Release of Soluble Transferrin Receptor From the Surface of Human Leukemic HL60 Cells

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Information regarding transferrin (Tf) receptor degradation is largely incomplete. HL60 cells were shown to release to their growth medium a Tf-binding protein which could be immunoprecipitated by anti-Tf receptor monoclonal antibodies (MoAbs) B3/25 and OKT9. Soluble Tf receptor was detected in the medium within one hour of replating of cells, and its release was inhibited at 4°C. The affinity of Tf for the soluble receptor released by cells (Kd = 2.3 x 10^{-10} mol/L) was slightly lower than its affinity for the detergent-solubilized cellular receptor ($K_d = 1.2 x 10^{-10}$ mol/L). 125I-Tf internalized and released by cells subsequently bound to Tf receptor released by the

same cells, and soluble Tf receptor in the conditioned medium (CM) inhibited 125I-Tf binding to intact cells. The soluble Tf receptor isolated from the CM was smaller (78,000 daltons) than the cell surface receptor (94,000 daltons) when analyzed by gel electrophoresis under reducing conditions. Isolated cell membranes readily released soluble receptor; however, this release could be blocked by protease inhibitors. The soluble Tf receptor may represent the extracytoplasmic domain of the cellular Tf receptor released from the surface of HL60 cells through proteolytic cleavage by a membrane-based protease.

MATERIALS AND METHODS

Materials. Human Tf, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), pyridoxal hydrochloride, iodiconic acid, ferric citrate, cyanogen bromide-activated Sepharose 4B, Sephadex G200, molecular weight (mol wt) standards, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), EDTA, and pronase were purchased from Sigma (St Louis). Leupeptin and pepstatin were obtained from Boehringer Mannheim Biochemicals (Indianapolis). Recombinant human insulin, Aquacide 111 (flame polyethylene glycol, PEG), and Tachisorb M IgG immunoadsorbent (goat antibody to mouse IgG conjugated to Pansorbin Staphylococcus aureus cells) were obtained from Calbiochem (La Jolla, CA). 125I-Sodium iodide was obtained from New England Nuclear (Boston). Saturation of Tf with iron and iodination of Tf-Fe were performed as previously described.

Fe-PIH was prepared as previously described by a modification of the method of Ponka et al. Monoclonal antibody (MoAb) B3/25 to the human Tf receptor was provided by Dr Ian Trowbridge. MoAb OKT9 to the human Tf receptor was purified from an OKT9-producing hybridoma cell line using Affi-Gel protein A (BioRad, Richmond, CA) affinity chromatography; 50 μg OKT9 was iodinated with 1 mCi 125I-sodium iodide with the chloramine T method.

Tissue culture. Human leukemic HL60 cells were adapted to grow in serum-free, Tf-free RPMI 1640 medium containing 5 μg/mL human insulin, 5 ng/mL sodium selenite, and 20 μmol/L Fe-PIH (Fe-PIH medium). Unless otherwise stated, these cells (Fe-PIH cells) and the medium conditioned by the growth of these cells (conditioned medium, CM) were used for the studies described below. Using a previously described radioimmunoassay (RIA) for Tf, we could not detect Tf in the CM or in the Fe-PIH cell lysates.

Concentration of CM. Cell-free medium conditioned by the growth of Fe-PIH cells was immediately filtered through a 0.2-μm filter and stored at -20°C. The CM was concentrated 100-fold at 4°C by placing it in 3,500 mol wt cut-off dialysis bags and then embedding the bags in Aquacide 111. The sample was dialyzed extensively against 10 mmol/L KPO4, 150 mmol/L NaCl pH 7.4 buffer (PBS), filtered through a 0.2-μm filter and assayed for protein content using an assay from Bio Rad.

125I-Tf binding to cells. Tf binding sites on intact HL60 cells grown in Fe-PIH medium or in serum-supplemented medium were measured with a previously described 125I-Tf binding assay, and maximal Tf binding was determined according to the method of Scatchard. In separate experiments, binding studies were performed with 0 to 150 μL concentrated (100-fold) CM added to the assay. Total incubation volume was kept constant at 500 μL.

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Reappearance of cell surface Tf receptors after pronase treatment of cells. The reappearance of Tf receptors on Fe-PIH cells after their removal by pronase treatment was examined as previously described. 11–13. Tf binding to cells was measured before and after pronase treatment and again after 30 and 60 minutes of reincubation of cells in fresh medium.

**Methods**

**Cell culture and conditioned medium.** Crude cell membranes were prepared by a modification of a previously described method. 11–13. HL60 cells were detached and washed twice with PBS, resuspended in fresh medium, and incubated at 37°C for 24 hours. The cell suspension was then washed twice with PBS and incubated in complete medium. The cell suspension was centrifuged at 1000 g for 10 minutes, and the supernatant was used as CM (100-fold concentrated). The CM was kept constant (100-fold) and the amount of OKT9 added was varied from 0.2 to 4 μg.

**Immunoprecipitation studies.** One hundred twenty-five microliters of different concentrations of conditioned medium (1 × 10^5, 10^5, 5 × 10^5, and 10^6) were incubated with 2.5 ng ^125I-Tf-Fe at 37°C for one hour. One microgram B3/25 or 2 μg OKT9 (anti-Tf receptor MoAbs) was then added (total volume 160 μL). After an additional hour of incubation at 37°C, 160 μL Tachisorb M was added and the mixture was incubated at room temperature for 20 minutes. The incubation tubes were centrifuged, and the radioactivity in the pellet and supernatant was counted to determine the amount of ^125I-Tf-Tf receptor complex precipitated by the MoAb (nonspecific cpm precipitated in control tubes was subtracted). In other tubes, 10 μg nonradioactive Tf-Fe was added to determine whether the binding of ^125I-Tf to the immunoprecipitable Tf receptor in the CM could be competitively inhibited. In separate experiments, the concentration of CM was kept constant (100-fold) and the amount of OKT9 added was varied from 0.2 to 4 μg.

**Detection of Tf receptor in CM by dot-blot method.** Five nanogram ^125I-Tf-Fe was incubated with 250 μL 100-fold concentrated CM for 90 minutes at 37°C. The mixture was applied to a 2.5 × 30-cm Sephadex G200 column and was eluted with PBS containing 20 μg/mL BSA. The cpm in the fractions (2 mL) was counted in an LKB Compugamma γ-counter. In a parallel experiment, 10 μg nonradioactive Tf was incubated with the mixture of ^125I-Tf plus CM before gel filtration. In another experiment, the concentrated CM alone was separated by gel filtration, and individual eluted fractions were analyzed for Tf receptor by the dot-blot method described below.

**RESULTS**

**Tf receptor expression on HL60 cells growing in Fe-PIH medium.** Although cells grown in Fe-PIH medium no longer required Tf for their growth, they continued to display approximately one half the number of cell surface Tf binding sites (123,000 sites/cell) than that present on cells growing in serum-supplemented medium (221,000 sites/cell). Growth of cells in Fe-PIH medium did not alter the affinity of the receptor for Tf. Removal of cell-surface Tf receptors by pronase treatment was followed by a rapid reappearance of these receptors, indicating that newly synthesized/intermedialized receptors were being translocated to the cell surface (Fig 1).

**Identification of a soluble Tf-binding protein in the conditioned medium.** To examine whether Tf receptors were being shed from cells, the concentrated CM was incubated with ^125I-Tf and the mixture was separated by gel filtration. As shown in Fig 2A, the elution peak of free ^125I-Tf was located at a column V_{e}/V_{o} of 1.75. However, after incubation of ^125I-Tf with the CM, an additional radioactive...
Fig 1. Reappearance of cell-surface Tf binding sites on Fe-PIH cells. Cells were assayed for \(^{125}\)I-Tf binding and treated with pronase at 4°C to remove cell-surface Tf receptors. Cells were then washed, an aliquot assayed for \(^{125}\)I-Tf binding (arrow), and the remaining cells were reincubated in fresh Fe-PIH medium at 37°C. Cells were removed after 30 and 60 minutes and assayed for \(^{125}\)I-Tf binding. The experiment shown is representative of four separate experiments.

A peak of larger mol wt was noted, which represented \(^{125}\)I-Tf bound to another protein. The specific nature of this binding is demonstrated in Fig 2B, in which the presence of a 2,000-fold excess of nonradioactive Tf competitively inhibited \(^{125}\)I-Tf binding to this protein and eliminated this larger mol wt radioactive peak.

**Immunoprecipitation of the soluble Tf-binding protein with anti-Tf receptor MoAbs.** The \(^{125}\)I-Tf-Tf-binding protein complex could be immunoprecipitated with two different anti-Tf receptor MoAbs, B3/25 and OKT9 (Fig 3A). These antibodies were used because they do not interfere with the binding of Tf to its receptor. \(^{125}\)I-Tf binding to the receptor was identified using \(^{125}\)I-OKT9 (Fig 4B). These studies demonstrate that release of Tf receptors is not the result of HL60 growth in defined medium but occurs regardless of the media used to support the growth of these cells.

**Comparison of \(^{125}\)I-Tf binding to detergent-solubilized cellular Tf receptors and soluble Tf receptor in CM.** Scatchard analysis of Tf binding to detergent-solubilized cellular Tf receptors and to Tf receptors in the concentrated condi-
Concentration of Conditioned Media Concentration of OKT9 (pg)

Fig 3. Immunoprecipitation of the Tf-binding protein in CM with anti-Tf receptor MoAbs. (A) One hundred twenty-five microliters of different concentrations of CM was incubated with 2.5 ng 125I-Tf. The mixture was then immunoprecipitated with 1 μg B3/25 or 2 μg OKT9. Nonspecific cpm precipitated in the control tubes was <5% and has been subtracted from the values shown. (B) Incubation conditions were identical to those in A except that the concentration of CM was kept constant (100-fold) and the concentration of OKT9 was varied.

The dissociation constants (k_d) for the receptor from either source were of the same order of magnitude (ie, 10^{-10} mol/L). However, the affinity of Tf for the cellular receptor (k_d = 1.216 × 10^{-10} mol/L) was slightly higher than its affinity for the soluble receptor in the CM (k_d = 2.306 × 10^{-9} mol/L). The high affinity of the soluble receptor for Tf enabled it to compete with cell surface Tf receptors for the binding of 125I-Tf. As shown in Fig 6, the presence of increasing concentrations of CM progressively inhibited 125I-Tf binding to HL60 cells.

Isolation and identification of the soluble Tf receptor released from cells. The soluble Tf receptor isolated from medium conditioned by a two-hour incubation of radiolabeled cells had an approximate mol wt of 78,000 when analyzed by gel electrophoresis under reducing conditions (Fig 7A). In contrast, the detergent-solubilized cell-surface Tf receptor analyzed under similar conditions had an approximate mol wt of 94,000 (Fig 7B). The cell-surface Tf receptor is known to exist as a disulfide-linked dimer. Therefore, the mol wts of the soluble and cellular receptors on reducing gels reflect differences in the size of their respective subunits (monomers).

The size of the soluble Tf receptor was also examined by Sephadex G200 gel filtration of the concentrated CM, with identification of the Tf receptor (nonreduced) in the eluted fractions by dot-blot analysis. The soluble receptor was eluted at a column V/v which corresponded to an approximate mol wt of 160,000 (not shown). No peaks of lower mol wt (representing receptor subunits) were detected; however, an additional peak (mol wt >600,000) was present in the void volume of the gel filtration column. Because of its large size, this peak was believed to represent aggregates of receptor protein. Therefore, these studies imply that the Tf receptor in the CM exists in a dimeric form and not as free receptor subunits.

Effect of protease inhibitors on release of soluble Tf receptor from cell membranes. Isolated cell membranes incubated in serum-free medium at 37°C readily released Tf receptor to the medium. However, in the presence of a...
of the Tf receptor is released from cells through the action of a cell membrane-based protease(s). These studies strongly suggest that the soluble form of the Tf receptor is released from cells through the action of a cell membrane-based protease(s).

Relationship between release of internalized 125I-Tf and release of soluble Tf receptor by cells. To examine whether receptors participating in Tf internalization and release were also being released from the cell surface along with Tf, cells were first allowed to bind 125I-Tf at 4 °C and then internalize and release 125I-Tf at 37 °C. The 125I-Tf released to the incubation medium was immunoprecipitated with the anti-Tf receptor MoAb OKT9. Because of differences in cellular Tf receptor density, the amount of 125I-Tf bound to cells and the actual cpm released by cells varied somewhat between experiments; therefore, the actual cpm released shown in Table I are from a representative experiment. However, the percentage of 125I-Tf released by cells was similar for all experiments. Table 1 shows that during the first 30 minutes of incubation there was a rapid loss of 125I-Tf from cells, but after that the amount of 125I-Tf bound to cells remained relatively constant. In contrast, the percentage of 125I-Tf immunoprecipitated by OKT9 progressively increased with the time of incubation. These results suggest that (a) Tf released by cells binds to Tf receptor released by the same cells, and (b) the release of Tf receptor from cells continues even after release of Tf ceases. These results further suggest that release of soluble Tf receptors probably occurs independent of cellular uptake and release of Tf-Fe.

DISCUSSION

The initial finding of high densities of Tf receptors on HL60 cells adapted to grow continuously in serum-free, Tf-free medium and the rapid reappearance of cell surface Tf receptors on pronase-treated cells prompted us to examine whether these receptors were being shed by cells. Three approaches were used to identify soluble Tf receptor in the CM: (a) gel filtration studies, which demonstrated that 125I-Tf bound specifically to a protein in the CM; (b) immunoprecipitation experiments using two different anti-Tf receptor MoAbs, which identified the Tf-binding protein in the CM as the Tf receptor; and (c) a sensitive dot-blot method, which demonstrated that cells grown in either Fe-PIH or serum-supplemented medium released Tf receptors shortly after being replated in fresh medium and that the amount released increased with duration of incubation and temperature.

In our studies, steps were taken to exclude contamination of the CM with cell fragments. CM was harvested by two consecutive centrifugations and was filtered twice through a 0.2-μm filter. Furthermore, the receptor-125I-Tf complex that eluted on gel filtration was within the inclusion volume of the filtration column (125I-Tf bound to cell fragments would have appeared in the void volume of the column). In addition, our more recent studies show that the Tf receptor remains in the supernatant following centrifugation of the CM at 100,000 g. Therefore, the Tf receptor in the CM appears to be a truly soluble protein.

In its native form, the cell-surface Tf receptor is a transmembrane receptor consisting of a dimer linked by an extracellular disulfide bridge. Each subunit of the receptor consists of a cytoplasmic domain (61 amino acids), a hydrophobic domain (28 amino acids), and an external extracellular domain (671 amino acids). The external domain binds Tf and anti-Tf receptor antibodies and can be cleaved from the cell surface by trypsin treatment of intact cells to yield a 70,000 dalton fragment. The Tf receptor isolated from detergent extracts of placenta and various tissue culture cell lines is a protein of mol wt 90,000 to 95,000 when analyzed by SDS-polyacrylamide gels under reducing conditions.
The Tf receptor shed by sheep reticulocytes during maturation in vitro appears to be released from these cells in vesicles in a nondegraded form and shows the same electrophoretic mobility on SDS-polyacrylamide gels as the receptor isolated from reticulocyte membranes (ie, two polypeptide bands of 186,000 and 93,000 daltons). In our studies, soluble Tf receptor release appeared to be mediated by a membrane-based protease(s), and the receptor existed in the CM as a truncated form of the dimeric receptor rather than as individual subunits. This finding appears to be consistent with proteolytic cleavage of the receptor occurring within the cell membrane, since such cleavage would be expected to spare the extracellular disulfide bond linking the two subunits and would result in release of a dimeric receptor. Under reducing conditions, the soluble Tf receptor subunit (monomer) was smaller (78,000 daltons) than its cellular counterpart (94,000 daltons). The smaller size of the soluble receptor may result from proteolytic cleavage of the larger intact cell-surface receptor. The size and the Tf and MoAb binding properties of the soluble receptor suggest that it may represent the external (extracytoplasmic) domain of the cell surface receptor.

The existence of a soluble form of the Tf receptor and the possibility that it may represent a cleavage product of the cellular Tf receptor is analogous to that observed with the folate receptor in KB cells. Recent studies showed the existence of a membrane-bound folate binding protein of
apparent mol wt 160,000 and a soluble folate-binding protein of mol wt 40,000. The latter appears to be derived from the larger membrane-bound protein. Similarly, other investigators have shown that a soluble form of the interleukin-2 (IL-2) receptor is released from activated lymphoid cells and that this receptor can be measured in the circulation in vivo. Like the soluble Tf receptor, the soluble IL-2 receptor is smaller than its cell-surface counterpart. Hence, release of soluble receptors from the surface of cells may be a feature common to several receptor-ligand systems.

Based on our studies, we propose the following modified model of Tf receptor kinetics in HL60 cells: Concomitant with the well-described process of receptor internalization, a fraction of receptors is also continuously released from the cell surface by proteolytic cleavage. These soluble receptors retain a high affinity for Tf and readily bind extracellular Tf. As a result, soluble Tf receptors in the CM can inhibit Tf binding to cells. The binding of Tf by soluble Tf receptors may therefore be of physiologic importance since such binding would preclude cellular uptake of extracellular Tf-Fe. Furthermore, receptor release appears to occur regardless of the presence of Tf in the medium and possibly is independent of Tf uptake and release by cells. Tf receptors released from HL60 cells may be derived from a pool of nonrecycling and perhaps "spent" receptors.

Tf receptors shed by maturing reticulocytes are currently believed to be the major source of circulating Tf receptors measured in vivo; however, the contribution of non-hemoglobin-producing cells to circulating Tf receptor levels is unknown. It remains to be determined whether soluble Tf receptors play an active role in ferrokinetics and can influence the uptake of Tf-Fe by certain cells. Future studies will undoubtedly address these questions.

REFERENCES

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