native serum...
from normal subjects exists as a truncated extracellular monomer.

REFERENCES

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