Transferrin receptors of human fibroblasts

Analysis of receptor properties and regulation

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Normal human skin fibroblasts cultured *in vitro* exhibit specific binding sites for ¹²⁵I-labelled transferrin. Kinetic studies revealed a rate constant for association (K_{on}) at 37°C of $1.03 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$. The rate constant for dissociation (K_{off}) at 37°C was $7.9 \times 10^{-2} \cdot \text{min}^{-1}$. The dissociation constant (K_D) was $5.1 \times 10^{-9} \text{ M}$ as determined by Scatchard analysis of binding and analysis of rate constants. Fibroblasts were capable of binding 3.9×10^5 molecules of transferrin per cell. Binding of ¹²⁵I-labelled diferric transferrin to cells was inhibited equally by either apo-transferrin or diferric transferrin, but no inhibition was evident with apo-lactoferrin, iron-saturated lactoferrin, or albumin. Preincubation of cells with saturating levels of diferric transferrin or apo-transferrin produced no significant change in receptor number or affinity. Preincubation of cells with ferric ammonium citrate caused a time- and dose-dependent decrease in transferrin binding. After preincubation with ferric ammonium citrate for 72 h, diferric transferrin binding was 37.7% of control, but no change in receptor affinity was apparent by Scatchard analysis. These results suggest that fibroblast transferrin receptor number is modulated by intracellular iron content and not by ligand–receptor binding.

The affinity of the plasma glycoprotein transferrin (Tf) for iron precludes the existence of free iron in plasma. Cells possess mechanisms to extract iron from Tf for intracellular synthesis of iron-containing proteins such as haemoglobin and cytochromes. The first step in the uptake of iron by cells requires binding of Tf to specific surface receptors. The interaction of Tf with receptors on reticulocytes (Jandl & Katz, 1963; Kailis & Morgan, 1974; Baker & Morgan, 1971), placenta (Wada *et al.*, 1979; G. M. P. Galbraith *et al.*, 1980a), activated lymphocytes (R. M. Galbraith *et al.*, 1980), hepatocytes (Young & Aisen, 1980), rat-embryo fibroblasts (Octave *et al.*, 1979, 1981, 1982; Rama *et al.*, 1981) and a variety of neoplastic cell lines (Larrick & Cresswell, 1979; Hu *et al.*, 1977; G. M. P. Galbraith *et al.*, 1980b; Hamilton *et al.*, 1979; Faulk *et al.*, 1980) has been described. The apparent subunit molecular weight of Tf receptors isolated from human, rat and rabbit reticulocytes and human placental tissue has been reported to be 90 000–95 000 (Leibman & Aisen, 1977; Sullivan & Weintraub, 1978; Hu & Aisen, 1978; Wada *et al.*, 1979; Enns *et al.*, 1981; Seligman *et al.*, 1979), but

lower estimates have been reported by others (Light, 1978; Steiner, 1980; Glass *et al.*, 1980).

There is compelling evidence that Tf is required for the growth of cells *in vitro* (Hutchings & Sato, 1978). Despite the demonstrated requirement for Tf, there is little information regarding the characteristics of the interaction between Tf and human fibroblasts.

In the present paper we define the characteristics of Tf binding to cultured human fibroblasts obtained from normal donors. Of particular interest were the observations that the fibroblast Tf receptor does not distinguish between apo-Tf and diferric Tf [$\text{Tf}(\text{Fe})_2$], and that the number of Tf receptors per cell is modulated by preincubation with ferric ammonium citrate. Receptor number and affinity were minimally affected by preincubation of fibroblasts with saturating levels of $\text{Tf}(\text{Fe})_2$ or apo-Tf. These studies suggest that the binding activity of fibroblast Tf receptors is altered by intracellular iron concentration, but not by previous exposure to ligand.

Experimental procedures

Materials

Human transferrin was purchased from the Calbiochem–Behring Corporation (La Jolla, CA,

Abbreviations used: Tf, transferrin; $\text{Tf}(\text{Fe})_2$, diferric transferrin; MEM, Eagle's minimal essential medium.

U.S.A.), Sigma Chemical Company (St. Louis, MO, U.S.A.), Cappel Laboratories (Downington, PA, U.S.A.) and Miles Laboratories (Elkhart, IN, U.S.A.). For most experiments, the transferrin from Calbiochem-Behring Corporation was used. Ferric ammonium citrate was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.) and dibasic ammonium citrate from Mallinkrodt Chemical Works (St. Louis, MO, U.S.A.). Bovine serum albumin (fraction V) was from Sigma. Carrier-free ^{125}I was bought from New England Nuclear Corp. (Boston, MA, U.S.A.). Tissue-culture dishes were from Costar (Cambridge, MA, U.S.A.). Foetal- and newborn-calf serum were obtained from Sterile Systems (Logan, UT, U.S.A.).

Cells

Human skin fibroblasts were obtained from normal individuals (with their informed consent) by skin biopsy. Cells were grown on plastic tissue culture dishes in MEM containing 10% (v/v) foetal-calf serum or 10% (v/v) newborn-calf serum, penicillin (200 units $\cdot$ ml $^{-1}$) and streptomycin (0.2 mg $\cdot$ ml $^{-1}$), at 37°C under CO $_2$ /air (1:19). Cells were used 4–7 days after passage, at which point cell densities varied between 0.5×10^5 and 3.0×10^5 cells per 35 mm-diameter plate.

Preparation of Tf(Fe) $_2$ and ^{125}I -labelled Tf(Fe) $_2$

Tf was assessed for purity by polyacrylamide-gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate, the procedure of Laemmli (1970) being adopted. Every preparation demonstrated a single band when the gel was stained with Coomassie Blue.

Tf was saturated with iron as described by Larrick & Cresswell (1979). The extent of iron saturation was assessed by spectroscopy at 465 nm, or by polyacrylamide-gel electrophoresis in the presence of urea as described by Makey & Seal (1976). Tf(Fe) $_2$ was iodinated by a modification of the chloramine-T procedure of McConahey & Dixon (1966). The iodinated Tf was purified by chromatography on Sephadex G-100, which was equilibrated and eluted with phosphate-buffered saline. The specific radioactivity of ^{125}I -labelled Tf(Fe) $_2$ varied from 280 to 620 c.p.m. $\cdot$ fmol $^{-1}$, or from 0.18 to 0.40 mCi $\cdot$ pmol $^{-1}$. Lactoferrin was isolated from human breast milk by the method of Querinjean *et al.* (1971).

Binding of ^{125}I -labelled Tf to cells

Fibroblasts were incubated on 35 mm-diameter Petri dishes for 60 min with MEM at 37°C before binding studies, to allow dissociation of any Tf-receptor complexes already present. To measure total binding, ^{125}I -labelled Tf(Fe) $_2$, in specific con-

centrations was added to cell cultures and incubated in a final volume of 1 ml of MEM containing 2–4 mg of bovine serum albumin $\cdot$ ml $^{-1}$. To measure non-specific binding, cells were incubated under the same conditions but in the presence of 1.25 μM -Tf(Fe) $_2$. After a specified incubation period, plates were placed on ice and the monolayers washed four times with 2 ml of cold phosphate-buffered saline. The monolayers were solubilized with 1.0% sodium dodecyl sulphate, and portions were taken for determination of radioactivity and protein concentration. Protein determinations were done as described by Lowry *et al.* (1951), with bovine serum albumin as a standard. All data are expressed as specific binding from which non-specific binding has been subtracted. Non-specific binding constituted less than 15% of total ^{125}I -labelled Tf bound.

Cytosolic iron content was determined by scraping cells from plates into 1 ml of phosphate-buffered saline. The cells were transferred to acid-washed glass test tubes, washed twice by centrifugation in phosphate-buffered saline, and resuspended in 2 ml of 0.01 M-Tris/HCl, pH 7.65, for 10 min at room temperature. Cells were homogenized with an acid-washed Dounce homogenizer. The sample was then centrifuged at 20 000 g at 4°C for 1 h. A portion of the supernatant was removed for iron and protein determination. Iron determinations were made by using a Perkin-Elmer atomic-absorption spectrophotometer with a heated graphite tube.

In preincubation experiments, ferric ammonium citrate, Tf(Fe) $_2$ or apo-Tf were added to cell cultures at designated times before binding studies. Tf concentrations are expressed in mol $\cdot$ litre $^{-1}$ (M), and ferric ammonium citrate concentrations in μg of iron $\cdot$ ml $^{-1}$. Before binding studies, cultures were incubated with MEM alone to allow dissociation of Tf from receptors.

Results

The binding of ^{125}I -labelled Tf(Fe) $_2$ to human skin fibroblasts was time-dependent and saturable. At 37°C, and at a ligand concentration of 6.5 nM, saturation occurred by 15–30 min. The rate constant for association (K_{on}) was $1.03 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$. Even if extremely low concentrations of radio-labelled ligand were used (0.3 and 0.9 nM), saturation was complete by 60 min. The amount of radioactivity associated with cells remained unchanged for at least 4 h (Fig. 1). The bulk of ligand bound to cells (88%) at 37°C could be released by incubation of cells with trypsin. Thus cells did not accumulate ^{125}I -labelled Tf(Fe) $_2$. Binding of ^{125}I -labelled Tf(Fe) $_2$ to cells incubated at 0°C was also time-dependent, with saturation occurring by 90 min. At this temperature, the rate constant for association was $4.3 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$.

Fig. 1. Binding of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ to human skin fibroblasts as a function of time at 37°C

Cultured human skin fibroblasts were washed and incubated with MEM for 60 min at 37°C . The medium was aspirated and replaced with medium equilibrated at 37°C . The cells were then incubated with ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ (6.6 nM) as described in the Experimental procedures section. At specific times, samples were taken for determination of cell-associated radioactivity and protein.

The rate of dissociation of ^{125}I -labelled Tf from cells was correspondingly temperature-dependent. At 37°C , more than 80% of cell-bound ligand exhibited a rate of dissociation which fits first-order kinetics, yielding a K_{off} of $7.9 \times 10^{-2} \text{ min}^{-1}$. The rate of dissociation was similar regardless of whether cells were incubated in a large volume of ligand-free media or in the presence of excess unlabelled ligand. Examination of the media for acid-soluble radioactivity did not reveal any evidence of ^{125}I -labelled Tf breakdown or catabolism. At 0°C , dissociation of cell-bound ligand exhibited first-order kinetics with a half-life ($t_{1/2}$) of 78 min and thus a K_{off} of $8.9 \times 10^{-3} \text{ min}^{-1}$ (Fig. 2).

The concentration-dependence of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ binding to cells was analysed by the method of Scatchard (1949). Examination of the data (Fig. 3) revealed that all the data points fit a straight line (correlation coefficient = -0.995), suggesting a single class of non-interacting receptors. The apparent binding capacity at 37°C , as defined by the intercept with the abscissa, was $1.56 \text{ fmol} \cdot (\mu\text{g of protein})^{-1}$ (S.E.M. = ± 0.12), or 3.9×10^5 molecules of Tf bound per cell. (Different culture preparations of the same cell line demonstrated minor differences in binding capacity, but no difference in ligand affinity.) The dissociation constant K_D ($K_D = 1/K_A$), as calculated from the slope, was $6.6 \times 10^{-9} \text{ M}$. This value is in good agreement with the K_D of $4.9 \times 10^{-9} \text{ M}$ calculated from the kinetic constants. Similar values for K_D , $3.8 \times 10^{-9} \text{ M}$, were also determined by

Fig. 2. Dissociation of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ from human skin fibroblasts as a function of time at 37 and 0°C . Cells were incubated with 4 nM - ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ for 60–90 min. The medium was removed, the cells were washed once and then incubated with MEM containing $1.25 \mu\text{M}$ -unlabelled $\text{Tf}(\text{Fe})_2$. At specific times, cell-associated radioactivity and protein were determined. The data are expressed as percentages of maximal binding, which is the amount of radioactivity bound to cells before incubation with non-radioactive media. O, 37°C ; ●, 0°C .

competition experiments with unlabelled ligand (Fig. 4). This latter result demonstrates that ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ and unlabelled $\text{Tf}(\text{Fe})_2$ exhibit similar binding characteristics. A mean K_D of $5.1 \times 10^{-9} \text{ M}$ was derived from the different methods of measurement.

When cells were incubated with ^{125}I -labelled $\text{Tf}(\text{Fe})_2$, and binding studies performed at 0°C , cell-associated radioactivity was consistently 40–46% of that obtained with comparably treated cells at 37°C (Fig. 5). Similar observations were reported by Karin & Mintz (1981) for Tf binding to mouse teratoma cells in culture.

Incubation of cells with high levels of albumin, apo-lactoferrin, or diferric lactoferrin did not affect binding of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ to cells. However,

Fig. 3. Representative Scatchard analysis of binding of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ to human skin fibroblasts

Various concentrations (0.9–24 nM) of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ were incubated with normal skin fibroblasts for 60 min at 37°C . The cells were washed and cell-associated radioactivity and protein were determined. The reciprocal of the slope defines the K_D ($6.6 \times 10^{-9}\text{M}$). Extrapolation of the line to the abscissa defined the binding capacity, which yielded a mean value of $1.56\text{ fmol} \cdot (\mu\text{g of protein})^{-1}$ (S.E.M. = ± 0.12) or 3.9×10^5 molecules of $\text{Tf} \cdot \text{cell}^{-1}$.

Fig. 5. Scatchard analysis of binding of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ to human skin fibroblasts at 0°C and 37°C . Various concentrations (0.9–24 nM) of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ were incubated with normal skin fibroblasts for 2 h at 0 or 37°C . The longer incubation time was necessary to ensure that binding at 0°C was complete. The cells were washed and cell-associated radioactivity and protein were determined. The K_D for cells incubated at 37°C was $5.6 \times 10^{-9}\text{M}$, with a binding capacity of $1.39\text{ fmol} \cdot (\mu\text{g of protein})^{-1}$. Cells incubated at 0°C exhibited a similar K_D ($4.4 \times 10^{-9}\text{M}$), and a binding capacity of $0.62\text{ fmol} \cdot (\mu\text{g of protein})^{-1}$, 44.6% of that measured at 37°C .

Fig. 4. Effect of increasing concentrations of apo-Tf (○) or $\text{Tf}(\text{Fe})_2$ (●) on binding of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ to human skin fibroblasts

Cells were incubated simultaneously with a fixed concentration of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ and various concentrations of apo-Tf or $\text{Tf}(\text{Fe})_2$. After 90 min at 37°C , monolayers were washed and cell-associated radioactivity and protein were determined. The data are expressed as cell-associated radioactivity relative to that bound by cells incubated with ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ alone.

apo-Tf prevented the binding of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ to cells. Apo-Tf and $\text{Tf}(\text{Fe})_2$ were equal in their

ability to compete with ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ for receptor occupancy (Fig. 4). Identical results were obtained using Tf from four different commercial sources, and in each instance the ability of unlabelled Tf to compete with radiolabelled ligand was independent of iron content. Identical results were obtained with cells incubated at 0°C and in the presence or absence of bivalent cations. These results demonstrate that, as far as receptor occupancy is concerned, fibroblasts do not discriminate between apo-Tf and $\text{Tf}(\text{Fe})_2$.

The amount of $\text{Tf}(\text{Fe})_2$ bound to cells, expressed either as binding per μg of protein or binding per cell, was not altered as cells grew from a sparse distribution to confluency. Cells were generally used before 3 days in confluent culture. Thus, within these limits, the growth state did not affect ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ binding.

^{125}I - $\text{Tf}(\text{Fe})_2$ binding to cells in the presence of 10% foetal-calf serum revealed that less than 5% of cellular receptors were occupied by the Tf present in the foetal-calf serum. No increase in Tf binding capacity was observed when cells grown in MEM/10% foetal-calf serum were incubated with MEM alone for up to 24 h. These results indicate that there is little interaction between bovine Tf and Tf receptors on human fibroblasts.

To determine if Tf-receptor number could be

Fig. 6. Decrease in ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ binding with time during incubation with ferric ammonium citrate. Human fibroblasts were grown in the presence or absence of ferric ammonium citrate ($28.1 \mu\text{g}$ of $\text{Fe} \cdot \text{ml}^{-1}$) for various periods of time. At indicated times, binding was measured by using a ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ concentration of 20 nM for 60 min . The plates were washed and cell-associated radioactivity was determined. Binding of cells preincubated with ferric ammonium citrate is expressed as a percentage of that of control cells.

Fig. 7. Effect of increasing concentrations of ferric ammonium citrate on ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ binding to human skin fibroblasts.

Human fibroblasts were grown in MEM/10% newborn-calf serum containing various concentrations of ferric ammonium citrate (0 – $28.1 \mu\text{g}$ of $\text{Fe} \cdot \text{ml}^{-1}$) for 72 h . Binding studies were done for 60 min at 37°C with a ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ concentration of 10 nM . Cells were washed and cell-associated radioactivity was determined. Binding is expressed as fmol of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ bound per μg of cellular protein.

Fig. 8. Scatchard analysis of binding of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ to human skin fibroblasts grown with or without ferric ammonium citrate.

Human fibroblasts were grown with or without ferric ammonium citrate ($28.1 \mu\text{g}$ of $\text{Fe} \cdot \text{ml}^{-1}$) for 72 h . Various concentrations (2.5 – 20 nM) of ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ were incubated with cells for 60 min at 37°C . The cells were washed and cell-associated radioactivity was determined. Cells preincubated with ferric ammonium citrate demonstrated a binding capacity 44.4% that of control cells. There was no difference in affinity of the receptor for ^{125}I -labelled $\text{Tf}(\text{Fe})_2$. ●, Control cells; ○, cells grown in the presence of ferric ammonium citrate.

42 to 240 h did not effect a significant decrease in Tf -receptor number. Cells grown in the presence of $\text{Tf}(\text{Fe})_2$ bound 94.0% (S.E.M. = $\pm 6.8\%$) as much ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ as control cells. Cells preincubated with apo-Tf demonstrated 87.3% (S.E.M. = $\pm 12.8\%$) as much ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ binding as control cells. However, preincubation of cells with ferric ammonium citrate at concentrations previously demonstrated to promote increased levels of cellular iron and accumulation of ferritin (Chu & Fineberg, 1969; Bass & Saltman, 1959) led to a time- (Fig. 6) and concentration-dependent (Fig. 7) decrease in Tf binding activity. Maximum decrease in binding capacity did not reach a stable level until 72 h after ferric ammonium citrate was added. Cells could be grown in the presence of ferric ammonium citrate with no adverse affects, and were maintained in ferric ammonium citrate for generations. Indeed, addition of ferric ammonium citrate to standard medium resulted in increased cell growth. Addition of ferric ammonium citrate to cells incubated at 0°C did not affect receptor– Tf interactions. Cells incu-

affected by exposure of cells to human Tf , cells were preincubated with either $\text{Tf}(\text{Fe})_2$ or apo-Tf and assayed for Tf binding activity. Preincubation of fibroblasts with saturating levels (0.7 – $20 \mu\text{M}$) of $\text{Tf}(\text{Fe})_2$ or apo-Tf for periods of time ranging from

bated with $28.1 \mu\text{g}$ of $\text{Fe} \cdot \text{ml}^{-1}$ in the form of ferric ammonium citrate bound only 37.7% (S.E.M. = $\pm 3.24\%$) as much ^{125}I -labelled $\text{Tf}(\text{Fe})_2$ as control cells. These cells had a cytosolic iron content of $4.6\text{--}7.0 \text{ ng} \cdot (\mu\text{g of cytosolic protein})^{-1}$, a value four to six times that of controls. Scatchard analysis of binding data (Fig. 8) demonstrated that decreased binding activity reflected a decrease in receptor number with no alteration in ligand receptor affinity. Cells grown in the presence of comparable concentrations of ammonium citrate alone demonstrated no alterations in either cell growth or receptor number.

Discussion

The present studies demonstrate that Tf binding to fibroblasts exhibits some similarities to Tf binding by other cell types. The affinity of fibroblast Tf receptors for Tf ($K_D = 5.1 \times 10^{-9} \text{ M}$) was similar to that reported for human choriocarcinoma cell lines (Hamilton *et al.*, 1979), placental tissue (Wada *et al.*, 1979), and activated lymphocytes (R. M. Galbraith *et al.*, 1980), but different from that reported for human lymphoblastoid cell lines (Larrick & Cresswell, 1979). Octave and co-workers have described the interaction of $[^3\text{H}]\text{Tf}$ with rat-embryo fibroblasts (Octave *et al.*, 1981). In contrast with our finding with human fibroblasts, Scatchard analysis of $[^3\text{H}]\text{Tf}$ binding to rat-embryo fibroblasts yielded a biphasic Scatchard plot, which Octave *et al.* (1981) interpreted as indicating two classes of transferrin receptors, one with high affinity and one with low affinity. Two classes of Tf receptors have not been described in any other tissue. Species differences or differences in experimental techniques may explain these findings.

Of particular interest was the observation that fibroblast Tf receptors do not distinguish between apo-Tf and $\text{Tf}(\text{Fe})_2$ as evaluated by competition experiments. We have also observed that Tf receptors on suspension-grown HeLa cells cannot distinguish between apo-Tf or $\text{Tf}(\text{Fe})_2$ (results not shown). Wada *et al.* (1979) suggested that Tf receptors of human choriocarcinoma cell lines also did not distinguish between apo-Tf and $\text{Tf}(\text{Fe})_2$, although results were not presented. In humans, the serum concentration of Tf is $1.5 \times 10^{-5}\text{--}2.5 \times 10^{-5} \text{ M}$ or over three orders of magnitude greater than the K_D for the Tf receptor. Thus Tf receptors should always be occupied with ligand. In general, only about 30% of plasma Tf molecules are saturated with iron. Thus apo-Tf is in excess and could limit the binding of $\text{Tf}(\text{Fe})_2$ to surface receptors. Others have described a preference of cellular receptors for $\text{Tf}(\text{Fe})_2$ over apo-Tf in reticulocytes (Jandl & Katz, 1963), hepatocytes (Young & Aisen, 1980) and T- and B-cell lymphoblastoid lines (Larrick & Cresswell,

1979). Differential affinity for $\text{Tf}(\text{Fe})_2$ or apo-Tf may be involved in regulating a tissue's access to iron.

At 0°C , the amount of Tf bound to cells was 40–46% of that bound at 37°C . Similar observations were reported by Karin & Mintz (1981). Two possibilities are compatible with these data. First, an intracellular pool of receptors may exist, which only becomes apparent at 37°C , a temperature which permits endocytosis and membrane recycling. Alternatively, at 0°C , the membrane is immobile and receptors may be present on cell surfaces in areas (i.e. membrane folds) which are inaccessible to the extracellular ligand. While evidence favours the first hypothesis (Karin & Mintz, 1981; Octave *et al.*, 1981), the second hypothesis has not been formally disproven. Regardless of which hypothesis is correct, measurement of receptor number at 0 or 37°C yields consistent results.

Our data demonstrate that incubation of cells with either $\text{Tf}(\text{Fe})_2$ or apo-Tf did not effect changes in receptor number. Other receptors are also typified by an inability of ligand to effect changes in receptor number or affinity. Examples of such receptors are: cobalamin–transcobalamin II receptors on fibroblasts (Youngdahl-Turner *et al.*, 1979), asialoglycoprotein receptors on hepatic parenchymal cells (Tanabe *et al.*, 1979), mannose-terminal glycoprotein receptors (Stahl *et al.*, 1980), macroglobulin–proteinase complex receptors on macrophages (Kaplan, 1980) and phosphovitellogenin and immunoglobulin receptors in yolk sac (Roth *et al.*, 1976). The common feature of these receptors is that their major, if not sole, function is ligand uptake (Kaplan, 1981). Consistent with this function is evidence demonstrating that these receptors are re-utilized (Kaplan, 1981). The same complement of receptors can mediate repeated rounds of ligand binding and uptake. This appears to be the case for Tf receptors on cultured skin fibroblasts.

Although receptor number appeared unaffected by previous exposure of cells to $\text{Tf}(\text{Fe})_2$, it was consistently decreased by more than 50% when cells were exposed to ferric ammonium citrate. The decrease in Tf binding was not a result of ferric ammonium citrate altering Tf receptor affinity, but represented a decreased number of receptors. These observations suggest modulation of Tf-receptor number results not as a direct consequence of ligand binding, but as a consequence of increased intracellular iron content. Studies in our laboratory indicate that human cell lines other than fibroblasts can also modulate the number of Tf receptors in response to increased cellular iron content (Ward *et al.*, 1982). In addition, studies demonstrate that cells grown in ferric ammonium citrate-supplemented media are able to bind more ^{125}I -labelled

epidermal growth factor than cells grown in un-supplemented media (J. H. Ward & J. Kaplan, unpublished work). This increase in ^{125}I -labelled epidermal-growth-factor binding may reflect the slightly enhanced growth of cells in ferric ammonium citrate-supplemented media. Certainly, ferric ammonium citrate did not lead to a decrease in binding of ^{125}I -labelled epidermal growth factor.

The pattern of regulation of fibroblast Tf receptors we observed is not consistent with the conventional understanding of down-regulation as exhibited by receptors for hormones such as insulin (Kosmakos & Roth, 1980), epidermal growth factor (Carpenter & Cohen, 1976) and human chorion-gonadotropin (Ascoli & Puett, 1978). For these, and other humoral agents, binding of ligand to receptor results in the disappearance of surface receptor-ligand complexes. The reappearance of unoccupied surface receptors results from receptor biosynthesis *de novo*.

Our results indicate that Tf receptors exhibit a pattern of regulation much more akin to that of the fibroblast low-density-lipoprotein receptor (Goldstein & Brown, 1977). These receptors mediate the uptake of low-density lipoproteins and provide for increased intracellular concentration of cholesterol. These receptors are re-utilized and are capable of multiple rounds of ligand accumulation. Increased intracellular cholesterol, but not ligand binding or internalization, results in a decreased number of low-density-lipoprotein receptors. Decreased receptor number results not from irreversible ligand-receptor internalization, but rather from inhibition of receptor biosynthesis. Modulation of Tf receptor number may also result from alteration in the rate of receptor biosynthesis as a consequence of increased intracellular iron concentration.

It is noteworthy that incubation of normal human fibroblasts with $\text{Tf}(\text{Fe})_2$ did not result in receptor number regulation. Preliminary studies demonstrate that HeLa cells can modulate Tf receptor number in response to Tf-mediated iron uptake. These results suggest that iron uptake may be regulated at loci subsequent to ligand-receptor binding.

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