

# Transferrin Synthesis by Small Cell Lung Cancer Cells Acts as an Autocrine Regulator of Cellular Proliferation

Michael Vostrejs, Patrick L. Moran, and Paul A. Seligman

Division of Hematology, University of Colorado Health Sciences Center, Denver, Colorado 80262

## Abstract

Since transferrin is required for cellular proliferation, we investigated transferrin synthesis by a small cell lung cancer line (NCI-H510) that survives in serum-free media without added transferrin. Immunoassays for human transferrin demonstrated that these cells contained immunoreactive human transferrin. Immunofluorescence studies showed that the protein is expressed on the surface of cells, presumably bound to transferrin receptor. Media conditioned by NCI-H510 cells support proliferation of human leukemic cells that would not survive in media lacking transferrin. [<sup>35</sup>S]Methionine incorporation documented transferrin synthesis by NCI-H510 cells as well as three other small cell lines. Transferrin synthesis by NCI-H510 cells increased more than 10-fold when cells entered active phases of the cell cycle, and this increase was seen before large increases in transferrin-receptor expression. Further experiments examining the effects of agents that affect iron metabolism show that the addition of transferrin-iron or hemin to the media is associated with a more rapid initial rate of proliferation and lower rates of transferrin synthesis than control cells. Gallium salts, which inhibit iron uptake, inhibited proliferation of these cells. If the cells recovered from this effect, transferrin synthesis remained greatly increased compared to control. We conclude that transferrin synthesis by these malignant cells is ultimately related to an iron requirement for cellular proliferation. It appears that this synthesized transferrin acts as part of an important autocrine mechanism permitting proliferation of these cells, and perhaps permitting tumor cell growth *in vivo* in areas not well vascularized.

## Introduction

Transferrin is a plasma protein that functions as the major iron transport protein (1). Many *in vitro* studies have demonstrated that plasma transferrin, which is synthesized in the liver, is an essential requirement for cellular proliferation (2, 3). Thus, a number of cell lines grown continuously in defined (serum-free) media all demonstrate that transferrin, along with several hormones and smaller organic compounds, is required for cellular proliferation (4, 5). Although it might be inferred from previous studies that transferrin functions as a hormone in a capacity separate from its role in iron transport, most studies have supported the notion that transferrin functions to main-

tain cellular proliferation by providing iron for processes that have not yet been defined (4-7).

The above observations lead to the conclusion that transferrin acts as a promoter of cell growth, based on its transport of iron in plasma. It could be hypothesized, therefore, that specialized cellular proliferation *in vivo* by tissues that are not well vascularized might be limited by insufficient delivery of transferrin-bound iron from plasma. There is good evidence, however, that under certain conditions some tissues synthesize transferrin, permitting specialized proliferation. One example, the sertoli cell of the testes, synthesizes transferrin to provide iron to proliferating spermatocytes (8). Recent experiments demonstrate another example of transferrin synthesis by specialized cells, where transferrin synthesis by lymphocytes is a function of the specific T<sub>4</sub> inducer subset of T lymphocytes (9). These studies suggest that T lymphocyte proliferation is dependent upon the expression of IL-2, followed by transferrin synthesis, IL-2 receptor expression, and finally transferrin receptor expression. It appears, therefore, that transferrin is involved in an autocrine pathway that is important for the induction of normal T cell lymphocyte proliferation in response to antigenic stimuli (9).

This recent demonstration of transferrin synthesis as an autocrine mechanism for normal lymphocyte proliferation has important implications in neoplasia, since the autocrine hypothesis proposes that malignant cells produce and secrete hormone-like substances that induce further autonomous proliferation (10). Many of these factors are polypeptide hormone-like substances isolated from tumor cells, with poorly defined functions other than as growth factors. Recent studies of small cell lung cancer cell lines grown continuously *in vitro* have demonstrated that several growth factors are produced by these cells (11-14). We investigated transferrin synthesis by one of these cell lines that survives in serum-free media without added transferrin. We have found that the cells produce a transferrin molecule with immunologic and biochemical characteristics similar to serum transferrin. The synthesized transferrin appears to act as part of an important autocrine mechanism, permitting continued proliferation of these cells under transferrin-free conditions.

## Methods

*Cells and tissue culture.* All small cell lung cancer cells (SCLC)<sup>1</sup> (obtained from A. Gazdar, National Cancer Institute, NCI) were maintained in 75 cm<sup>2</sup> flasks in defined media. For the NCI-H510 cells, the cells were maintained in HIES media (RPMI 1640 with L-glutamine, 10 nM hydrocortisone, 5 µg/ml bovine insulin, 10 nM β-estradiol, and 30 nM sodium selenite), while the other SCLC were maintained in

Address reprint requests to Dr. Seligman, Division of Hematology, University of Colorado Health Sciences Center, 4200 E. 9th Avenue, Denver, CO 80262.

Received for publication 7 May 1987 and in revised form 21 January 1988.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/88/07/0331/09 \$2.00

Volume 82, July 1988, 331-339

1. *Abbreviations used in this paper:* HIES media, RPMI 1640 with L-glutamine, 10 nM hydrocortisone, 5 µg/ml bovine insulin, 10 nM β-estradiol, and 30 nM sodium selenite; MII, median immunofluorescent intensity; SCLC, small cell lung cancer cells.

HITES media (HIES + 10–100 µg/ml human serum transferrin, Sigma Chemical Co., St. Louis, MO). For specific experiments measuring immunological transferrin or transferrin synthesis, all cells were grown in HIES media. Most cells analyzed, including the NCI-H510 cells, represented “classic” small cell lung cancer lines, in that they grew as densely packed small floating aggregates (13, 14). For cell counts as well as other experiments in which suspensions of single cells were needed, the cells were slowly expressed through a 25-gauge needle at the end of a 1-ml syringe, this technique resulting in a suspension of cells in which > 90% of the cells are single cells with > 90% viability as assessed with trypan blue (15). Cell counts from single suspensions were compared in growth studies with “colony counts” (14), and proportional changes during cell growth utilizing this other technique were identical to the single-cell suspension counts.

**Radioimmunoassay for human transferrin.** Various samples of cells or media were assayed for human transferrin using a radioimmunoassay we have previously described (16) utilizing ammonium sulfate precipitation to separate precipitated <sup>125</sup>I-human transferrin bound to rabbit anti-human transferrin antibody from free <sup>125</sup>I-human transferrin. A standard curve utilizing nonradioactive human transferrin determined that this assay could sensitively measure from 1 to 100 ng of transferrin present in various samples.

When samples of cells were assayed for immunoreactive human transferrin, the cells were washed extensively by centrifugation, reconstituted in one-tenth of their original volume in 10 mM KPO<sub>4</sub>, pH 7.5, 150 mM NaCl (PBS), and the sample was frozen. The frozen sample was then thawed and the suspension was subjected to sonication utilizing three 15-s bursts. The suspension was then centrifuged at 30,000 g to remove particulate matter and the supernatant was concentrated in a microconcentrator (Centricon-10; Amicon Corp., Danvers, MA) 100-fold before assay for transferrin. When media or various column fractions were assayed for immunoreactive human transferrin, samples were concentrated in a similar manner.

**Gel filtration of conditioned media.** 2 liters of conditioned media were collected over several months. The medium, which was obtained from NCI-H510 cells grown in HIES media, was frozen at –80°C. When the 2 liters of medium had been collected, it was concentrated in an Amicon filtration apparatus with a YM10 membrane to 2 ml. To this concentrated sample a trace amount of <sup>125</sup>I-human transferrin (equivalent to ~ 1 ng of human transferrin) was added and applied to a Sephadex G-150 column (2 × 50 cm) equilibrated with 10 mM KPO<sub>4</sub>, 150 mM NaCl. The mixture was chromatographed and the samples collected at 4°C, as previously described (16).

**<sup>59</sup>Fe labeling of transferrin eluted from NCI-H510 cells.** The transferrin utilized for <sup>59</sup>Fe labeling was eluted from NCI-H510 cells grown for 8 wk in 150 cm<sup>2</sup> flasks to which fresh medium was added bi-weekly. Cells grown in this fashion, with small amounts of frequently added fresh media, grow in larger aggregates and can attain large numbers so that at the end of 8 wk, 10<sup>9</sup> cells were produced in four T-flasks. These cells were harvested by centrifugation and slowly expressed in small aliquots through a 25-gauge needle, resulting in a single cell suspension. Transferrin is then eluted by suspending the cells in 10 ml of PBS buffer containing 5 µg/ml of BSA. This mixture is incubated for 45 min at 37°C. The cell pellet is then separated by centrifugation and the supernatant is dialyzed against 10 mM sodium citrate, pH 5.0, 150 mM NaCl, and 10 mM EDTA. This step is performed to remove any excess amounts of iron that may be present in the PBS buffer as well as iron bound to the transferrin. The supernatant is dialyzed extensively against 10 mM Tris-HCl, pH 7.5, 150 mM NaCl to remove any residual EDTA. The dialysate was then concentrated to 2 ml (Amicon filtration apparatus with a YM10 membrane). The immunoassay for human transferrin indicated that this concentrated sample contained ~ 2.4 µg of transferrin. Utilizing a previously described method (15, 18), a 3-M excess of <sup>59</sup>Fe-NTA was added to the sample in order to saturate the transferrin with radioactive iron. This mixture, with the addition of a trace amount of <sup>125</sup>I-transferrin-Fe, was then applied to a Sephadex G-150 column equilibrated in 10 mM Tris, pH 7.5, 150 mM NaCl, as described above. Fractions were collected as described above

and assayed for <sup>59</sup>Fe radioactivity as well as immunoreactive transferrin. In a separate experiment, PBS buffer with BSA, concentrated, dialyzed, and with <sup>59</sup>Fe added as described above, did not contain measurable immunoreactive transferrin and on chromatography a small radioactive peak representing <sup>59</sup>Fe bound to protein was present, but represented < 3% of the radioactivity seen when the sample containing immunoreactive transferrin was chromatographed. From the sample containing the immunoreactive transferrin, separate aliquots of each fraction representing the peak <sup>59</sup>Fe-transferrin (as well as immunoreactive transferrin; see Results) were then pooled and to this mixture, solid urea was added slowly until a 7-M concentration was achieved. This solution was incubated for 2 h at room temperature and then reappplied to the same Sephadex G-150 column, this time equilibrated in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, plus 7-M urea. Fractions were then collected as described above, with the notable exception that the column was run at room temperature due to the high viscosity of the urea buffer. The fractions from this column were then assayed for <sup>59</sup>Fe radioactivity.

### Measurements of transferrin receptor and transferrin

**Immunofluorescence and DNA content of cells.** Surface bound transferrin, as well as transferrin receptor, were measured utilizing a qualitative immunofluorescent assay we have previously described for transferrin receptor (15). For these assays, approximately 1 to 3 million cells were washed with ice-cold PBS, resuspended in 100 µl of PBS and first incubated with 100 µl of polyclonal rabbit anti-human transferrin receptor antisera or anti-human transferrin antisera for 1 h. The cells were then washed by centrifugation to remove excess antibody and resuspended in 100 µl of PBS, followed by the addition of 100 µl of second antibody, goat antirabbit IgG labeled with fluorescein isothiocyanate (Gibco Laboratories, Grand Island, NY), with the second incubation performed at 4°C for 30 min. Some cell populations were analyzed separately for cellular DNA by a propidium iodide staining technique we have previously utilized (15). Samples of cells were then run through a fluorescent-activated cell sorter, the EPICS flow cytometer (Coulter Electronics, Hialeah, FL). For immunofluorescence studies defining surface transferrin, an automated analysis of each cell population calculated a point on the x axis that represented the median immunofluorescent intensity (MII) (15), as well as the percentage of cells with positive staining as determined by a control cell population incubated with control rabbit sera instead of antisera.

**Pulse labeling and purification of radioactive transferrin.** Depending on the experiment, 10<sup>6</sup> to 10<sup>7</sup> cells were harvested, centrifuged at 500 g, and reconstituted in 5 ml of methionine-free HIES media. After an incubation at 37°C for 90 min, 0.5 mCi of [<sup>35</sup>S]methionine (ICN Nutritional Biochemicals, Cleveland, OH) was then added to the flask, followed by a 5-h incubation at 37°C. The contents of each flask was then centrifuged at 500 g at 4°C, and reconstituted in PBS, washed again twice by centrifugation. The cell pellet was then resuspended in 500 µl of PBS and sonicated twice for 15 s and frozen for 24 to 72 h at –80°C. The cell lysates to be placed in a given gel were thawed simultaneously and the synthesized radioactive transferrin was purified using antitransferrin Sepharose as previously described (16). With this technique transferrin bound to the antibody-Sepharose is eluted with 10 mM glycine, pH 2, 150 mM NaCl and the elution buffer is quickly neutralized utilizing KPO<sub>4</sub>, pH 7.5, in a concentration of 40 mM.

**SDS polyacrylamide gel electrophoresis and autoradiography.** SDS polyacrylamide slab gel electrophoresis using 10% acrylamide was performed as previously described (16) using the method of Laemmli (17). Samples were heated at 100°C for 2 min with 1%-mercaptoethanol before application to the gels. Gels were dried after fixation, staining for protein, and enhancement for radiography by impregnation with 1 M sodium salicylate and 0.5% glycerol at pH 8.0. Kodak X-Omat AR5 film was exposed to each gel for 4–14 d at –80°C and, in some cases, the protein bands were quantitated using a scanning densitometer.

**Growth of NCI-H510 cells in culture with media containing various agents that affect iron uptake.** Certain monoclonal antibodies described in Results were purified from mouse ascites utilizing an ammo-

nium sulfate precipitation. These partially purified monoclonal antibodies were all added to cells grown in HIES media to make various protein concentrations.

In other experiments, a sterile 2-mg/ml solution of gallium nitrate (Aldrich Chemical Co., Milwaukee, WI) was added to cells in HIES media to obtain concentrations of 10–100  $\mu\text{g}/\text{ml}$ . Similarly, hemin (Sigma Chemical Co.) was added to a concentration of 5 mM. In all growth experiments, cells grown in various agents were compared to control cells grown in HIES medium alone.

## Results

*Cell growth, transferrin receptor expression, and immunoreactive cellular transferrin on NCI-H510 cells cultured in HIES medium.* Table I shows the results of cell counts obtained on NCI-H510 cells subcultured (day 0) at  $0.8 \times 10^6$  cells/ml. As shown on the table, these cells grow relatively slowly until day 11, when an increase in the proportion of cells in S-phase is seen. This increase in the number of cells in active phases of the cell cycle is manifested by an increase to  $\sim 2.2 \times 10^6$  cells/ml by day 21, when the cells reach a plateau with no further increase in cell number. Also, as shown in Table I, transferrin-receptor expression on these cells increases as more cells enter S-phase of the cell cycle, which is in agreement with our previous studies (15, 18). This increase in transferrin-receptor expression is expressed as maximal transferrin binding based on  $^{125}\text{I}$ -transferrin binding studies (15). In concert with increased transferrin-receptor expression, the radioimmunoassay for human transferrin of whole cell lysates shows an increase in immunoreactive transferrin present. As noted, qualitative immunofluorescent studies, also shown in the table, indicate that these increases in total cellular immunoreactive transferrin are reflected by changes in transferrin expression on the cell surface. This surface transferrin (that is presumably bound to transferrin receptor) is not only present on a greater percentage of cells, but is also associated with a shift in the median immunofluorescent intensity, indicating that there are more transferrin molecules on the surface of each cell.

Thus, these data provide good evidence that NCI-H510 cells express increased numbers of transferrin receptors as cells enter active phases of the cell cycle, in spite of the fact that these cells are grown in media that does not contain transferrin. Additionally, utilizing immunoassays for human transferrin, and even though these cells have been subcultured extensively in HIES media, it appears that the cells contain a protein immunologically similar to human transferrin, and, as noted in the immunofluorescent studies, the protein is expressed on

the surface of cells, presumably bound to transferrin receptor. In further studies (not shown), we determined that the media conditioned by NCI-H510 cells showed increasing concentrations of immunoreactive transferrin over time with the highest concentrations of  $\sim 50$  ng/ml media found when the cells had reached their greatest density. Whether this transferrin present in the media was the product of transferrin synthesized by the cells, representing transferrin mainly eluted from dead cells or cellular debris present in the media, or representing a biochemically altered transferrin molecule, was unknown. Further studies were therefore undertaken to examine the functional and biochemical properties of this transferrin molecule present in the media.

*HIES media conditioned by growth of NCI-H510 cells supports proliferation of human promyelocytic leukemia cells (HL60).* We found that HIES media that was conditioned by growth with NCI-H510 cells for 30 d contained 52 ng/ml of human transferrin. We set out to determine if this conditioned media would support the growth of another cell line that required transferrin for proliferation. As shown in Fig. 1, human promyelocytic leukemia cells (HL60), washed extensively and with surface transferrin eluted as described in Methods, will survive for 48 h in unconditioned HIES medium, but the cells do not divide, and within 72 h obvious cell death occurs. However, HL60 cells grown in HIES medium conditioned by NCI-H510 cells exhibit cellular proliferation. Counts obtained at 72 h ( $1.6 \times 10^6$  cells per ml) are  $\sim 85\%$  of the cell counts obtained by growing HL60 cells in HIES medium supplemented with 5  $\mu\text{g}/\text{ml}$  of human transferrin (15). Since it was possible that other growth factors present in the conditioned medium supported proliferation of HL60 cells, the following experiments were undertaken to provide evidence that the transferrin present was the important component in the conditioned medium that sustained growth. We determined that the addition of 50 ng/ml of transferrin to unconditioned media would support the growth of HL60 cells with the cell counts ( $1.5 \times 10^6/\text{ml}$ ) obtained at 72 h similar to the cell counts obtained with the conditioned media. Additionally, when the conditioned media was applied to antitransferrin Sepharose (permitting the removal of  $> 90\%$  of the transferrin present in the media) cell counts obtained at 72 h for HL60 cells grown in this preparation were  $0.75 \times 10^6/\text{cells per ml}$ .

*Sephadex G-150 chromatography of conditioned media.* We performed further studies of the transferrin present in the media by chromatographing HIES medium conditioned by growth of NCI-H510 cells on Sephadex G-150. Measurements

Table I.

Time in culture	Cell count	DNA			Maximal transferrin binding	Total cellular transferrin (Immunoassay)	Surface transferrin immunofluorescence	
		% in G1	% in S	% in G <sup>2</sup> M			% positive cells	MII <sup>†</sup>
d	$10^6/\text{ml}$				ng/ $10^6$ cells	ng/ $10^6$ cells		
4	0.8*	72	16	12	2.0	0.35	95	1
8	0.8	71	15	14	3.0	0.75	97	1.4
11	1.0	59	26	15	6.2	1.24	99	1.8
21	2.2	74	14	12	1.4	0.22	66	0.6

\* All values represent the mean of two experiments. Cells were plated at  $0.8 \times 10^6/\text{ml}$  on day 0. <sup>†</sup> Median immunofluorescent intensity of population of cells as ratio of value obtained at day 4.

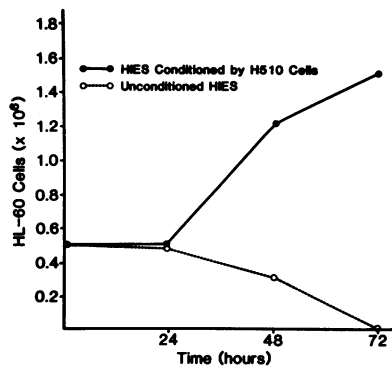


Figure 1. Growth of HL60 cells subcultured at  $0.5 \times 10^6$  cells/ml in either HIES media conditioned by NCI-H510 cells (●—●), as described in Methods, or unconditioned HIES media (○---○).

obtained for each fraction of immunoreactive transferrin using the radioimmunoassay resulted in a single, but broad, peak of immunoreactive transferrin (Fig. 2). There was a plateau in the lower molecular weight fractions, indicating that there might be a lower molecular weight species of immunoreactive transferrin present in this medium. The  $^{125}\text{I}$  human transferrin standard peak coincided with the peak of immunoreactive human transferrin protein (Fig. 2), indicating the majority of the immunoreactive transferrin had the same molecular weight as "native" transferrin, but the radioactive standard exhibited a symmetrical peak that did not contain a plateau in the lower molecular weight fractions. In other experiments, utilizing crossed immunoelectrophoresis, there were identical precipitin lines seen using either the conditioned medium or a transferrin standard against rabbit antihuman-transferrin antisera, indicating that the immunoreactive transferrin present in the conditioned media was not greatly dissimilar, immunologically, from native transferrin (data not shown).

The transferrin produced by H510 cells has the ability to bind iron. Transferrin, eluted from H510 cells and dialyzed extensively to remove contaminating nonradioactive iron, was saturated with  $^{59}\text{Fe}$  as described in Methods. Fig. 3 A shows the results of chromatography of the mixture. Immunoassays and radioactive counts, performed in each fraction show that the  $^{59}\text{Fe}$  as well as immunoreactive transferrin both result in single but broad peaks with plateaus in the lower molecular weight fractions. Since the curves are almost superimposable, these

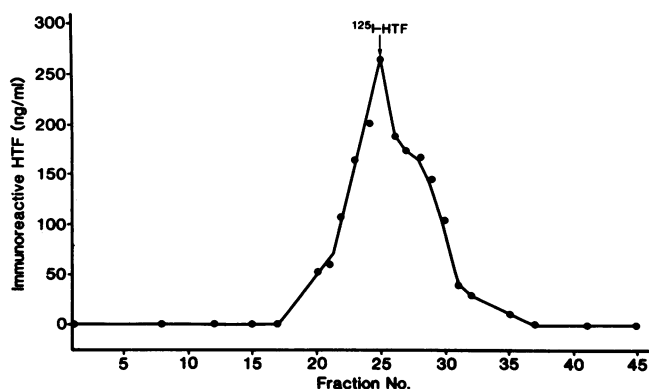


Figure 2. Sephadex G-150 chromatography of HIES media conditioned by growth of NCI-H510 cells. The immunoreactive human transferrin (HTF) peak coincides with an  $^{125}\text{I}$ -human transferrin standard as indicated by the arrow.

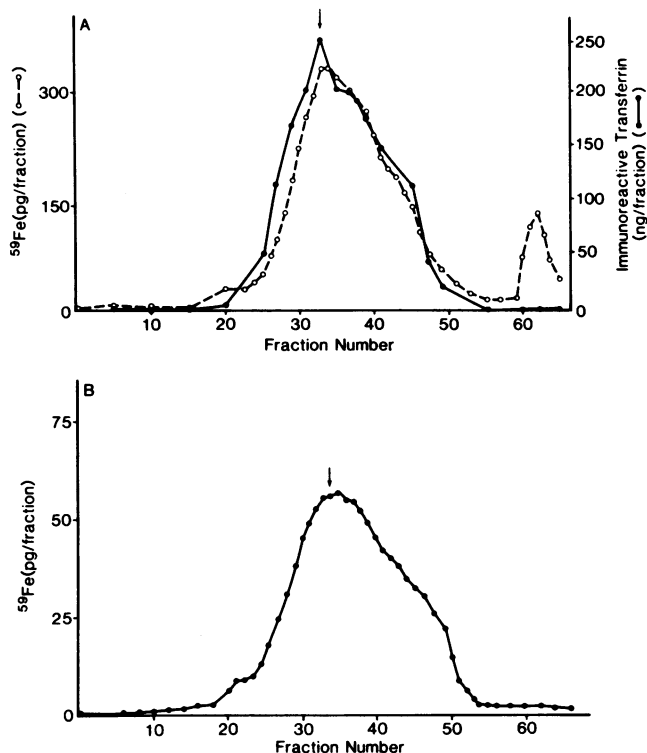
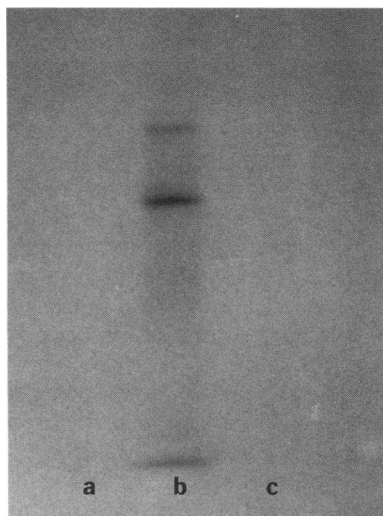


Figure 3. (A) Sephadex G-150 chromatography of immunoreactive transferrin eluted from H510 cells, concentrated and dialyzed extensively, and incubated with  $^{59}\text{Fe}$ -NTA as described in Methods. The arrow indicates the  $^{125}\text{I}$ -transferrin-Fe standard. The immunoreactive transferrin peak coincides with the main  $^{59}\text{Fe}$  peak. The smaller  $^{59}\text{Fe}$  peak seen in the much lower molecular weight fractions presumably represents free  $^{59}\text{Fe}$ -NTA. (B) Aliquots of the  $^{59}\text{Fe}$  major peak shown above were incubated in 7 M urea and rechromatographed on the Sephadex G-150 in 7 M urea as described in Methods. More than 75% of the radioactivity added was recovered from the column. The arrow again indicates the  $^{125}\text{I}$ -transferrin-Fe standard.

data indicate that the transferrin present on the surface of H510 cells has the ability to bind iron. Based on calculations of iron present with immunoreactive protein in individual fractions, it appears that both the high as well as lower molecular weight transferrin species have the ability to bind 2 mol of iron. Fig. 3 B shows the result of chromatography performed in 7 M urea of pooled peak fractions from Fig. 3 A. The resultant peak also exhibits a plateau in the lower molecular weight fractions and is similar to the peak seen in Fig. 3 A from which aliquots were taken. Thus, it appears that the transferrin with molecular weight identical to the  $^{125}\text{I}$  transferrin as well as the transferrin present in the lower molecular weight fractions, both have the ability to bind  $^{59}\text{Fe}$ . This bound iron even in lower molecular weight fractions remained bound in the presence of 7 M urea, a property of transferrin-bound iron that has been previously described by Mackey and Seal (19).

$^{35}\text{S}$  Methionine incorporation into transferrin synthesized by NCI-H510 cells. As detailed in Methods, we studied  $^{35}\text{S}$ -methionine incorporation into transferrin synthesized by NCI-H510 cells. Fig. 4 shows an autoradiograph made from exposure of x-ray film to a 10% SDS polyacrylamide gel. In the experiment, the H510 cells pulsed with  $^{35}\text{S}$  methionine were lysed and subjected to an antitransferrin Sepharose column

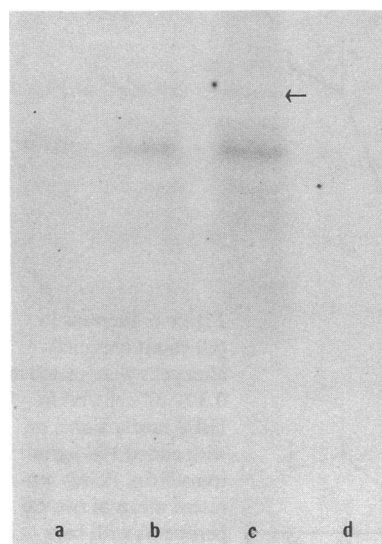


**Figure 4.** Autoradiography of [<sup>35</sup>S]methionine incorporated into transferrin. NCI-H510 cells (10<sup>7</sup>) were exposed to [<sup>35</sup>S]methionine for 5 h, then subjected to anti-transferrin Sepharose, and the purified <sup>35</sup>S incorporated into transferrin is applied to an SDS polyacrylamide gel as described in Methods. Lane B shows the migration of [<sup>35</sup>S]-methionine under these conditions, and two radioactive bands are seen, where the top, less dense band comigrates with a transferrin stan-

dard (80,000 mol wt), while the lower band is calculated at about 58,000 molecular weight. In lane A, the same cell lysate containing [<sup>35</sup>S]methionine has been applied to a control Sephadex column. In lane C, a 10-mg/ml solution of human transferrin was applied to the anti-transferrin Sepharose column before application of the cell lysate.

and applied to the gel. On the autoradiograph (Fig. 4), two radioactive bands indicating <sup>35</sup>S incorporation into immunoreactive transferrin visualized. The upper, less dense band comigrates with the position of the transferrin protein standard on the SDS gel (80,000 mol wt). The lower, darker band, based on the migration of transferrin and bovine serum albumin, is ~ 60,000 mol wt, indicating, as with the medium, the immunoreactive transferrin found in the cells contains a smaller molecular weight species. Additionally, the smaller molecular weight species was also seen in a recent study that documented transferrin synthesis by inducer T lymphocytes (9). For H510 cells, as with the lymphocyte studies, the 80,000-mol wt protein and the smaller species are both specifically inhibited from binding when unlabeled human transferrin is added to cell lysates before application to the antitransferrin Sepharose (Fig. 4). Though other small cell lung cancer lines could not be subcultured for long periods of time in HIES medium, we found that at least three other small cell lung cancer lines (designated NCI-N417D, NCI-H69, and NCI-H345) exhibited transferrin synthesis, with similar [<sup>35</sup>S]methionine incorporation into two bands of different molecular weight (as shown in Fig. 4) when the cells were subcultured for 5 d in HIES medium. In contrast, three other cell lines, including one small cell lung cancer cell line (NCI-H209) subcultured for 5 d and a human promyelocytic leukemia cell line (HL60) after 2 d in HIES medium, did not exhibit evidence of transferrin synthesis.

*Transferrin synthesis related to various stages of growth of H510 cells.* Since the previous experiments documented that human transferrin was synthesized by NCI-H510 cells, we sought to determine how transferrin synthesis was related to cellular proliferation. Fig. 5 shows an autoradiograph of gel when four sets of cells were exposed to [<sup>35</sup>S]methionine at various times after subculture, under the exact conditions shown in Table I. Although the smaller molecular weight species is the denser in each sample, and using densitometry the



**Figure 5.** Autoradiography of [<sup>35</sup>S]methionine incorporated into transferrin by NCI-H510 cells at various times after subculture. In this experiment, identical numbers of cells for each set are pulsed for 5 h with [<sup>35</sup>S]methionine and the cell lysate is subjected to anti-transferrin Sepharose. The purified radioactive transferrin is applied to an SDS slab gel. The lanes represent cell subcultured for: (A) 4 d; (B) 8 d; (C) 11 d; and (D) 21 d. The culture conditions are shown in Table I. Densitometry

tracings of the various samples show the following ratios as compared to day 4 cells: day 8 cells, 5.1, day 11 cells, 12.7, and day 21 cells, < 0.5. The arrow indicates migration of the faint higher molecular weight band.

ratios obtained compare the optical density of the smaller species, any change in the density is proportional for both peaks, and almost identical ratios are obtained when the 80,000-mol wt bands are compared for samples B and C in Fig. 5. It can be seen that the relative rate of transferrin synthesis reaches a peak as more cells enter S-phase of the cell cycle (Fig. 5 and Table I). Additionally, it is clear that a marked increase in transferrin synthesis is seen even before large increases in either transferrin receptor expression or cells entering S-phase of the cell cycle (comparing the 8-d values found in both Fig. 5 and Table I). Additionally, transferrin synthesis is below the limits of detection when cells are at high density and not actively proliferating, even though at this time the medium contains the largest amounts of transferrin.

*Effects of plating density and different agents on proliferation of NCI-H510 cells.* When NCI-H510 cells, grown in HIES medium, are plated at a relatively low density ( $0.3 \times 10^6$  cells/ml), their growth is almost totally inhibited (Fig. 6). When transferrin is added to the medium under identical conditions, the cells can then proliferate normally, clearly showing that transferrin is important in allowing for growth of these cells (Fig. 6). We compared the effects of three different monoclonal antibodies on the growth of NCI-H510 cells plated at somewhat higher density ( $0.5 \times 10^6$  cells/ml) (Fig. 7). As expected, mouse monoclonal antibody directed against a hamster surface protein (designated NS1) added to cells at 100  $\mu$ g/ml of protein does not effect the growth of cells as compared to control cells in HIES medium (Fig. 7). However, two other monoclonal antibodies, added at the same protein concentration, directed against the human transferrin receptor and human transferrin, respectively, both markedly inhibit NCI-H510 cellular proliferation (Fig. 7).

The antihuman transferrin-receptor monoclonal antibody designated 3A7 has been previously utilized to inhibit the growth of HL60 cells, and appears to specifically inhibit transferrin-mediated iron uptake by blocking transferrin binding to

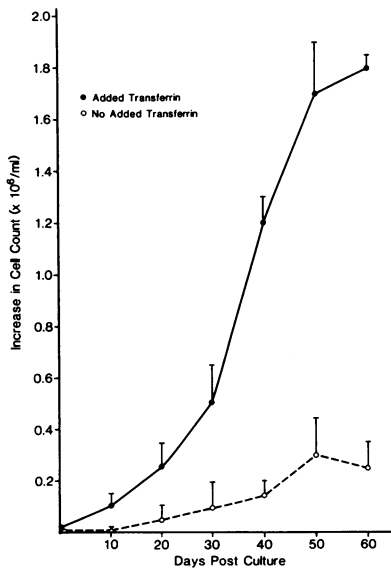


Figure 6. Increase in cell count obtained after cells were plated at  $0.3 \times 10^6$  cells/ml in HIES media alone, or with added  $100 \mu\text{g/ml}$  transferrin. Points represent mean of two experiments with bars representing the range.

the transferrin receptor and is associated with marked down-regulation of receptor on the cell surface (15). The antihuman transferrin monoclonal antibody designated TF1 has been previously described (20), although its specific effects on transferrin-mediated iron uptake have not been characterized. Cells exposed to  $100 \mu\text{g/ml}$  of protein containing the above monoclonal antibodies exhibit a higher percentage of dead

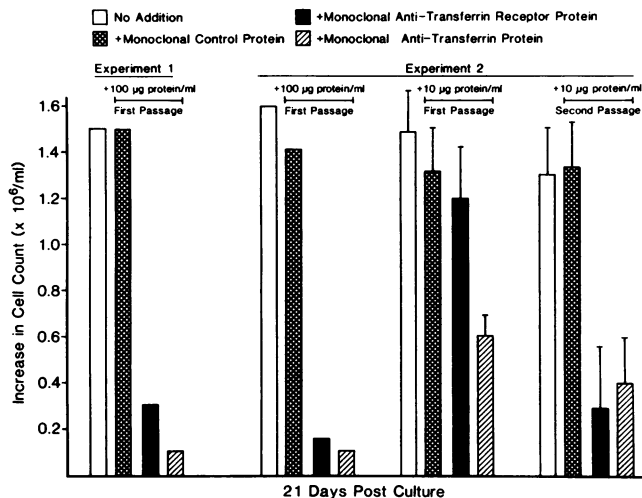
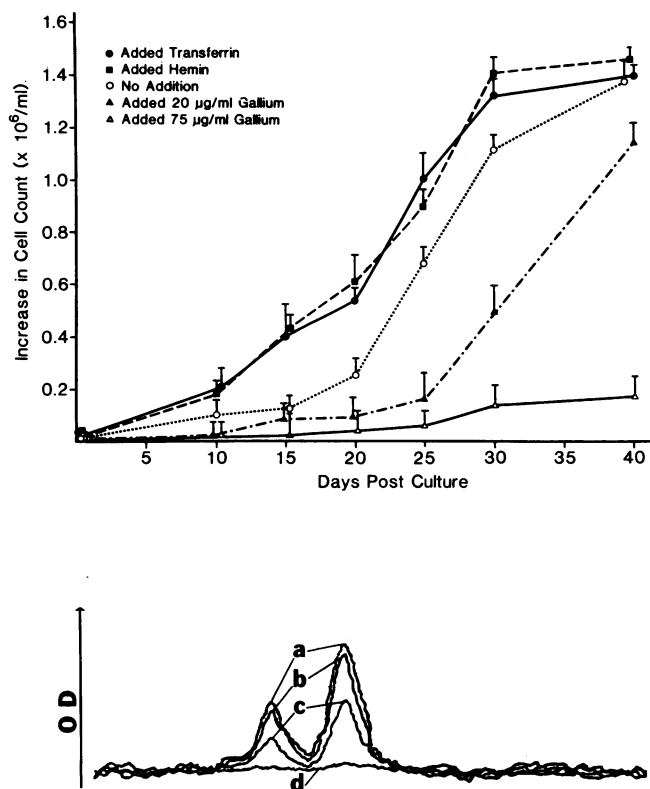


Figure 7. Increase in cell count at 21 d postculture after cells were plated at  $0.5 \times 10^6$ /ml in either HIES media or with the addition of two different concentrations of protein containing various monoclonal antibodies. The mouse monoclonal antibodies include the monoclonal control antibody (designated NS1), directed against a hamster surface antigen, a monoclonal antibody directed against the human antitransferrin receptor protein (designated 3A7), and the monoclonal antimonoconal antibody directed against the human antitransferrin protein (designated TF1). The experiments utilizing  $100 \mu\text{g}$  protein per ml were performed several months apart, whereas the experiment utilizing  $10 \mu\text{g}$  protein/ml were performed in duplicate for both the first passage and then a second passage after the cells were replated again at  $0.5 \times 10^6$  cells/ml and recounted at day 21 postculture. The arrow bars represent the range of the cell counts obtained for the duplicates.

cells at day 21, and whether the two monoclonal antibodies have a direct cytotoxic effect or effect the biologic function of the cells by inhibiting transferrin-iron uptake may not be entirely clear.

Therefore, when all the monoclonal antibodies are plated at protein concentrations of  $10 \mu\text{g/ml}$  (Fig. 7); after the first passage, cells exposed to the antitransferrin receptor monoclonal antibody proliferate normally and there is moderate inhibition of proliferation with the antitransferrin antibody. However, when cells are replated at lower density in the same concentration of antibody, growth is more inhibited by the two antibodies directed against the human proteins, particularly 3A7. The results of this experiment are similar to the results obtained in studies performed by us utilizing lower doses of transferrin-gallium (15), an agent that specifically inhibits iron uptake, and studies performed by Taetle et al. (21) that exposed HL60 cells to "transferrin-free medium." In both studies cells grew fairly normally during the first passage, but growth was significantly inhibited by the second passage, suggesting that the cells obtained enough iron (possibly from small amounts of residual transferrin-Fe) to initially maintain a normal cellular proliferation. These studies suggest that monoclonal antibodies directed against transferrin as well as transferrin receptor inhibit an important biologic function are similar to other studies measuring the effects of inhibition of iron uptake by cells.

These data taken together with the data presented in Fig. 5 and Fig. 6 lend strong support to the notion that synthesis and uptake of transferrin is a requirement for maintenance of cellular proliferation by NCI-H510 cells in HIES medium. We therefore sought to examine the effects of specific agents that would effect iron utilization by H510 cells to further examine this transferrin requirement. Fig. 8 A shows differences in rate of growth among cells treated with various agents. Cells grown in medium with added transferrin-iron (HITES), as well as cells grown in hemin, show an initially faster growth rate than control cells grown in HIES medium with significant differences ( $P < 0.01 - < 0.05$ ) seen at 15 and 20 d postculture, indicating that either delivery of iron by transferrin, or providing iron in soluble form that can freely enter cells, permits a more optimal rate of growth. Alternatively, cells grown in HIES medium with added gallium nitrate exhibit a slower growth rate. Gallium specifically inhibits iron uptake (15), and at  $75 \mu\text{g}$  of gallium/ml (Fig. 8 A) there is marked growth inhibition with  $\sim 20\%$  of the cells trypan blue positive at day 40. As shown in the figure, gallium at  $20 \mu\text{g/ml}$  shows an initially slower growth rate, but eventually recovery of proliferation to control levels is achieved (Fig. 8 A). These changes in initial growth rate related to decreased iron availability are also reflected by differences in transferrin synthesis. Thus, as shown in Fig. 8 B, when cells have been subcultured for 15 d, a time when control cells show the greatest increase in transferrin synthesis, there is a marked decrease in transferrin synthesis by cells exposed to transferrin-Fe or hemin, and a slight increase in transferrin synthesis in cells exposed to  $20 \mu\text{g/ml}$  gallium. Although cells provided with adequate amounts of available iron continue to exhibit decreased transferrin synthesis as compared to control, cells exposed to lower dose gallium consistently show increased transferrin synthesis, even at day 30, a time when control cells exhibit little or no transferrin synthesis, whereas cells treated with  $20 \mu\text{g/ml}$  gallium maintain a relatively high rate of transferrin synthesis (data not shown).



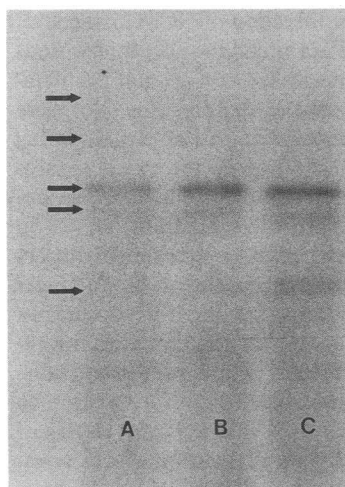
**Figure 8.** (Top) Cell counts obtained at various time points after subculture with cells plated at  $0.4 \times 10^6$  cells/ml for NCI-H510 cells grown in HIES media alone (control cells) and with added human transferrin (100  $\mu\text{g/ml}$ ), gallium (20  $\mu\text{g/ml}$  as gallium nitrate), gallium (75  $\mu\text{g/ml}$  as gallium nitrate) or 5 mM hemin. Points represent mean of three experiments, where standard deviation for each point is represented by the bars. (Bottom) Densitometry tracing of autoradiograph of a gel containing purified transferrin from day 15 cells. (a) gallium-treated cells; (b) control cells; (c) cells treated with hemin; (d) cells exposed to transferrin-iron.

*Further characterization of the transferrin molecule synthesized by NCI H-510 cells.* To determine the relationship between the large and smaller molecular weight transferrin species, several studies have been performed. First, two aliquots of cells were removed from the same T-flask, and one aliquot was pulsed with [ $^{35}\text{S}$ ]methionine for 5 h as described in Methods, the other aliquot was "pulsed" for 5 h followed by 5-h "chase" with nonradioactive methionine added to the medium. Purified [ $^{35}\text{S}$ ]methionine incorporated into transferrin for each aliquot was subjected to autoradiography as described in Methods. Although the cells that had been "chased" exhibited very slightly less dense bands on the autoradiograph, the proportional density of small molecular weight species as compared to the large molecular weight species for both cell aliquots was the same, indicating that under the conditions of this pulse-chase experiment as well as other experiments for different time periods, the smaller molecular weight species did not appear to be a product of the larger species. Attempts to purify the [ $^{35}\text{S}$ ]methionine incorporated into immunoreactive transferrin from the media under the conditions described above were not successful, probably since the cells utilized were actively proliferating (with high densities of transferrin receptors) and it may be presumed that the majority of the

synthesized transferrin is bound to the cell surface. The medium from cells pulsed for 24 h with and without a 24-h chase did contain a small amount of [ $^{35}\text{S}$ ]methionine incorporated into transferrin, and the very faint bands seen on the autoradiograph did not show any changes in the proportion of the two species when comparing the pulsed cells with cells that were also chased. On the other hand, further experiments suggested that the smaller species might, to a large extent, be produced by the larger species during the process of sample preparation since cells pulsed for 5 h as described in Methods but reconstituted in 10 mM Tris, pH 7.5, 150 mM NaCl buffer, lysed, and subjected to transferrin-Sepharose for purification of [ $^{35}\text{S}$ ]methionine-labeled transferrin the same day and immediately applied to SDS polyacrylamide gel electrophoresis showed a somewhat different pattern on autoradiography. As shown in Fig. 9, the autoradiograph produced by three sets of cells at various times after subculture (in a similar experiment to that shown in Fig. 5) as expected, shows marked changes in density of the bands with the highest rate of synthesis seen at day 11. What is also clear is that when utilizing a number of molecular weight standards, the more dense band in all three samples is calculated as 80,000 mol wt and migrates in an identical fashion to the transferrin standard. The lower molecular weight less dense band is calculated to be 63,000 mol wt. These data, taken along with the Sephadex G-150 chromatographic data presented in Figs. 2 and 3 also suggest that the predominant form of transferrin found in tissue culture is the larger molecular weight species with the molecular weight identical to native serum transferrin.

## Discussion

This manuscript presents data that conclusively demonstrate transferrin synthesis by human small-cell lung cancer cell lines in vitro. One SCLC line (designated NCI-H510) exhibits cellular proliferation in media lacking transferrin, a characteristic that is different from the other SCLC cell lines studied thus far. However, based on [ $^{35}\text{S}$ ]methionine incorporation into transferrin, we have found that at least three other SCLC lines



**Figure 9.** Autoradiography of [ $^{35}\text{S}$ ]methionine incorporated into transferrin by NCI-H510 cells at various times after subculture utilizing cells similar to those shown in Fig. 5. The methodology is different in this experiment in that the cells are lysed in a different buffer system, purified the same day and then the sample is applied almost immediately to SDS-polyacrylamide gel electrophoresis. The lanes indicate cells at A: 4 d postculture, B: 7 d postculture; and C: 11 d postculture. As expected,

cells at day 11 postculture synthesize the most transferrin. The arrows to the left of the figure indicate the molecular weight standards that from the top are: beta-galactosidase, 116 kD; phosphorylase, 97 kD; transferrin 79 kD; bovine albumin, 66 kD; and IgG albumin 45 kD.

subcultured for short periods of time in media lacking transferrin also have some ability to synthesize transferrin. These data taken together provide evidence that certain human malignant cells have the ability to produce transferrin, a plasma protein necessary for cell growth. Transferrin synthesized by these malignant cells has marked functional and biochemical similarities to transferrin synthesized by T-lymphocytes (9), as well as native serum transferrin and is distinguished from previously reported molecules such as the 6,000 mol wt chicken lymphoma cell oncogene product with homology to transferrin (22), and the "transferrin-like" molecule present in media conditioned by a mouse lymphoma cell line (23). Similar to previous studies that utilized other techniques for documenting transferrin synthesis by T lymphocytes, the [<sup>35</sup>S]-methionine incorporation data indicated that the synthesized transferrin was composed of two immunoreactive species, one with a calculated molecular weight identical to transferrin, and the other smaller species of ~ 60,000 mol wt. Our studies suggest that the smaller species, although found to some extent in tissue culture, is also formed from the larger species during the sample preparation necessary for analysis of [<sup>35</sup>S]-methionine incorporation into transferrin. Further biochemical characterization of the smaller species found in both SCLC, as well as other cells including human lymphocytes requires further study.

The transferrin produced by these lung cancer cells acts as an autocrine promoter of cellular proliferation, in a similar fashion to a number of other growth factors synthesized by malignant cells (11-14). That autocrine secretion of transferrin promotes cell growth of NCI-H510 cells is supported by the fact that transferrin synthesis markedly increases shortly before and during the period when the cells are entering active phases of the cell cycle, a time when it appears that transferrin, and particularly iron, is necessary for cell division to proceed. Whether the stimulus for transferrin synthesis is related to recruitment of a certain subpopulation of cells or dependent on synthesis of other growth promoters has not been determined. Our studies do suggest, however, that an increase in transferrin synthesis appears to precede increased expression of transferrin receptors, and this latter event is clearly important in maintaining cellular proliferation of NCI-H510 cells.

In further experiments, we examined the effects of various agents that would affect iron metabolism on cellular proliferation of NCI-H510 cells. At low plating density, growth of these cells is dependent on added transferrin, and at higher plating density, the addition of transferrin-iron to the media is associated with a more rapid initial rate of proliferation, as opposed to cells grown in transferrin-free medium. Based on extremely low rates of transferrin synthesis, transferrin synthesis as a requirement for proliferation appears to be no longer necessary. In further experiments to examine if the transferrin requirement was, as might be expected, related to an iron requirement, we measured the effects of hemin and gallium salts. The addition of hemin, which allows for delivery of iron to cells in a soluble state, was associated with the same degree of cellular proliferation, but less transferrin synthesis as compared to cells grown without hemin. Gallium salts, which have been shown previously to inhibit proliferation by a mechanism associated with inhibition of cellular iron uptake (24), had a dose-related effect on inhibition of proliferation. As cells showed recovery from lower dose gallium effects by exhibiting proliferation, transferrin synthesis remained increased when

compared to cells grown in transferrin-free media without gallium. These experiments taken together indicate that transferrin synthesis as a requirement for proliferation of NCI-H510 cells is ultimately related to an iron requirement as cells enter active phases of the cell cycle.

Since transferrin synthesis by certain small cell lung cancer cell lines in vitro allows for the appropriate delivery of iron to cells so as to sustain cellular proliferation, further studies are important in order to determine if transferrin synthesis occurs in vivo. Transferrin, along with the synthesis of other autocrine promoters of cell growth, may permit tumor cell growth in vivo in areas not well vascularized. Synthesis of this important plasma growth factor may explain why small cell lung cancer has an extremely short doubling time for a tumor without much vascularization (25). Additionally, specific agents that would affect iron metabolism, including monoclonal antibodies against transferrin or transferrin receptors, as well as gallium nitrate (24, 26), may provide new strategies in the treatment of small cell lung cancers.

### Acknowledgments

We wish to thank Susan A. Veach for her preparation of the manuscript, and Drs. Frank Cuttitta and Paul A. Bunn, Jr., for helpful discussions.

Supported by a grant from the National Institutes of Kidney, Urologic, and Hematologic Diseases, Division of Health and Human Services RO1 AM-27039 and the Cancer League of Colorado, Inc.

### References

1. Aisen, P., and E. B. Brown. 1975. Structure and function of transferrin. *Prog. Hematol.* 9:25-56.
2. Tormey, D. C., and G. C. Meuller. 1972. Biologic effects of transferrin on human lymphocytes in vitro. *Exp. Cell Res.* 74:220-226.
3. Hayashi, I., and G. H. Sato. 1976. Replacement of serum by hormones permits growth of cells in a defined medium. *Nature (Lond.)* 259:132-143.
4. Cherington, P. V., B. L. Smith, and A. B. Pardee. 1979. Loss of epidermal growth factor requirement and malignant transformation. *Proc. Natl. Acad. Sci. USA.* 76:3937-3941.
5. Rudland, P. S., H. Durbin, D. Clingan, and L. J. deAsua. 1977. Iron salts and transferrin are specifically required for cell division of cultured 3T6 cells. *Biochem. Biophys. Res. Commun.* 75:556-562.
6. Robbins, E., and T. Pederson. 1970. Iron: Its intracellular localization and possible role in cell division. *Proc. Natl. Acad. Sci. USA.* 66:1244-1251.
7. Taetle, R., K. Rhyner, J. Castagnola, D. To, and J. Mendelsohn. 1985. Role of transferrin, Fe, and transferrin receptors in myeloid leukemia cell growth. *J. Clin. Invest.* 75:1061-1067.
8. Skinner, M. K., and M. D. Griswold. 1980. Sertoli cells synthesize and secrete transferrin-like protein. *J. Biol. Chem.* 255(20):9523-9525.
9. Lum, J. B., A. J. Infante, D. M. Makker, F. Yang, and B. A. Bowman. 1986. Transferrin synthesis by inducer T lymphocytes. *J. Clin. Invest.* 77:841-849.
10. Sporn, M. D., and G. J. Todaro. 1980. Autocrine secretion and malignant transformation of cells. *N. Engl. J. Med.* 303:878-880.
11. Cuttitta, F., D. N. Carney, J. Mulshine, T. W. Moody, J. Fedorko, A. Fischler, and J. D. Minna. 1985. Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. *Nature (Lond.)* 316:823-826.
12. Carney, D. N., H. Oie, T. Moody, A. Gazdar, F. Cuttitta, and J.



- Minna. 1983. Bombesin: an autocrine growth factor for human small-cell lung cancer cell lines. *Clin. Res.* 31:404a. (Abstr.).
13. Carney, D. N., A. F. Gazdar, G. Bepler, J. G. Guccion, P. J. Marangos, T. W. Moody, M. H. Zweig, and J. D. Minna. 1985. Establishment and identification of small-cell lung cancer cell lines having classic and variant features. *Cancer Res.* 45:2913-2923.
14. Gazdar, A. F., D. N. Carney, M. M. Nau, and J. D. Minna. 1985. Characterization of variant subclasses of cell lines derived from small-cell lung cancer having distinctive biochemical, morphological, and growth properties. *Cancer Res.* 45:2924-2930.
15. Chitambar, C. R., E. J. Massey, and P. A. Seligman. 1983. Regulation of transferrin receptor expression on human leukemia cells during proliferation and induction of differentiation. *J. Clin. Invest.* 72:1314-1325.
16. Seligman, P. A., R. B. Schleicher, and R. H. Allen. 1979. Isolation and characterization of the transferrin receptor from human placenta. *J. Biol. Chem.* 254:9943-9946.
17. Frazier, J. L., J. H. Caskey, M. Yoffe, and P. A. Seligman. 1982. Studies of the transferrin receptor on both reticulocytes and nucleated human cells in culture. Comparison of factors regulating receptor density. *J. Clin. Invest.* 69:853-865.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
19. Makey, D. G., and U. S. Seal. 1976. The detection of four molecular forms of human transferrin during the iron binding process. *Biochim. Biophys. Acta.* 453:250-256.
20. Sullivan, N., B. Kao, and Y. E. Miller. 1985. Monoclonal antibodies to human transferrin: conservation of epitopes within primates. *Am. J. Hum. Genet.* 37:A142.
21. Taetle, R., R. Kaspar, J. Castagnola, D. To, and J. Mendelsohn. 1985. Role of transferrin, Fe, and transferrin receptors in myeloid leukemia cell growth. *J. Clin. Invest.* 75:1061-1067.
22. Goubin, G., D. S. Goldman, J. Luce, P. E. Neiman, and G. M. Cooper. 1983. Molecular cloning and nucleotide sequence of a transforming gene detected by transfection of chicken B-cell lymphoma DNA. *Nature (Lond.)*. 302:114-119.
23. Kitada, S., and E. F. Hays. 1985. Transferrin-like activity produced by murine malignant T-lymphoma cell lines. *Cancer Res.* 45:3537-3540.
24. Chitambar, C. R., and P. A. Seligman. 1986. Effects of different transferrin forms on transferrin receptor expression, iron uptake, and cellular proliferation of human leukemic HL60 cells. *J. Clin. Invest.* 78:1538-1546.
25. Mizuno, T., A. Masaoka, H. Ichimura, K. Shibata, H. Tanaka, and H. Niwa. 1984. Comparison of actual survivorship after treatment, with survivorship predicted by actual tumor-volume doubling time from tumor diameter at first observation. *Cancer (Phila.)*. 53:2716-2720.
26. Warrell, R. P., C. J. Coonley, D. J. Strauss, M. J. Lacher, and C. W. Young. 1983. Clinical evaluation of gallium nitrate (NSC-15200) by 7 day continuous infusion in advanced malignant lymphoma. *Cancer (Phila.)*. 51:1982-1987.