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Modulation of Cell Surface Iron Transferrin Receptors by Cellular Density and State of Activation

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This report describes investigations of plasma membrane transferrin receptors on a variety of lymphoid cell lines and normal peripheral blood lymphocytes during activation and cell growth cycles. Transformed lymphoid cell lines have as many as 1,000 times the number of receptors found on normal resting lymphocytes. The number of iron transferrin receptors on continuous cell lines as well as normal human fibroblasts is down-regulated during the transition from log-phase growth to stationary plateau growth. When normal lymphocytes are transformed by mixed lymphocyte culture or mitogens, they rapidly express a 50-fold increase in the number of transferrin binding sites. This appearance of iron transferrin receptors anticipates nuclear changes during cell activation and subsequent mitosis of normal cells.

Key words: growth factors, transferrin receptors, mitogenesis, mixed lymphocyte culture, cell cycle

A number of investigators have shown that transferrin is among a small group of essential protein growth factors necessary to support serum-free growth of cell lines [1–6]. These studies suggest an important role for transferrin in the metabolism of a number of cell types, a role which is poorly understood at the present time. During the course of biochemical studies of iron transferrin receptors on lymphoblastoid cell lines [7] we noted variation in the number of receptors with growth state of the cell cultures. Although the number of iron transferrin receptors on erythrocyte precursors has been shown to decrease during maturation in the bone marrow [8], variation in numbers of transferrin receptors on other cell types in various metabolic states has not been studied. Furthermore the metabolism of these important cell surface molecules has not been studied in relation to the change from normal to malignant cell growth. Because resting lymphocytes express very few receptor sites and continuous lymphoid cell lines express up to 1,000 times as many receptors the present communication describes investigations of iron transferrin receptors during transformation, during in vitro growth and during the cell cycle.

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MATERIALS AND METHODS

Reagents

Purified transferrin and crystalline bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis). $Na^{125}I$ (carrier-free) was obtained from New England Nuclear (Boston).

Cell Lines

Human lymphoblastoid cell lines which were used had the following origins: HSB, CEM, T cell lymphoma; SB, JOKO, in vitro transformation with Epstein-Barr virus; K562, chronic myelogenous leukemia; U937, histiocytic lymphoma. Cell lines are grown in RPMI-1640 medium containing 10% fetal calf serum. They are maintained in 250-ml polystyrene tissue culture flasks (Corning) at 37°C in a humidified incubator with 5% CO_2.

Normal Human Peripheral Lymphocytes

Blood was drawn by venipuncture into heparinized plastic syringes (40 units/ml). It was diluted 10:35 with 0.95% NaCl, layered on Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, Maryland) and spun at 400 g for 30 min. The harvested peripheral mononuclear cells were harvested from the interface above the Ficoll-Hypaque and washed twice in culture medium to remove platelets. All peripheral blood lymphocytes (PBLs) were cultured in the same medium as that used for cell lines.

Mixed Lymphocyte Cultures

For two-way mixed lymphocyte culture (MLC) activation of peripheral blood lymphocytes equal numbers of lymphocytes from two individuals were cultured at a final concentration of 1.5×10^6 cells per milliliter. For one-way MLC activation stimulator cells received 1,500 R prior to being added to an equal volume of responder cells with a final concentration of 1.5×10^6 cells per milliliter.

Mitogen Stimulation

PBLs were mitogen-stimulated by culturing cells at 1.5×10^6/ml in the presence of phytohemagglutinin (PHA) (2 μg/ml) (Kidney Bean Phytohemagglutinin, Burroughs Wellcome Co., Research Triangle Park, North Carolina) or Con A (2 μg/ml) (Concanavalin A Jack Bean lectin, Miles Laboratories, Elkhart, Indiana).

Thymidine Labeling

When cells were prepared for MLC or mitogen activation, 0.2-ml aliquots were plated into round-bottom microtiter plates for subsequent thymidine labeling. Sixteen hours prior to a time point 1 μCi of ^{3}H-thymidine was added to quadruplicate wells. Cells were harvested with a Skatron cell harvester and counted in a Beckman liquid scintillation counter.

Fibroclast Cultures

Passage seven human skin fibroblasts were cultured in 75-cm^2 Falcon tissue culture flasks in Eagle's minimal essential medium (MEM) supplemented with 4 mM glutamine, 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). For assay cells were removed with 0.05% sodium bicarbonate (w/v) in phosphate-buffered saline con-

taining 0.02% ethylenediaminetetraacetic acid (EDTA) (w/v). Cells were washed twice in culture medium and assayed for transferrin binding as described above.

Cell Synchronization

A double-thymidine block technique [11, 12] was used to arrest CEM (T cell line) in late G1. Cells were grown in 2 mM thymidine (Sigma Chemical Co., St. Louis) for 16 h, followed by growth for 10 h in thymidine-free medium. Thymidine was added for an additional 14 h and the cells were washed free of thymidine. Transferrin receptors of the synchronized population of cells were measured by the ^{125}I-FeTF binding assay. Cell mitoses were counted in Dif-Quik-stained cytofuge preparations (Harleco, Gibbstown, New Jersey).

Preparation of Transferrin

A 10 mg/ml solution of transferrin in phosphate-buffered saline was saturated with iron by incubation with ferric ammonium citrate (0.1 mg/ml) in 0.01 M Na HCO_3 for 4 h at room temperature. Excess ions were removed by dialysis versus phosphate-buffered saline over night at 4°C and the final concentration of iron-saturated transferrin was measured by absorbance at 454 nm.

Iron-saturated transferrin was labeled by a modified chloramine T method [9]. Briefly, 1–2 mCi Na-^{125}I, 100–200 μg saturated transferrin, and 2.5 μg chloramine T were incubated at room temperature in 30 μl 0.05 M phosphate buffer pH 7.4. After 2 min, the reaction was stopped by the addition of 10 μl sodium metabisulfite (0.625 mg/ml) and 100 μg KI in water (20 mg/ml). Excess iodine was removed by Sephadex G-50 gel filtration chromatography in phosphate-buffered saline. We found that 99% of the labeled transferrin (^{125}I-FeTF) was immunoprecipitable by specific antitransferrin antibody. Labeled transferrin gave a single peak of apparent molecular weight 80,000 on SDS-PAGE run under reducing conditions [10].

Binding Assay

To assess binding of transferrin to cells, various amounts of ^{125}I-FeTF were added to 2.0×10^6 cells in a final volume of 200 μl of RPMI-1640 containing 15 mM Hepes, pH 7.4. Cells were incubated at 37°C for 20 min. Binding was terminated by addition of 12 ml cold phosphate-buffered saline to the conical 15 ml polystyrene tubes used for the incubations. Cells were pelleted and resuspended in additional cold buffer three times and transferred to 12- × 75-mm glass tubes. After the cells were centrifuged a final time they were counted in a Nuclear-Chicago Model 1185 gamma counter. The washing procedure consistently removed all but about 50 cpm from an initial input as high as 10^6 cpm.

RESULTS

Transferrin Receptors on Normal and Transformed Lymphoid Cells

Table I presents a comparison of the relative amount of binding of iron-saturated transferrin to normal peripheral blood lymphocytes and to cell lines derived from T and B lymphocyte subpopulations. Normal mixed peripheral blood mononuclear cells express very few receptor sites. T lymphoblastoid cell lines derived from patients with acute leukemia bind 3–7 times as much ^{125}I-FeTF as B lymphoblastoid cell lines induced

TABLE I. Binding of ^{125}I-FeTF to Various Human Myeloid and Lymphoid Cells

Name of cell	Origin	^{125}I-FeTF bound (ng/10^6 cells)
CEM	T lymphoblastoid	21.7
HSB	T lymphoblastoid	12.3
SB	B lymphoblastoid	3.5
JOKO	B lymphoblastoid	4.0
K562	Myeloid/erythroid	38.7
U937	Macrophage-like	5.4
Normal PBL[a]		0.1

[a]Mean from eight people.

by Epstein-Barr virus transformation of B cells. U937, a human macrophage-like cell line derived from the pleural effusion of a patient with histiocytic lymphoma [13], binds approximately as much ^{125}I-FeTF as the B cell lines. The cell line binding the most transferrin was K562, derived from a patient with chronic myelocytic leukemia. This is of interest because this human myeloid cell line can be induced to produce hemoglobin and may in fact be of early erythroid origin [14].

Density-Dependent Down-Regulation of Transferrin Receptors

Because of the large difference in transferrin receptor number between transformed and normal cells, it was of interest to determine if the greater receptor number was due to the higher metabolic rate of the neoplastic cells. Using the T cell line CEM, binding of transferrin was measured during log-phase growth at relatively low cell density and during the slowing of growth with the approach of stationary phase (Fig. 1). As the cells slow their metabolic rate and cease dividing, there is a gradual decline in the number of binding sites for iron transferrin. However, even under conditions in which the cells are in stationary phase they express no less than 10% of the number of log-phase binding sites. The amount of ^{125}I-FeTF binding to the stationary lymphoblastoid cell line is approximately 50–100 times that binding to resting lymphocytes.

To determine if a similar phenomenon of down-regulation has relevance to normal cells, transferrin binding was measured on secondary cultures of human skin fibroblasts. Following cell passage these cells grow slowly. At subconfluency their rate of growth is more rapid and they display density-dependent topoinhibition when the culture when the culture becomes confluent. There is a marked decrease in the number of binding sites on cells grown to confluency compared to subconfluent or low-density, recently passaged, cells (Table II).

Normal Lymphocytes Transformed by Mitogens or During MLC Exhibit a Marked Increase in Transferrin Receptors Prior to DNA Synthesis

The plant lectins kidney bean phytohemagglutinin (PHA) and jack bean concanavalin A (Con A) have been used to probe the molecular events associated with lymphoid cell activation. Lymphocytes cultured in the presence of these substances undergo cell division and exhibit many of the cell surface changes characteristic of normally cycling cells. When the kinetics of transferrin binding and cellular DNA synthesis (measured by ^{3}H-

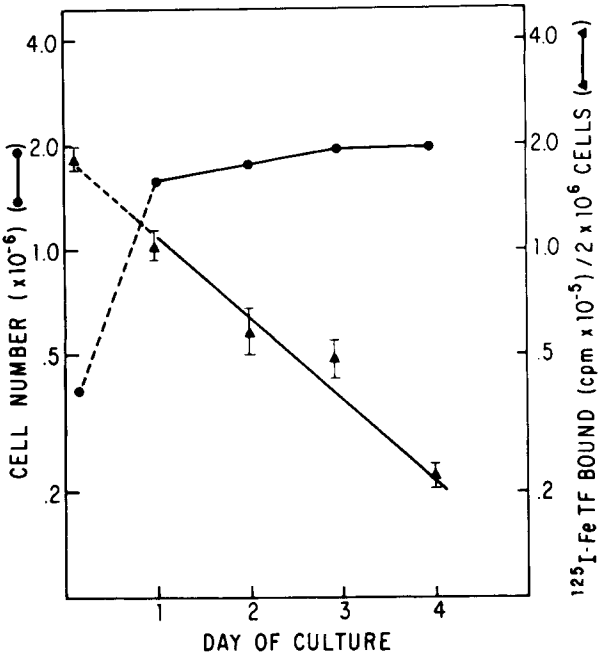

Fig. 1. Modulation of ^{125}I-FeTF binding: CEM cells (T lymphoblastoid) approaching stationary phase (sold lines) are compared to log-phase cells (left of broken lines).

TABLE II. Down-Regulation of Transferrin Receptors on Human Skin Fibroblasts

Cell Density	Cells harvested/cm^2	^{125}I-FeTF bound (ng/10^6 cells)
Confluent	3.0 X 10^4	3.9
Subconfluent	1.7 X 10^4	50.0
Sparse	0.9 X 10^4	29.7

thymidine incorporation) was studied (Fig. 2), there was a marked increase in the number of receptors within 24 h of contact with both lectins, and the peak of receptor expression anticipated the DNA synthesis peak by almost 24 h. Cells cultured in the absence of mitogens demonstrated a small increase in binding consistent with low-level nonspecific activation or bound transferrin released after transfer from in vivo.

Figure 3 presents a comparison of the kinetics of appearance of iron transferrin receptors during one-way and two-way MLCs. The total numbers of cells in each experiment are equal, but in the one-way MLC stimulator cells were irradiated to prevent cell division. In the two-way MLC the transferrin receptor number peaks earlier and is approximately twice the one-way MLC response. In the one-way MLC the transferrin receptor number anticipates DNA synthesis and subsequent cell mitosis in a manner similar to

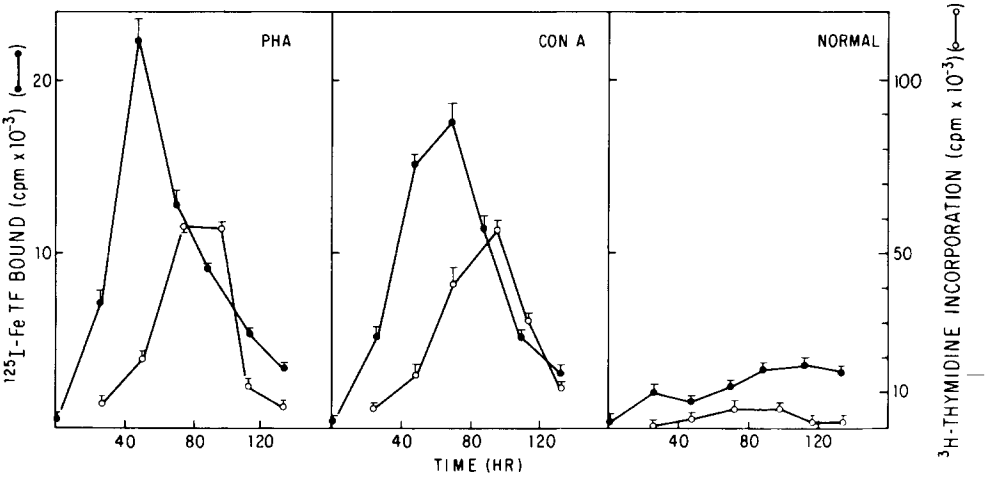

Fig. 2. Mitogen-stimulated peripheral mononuclear cells demonstrate a marked increase in the number of ^{125}I-FeTF binding sites (●—●), which anticipates ^{3}H-thymidine incorporation (○—○).

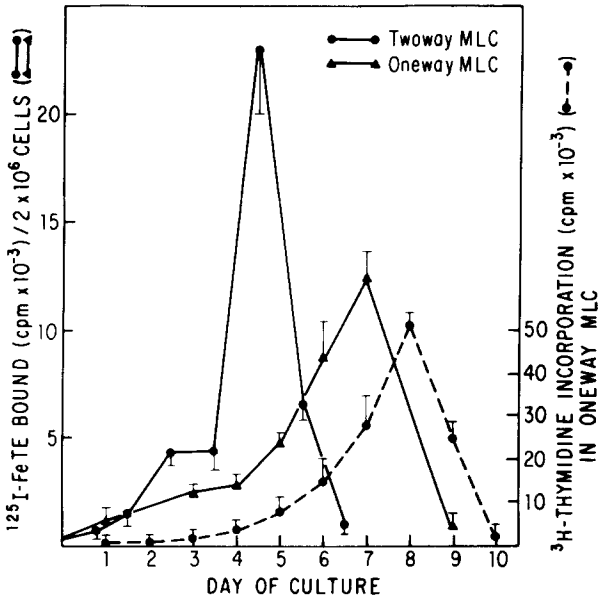

Fig. 3. Increase in the number of ^{125}I-FeTF binding sites during mixed lymphocyte cultures.

that described for mitogen activation. Because cells activated by plant lectins or allogeneic lymphocytes (MLC) express transferrin receptors prior to the ^{3}H-thymidine incorporation peak, it is possible to substitute binding of transferrin as the end point for these assays.

Increase in Transferrin Receptors in the Late G1 Phase of the Cell Cycle

The appearance of iron transferrin receptors was studied in synchronized populations of lymphoblastoid cells because the kinetic studies of mitogen-activated cells suggested a possible correlation with the cell cycle. These studies showed that the blocked synchronized cells (0.2% mitoses) had 54% of the binding sites of the unsynchronized randomly growing cells. When the thymidine blockage was removed the number of transferrin receptors remained constant for 24 h. At 31 h, when 8% of the cells were in mitosis, the number of iron transferrin binding sites had increased to 93% of control levels.

DISCUSSION

These studies demonstrate that the number of cellular iron-transferrin binding sites is modulated by the growth rate of cells. Cells which are rapidly proliferating, such as a variety of leukemia and continuous lymphoid cell lines, have many receptors. Normal resting lymphocytes have very few receptors. Density-dependent arrest of normal fibroblasts was accompanied by a significant decline in the number of receptors. Likewise slowly proliferating cells in stationary-plateau cultures demonstrated one-tenth as many receptors as rapidly growing log-phase lymphoblastoid cells. These studies suggest that rapidly dividing cells which require one of the metals which transferrin is capable of delivering will express more cell surface receptors than slowly growing or nonproliferating cells.

Early studies of mitogens showed that cultured human lymphocytes release a substance which is required for an optimal PHA response [15]. Tormey et al [16] demonstrated that a protein factor from human serum could substitute for this substance and identified it as transferrin. These same investigators subsequently reported that a subpopulation of mitogen-stimulated lymphocytes as well as cells activated by other means develop a requirement for transferrin 5–6 h prior to the onset of DNA synthesis in late G1 [17]. Our preliminary cell synchronization studies and mitogen studies showing increased numbers of transferrin receptors prior to DNA synthesis complement these findings. In contrast to these studies the original studies describing appearance of insulin receptors on mitogen-stimulated lymphocytes showed that their appearance was a late event which coincided with uptake of ^{3}H-thymidine [18]. It can be hypothesized that during late G1, prior to commitment to DNA synthesis, the cell has a programmed expression of transferrin receptors because critical metabolic pathways require one of the metals transferrin transports. This could be iron [19] (multiple critical biochemical pathways require heme proteins) or zinc [20], which is required as a cofactor in nucleic acid metabolism. Whether transferrin itself has a mitogenic or growth factor role in cell proliferation independent of its transport role for metals is under current investigation. None of the published studies on the requirement of transferrin for serumless growth of cells in vitro suggest what its precise role in cell growth is [1–6].

Investigations of other receptor molecules demonstrate no single pattern of response to a state of increased cellular growth. For example, studies by Smith et al [21] do demonstrate an increase in glucocorticoid receptors (by a factor of 2–6) in blast transformation and Lippman et al [22] have reported that lymphoblasts from patients

with acute lymphoblastic leukemia have higher levels of receptors than normal peripheral lymphocytes. Studies of BALB/3T3 mouse embryo fibroblasts showed that insulin binding was low in growing cells and increased 2–9 times in confluent stationary cultures [23]. The same authors showed that viral transformation caused the number of insulin receptors to decrease. Westermark compared epidermal growth factor binding in sparse and dense cultures of normal human glial cells [24]. Sparse cultures had 20,000 sites per cell and dense, contact-inhibited cells had 35,000 sites per cell.

The expression of an increased number of iron transferrin receptors may be a useful marker of cellular activation for studying subpopulations of cells in vitro. Mitogen stimulation of lymphocytes or the blastogenesis which accompanies an MLC results in the expression of as many as 50 times the number of receptors expressed by resting peripheral mononuclear cells. Not only is this enhanced expression of a cell surface marker many times the increase reported for insulin receptors [25] but the demonstrated high affinity of the receptor for transferrin [7] (Kaffinity = $10^{11}\,M^{-1}$) suggests that it may be possible to separate activated and unactivated subpopulations by affinity techniques.

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REFERENCES

1. Hayashi I, Sato GH: Nature 259:132, 1976.
2. Hamilton WG, Ham RG: In Vitro 13:537, 1977.
3. Hutchings SE, Sato GH: Proc Natl Acad Sci USA 75:901, 1978.
4. Allegra JC, Lippman ME: Can Res 38:3823, 1978.
5. Rizzino A, Sato GH: Proc Natl Acad Sci USA 75:1844, 1978.
6. Iscove NN, Melchers F: J Exp Med 147:923, 1978.
7. Larrick JW, Cresswell P: Biochim Biophys Acta 583:483, 1979.
8. Kailis SG, Morgan EH: Br J Haem 28:37, 1974.
9. Hunter WM, Greenwood FC: Nature 194:495, 1962.
10. Laemmli AK: Nature 227:680, 1970.
11. Bootsma D, Budke L, Vos O: Exp Cell Res 33:301, 1964.
12. Griffin MJ, Ber R: J Cell Biol 40:297, 1969.
13. Sundstrom C, Nilsson K: Int J Can 17:565, 1976.
14. Anderson LC, Jokinen M, Galinberg CG: Nature 278:364, 1979.
15. Sasaki MS, Norman A: Nature 210:913, 1966.
16. Tormey DC, Imrie RC, Mueller GC: Exp Cell Res 74:163, 1972.
17. Tormey DC, Mueller GC: Exp Cell Res 74:220, 1972.
18. Krug U, Krug F, Cuatrecasas P: Proc Natl Acad Sci USA 69:2604, 1972.
19. Worwood M: Sem Haematol 14:3, 1977.
20. Phillips JL: Cell Immunol 35:318, 1978.
21. Smith KA, Crabtree GR, Kennedy SJ, Munck AU: Nature 267:523, 1977.
22. Lippman ME, Halterman RH, Leventhal BG, Perry S, Thompson EB: J Clin Invest 52:1715, 1973.
23. Thomopoulos P, Roth J, Lovelace L, Pastan I: Cell 8:417, 1976.
24. Westermark B: Proc Natl Acad Sci USA 74:1619, 1977.
25. Helderman JH, Strom TB: Nature 274:62, 1978.