Role of Transferrin, Fe, and Transferrin Receptors in Myeloid Leukemia Cell Growth

Studies with an Antitransferrin Receptor Monoclonal Antibody

Raymond Taetle, Kaspar Rhyner, Jan Castagnola, Dong To, and John Mendelsohn

Departments of Medicine and Pathology, University of California Medical Center, San Diego, California 92103

Abstract

In previous studies, antitransferrin receptor antibody 42/6 inhibited growth of normal granulocyte/macrophage progenitors and some malignant myeloid cells. In these studies, leukemia cell lines cultured without serum and fresh leukemia cells were used to investigate the roles of Fe, transferrin receptors, and transferrin in leukemia cell growth, and mechanisms of 42/6 inhibition and resistance. HL60 and KG-1 leukemia cells grown in serum-free medium were inhibited by 42/6. In contrast to results in fetal calf serum (FCS), soluble Fe (ferric nitriloacetate) reversed 42/6 growth inhibition of serum-free HL60 cells. When HL60 cells were adapted for growth in serum-free, transferrin-free medium, they became refractory to 42/6 growth inhibition. By using radiolabeled transferrin and 42/6, HL60 cells cultured in FCS and transferrin displayed similar quantities of transferrin receptors (29,000–30,000/cell) and similar K_d 's ($3.8\text{--}4.9 \times 10^{-9}$ M). Cells grown in transferrin-free medium showed a similar K_d (3.1×10^{-9} M), but fewer transferrin binding sites (5,000/cell). Transferrin-independent cells contained a log higher concentration of intracellular ferritin. For both FCS and serum-free HL60 cells, calculated affinities for 42/6 were lower ($5.7\text{--}10.0 \times 10^{-9}$ M), but the number of binding sites was three- to fourfold higher. To investigate further the relationship between receptor display and antibody inhibition in proliferating normal and malignant myeloid cells, simultaneous immunofluorescence was used to determine the cell cycle status of transferrin receptor-positive cells. Malignant cells in S + G₂/M displayed ~50% of the amount of transferrin receptors detected in normal dividing colony-stimulating factor-stimulated marrow cells. Receptor display by dividing cells from two patients with acute nonlymphocytic leukemia was variable. When HL60 cells were exposed to dimethyl sulfoxide, transferrin receptor display decreased, and 42/6 growth inhibition was abrogated or greatly diminished. The presence of 42/6 did not prevent dimethyl sulfoxide-induced HL60 differentiation in serum-containing or serum-free cultures. We conclude that human leukemia cells require Fe for growth and that 42/6 inhibits transferrin-dependent cells by Fe deprivation. Some dividing normal and differentiating malignant cells display reduced transferrin receptors, and can also escape antibody inhibition. The increased ferritin levels and decreased transferrin receptors in transferrin-independent

HL60 cells confirm the inverse relationship between cell ferritin content and transferrin receptor display. These studies indicate a critical role for Fe in leukemia cell growth and possible roles in cellular differentiation.

Introduction

The major human Fe transport protein is the glycoprotein transferrin (Tf)¹ (1). Tf is necessary for normal and malignant cell growth in serum-free culture (2), and cells display specific Tf receptors (TfR) (3–8). Recently, monoclonal antibodies were used to detect TfR display by cultured cells (9, 10) and in human tissues (11). One such antibody, 42/6 (12), blocks Tf binding to specific receptors and inhibits growth of human hemopoietic cells in culture (12, 13). A similar antibody to murine TfR has been described (14). These reagents provide innovative tools for investigating roles of Tf and Fe in hemopoietic cell growth.

We have previously studied effects of 42/6 and other anti-TfR on growth of normal and malignant myeloid cells (13). 42/6 was a potent inhibitor of acute nonlymphocytic leukemia (ANLL) cell line and granulocyte/macrophage progenitor growth. However, 42/6 did not inhibit growth of some ANLL blast colonies, and free Fe was able to reverse growth inhibition of some ANLL cell lines but not others. Further, these studies were conducted using conventional fetal calf serum (FCS) cultures and others have questioned the efficiency of bovine Tf-human TfR interactions (15, 16).

In the present studies, we used serum-free cultures of specially adapted ANLL cell lines to further investigate roles of Tf, Fe, and TfR in ANLL cell growth, and mechanisms of 42/6 growth inhibition.

Methods

Cell lines. The promyelocytic leukemia cell line, HL60 (17), was kindly provided by Dr. Stephen Collins, University of Washington, Seattle, WA, and KG-1 myeloid leukemia cells (18) by Dr. Phillip Koeffler, University of California at Los Angeles, CA. Stock cultures were maintained in RPMI-1640 with 10% FCS (JMI International, Kansas City, KA).

Serum-free cultures. HL60 cells were maintained in serum-free cultures with RPMI-1640 containing bovine insulin (5 µg/ml), ethanolamine 5.0 µM, selenium 2.5 nM, and 25 µg/ml Fe-saturated Tf (purified human Tf; Sigma Chemical Co., St. Louis, MO) or 25 µg/ml unsaturated Tf (apotransferrin; Sigma Chemical Co.) (ETIS medium—ethanolamine, transferrin, insulin, and selenium). Cells were

Dr. Rhyner is on leave from the Department of Medicine, University Hospital, Zurich, Switzerland. Address correspondence to Dr. Taetle.

Received for publication 6 August 1984 and in revised form 26 November 1984.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/85/03/1061/07 \$1.00

Volume 75, March 1985, 1061–1067

1. *Abbreviations used in this paper:* ANLL, acute nonlymphocytic leukemia; DMSO, dimethyl sulfoxide; EIS-Fe, medium containing ethanolamine, insulin, and selenium with 5 µM ferric nitriloacetate; ETIS, medium containing ethanolamine, transferrin, insulin, and selenium; FCS, fetal calf serum; Tf, transferrin, TfR, transferrin receptor; 42/6, antibody 42/6.

adapted to serum-free culture by stepwise reduction in FCS concentration. These cells were further adapted to culture without Tf by serial Tf reduction and culture with 5 μ M ferric nitriloacetate (FeNTA) (EIS-Fe medium—ethanolamine, insulin, and selenium with FeNTA). KG-1 cells were adapted to serum-free and Tf-free culture in a similar manner. Cells were passed at intervals of 2–3 d for > 6 mo in ETIS. HL60 and KG-1 cells were grown in EIS-Fe for >3 mo.

For growth assays, 2×10^5 /ml cells were seeded into 25-cm² tissue cultures flasks (Falcon Plastics, Cockeysville, MD), and viable cell counts were performed using trypan-blue dye exclusion.

Bone marrow and leukemia cell cultures. Bone marrow from four normal donors and peripheral blood from one patient each with acute myelomonocytic leukemia and chronic myelogenous leukemia in blast crisis were obtained in heparin. Cells were characterized and separated on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described (13, 19). The mononuclear cells were collected by aspiration and washed three times in Iscove's modification of Dulbecco's minimum essential medium (Grand Island Biological Co., Grand Island, NY). The cells were plated at 10^6 /ml in Iscove's modification of Dulbecco's medium with 10% FCS and 10% placenta-conditioned medium as previously described (19). When cultures reached a density of 2×10^6 /ml, cells were resuspended at the initial cell concentration with fresh media containing placenta-conditioned medium. Viable cell counts were performed on days 1, 3, 6, 9, and 12, and cells were removed on these days for morphology, surface marker staining, and cell cycle analysis. In some studies, cell numbers limited the studies that could be performed.

Induction of ANLL cell differentiation. HL60 cells were induced for granulocytic differentiation by treatment with various concentrations of dimethyl sulfoxide (DMSO) (17). Differentiation was assessed by morphology, reactivity with monoclonal antibody to polymorphonuclear leukocyte 29 (kindly provided by Dr. Edward Ball, Dartmouth University, Hanover, NH), and nitroblue tetrazolium dye reduction (20).

In two studies, HL60 cells were incubated with 1.0% DMSO for 24 or 48 h, washed and plated with DMSO or DMSO + 10 μ g/ml of 42/6. Viable cell counts were performed every other day.

Characterization of cells in culture. To characterize cells for morphology, cytospin preparations were stained with Wright-Giemsa stain and 500 cell differential counts were performed. Cells were stained by immunofluorescence with monoclonal anti-TfR and other antibodies as previously described (13, 21). TfR detected by monoclonal antibodies are referred to below as "immunologically detectable" TfR. 5×10^5 cells were incubated with saturating concentrations of antibodies 42/6 or B3/25 (kindly provided by Dr. Ian Trowbridge, The Salk Institute for Biologic Studies, La Jolla, CA) with 0.1% Na azide at 4°C for 30 min. The cells were washed twice in phosphate-buffered saline (PBS) (pH 7.4) with 1% FCS and incubated with fluorescein-conjugated, goat anti-mouse antibody (Tago, Inc., Burlingame, CA) for 30 min at 4°C. The cells were washed twice more in PBS with FCS, and resuspended in 1% buffered formalin. A mixture of mouse immunoglobulins served as a control. Positive cells were enumerated on a fluorescence microscope or by using a cytofluorograph (model 50H; Ortho Diagnostic Systems Inc., Westwood, MA).

Simultaneous staining for TfR and DNA content was performed using propidium iodide. Cells stained for surface markers and fixed in 25% ethanol were diluted to 4 ml with PBS, vortexed, centrifuged, and resuspended in 100 μ l PBS. A quantity of 500 μ l 1.12% Na citrate containing RNase (10 μ g/ml) and 0.5 ml propidium iodide (25 μ g/ml) was added and the cells incubated for 10 min at 37°C. The cells were immediately read for DNA content. Cell cycle status was determined by using a computer program (22) or by visual determination. Validity was confirmed by simultaneous propidium iodide staining for DNA content and monoclonal antibody staining of incorporated bromodeoxyuridine (Becton, Dickinson & Co., Sunnyvale, CA). Results obtained agreed closely ($r = +0.96$, $P < 0.001$).

Additions to cultures. Fe-saturated Tf, relatively unsaturated Tf (apotransferrin), ethanolamine, DMSO, bovine serum albumin, and

insulin were obtained from Sigma Chemical Co. FeNTA was prepared as described by Trowbridge and Lopez (12). Fe in this form is referred to below as "free Fe" in contrast to Fe complexed to Tf. Monoclonal anti-TfR 42/6 was purified as previously described (12) and kindly provided by Dr. Ian Trowbridge. 42/6 was added to cultures at saturating concentrations of 10–20 μ g/ml. Unless otherwise indicated, antibody was always added at initiation of culture, and cells were not re-fed or replated.

Binding of Tf and 42/6 to HL60 cells. Determination of labeled Tf and 42/6 binding was performed as previously described (23). Briefly, 100 μ g of either human Tf or 42/6 was radiolabeled by a modified chloramine T method (24) that used a 30-s ¹²⁵I incubation with 5 μ g of chloramine T for Tf and 5 μ g for 42/6. Free isotope was removed by G25 (Pharmacia Fine Chemicals) chromatography. Assuming complete protein recovery, the specific activity for labeled Tf was 1.5×10^4 cpm/ng and 1.78×10^3 cpm/ng for 42/6. Integrity of labeled proteins was confirmed by analysis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (25) and competition studies that used cold material.

Log phase HL60 cells grown in FCS, ETIS, or EIS-Fe were washed twice, incubated for 2 h at 37°C in RPMI media, washed at 4°C, and resuspended in binding buffer (0.2% bovine serum albumin containing 0.02% Na azide). All subsequent steps were performed using this buffer at 4°C to prevent internalization of Tf or antibody. A quantity of 10^6 cells in 20 μ l was added to presoaked (0.25% gelatin in buffered saline, pH 7.4, containing 1% albumin and 5% FCS) microtiter plates (No. 107; V and P Enterprises, San Diego, CA) containing glass filters and whose well bottoms had previously been punctured. After 30 min, cells were washed twice with gel buffer, and filter plates dried by vacuum. ¹²⁵I-labeled Tf or ¹²⁵I-labeled 42/6 were added in 50 μ l at increasing concentrations. Nonspecific binding was determined by addition of 200-fold excess of cold antibody or Tf. Cells were incubated for 2 h at 4°C and washed five times; the filters were dried and counted in a gamma counter (Searle 1185; G. D. Searle & Co., Skokie, IL). Data were analyzed by Scatchard analysis (23) using regression lines determined from least squares analysis.

Assay for intracellular ferritin. The ferritin content of HL60 cells was assessed by radioimmunoassay. Cell lysates were prepared using a modification of previously described techniques (26) by several cycles of freezing and thawing on acetone/dry ice. Complete lysis of 10^7 FCS, ETIS, and EIS-Fe cultured HL60 cells in 0.1 M phosphate buffer, pH 7.4, containing 0.1% human albumin and 0.02% azide, was confirmed by visual examination. The final volume was adjusted to 2 ml after ultracentrifugation by using the same buffer. Radioimmunoassay with ¹²⁵I-rabbit, anti-human ferritin was performed according to the manufacturer's recommendations (Becton, Dickinson & Co., Orangeburg, NY).

Results

Role of Fe in anti-TfR inhibition. In previous studies, soluble Fe reversed 42/6 inhibition of FCS-cultured KG-1 but not HL60 cells (13). To determine whether results were due to different abilities to grow with soluble Fe, we examined the ability of the cells to grow in EIS media with 5 μ M FeNTA. When KG1 or HL60 cells were transferred from FCS to EIS-Fe, the cells grew for 2–3 wk at extremely slow rates, but eventually died. Therefore, the ability of the cells to grow with free Fe did not distinguish lines in which 42/6 inhibition was reversible with Fe.

Initially, when HL60 and KG-1 cells were transferred directly to serum-free ETIS, the cells did not grow in continuous culture. However, after cells were serially cultured with progressively lower FCS concentrations, the cells were successfully cultured in serum-free ETIS for > 6 mo. Proliferation was slightly, but consistently better with unsaturated than saturated

Tf. The doubling time of HL60 cells in ETIS (33 h) (Fig. 1) was similar to that in FCS (30 h).

By a similar process, HL60 and KG-1 cells were transferred to serum-free, Tf-free medium. As noted above, initially cells grown with 5–20 μ M FeNTA grew for only a few weeks, and then died. After adaptation to Tf-free culture, cells were grown for > 3 mo in EIS-Fe. Growth with FeNTA was dose-dependent, and optimal growth was obtained with 5–20 μ M FeNTA. Inhibition was observed with concentrations > 20 μ M and reduced growth with concentrations < 2.5 μ M. The doubling time of HL60 cells in EIS-Fe was slightly longer than cells in ETIS (Fig. 1) (~40 h). Cells adapted to EIS-Fe or ETIS or cultured with FCS did not grow when cultured in EIS medium without Fe (Fig. 1). Thus human myeloid leukemia cells could be adapted to Tf-free medium, but remained dependent on exogenous Fe.

As reported previously (13), HL60 and KG-1 cells grown with FCS were inhibited by 42/6 and FeNTA did not reverse this inhibition (not shown). ETIS-cultured HL60 (Fig. 2 A, one of five representative experiments) and KG-1 (three studies,

Figure 1. Growth of HL60 cells in serum-free medium ETIS (Δ) and Tf-free medium containing EIS-Fe ($\square$). Cells cultured with EIS ($\circ$) only could not be continuously grown in culture.

Figure 2. (A) Representative experiment showing effects of 20 μ g/ml of 42/6 on growth of HL60 cells cultured in ETIS with or without 20 μ M FeNTA. (B) Effect of 42/6 on Tf-independent HL60 cells grown in EIS and FeNTA.

data not shown) cells were inhibited by 42/6. Antibody inhibition was similar in the presence of either saturated or relatively unsaturated Tf. As in previous studies (13), addition of a 100-fold excess of Fe-saturated or relatively unsaturated human Tf failed to reverse the inhibition (data not shown). In contrast to studies with FCS, 5 μ M FeNTA now reversed 42/6 inhibition of HL60 cells grown in ETIS (Fig. 2 A). Thus, 42/6 inhibited both cell lines grown in serum-free medium, and HL60 cells adapted to ETIS could now use free Fe to overcome this inhibition.

Tf-independent cells provided a tool to study the role of Fe in 42/6 inhibition. If 42/6 inhibition were due to Fe deprivation, Tf-independent cells should no longer be inhibited by the antibody. As shown in Fig. 2 B, 42/6 had no effect on growth of HL60 cells in EIS-Fe. Growth of KG-1 cells in EIS-Fe was also not affected by 42/6 (not shown). Therefore, cells adapted to serum-free culture were able to utilize Fe to overcome 42/6 inhibition, and Tf-independent cells were unaffected by the antibody.

Tf and 42/6 binding to HL60 cells. Although these studies argue strongly that 42/6 acts by depriving cells of Fe, culture of HL60 cells in Tf-free medium might change either the affinity or number of specific TfR, thus altering interactions between TfR, Tf, and 42/6. 125 I-Tf binding demonstrated specific receptors on both FCS- and ETIS-cultured HL60 cells (Fig. 3 A). Tf affinity and receptor number were also similar (Table I). Tf receptors were present on EIS-Fe cells (Fig. 3 B) and affinity was also similar (Table I). However, the number of TfR sites was reduced by nearly 1 log (Table I). Thus, serum-free culture did not alter Tf affinity, but Tf-free culture resulted in decreased TfR number.

To assess whether HL60 cells cultured with FCS, ETIS, or EIS-Fe differed in cytosolic ferritin content, cell lysates were prepared from each cell line. Lysates of FCS and ETIS cells contained 38 and 40 ng/ml ferritin. In contrast, EIS-Fe cell lysates contained 435 ng/ml. Therefore, EIS-Fe cultured HL60 cells displayed fewer TfR and decreased TfR were associated with increased cytosolic ferritin.

Studies with labeled 42/6 also showed specific binding to all 3 HL60 lines (Fig. 4). For these calculations, the molecular weight of immunoglobulin A (IgA) was assumed to be 180,000. Affinities were slightly less than those for Tf (Table I). The number of 42/6 binding sites appeared consistently higher than those for Tf (Table I). Similar to results with Tf, EIS-Fe cells showed reduced 42/6 binding sites. These data suggest the 42/6 preparation binds more molecules to each TfR than Tf, or that the antibody binds to other proteins.

Figure 3. Specific binding of labeled Tf to HL60 cells grown in (A) FCS (●), ETIS (○) or (B) EIS-Fe (□). Points represent the means of two experiments using duplicate samples.

Relationship between TfR display and 42/6 inhibition. Although FeNTA apparently overcame 42/6 inhibition in some settings, these observations did not explain failure to inhibit some ANLL and granulocyte/macrophage progenitor colony-forming cells (13). Recent studies suggest varying levels

Table 1. Binding of Labeled Tf and 42/6 by HL60 Cell Lines

Culture	Tf binding		42/6 binding	
	Sites/cell	K_d	Sites/cell	K_d
		<i>M</i>		<i>M</i>
FCS	2.9×10^4 *	3.8×10^{-9} *	1.2×10^5 *	9.6×10^{-9} *
ETIS	3.0×10^4	4.9×10^{-9}	1.0×10^5	10^{-8}
EIS-Fe	5.0×10^3	3.1×10^{-9}	2.1×10^4	5.7×10^{-9}

* $\bar{x}$ of two experiments.

Figure 4. Specific binding of labeled 42/6 to FCS (●), ETIS (○), or EIS-Fe (□) HL60 cells. Points are means of two experiments using duplicate samples.

of TfR display by dividing T lymphoblasts (27). To examine this phenomenon in dividing normal and malignant myeloid cells, normal marrow and myeloid leukemia cells were simultaneously stained for TfR and DNA content, and TfR display by cycling cells in S + G₂/M phase cells was analyzed. When normal marrow cells were simultaneously stained for cell cycle status and immunologically detectable TfR, only a percentage of S phase cells expressed TfR (Table II). In these cultures, net cell growth was apparent; the percentage of S phase cells rose from $3 \pm 1\%$ ($\bar{x} \pm SE$, three experiments) to $10 \pm 5\%$ on day 6 and $13 \pm 2\%$ on day 12. Cell density corrected for dilution rose continuously from 1×10^6 to $5 \pm 0.6 \times 10^6$ /ml. In patient 1 with ANLL (Table II), the percentage of TfR-positive cells rose with colony-stimulating factor stimulation, but then fell back to baseline. In this patient's cultures, S phase cells increased from 2 to 18% on day 3 and decreased to 4% on day 6. In patient 2 with chronic myelogenous leukemia in blast crisis, TfR display by S phase cells remained relatively constant. The percentage of cells in S phase was 1, 3, 5, and 5% on days 0, 3, 6, and 9, respectively. These data indicate proliferating myeloid cells display widely varying levels of immunologically detectable TfR.

Because these primary cultures contain heterogenous populations of cells, the relationship between TfR display and 42/6 inhibition is difficult to study. Studies with HL60 show that TfR display is reduced by DMSO and phorbol ester-induced differentiation (28, 29), and serves as a model for growing myeloid cells with varying TfR display. In two studies performed here that used 42/6 and B3/25, immunologically detectable TfR that used 42/6 and B3/25 were present on 92% of control HL60 cells compared with 83% of cells exposed to DMSO for 24 h, but median fluorescence intensity decreased from 116 to 72 U on the DMSO-treated cells. After 48 h, the percentage of positive cells on controls was 85%, but dropped to 51% with DMSO exposure. Fluorescence intensity again decreased from 80 to 53 U. By the 5th d of culture with DMSO, the percentage of immunoreactive TfR-positive cells fell to 13%.

The agents that cause a drop in HL60 TfR display induce differentiation. Therefore, before studying effects of 42/6 on proliferation of DMSO-treated cells, it was necessary to determine whether the antibody affected the ability of the cells to undergo differentiation. Treatment of FCS-cultured, HL60 cells with 1.25% DMSO for 5 d caused morphologic differen-

Table II. TfR Display by Dividing Normal Marrow and ANLL Cells

	Day of Culture				
	0	3	6	9	12
	%	%	%	%	%
Normal marrow					
Percentage of TfR-positive cells					
B3/25	13±6*	31±3	38±14	32±6	32±5
42/6	5±1	21±8	27±5	24±5	26±2
S + G ₂ /M cells					
B3/25	64±7‡	56±11	62±9	45±17	38±3
42/6	44±12	47±8	50±4	35±11	23±1
Patient 1 ANLL					
Percentage of TfR-positive cells					
B3/25	5§	69	26		
42/6	2	65	20		
S + G ₂ /M cells					
B3/25	33	87	28		
42/6	21	81	22		
Patient 2 chronic myelogenous leukemia in blast crisis					
Percentage of TfR-positive cells					
B3/25	40	29	17	29	
42/6	26	20	17	15	
S + G ₂ /M cells					
B3/25	61	45	54	64	
42/6	48	35	52	51	

* Percentage of total cells, $\bar{x} \pm SE$, in three experiments.

‡ Percentage of TfR-positive cells in S + G₂/M phases determined by simultaneous surface antigen and propidium iodine staining (see text).

§ Percentage of total cells.

tiation by 80–89% of cells to myelocytes and more mature cells. Consistent with the acquisition of mature granulocyte antigens, labeling with monoclonal antibody to polymorphonuclear leukocyte 29 also increased from 13 to 66%. The percentage of nitroblue tetrazolium-positive cells increased from 0 to 86%. Addition of 42/6 DMSO and FCS-containing cultures did not inhibit morphologic or functional granulocyte differentiation.

In serum-free ETIS cultures, DMSO induction was less effective. Only 28% of cells developed nitroblue tetrazolium reduction, and this number was not changed by 42/6. Similarly, 16–18% EIS-Fe cells developed nitroblue tetrazolium reduction, and differentiation was similar with 42/6. Thus, although 42/6 profoundly inhibited growth of some HL60 cells, it did not prevent cellular differentiation.

In two studies, FCS HL60 cells were grown with 1.25% DMSO, and day 5 cell growth was 76% of that of FCS controls. In contrast, addition of 20 μ g/ml 42/6 reduced cell growth to 2% of control levels. However, when cells were grown with

DMSO and 42/6, cell growth was 66% of FCS controls (or 87% of DMSO cultures without 42/6). Thus, leukemia cells that were induced to differentiate decreased their TfR display, and were able to undergo several cycles of cell division despite the presence of 42/6.

In ETIS media, the situation was slightly more complex. In the presence of 1.0–1.25% DMSO, cell growth decreased slightly (Fig. 5). In contrast to results obtained with FCS cultures where growth eventually ceases with DMSO, with more prolonged exposure to DMSO, cell growth slowed even further, but did not cease. These results presumably reflect incomplete and slower cellular differentiation in serum-free cultures, and continued cell growth by cells unaffected by DMSO. When 42/6 was added to control HL60 cultures after 24 or 48 h in culture, cell growth ceased (Fig. 5), and when cultures were initiated with DMSO and 42/6, growth inhibition was still observed (data not shown). However, when 42/6 was added after 24–72 h of exposure to DMSO, cell growth continued for at least 2 d (Fig. 5). The later the 42/6 was added, the less inhibition observed. When DMSO-treated cells were washed and placed in Tf-free cultures, cell growth ceased, indicating DMSO-treated cells remained dependent on Tf for growth. Thus, HL60 cells induced to differentiate and to decrease TfR display became refractory to 42/6 inhibition.

Discussion

TfR are displayed by proliferating normal and malignant cells (3–11), and deprivation of Tf causes cell-cycle dependent arrest of lymphocyte proliferation (5, 6). Anti-TfR monoclonal antibodies also inhibit growth of normal and malignant cells (12, 13, 30), but their mechanism of action is unclear. Some anti-TfR inhibit Tf binding (12, 13), but others cause growth inhibition without this finding (13). Studies using rat, anti-mouse TfR suggest a poor relationship between inhibition of Fe uptake and growth inhibition (14). In previous studies that used 42/6, Fe reversed growth inhibition of some cells (13, 30), but not others (12, 13). Finally, tumor-associated proteins, including melanoma p97 and the B-lym onc gene product (31), are homologous to Fe-binding proteins. These observations suggest Tf might act as a “growth factor-like” molecule, and anti-TfR by preventing this activity. Therefore, although Tf appears important for normal and malignant cell growth, its

Figure 5. Effects of 42/6 on HL60 cells grown in ETIS with or without 1.25% DMSO. Cells were cultured for 24–72 h and 42/6 (○) added at varying times (arrows). Cultures without 42/6 (●) represent duplicate (control) or triplicate (DMSO) results.

exact role, the role of Fe, and mechanism of 42/6 inhibition were poorly defined.

Our studies using serum-free HL60 cultures show Fe is required for cell growth. Although HL60 cells could be grown without Tf, they could not be maintained in RPMI-1640 medium without supplemental Fe. These results are similar to studies with the ANLL line K562, which has been grown for several years in protein-free, F10 medium containing FeSO₄ (32). We have now grown HL60 and KG-1 cells in RPMI medium supplemented with FeNTA for several months (unpublished observations). Thus, ANLL cell lines require available Fe, provided either via Tf and its receptor, or, for some cells, as soluble Fe. Whether cells require Fe for synthesis and function of enzymes, or whether Fe transport provides a cycle-specific signal for growth (5, 6), can now be studied using Fe deprivation of Tf-independent cells. A possible mechanism of inhibition is suggested by a recent study showing that lymphocyte growth in presence of the Fe chelator, desferoxamine, is inhibited, and that the mechanism may be nucleotide depletion secondary to ribonucleotide reductase inhibition (33).

The physiologic relevance of free Fe use is questionable. A major function of Tf is binding and prevention of toxicity due to free Fe (1). Perhaps in unusual circumstances, such as intramedullary destruction of cells during ANLL induction therapy, small amounts of free Fe might exist. If so, some ANLL cells might use this Fe for growth, while normal cells would be inhibited or killed. This seems unlikely. Instead, these studies and those on K562 represent remarkable in vitro adaptations by malignant human cells.

Studies using 42/6 define three levels of free Fe use: (a) HL60 cells cultured with FCS are inhibited by the antibody and cannot use free Fe for growth (13). (b) Serum-free HL60 cells, KG-1 cells with FCS (13), and normal, mitogen-stimulated lymphocytes in autologous serum (30) are inhibited by the antibody, but can overcome inhibition with free Fe. However, these cells remain dependent on Tf for growth. (c) Tf-independent cells are not inhibited by the antibody, and can use Fe for continuous proliferation. These studies suggest 42/6 disrupts free Fe provision to Tf-dependent growing cells and inhibits their growth, but some cells take in enough free Fe to keep growing. Presumably, KG-1 cells grown with FCS fall into this category (13). The amount of Fe involved must be small, in that protein-independent K562 cells grow with only the small amount of FeSO₄ that is soluble at pH 7.4 (32).

Although these data suggest Fe deprivation is the mechanism of 42/6 inhibition, in vitro manipulation of Tf dependence also has consequences for TfR display. Similar to HeLa cells (15), HL60 cells grown with Fe demonstrate increased ferritin content and decreased surface TfR. Studies with labeled 42/6 confirmed the reduction of TfR on EIS-Fe cells.

Studies using labeled 42/6 also showed increased antibody binding sites on all HL60 cells compared with the findings of studies using labeled Tf. These observations suggest multiple 42/6 molecules or multimers bind to a single TfR chain, or the antibody cross-reacts with other antigens. The binding site calculations performed here assumed IgA was monomeric. Since multimeric forms exist in IgA preparations, binding of multimers may explain the apparent increased numbers of bound antibody molecules. It is unlikely the antibody cross-reacts with other antigens, because immunologically detectable TfR are not present on cells such as resting, normal lymphocytes known to display small numbers of TfR (30).

These studies with cell lines did not explain why 42/6 failed to inhibit colony formation by some fresh, nonadapted ANLL cells (13). To test the hypothesis that normal and malignant myeloid cells divide with varying TfR numbers, colony-stimulating factor-stimulated marrow and ANLL cells were simultaneously stained for TfR and DNA content. For normal cells, the percentage of TfR-positive cells in S phase was ~50% indicating variable TfR numbers on dividing cells (Table II). TfR display by ANLL cells was even more variable. Detection of receptors by immunofluorescence is a relatively crude technique, and the minimum number of TfR detected by these methods is unknown. However, the data suggest significant heterogeneity in TfR number on dividing myeloid cells. These results are similar to those obtained by Musgrove et al. (27), who by using immunofluorescence staining of the CEM T leukemia cell line, also detected TfR on only a percentage of S phase cells.

Primary cultures of myeloid cells are too heterogeneous a system to test relationships between TfR display and 42/6 inhibition. Previous studies showed induction of HL60 cells for granulocyte or macrophage differentiation caused a fall in immunologically detectable TfR (28, 29). When HL60 cells were incubated with DMSO for 24–48 h to decrease TfR, and then exposed to 42/6, cells were able to undergo several cycles of cell division and increase in number. In contrast, control cultures not exposed to DMSO failed to grow from the outset when cultured with antibody. Fresh leukemia cells also undergo differentiation in cultures such as the ones used here (19). For the two patients studied here, initial blast cell percentages were 80 and 75%; at termination of the culture, 37–74% of cells were myelocytes or macrophages. Therefore, HL60 differentiation reduces TfR display and makes cells refractory to 42/6 inhibition. Dividing normal and fresh leukemia cells display varying numbers of TfR, and might also escape 42/6 inhibition by decreasing TfR display through differentiation.

Monoclonal anti-TfR have broadened understanding of Tf and Fe roles in cellular proliferation (10). Our studies indicate a major Tf function is Fe provision to dividing malignant cells and support the link between decreased TfR and differentiation. Cells escape inhibition by anti-TfR 42/6 either by using non-Tf sources of Fe, or decreasing TfR display. The exact mechanism in the latter case is unclear, but further studies will define relationships between Fe loading, decreased TfR display, differentiation, and biochemical mechanisms of Tf independence.

Acknowledgments

This study was supported by U. S. Public Health Service grants CA-32094, CA-34077, and CA-32100 awarded by the National Cancer Institute.

References

1. Bothwell, H., R. W. Charlton, J. D. Cook, and C. H. Finch. 1979. Iron Metabolism in Man. Blackwell Scientific, Oxford. 284–306.
2. Barnes, D., and G. Sato. 1980. Serum-free cell culture: a unifying approach. *Cell* 22:649–655.
3. Ekblom, P., I. Thesleff, S. L. Veli-Pekka, and I. Virtanen. 1983. Distribution of the transferrin receptor in normal human fibroblasts and fibrosarcoma cells. *Int. J. Cancer* 31:111–117.
4. Hamilton, T. A., H. G. Wada, and H. H. Sussman. 1979. Identification of transferrin receptors on the surface of human cultured cells. *Proc. Natl. Acad. Sci. USA* 76:6406–6410.

5. Tormey, D. C., R. C. Imrie, and G. C. Mueller. 1972. Identification of transferrin as a lymphocyte growth promoter in human serum. *Exp. Cell. Res.* 74:163-169.
6. Tormey, D. C., and G. C. Mueller. 1972. Biological effects of transferrin on human lymphocytes in vitro. *Exp. Cell. Res.* 74:220-226.
7. Mattie, E., R. Krishnamurty, D. S. Shapiro, H. H. Sussman, and R. D. Klausner. 1984. Biosynthetic regulation of the human transferrin receptor by desferrioxamine in K562 cells. *J. Biol. Chem.* 259:2689-2692.
8. Frazier, J. L., J. O. Caskey, M. Yoffe, and P. A. Seligman. 1982. Studies of the transferrin receptor on both human reticulocytes and nucleated human cells in culture. Comparison of factors regulating receptor density. *J. Clin. Invest.* 69:853-865.
9. Sutherland, R., D. Delia, C. Schneider, R. Newman, J. Kemshead, and M. Greaves. 1981. Ubiquitous cell surface glycoprotein on tumor cells is proliferation-associated receptor for transferrin. *Proc. Natl. Acad. Sci. USA.* 78:4515-4519.
10. Trowbridge, I. S., and R. A. Newman. Monoclonal antibodies to transferrin receptors. In *Antibodies to Receptors: Probes for Receptor Structure and Function*. Chapman & Hall, London. In press.
11. Gatter, K. C., G. Brown, I. S. Trowbridge, R. E. Woolstone, and D. Y. Mason. 1983. Transferrin receptors in human tissues: their distribution and possible clinical relevance. *J. Clin. Pathol. (Lond.)*. 36:539-545.
12. Trowbridge, I. S., and F. Lopez. 1982. Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor cell growth in vitro. *Proc. Natl. Acad. Sci. USA.* 79:1175-1179.
13. Taetle, R., J. M. Honeysett, and I. S. Trowbridge. 1983. Effects of anti-transferrin receptor antibodies on growth of normal and malignant myeloid cells. *Int. J. Cancer.* 32:343-349.
14. Trowbridge, I., J. Lesley, and R. Schulte. 1982. Murine cell surface transferrin receptor: Studies with an anti-receptor monoclonal antibody. *J. Cell. Physiol.* 112:403-410.
15. Ward, J. H., J. P. Kushner, and J. Kaplan. 1982. Regulation of HeLa cell transferrin receptors. *J. Biol. Chem.* 257:10317-10323.
16. Testa, U., M. Titeux, F. Louachie, P. Thomopoulos, H. Rochart, and J. Breton-Gorins. 1983. Transferrin is not an obligatory growth factor for human erythroleukemic cell lines. *Blood.* 62:127a. (Abstr.)
17. Collins, S. J., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo. 1978. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. *Proc. Natl. Acad. Sci. USA.* 75:2458-2462.
18. Koefler, H. P., and D. W. Golde. 1980. Human myeloid leukemia cell lines: a review. *Blood.* 56:344-350.
19. Taetle, R., A. Caviles, and J. Koziol. 1983. Response of human myeloid leukemia cells to various sources of colony stimulating activity and PHA-conditioned medium. *Cancer Res.* 43:2350-2357.
20. Chitambar, C. R., E. T. Massey, and P. A. Seligman. 1983. Regulation of transferrin receptor expression on human leukemic cells during proliferation and induction of differentiation. *J. Clin. Invest.* 72:1314-1325.
21. Taetle, R., D. To, A. Caviles, S.-W. Norby, and J. Mendelsohn. 1983. Characterization of normal, peripheral blood lymphocyte colony-forming cells: cell cycle status, surface markers and cellular growth requirements. *Blood.* 61:548-555.
22. Dean, P. N., and J. H. Jett. 1974. Mathematical analysis of DNA distribution derived from flow microfluorometry. *J. Cell. Biol.* 60:523-530.
23. Kawamoto, T., J. D. Sato, J. Polikoff, G. H. Sato, and J. Mendelsohn. 1983. Growth stimulation of A431 cells by epidermal growth factor: Identification of high affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 80:1337-1341.
24. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.)*. 194:495-496.
25. Laemmli, U. K. 1970. Cleavage and structural proteins during the assembly of the head of bacteriophage, T₄. *Nature (Lond.)*. 227:680-681.
26. Taetle, R. 1981. Acidic isoferitins (leukemia-associated inhibitory activity) fail to inhibit blast proliferation in acute myelogenous leukemia. *Blood.* 58:653-657.
27. Musgrove, E., C. Rugg, I. Taylor, and D. Hedley. 1984. Transferrin receptor expression during exponential and plateau phase growth of human tumour cells in culture. *J. Cell. Physiol.* 118:6-12.
28. Omary, M. B., I. S. Trowbridge, and J. Minowada. 1980. Human cell-surface glycoprotein with unusual properties. *Nature (Lond.)*. 286:888-891.
29. Matsui, T., Y. Nakao, N. Kobayashi, M. Kissihara, S. Ishizuka, S. Watanabe, and T. Fujita. 1984. Phenotypic differentiation-linked growth inhibition in human leukemia cells by active vitamin D₃ analogues. *Int. J. Cancer.* 33:193-202.
30. Mendelsohn, J., I. Trowbridge, and J. Castagnola. 1983. Inhibition of human lymphocyte proliferation by monoclonal antibody to transferrin receptor. *Blood.* 62:821-826.
31. Diamond, A., G. M. Cooper, J. Ritz, and M. A. Lane. 1983. Identification and molecular cloning of the human B-lym transforming gene activated in Burkitt's lymphoma. *Nature (Lond.)*. 305:112-116.
32. Okabe, T., M. Fujisawa, and F. Takaku. 1984. Long-term cultivation and differentiation of human erythroleukemia cells in a protein-free chemically defined medium. *Proc. Natl. Acad. Sci. USA.* 81:453-455.
33. Lederman, H. M., A. Cohen, J. W. W. Lee, M. H. Freedman, and G. W. Gelfand. 1984. Desferoxamine: a reversible S-phase inhibitor of human lymphocyte proliferation. *Blood.* 64:748-753.