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GRANT NUMBER DAMD17-94-J-4189

TITLE: Role of the Mammary Adenocarcinoma Cell Transferrin Response in Breast Cancer Metastasis

PRINCIPAL INVESTIGATOR: Philip G. Cavanaugh, Ph.D.

CONTRACTING ORGANIZATION: University of Texas at Houston
Houston, Texas 77030

REPORT DATE: September 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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DTIC QUALITY INSPECTED 4

19990811 108

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Final (22 Aug 94 - 21 Aug 98)

4. TITLE AND SUBTITLE
Role of the Mammary Adenocarcinoma Cell Transferrin Response in Breast Cancer Metastasis

5. FUNDING NUMBERS
DAMD17-94-J-4189

6. AUTHOR(S)
Philip G. Cavanaugh, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas at Houston
Houston, Texas 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Prior to the grant, we had found that breast cancer expression of the transferrin receptor (TfR) and concomitant response to transferrin correlated with the metastatic capabilities of the cells. The purpose of the grant was to test this by purposefully altering breast cancer cell TfR expression, and to determine the effects of this on their metastatic behavior. In addition, the creation of and assessment of self-Tf producing cells was proposed as well as the testing of some known TfR modulators on breast cancer cell TfR level. In a rat breast cancer cell model, lab-induced elevation of cellular TfR by selection or transfection techniques caused an increase in metastatic capability, proving the major aims of the grant. Attempts to decrease TfR expression by antisense TfR transfection were met with major roadblocks, however, successful accomplishment of this was performed *in vitro*. A plasmid containing the human Tf cDNA was unable to confer Tf production in transfected cells. Interleukins which have major TfR level modulating effects on certain immune and other cells were found not to fluctuate breast cancer cell TfR levels. The importance of breast cancer response to Tf and expression of TfR in metastasis were proven in a rat model. Further development of and attention towards therapy aimed at the Tf/TfR system are warranted.

14. SUBJECT TERMS Breast Cancer, Transferrin, Transferrin Receptor, Metastasis, Growth Factors, Prognostic Indicators

15. NUMBER OF PAGES 53

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Philip Cavanaugh 11/5/98

PI - Signature Date

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Cavanaugh, P.G., and Nicolson, G.L. The selection of a metastatic rat mammary adenocarcinoma cell line from a low metastatic parental population by an in vitro process based on cellular ability to proliferate in response to transferrin. Journal of Cellular Physiology 174: 48-57, 1998.

Pages 27-36

Cavanaugh, P.G., Jia, L., and Nicolson, G.L. Transferrin receptor overexpression enhances transferrin responsiveness and the metastatic capability of a rat mammary adenocarcinoma cell line. Breast Cancer Research and Treatment (Accepted with Revision).

Pages 37-53

Introduction:

This project formed out of the interest in defining characteristics of metastatic breast cancer cells, and thus establishing properties of these cells which delineate them from those which exhibit a less aggressive, less metastatic phenotype. The general observation that most types of tumor cells tend to metastasize preferentially to only certain organs formed a foundation for the studies. It was thought that each normal organ contained a specific repertoire of adhesive, chemotactic, and growth factors required for its particular function. The ability of tumor cells to respond to these multiple stimuli in the normal organ environment is thought to be involved in successful metastasis to those particular organ sites (1-6). One subset of responses deemed responsible for such successful organ colonization are those that enable tumor cell reactivity towards normal target organ-situated growth factors.

Thus, one hypothesis formed was that cells metastatic to a certain site had attained the necessary components and mechanisms to proliferate upon exposure to that particular normal target organ's collection of growth factors. Resultant rapid growth following localization to this organ then dictated it as the preferred site for metastasis. In a specific study of the role of these types of responses in breast cancer metastasis, we focused on the rat 13762NF mammary adenocarcinoma system. Here, certain sublines are capable of metastasizing to the lung from a mammary fat pad site, whereas other sublines do not metastasize at all. In keeping with the above, one hypothesis considered was that the metastatic cells had become competent to respond to lung-situated growth factors. In support of this, we found that media conditioned by viable, normal, perfused rat lung fragments possessed mitogenic activity for the lung-metastasizing cells, but no such activity in the media was evident for the non-lung metastasizing cell lines from that system (7, 8). The responsible lung-metastatic breast cancer cell proliferation-inducing factor was purified from the conditioned media (7, 8) and was eventually identified as transferrin (Tf; 9). The results indicated that response to transferrin may be a property of highly metastatic breast cancer cells and that the lung may serve as a site of high transferrin localization.

Transferrin is a ubiquitous iron binding protein which exists in relatively high concentrations in blood. All iron transport and delivery is mediated by transferrin, as free iron or iron salts are harmful and are essentially absent *in vivo.* Transferrin exerts a proliferative effect on cells in culture by supplying iron for necessary synthesis processes required for cell growth and division. Transferrin mediates the delivery of iron into cells via binding to the cell surface transferrin receptor (TfR; 10,11). The proliferative effect of transferrin on cells is thought to be due primarily to its iron transport function, which results in the maintenance of key enzymes required for proliferation, such as ribonucleotide reductase (RR; 12).

However, some studies have suggested that iron transport alone cannot explain the growth stimulating activity of transferrin (13-15). Along these lines, iron delivered by transferrin mediates processes that soluble iron cannot (16), and stimulation of TfR through transferrin-independent means induces several activities in T cells (17). In addition, non-growth properties of transferrin have been described, such as its ability to promote angiogenesis by stimulating endothelial cell invasion and migration (18), and to effect the adhesion and migration of chicken mesoderm explants (19). Whether or not the effect of Tf on the breast cancer cell lines that we had studied was due to simple nutrient transport function or to more of the exotic latter mechanisms was unknown. This continues to be an object of study.

The results obtained with the 13762NF lines led us to investigate the amount of TfR expression on a number of breast and melanoma cell lines. Here, we found a correlation between the expression of this receptor and previously reported metastatic potential (7,8,20,21). In exploring the growth response of various tumor cell lines to transferrin, an incomplete correlation was found as certain high-TfR expressing lines exhibited no proliferative response to Tf (20,21).

Therefore, high TfR expression alone did not endow cells with the ability to proliferate in culture subsequent to transferrin exposure.

Results from a number of other laboratories, indicate that the expression of TfR in human tumor cells associates with tumor grade, stage, progression, and metastasis. Neoplasms where this correlation has been found include breast carcinomas (22,), bladder transitional cell carcinomas (23), and malignant melanoma (24). In addition, high levels of TfR have been observed in a metastatic lesion of a maxillary neoplasm, but not in the parental tumor (25), and the expression of TfR was higher in a human melanoma line selected for metastatic capability in nude mice than in the poorly metastatic tumor cells of the parental population (26). Others have reported that tumor cell growth response to transferrin (as opposed to mere expression of TfR) also associates with metastatic capability, indicating that transferrin-responsiveness may be involved in secondary site-specific tumor cell growth (27-29).

Many agents can alter TfR expression in normal cells. This is most notable in certain immune cells as rapid proliferation is required subsequent to challenge by the proper immunogen. Elevation of TfR and initiation of proliferation is the result of immune cell cross-talk and is initiated by interleukin-2 or interleukin 1a.

The purpose of the grant was to confirm the observations seen with the lung conditioned media experiments by purposefully increasing or decreasing breast cancer cell expression of the transferrin receptor, with the hopes that growth response to Tf would likewise be augmented or diminished. Metastatic capability of altered cells would be studied and any alterations in this would dictate TfR expression and response to Tf as components of metastatic behavior in breast cancer. In addition, minor projects outlined in the grant proposal were to attempt to induce transferrin synthesis in cultured breast cancer cells and to examine the effect of this on their metastatic capability, as well as to examine the possibility that certain interleukins known to alter immune cell TfR expression might likewise influence breast cancer cell TfR expression.

Understanding of tumor cell properties which earmark metastatic, aggressive cells is one of the most useful accomplishments that can be performed in basic research. Many potential breast cancer markers which associate with the metastatic or aggressive phenotype have been identified and are being explored (30,31). However, no one, or very few researchers, are studying breast cancer cell response to transferrin as a possible factor in breast cancer growth and metastasis. Strategies to combat cancer using reagents directed at the transferrin/TfR system are currently being explored, and these are most successful when used to treat tumors of hematopoetic origin (32-34). Clarification of the role of transferrin-responsiveness in breast cancer metastasis by this grant would aid in the development of further therapeutic treatments directed at this system and would aid in the eradication of mammary adenocarcinoma metastases.

BODY

Specific aim 1: Reduction of breast cancer cell TfR expression by transfection with a plasmid containing antisense TfR cDNA.

Assumption: Transfection of a high-TfR expressing breast cancer line with an antisense-TfR plasmid would decrease cellular TfR expression, growth response to Tf, and metastatic capability.

Methods: A full-length antisense TfR plasmid was constructed at the beginning of these studies. This construct failed to reduce TfR expression in a number of test cell lines. This was reported on in past progress reports. A new antisense TfR plasmid was formed in the summer of 1997: the insert consisted of a shorter, incomplete TfR fragment and the host plasmid was the highly utilizable pcDNA3.1 eukaryotic expression plasmid. The formation of this construct was reported on in detail in last years progress report. Briefly, the TfR coding region was removed from pcDTR1 using EcoRV and Xba I. The fragment was isolated by agarose electrophoresis and electroelution, and was further digested with Kpn I; the resulting fragment possessed an Eco RV site at the 5' end and a Kpn I site at the 3' end. The cloning site of pcDNA3.1 has novel Kpn I and Eco RV sites, but they are oriented in the 5' - 3' direction, respectively. The abbreviated TfR fragment was ligated into pcDNA3.1 cut with these enzymes. As such, the only possible orientation for the insert was in the antisense direction.

The human T47D breast cancer line has been found by us to express extremely high levels of TfR. Therefore, this line was chosen as a subject for transfection with the new antisense plasmid. As determined by western blot analysis, the expression of TfR on the initial uncloned, unsorted transfectants was approximately 60% of that in vector transfected controls (Figure 1). The former population has shown preliminary evidence of a decreased transferrin-induced proliferative response (Figure 1). It must be emphasized here that this response is modest and is not nearly that of much lower TfR-expressing cells. These data appear promising and further testing of the transfectants is underway.

Results and discussion: This work appears promising, however, the successful choice of plasmids, inserts and cell lines fell into place too late in the funding period to make *in vivo* behavior statements. A number of cell lines were transfected with the new plasmid, however, the T47D was the only one to demonstrate the desired effect. With the T47D cells, the demonstration of successful alteration of *in vitro* behavior was performed.

Specific aim 2: Effect of transfection-induced TfR up-regulation on breast cancer metastatic capability.

Assumption: The generation of higher TfR-expressing cells with a greater response to Tf from a parental population diminished in both qualities will result in a cell line with greater metastatic capability. The results from two cell lines where this was successfully performed are presented.

MTLn2 cells:

Transfection of poorly metastatic rat MTLn2 mammary adenocarcinoma cells with the gene encoding the transferrin receptor results in an increased ability of those cells to proliferate in response to transferrin and an increased ability to metastasize to the lungs of nude mice. A manuscript detailing these results was submitted to Cancer Research in January of 1998. Despite mild comments by reviewers, the editor did not accept it for publication. It was re-submitted to Breast Cancer Research and Treatment in May of 1998 and has been accepted with revision. Additional *in vivo* experiments are currently underway to satisfy reviewer's concerns.

The version of the paper originally submitted to Breast Cancer Research and Treatment is included in the appendix.

Methods: A eukaryotic human TfR expression plasmid was made by excising the TfR cDNA from pcDTR1 and cloning it into pcDNA1Neo. Rat MTLn2 cells were transfected with this and high human TfR expressors were selected by FACS. The sorted cells were analyzed for growth response to Tf, for Tf internalization capability, for cell surface TfR expression, and cell surface Tf binding. The cells were tested for their ability to metastasize to the lungs of nude mice from a primary mammary fat pad tumor site.

Results: all properties measured were increased as a result of the transfection process. It was found that the transfected population formed twice as many lung metastases as did the vector transfected controls, and that the lung tumor burden in the former was 6- 7 fold higher than in the latter.

Discussion: These results also support a role for transferrin responsiveness and TfR expression in the ability of certain breast cancer cells to metastasize.

MDA 468 cells:

Methods: A eukaryotic human TfR expression plasmid was made by excising the TfR cDNA from pcDTR1 and cloning it into pcDNA3.1. Details of the formation of this plasmid were outlined in the 1997 progress report. This construct was transfected into human MDA468 cells using Lipofectamine, G418 resistant cells were isolated and high human TfR expressors were selected by FACS. Cells transfected with pcDNA3.1 alone and rendered G418 resistant were used as controls. The sorted and control cells were analyzed for growth response to Tf, and for cell surface TfR expression.

Results and Discussion: Transfection of the human MDA 468 breast cancer line with the new TfR expression plasmid developed in the summer of 1997 resulted in a cell line with increased levels of cell surface TfR and increased proliferative response to transferrin (Figure 2). The grant period ended prior to the ability to test this line for metastatic capability in nude mice.

Specific aim 3: Transfection-mediated creation of breast cancer cell lines which synthesize their own transferrin.

Assumptions: Enabling breast cancer cells which express high levels of TfR to produce their own transferrin would create an autocrine stimulatory loop. The continuous availability of high levels of self-produced Tf would provide for maximum growth effect and would create a yet more aggressive cancer cell.

Methods: the creation of a Tf-cDNA containing eukaryotic expression plasmid was detailed in last year's progress report. Briefly, the human Tf coding region was cut from R27A using Fsp I and Ssp I, producing a blunt end DNA fragment. This was cloned into Eco RV cut and alkaline phosphatase treated pcDNA3.1. Minipreps were made from ampicillin-resistant, ligation product -transformed E. coli colonies. Plasmid DNA was assessed for sense or antisense Tf cDNA inserts by cutting minipreps with Xba I and Pst I. Both constructs were found. Bacterial colonies containing the sense construct were expanded and plasmids isolated for transfection studies.

Four lines (MDA-MB 231, MTLn3, MCF-7, and MDA-MB 468) were transfected with the Tf construct. The highly metastatic rat MTln3 breast cancer line was chosen as a test line for the sense Tf plasmid transfections, since the use of a rat line negated the possibility that any endogenous human Tf would be present in the transfected population. The line possesses high

levels of rat TfR and exhibits excellent growth response to Tf. All cells were transfected using Lipofectamine which resulted in the formation of large numbers of G418 resistant cells.

Transfected cells were assessed uncloned, with the hope that sensitive assay procedures would point out any Tf production in lieu of laborious cloning procedures. An anti-human Tf antibody was obtained which does not recognize bovine or rat Tf. Immunofluorescent staining of vast numbers of permeablized transfected cells with this antibody did not reveal any fluorescent cells and thus no Tf synthesis. Cultures of transfected cells were rendered serum free for 48h and the resultant media concentrated 10 fold using a 10,000 mw cut off filter. Western blot analysis of transfected cell lysates or concentrated conditioned media did not result in the display of any human Tf using a sensitive ECL procedure. Results for the MTLn3 line are shown in figure 3; those obtained with other cell lines were similar. These indicated that the cells were not producing any Tf protein.

Results and Discussion: The Tf cDNA containing plasmid produced in 1997 was unable to confer Tf synthesis in four test cell lines. A region of bacterial DNA in the 5' region of the insert may be to blame for this. As further cloning of the Tf region from R27A is difficult, other Tf inserts will be obtained from laboratories that have successfully produced such.

Specific aim 4: Effect of selection-mediated TfR up-regulation on breast cancer metastatic capability.

Assumption: the selection of highly metastatic rat MTLn2 mammary adenocarcinoma cells from the poorly-metastatic parental population by an *in vitro* process based on cellular ability to survive and proliferate in low serum containing media supplemented with low levels of transferrin.

Work in this area has been reported on extensively in the past. A manuscript concerning the results with this experiment was published in January of this year. New data concerning the basic selection and metastasis story was generated only in response to reviewer's requests. The manuscript is included in the appendices as a reprint.

Methods: MTLn2 cells were placed in a stress culture situation where all cells normally died. If this stress culture was supplemented with low levels of rat transferrin, a few colonies were seen to arise, presumably due to their ability to use transferrin in such as way as to ensure their survival and proliferation. These colonies were harvested and exposed to twenty like cycles of culture in these conditions.

Results: When compared to the parental population, the final selected cells displayed increased numbers of transferrin receptors (TfR), an increase ability to grow in response to transferrin (Tf), and an increased ability to form lung metastases in rats.

Discussion: The results indicate that those few cells in a mixed parental breast cancer population which possess the ability to utilize transferrin as a sole survival and proliferation agent, are the more aggressive, metastatic cells. In some systems, ability to respond to transferrin is an earmark of highly metastatic breast cancer cells.

Specific Aim 5: The effect of interleukins known to influence TfR expression on TfR expression in breast cancer cells.

Assumptions: There are many agents that regulate transferrin receptor expression including environmental iron. Certain interleukins have been observed to be potent regulators of transferrin receptor expression in fibroblasts (35) and some T-cell lines (36) .

Interleukins are the regulatory factors of the immune system. They also interact with cells of the vascular endothelium, fibroblasts, keratinocytes, adipocytes, and cells of the central nervous system (35,36). Most interleukins are also growth factors (35, 36). Melanoma cells have been shown to express a functional IL-2 receptor, and in these cells, treatment with IL-2 modifies the expression and or induction of several surface molecules (37). Some human squamous cell carcinomas of the head and neck have also been shown to possess IL-2 receptors (38). Also, IL-2 has been shown to augment the proliferation of some human mammary adenocarcinoma cell lines (39). The following experiments were performed by us to see if IL−1α or IL-2 stimulated the proliferation or measurably increased the cell surface numbers of transferrin receptors on human mammary adenocarcinoma cells, in an effort to see if an interplay between possible interleukin response and TfR expression might influence metastatic behavior.

Interleukins:

IL−1α and IL-2 were obtained from R and D systems (Minneapolis, MN). The cytokines were reconstituted in PBS containing 10 mg/ml bovine serum albumin (BSA). Stock solutions (100 ng/ml for IL-2, 10 ng/ml for IΛ−1α) were stored at -70° C. For use in tissue culture, stock solutions of cytokines were diluted in 25 mM Hepes buffered DMEM (pH 7.5) containing 10 mg/ml BSA.

Cell lines:

Two human mammary adenocarcinoma cell lines (MCF-7, MCF-7/LCC2) and one rat mammary adenocarcinoma (MTLn2) cell line were used. The MCF-7 line is moderately metastatic in nude mice, and responds well to Tf in tissue culture (20). The MCF7/LCC2 line a more metastatic variant of the MCF-7 and expresses high levels of TfR (20). The MTLn2 is a low metastatic, low TfR expressing line. Lines were grown in DMEM/F12 (1:1, v:v) media (MCF-7, MCF-7/LCC2) or a-MEM (MTLn2) containing 5 % fetal bovine serum (FBS) in a 37° C, humidified 5 % CO_2 atmosphere.

Cell Proliferation assays:

Cells were removed from stock cultures using 0.25 % trypsin, 2 mM EDTA and were plated at 20,000 cells/well in 12-well plates in 2 mL DMEM/F12 media containing 1% FBS. After 24 h, the media was changed to 2 ml DMEM/F12 with low (0.3%) FBS. Wells were treated with IΛ−1α, IL-2, and IΛ−1α or IL-2 and/or Tf. The control wells received equal amounts of cytokine and/or Tf solvent. After a 4-7 day incubation, cells were quantitated using a crystal violet stain assay. Wells were rinsed with PBS and cells were fixed with 0.5 mL of 5 % glutaraldehyde. The wells were rinsed with water and allowed to dry. Fixed cells were stained with 0.5 mL of a 1:1 mixture of 100 mM CAPS (pH 9.5) and 0.2 % crystal violet. Stained cells were rinsed with water and allowed to dry. Stained cells were solubilized with 0.5 mL of 10 % acetic acid, transferred to cuvettes, and absorbance at 590 nm of the solution in each well was determined. In this assay, A_{590} correlates with cell number up to 400,000 - 500,000 cells/well (7,8).

Interleukin induced changes in TfR expression:

Cell treatment:

Cells were plated at identical density in multiple six well plates and were grown to 70 - 80 % confluency. Media was changed to that containing 1.0% FBS and all six wells of a given plate were treated with an interleukin or with the appropriate amount of interleukin solvent. Two days

later, one well of each plate was trypsinized and cell density determined using a model ZM Coulter Counter.

Isolation of TfR using immobilized transferrin:

Cells in the remaining five wells were rinsed 3-4 times with PBS and were equilibrated to 4°C. PBS containing 1 mg/ml NHS-LC-biotin was added to all wells (0.2 ml/well), and the plates were incubated at 4°C for two h. Cells were washed 4 times with PBS and were lysed in 2 ml/well PBS containing 2.0% Triton X-100, 0.1 mM PMSF, 0.1 mM TLCK, 0.1 mM iodoacetamide, 10 µg/ml aprotinin, and 10 µg/ml leupeptin while shaking. The lysate was centrifuged at 13,000g for 10 minutes. Supernatants were harvested and volumes of supernatant corresponding to the same amount of cells from each group were treated with 0.3 ml transferrin-agarose (50 % slurry) at 37°C for 2 h. The agarose was removed by centrifugation and washed 3 times by resuspension and repeated centrifugation in PBS containing 2.0% Triton X-100 and 0.5 M NaCl, followed by one wash in PBS only. The bound proteins were removed from the transferrin-agarose with 200 µL SDS-PAGE treatment solution at 95°C for 5 minutes. The samples were then separated on a 9% SDS-PAGE gel and blotted onto a PVDF membrane using 25 mM Tris - 180 mM Glycine transfer buffer. The PVDF membrane was blocked with PBS containing 10% non-fat dry milk and 0.5% Tween 20 at 25°C for 2 hours. The block was then replaced with fresh block contain 1:2000 streptavidin-HRP and was incubated at 25°C for 1 hour. The membrane was then washed 4X with 100 ml PBS containing 0.5% Tween 20. Amersham ECL substrate was applied and light emitting bands were detected by autoradiography.

Results:

Cell proliferation experiments:

MCF-7 cells displayed no increased rate of proliferation when exposed to IL-2 (Figure 4a) or IΔ−1α (Figure 4b). In one experiment, a decrease in cell numbers was seen after IΛ−1α treatment (Figure 4b). When treated with low levels of human holo transferrin, a mild but insignificant increase in MCF-7 cells numbers was seen (Figure 4c). Addition of IL-2 to wells treated with these levels of Tf did not cause an increase in cell numbers when compared to Tf only (Figure 4c). Interleukin levels used were 2 - 20 times the ED_{50} as described by the supplier.

The rat MTLn2 line also displayed no proliferative response to IΔ−1α or IL-2 (Figure 5). These interleukins were unable to increase the proliferative effect of low levels of Tf on these cells (data not shown).

The highly metastatic MCF7/LCC2 line demonstrated no ability to proliferate in response to IL-2 (Figure 6). An earlier result indicated that these cells may have proliferated in response to IL-2 (data not shown), however, this result could not be duplicated.

TfR measurements:

An extremely small amount of TfR was recovered from MTln2 cells, under control conditions (Figure 7). No apparent increases in TfR amount was seen on a per cell basis when the cells were treated with IΔ−1α or IL-2 (Figure 7). The fact that these cells express low levels of TfR was verified by immunofluorescent staining (data not shown).

In one experiment, the treatment of MCF7 cells with IL-2 resulted in a two fold increase in the amount of TfR obtained on a per cell basis using the affinity isolation procedure (Figure 8a).

This indicates a possible up-regulation of TfR in these cells in response to IL-2. However, this result was not consistent as other trials indicated no up-regulation (Figure 8b)

Treatment of the MCF7/LCC2 line with IΛ−1α or IL-2 did not cause any changes in their cell surface TfR expression, as the amount of biotinylated TfR recovered from all groups on a per cell basis was the same (Figure 8).

Discussion:

We found the human mammary adenocarcinoma cells studied to be largely unresponsive to IΛ−1α and IL-2, with the exception of a possible IL-2 induced increase in cell surface TfR in MCF-7 cells. Others have seen a proliferative response for human mammary adenocarcinoma upon IL-2 exposure, however, this response was seen at a very narrow dose range and in tissue culture conditions which would favor a high basal proliferation rate (39).

There is little information on the interleukin receptor content of tumor cells. There are reports of IL-2 receptors existing on certain melanoma cells (37), and on squamous cell carcinomas of the head and neck (38). Although certain human mammary adenocarcinoma lines have been shown to respond to IL-2, information as to the existence of interleukin receptors on mammary adenocarcinoma cells is lacking.

Studying the effects of various interleukins on tumor cells is of interest in terms of understanding basic tumor cell behavior and in interpreting results of interleukin treatment of cancer patients. Studying the effects of cytokines on cells in vitro does not necessarily implicate the effects the will have on the same cells in vivo when other cytokines are present in various amounts. This is an area of cancer research that is largely unexplored, and where further experimentation is warranted.

Conclusions:

With the 13762NF rat mammary adenocarcinoma system, up-regulation of cell surface TfR by transfection or selection techniques causes a concomitant increase in cellular growth response to transferrin and results in increased metastatic capability. This proves the essential elements that the grant was designed to demonstrate and provides the seeds for further research into this system's importance in breast cancer aggressiveness. The display of similar results in human breast cancer cell lines was hampered by their inability to respond to transfection. In many cases, transfection induced the elevation of TfR, but did not alter cellular proliferative response to Tf. In the last year of the funding period, transfection with newly developed plasmids provided the required responses in the human MDA 468 line. If granting futures and the environment permit, further work with these cells will hopefully provide results in keeping with those of the rat lines. The lack of universality in response to transfection was more problematic than expected. The lack of uniformity of the recognizance of the precise metastatic capability of human breast cancer lines as well as lab to lab variation in the morphology and behavior of these was also problematic. The antisense TfR transfection experiments were met with unforeseen problems as the original plasmid designed for these proved ineffective and a new construct had to be designed from the ground up. The use of this has resulted in some bench success in one cell line, but still many lines transfected show none of the desired alterations. The autocrine transferrin secretion protocol proved to be more of a cloning challenge than expected. The Tf cDNA is difficult to excise in one piece from its parental plasmid. The strategy used as outlined by the plasmid development in the 1997 progress report was one pursued after consultation with a number of molecular biologists. However, this has not yet resulted in any cell lines exhibiting bona-fide human Tf production. The interleukin trials were designed as a part of a continuing curiosity on my part as to whether or not metastatic tumor cells might share some of the same characteristics of certain activated leukocytes. Since studies into transferrin receptor expression were already being pursued, the modification of these to investigate as to whether or not agents which (according to the literature) affect TfR expression on those leukocytes did the same on breast cancer cells was explored. As such, this was a stab in the dark aim and the results indicated that there was no TfR alteration induced in breast cancer cells by interleukins which do the same to leukocytes.

The principal investigator would like to thank the Department of Defense for providing the funds. Although not as many publications hoped for have currently resulted from this work, others are in progress and will be produced in the near future, as part of this grant's effort and funding. In addition, many new assays (outlined in the 1997 progress report) have been developed because of this work. Also, a number of new plasmids were constructed, and one collaborator (Dr. Chitambar) has already been provided with one of the plasmids created, for use in other studies.

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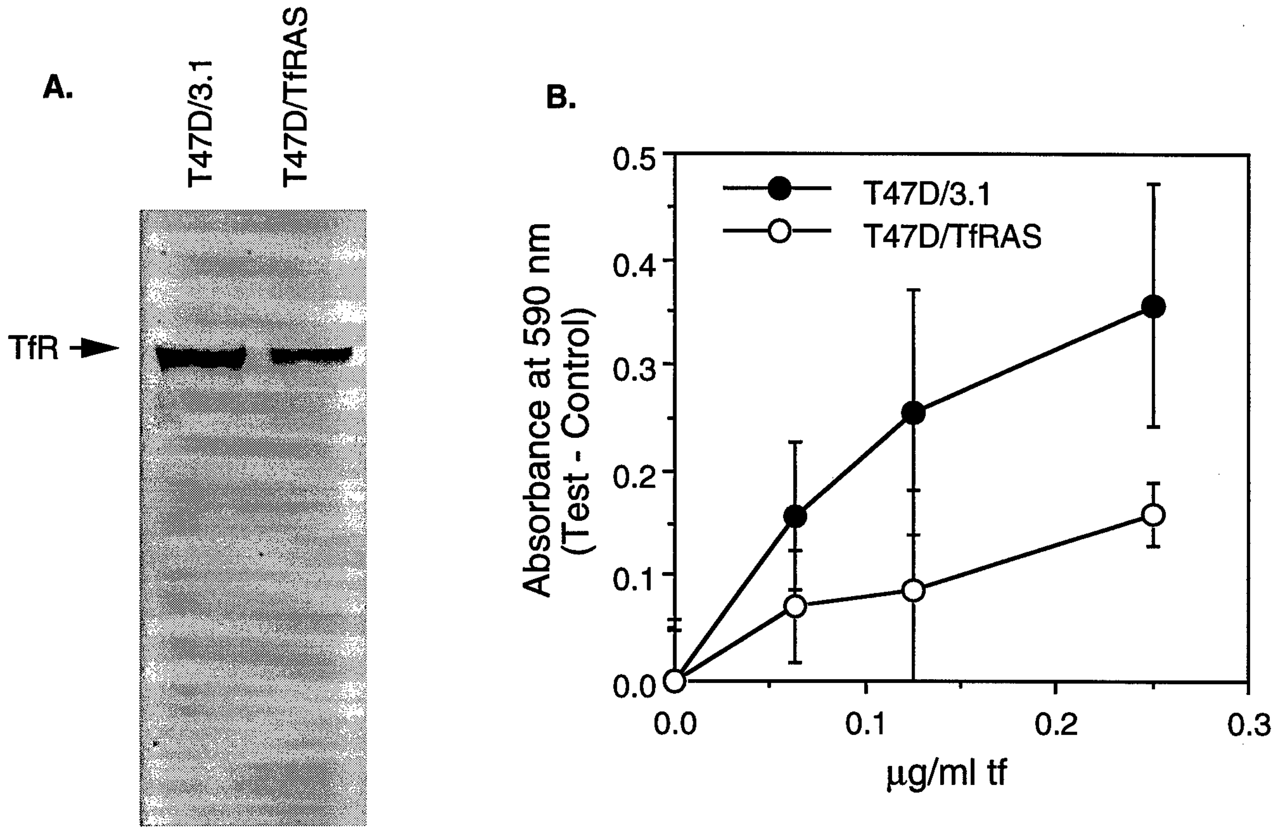
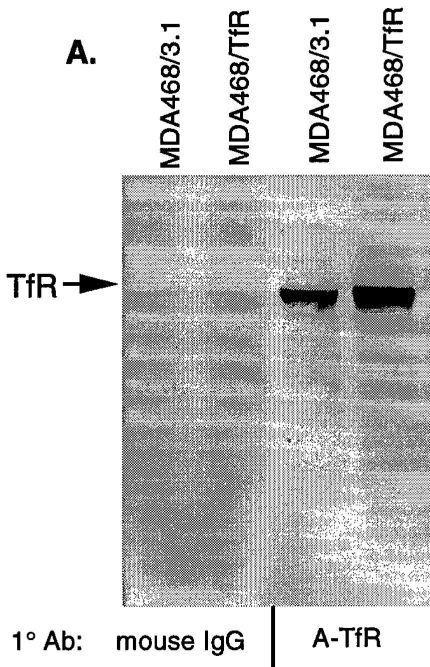
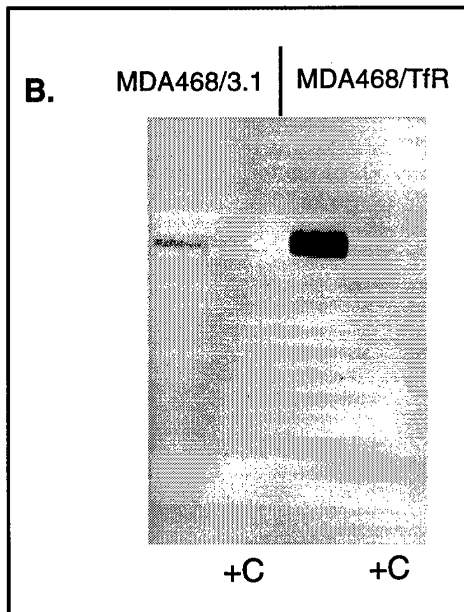


Figure 1. A: Results of western blot analysis of TfR expression on T47D cells transfected with pcDNA3.1 (T47D/3.1) or pcDNA3.1 with the TfR antisense insert (pcDNA3.1/TfRAS plasmid; cells = T47D/TfRAS). **B:** Results of growth response of either line towards transferrin in media containing 0.3 % FBS





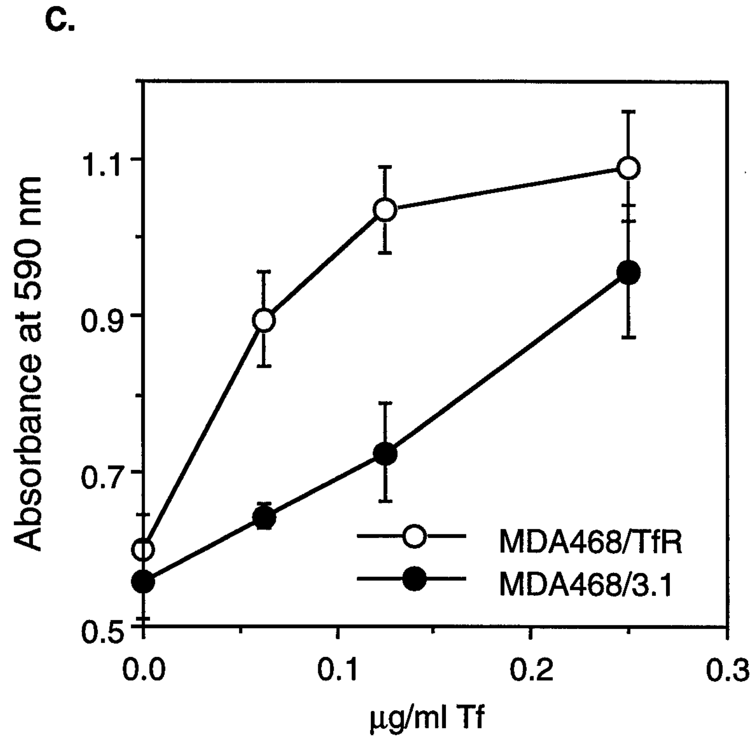


Figure 2 A: Results of western blot analysis of TfR in MDA468 cells transfected with pcDNA3.1 or pcDNA3.1/TfR **B:** Display of FITC-Tf binding: cells were incubated for 2 h at 4° C with 0.5 μg/ml of FITC-transferrin, with or without excess un-conjugated transferrin (+C). Cells were washed, lysed, electrophoresed, blotted, and FITC content measured by exposure to anti-FITC followed by ECL. **C:** Growth reponse of both lines towards transferrin in serum free conditions.

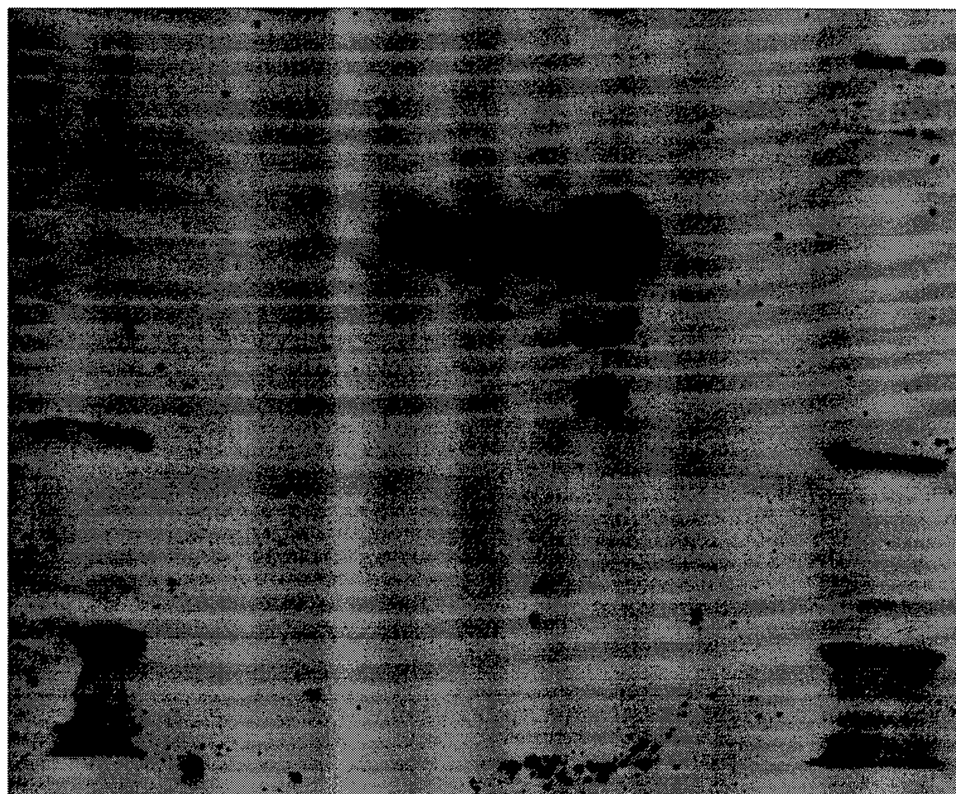


Figure 3. Results of analysis for the presence of human transferrin (Tf) in cell lysates or conditioned media from G418 resistant MTLn3 cells transfected with a plasmid carrying the human Tf cDNA. Lane 1 was loaded with 2 X 10^6 cells and lane 2 with 200 uL of 10X concentrated conditioned media. Lanes 9 and 10 are repeats of 1 and 2. Lane 3 contained 50 uL of media + 5% FBS, to ensure no reactivity of the anti-human Tf used with bovine Tf. Lanes 4-6 were loaded with the indicated amounts of pure human Tf. Samples were electrophoresed, blotted onto nitrocellulose, blocked, treated with anti-human Tf, then with anti-mouse IgG-HRP. HRP-containing bands were detected using an ECL substrate.

Figure 4a.

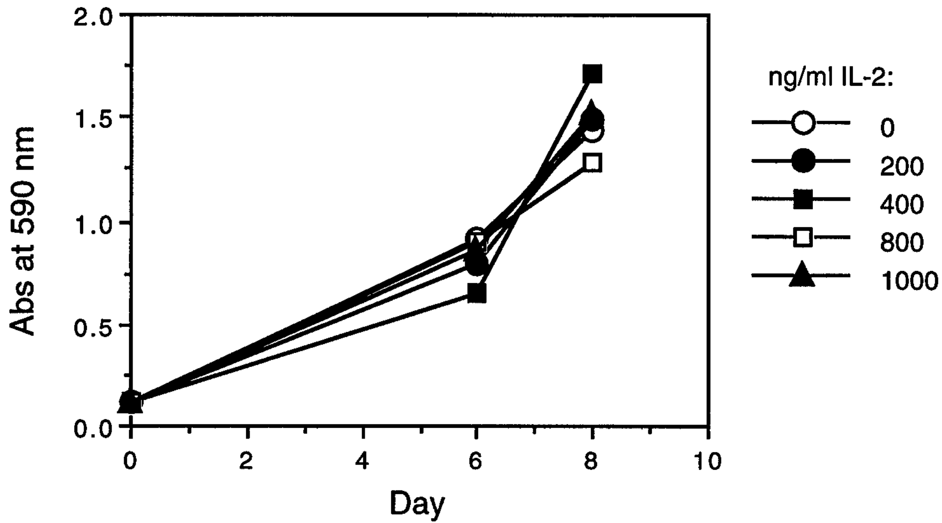
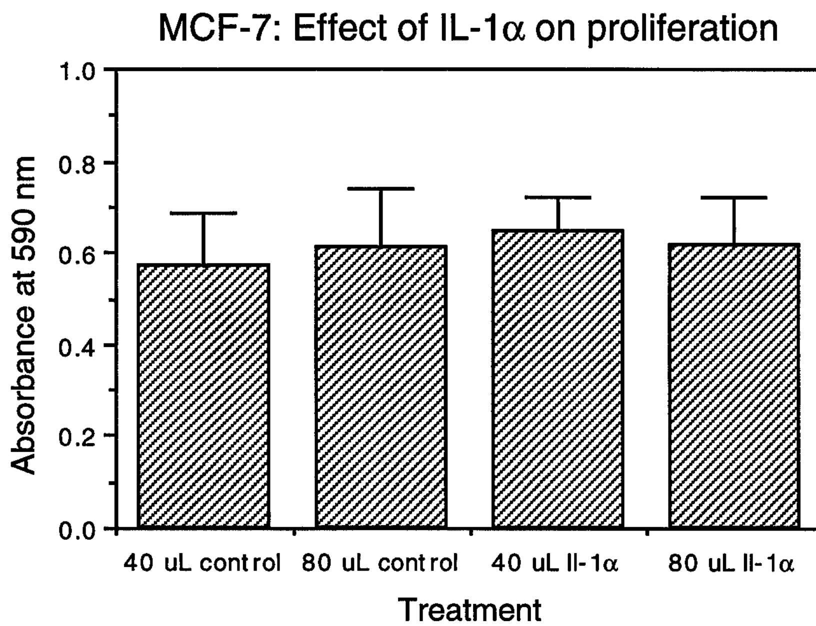


Figure 4b.



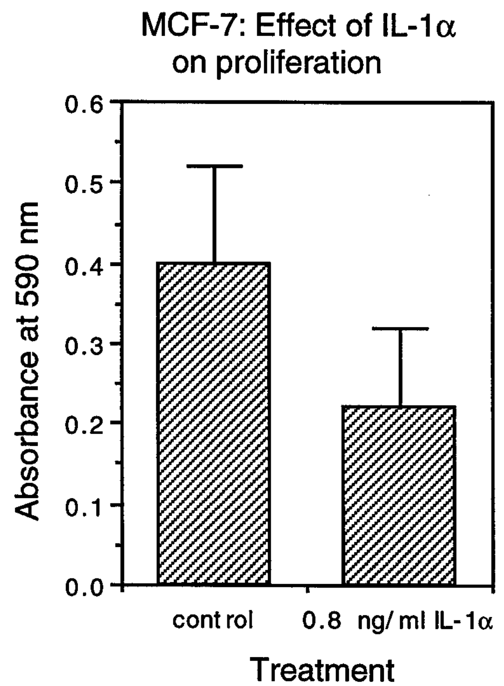


Figure 4c.

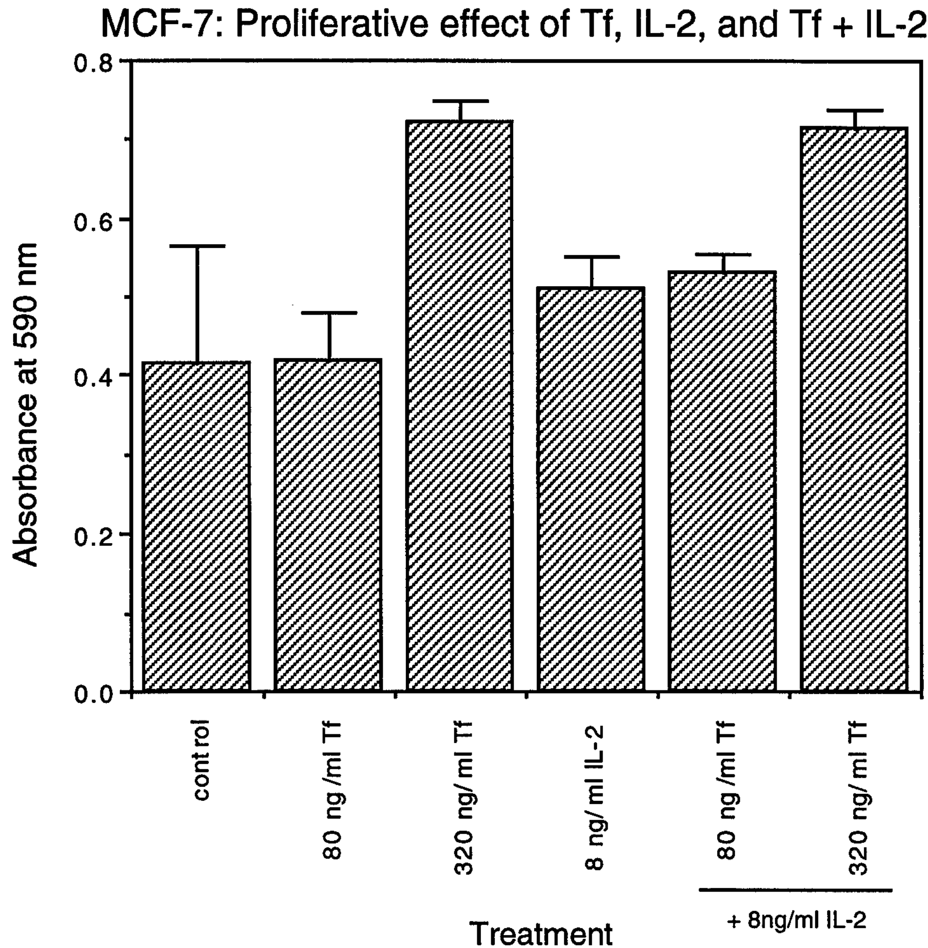


Figure 4a. Effect of IL-2 on the proliferation of MCF-7 cells. Cells were plated at 20,000/well in 12 well plates. One day after plating, media was changed to that containing 0.3% FBS, and interleukin-2 or appropriate control was added. On ensuing days, cell numbers were quantitated using a crystal violet stain assay.

b. Effect of IL-1α on the proliferation of MCF-7 cells. Cells were plated at 20,000/well in 12 well plates. One day after plating, media was changed to that containing 0.3% FBS, and IL-1α was added. Five days later, cell numbers were quantitated using a crystal violet stain assay. Bars represent mean ± SD for data from three wells. For this experiment, 40 μL and 80 μL controls are the controls for the 100 pg/ml and 200 pg/ml IL-1α doses, respectively.

c. Effect of IL-2 and Tf on the proliferation of MCF-7 cells. Cells were plated at 20,000/well in 12 well plates. One day after plating, media was changed to that containing 0.3% FBS, and IL-2 and/or Tf was added. Five days later, cell numbers were quantitated using a crystal violet stain assay. Bars represent mean ± SD for data from three wells.

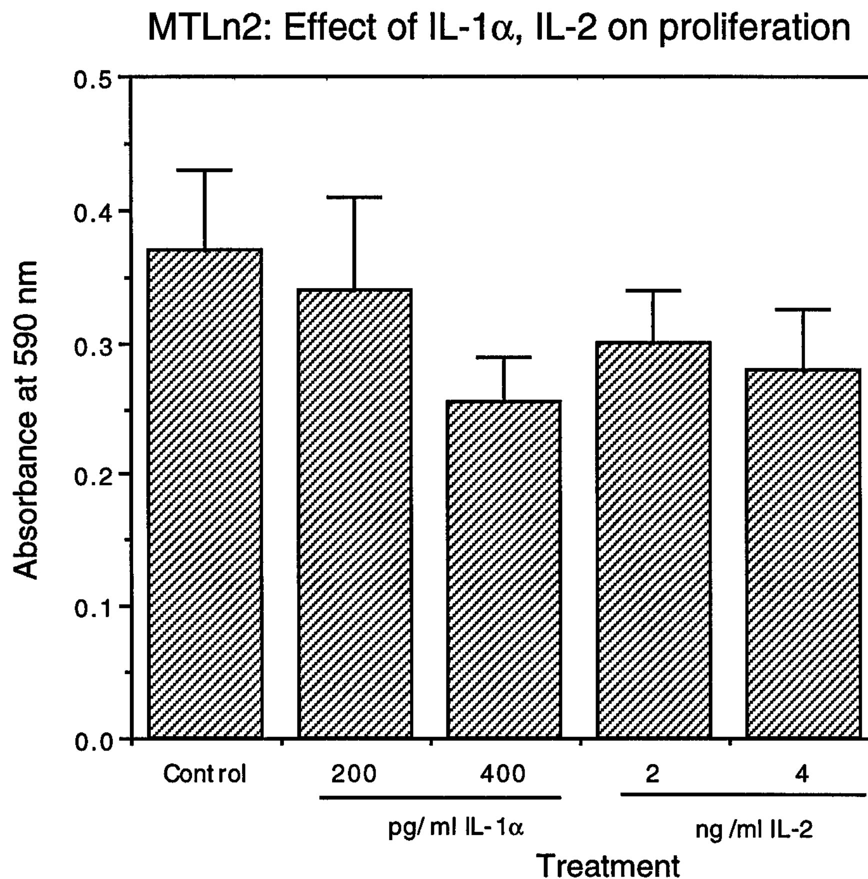


Figure 5. Effect of IL-2 and IL-1α on the proliferation of MTLn2 cells. Cells were plated at 20,000/well in 12 well plates. One day after plating, media was changed to that containing 0.3% FBS, and IL-2 or IL-1α was added. Five days later, cell numbers were quantitated using a crystal violet stain assay. Bars represent mean ± SD for data from three wells.

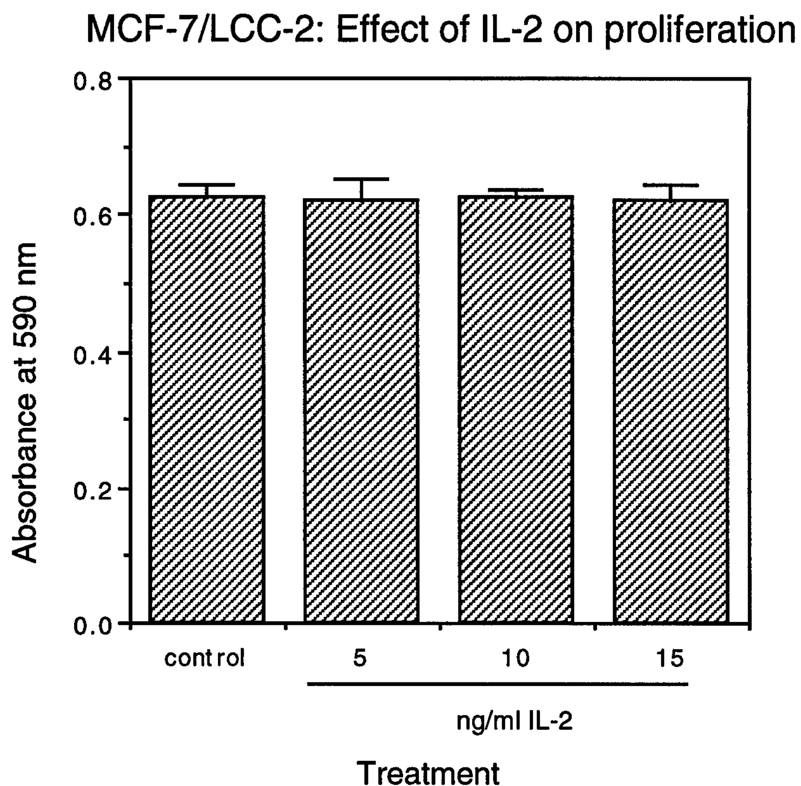


Figure 6. Effect of IL-2 on the proliferation of MCF7/LCC2 cells. Cells were plated at 20,000/well in 12 well plates. One day after plating, media was changed to that containing 0.3% FBS, and IL-2 or control solution was added. Five days later, cell numbers were quantitated using a crystal violet stain assay. Bars represent mean ± SD for data from three wells.

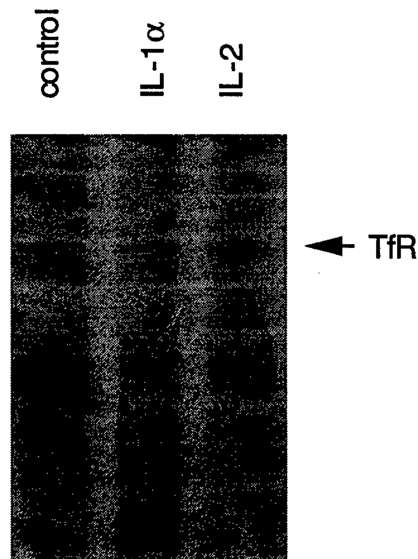


Figure 7. Results of the affinity isolation of biotinylated TfR from MTln2 cells treated with IL-2 or IL-1α. Cells were grown to 70-80% confluency in six well plates. Media was changed to that contining 1% FBS and interleukin solvent, IL-α stock solution (final concentration = 250 pg/ml), or IL-2 stock solution (final concentration = 2.5 ng/ml) was added to all six wells of a given plate. Two days later, one well was used to determine cell density and the other five were surface biotinylated, lysed and equal cell equivalents of the lysate treated with Tf-agarose. Agarose-binding proteins were separated by SDS-PAGE and blotted. Blots were treated with streptsavidin-HRP, and HRP-containing bands were detected by ECL.

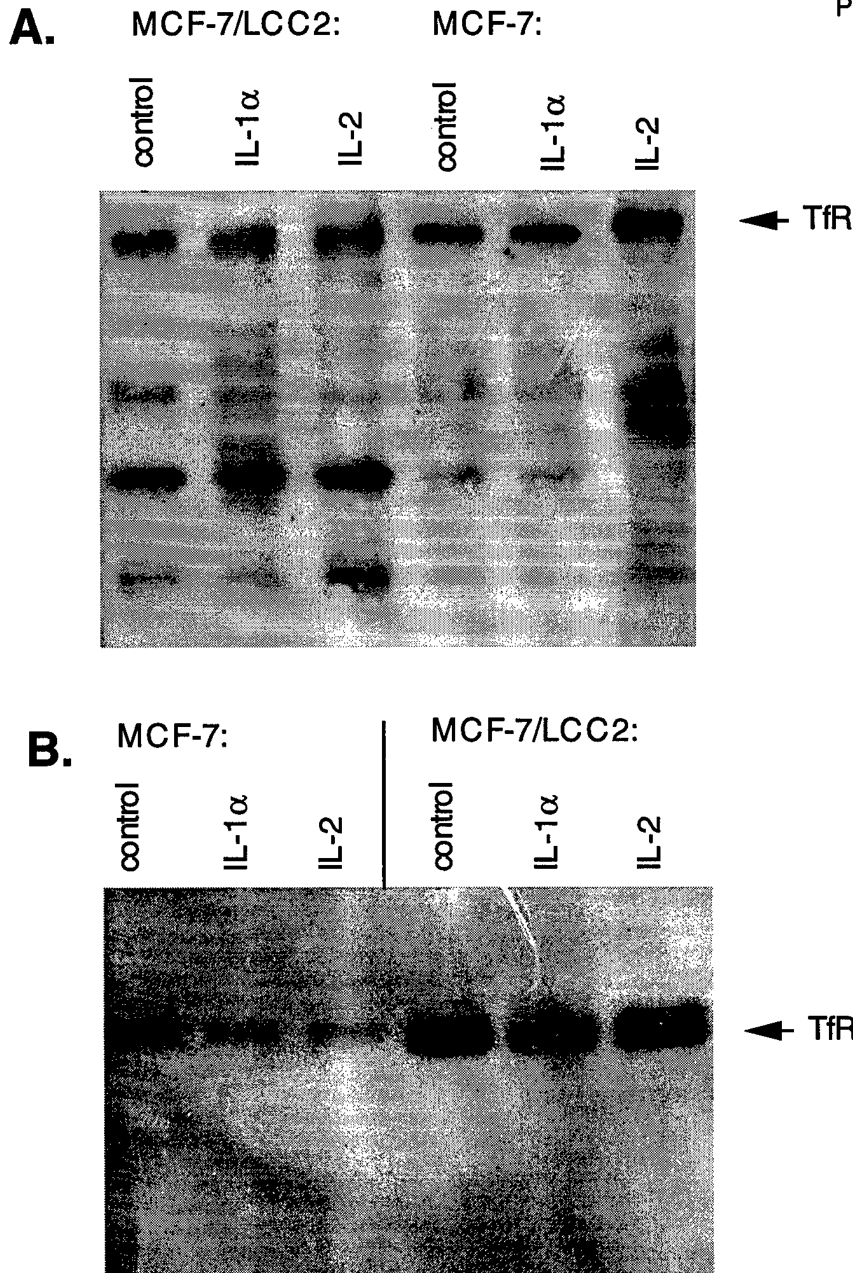


Figure 8. Results of the affinity isolation of biotinylated TfR from MCF-7 and MCF7/LCC2 cells treated with IL-2 or IL-1α. Cells were grown to 70-80% confluency in six well plates. Media was changed to that contining 1% FBS and interleukn solvent, IL-α stock solution (final concentration = 250 pg/ml), or IL-2 stock solution (final concentration = 2.5 ng/ml) was added to all six wells of a given plate. Two days later, one well was used to determine cell density and the other five were surface biotinylated, lysed and equal cell equivalents of the lysate treated with Tf-agarose. Agarose-binding proteins were separated by SDS-PAGE and blotted . Blots were treated with streptsavidin-HRP, and HRP-containing bands were detected by ECL .

Selection of Highly Metastatic Rat MTLn2 Mammary Adenocarcinoma Cell Variants Using In Vitro Growth Response to Transferrin

PHILIP G. CAVANAUGH* AND GARTH L. NICOLSON
Department of Tumor Biology, University of Texas M.D. Anderson Cancer Center, Houston, Texas

We previously found that the proliferative response to transferrin and the expression of transferrin receptors (TfR) on the cell surface of various rat 13762NF mammary adenocarcinoma cell sublines correlated with their spontaneous metastatic capability. To further assess the involvement of transferrin and TfR in metastasis, transferrin-responsive cells were selected from the poorly-metastatic, low-transfferin responsive 13762NF MTLn2 subline. When maintained in low serum (0.3%) conditions, MTLn2 cells failed to survive. However, if like medium was supplemented with 0.5 μg/ml rat transferrin, some colonies emerged, presumably due to their ability to proliferate in response to the added transferrin. The surviving cells were expanded and exposed to ten or 20 similar cycles of transferrin growth selection to obtain the sublines MTLn2-Tf10 and MTLn2-Tf20, respectively. The MTLn2-Tf20 cells proliferated in response to transferrin at a rate similar to that of the high metastatic 13762NF sublines. Using immunofluorescent staining, Scatchard analysis, and affinity isolation of TfR, we discovered that the MTLn2-Tf20 cells had 5 to 6 times more TfR than did the parental MTLn2 line. When injected into the mammary fat pads of rats, the MTLn2-Tf20 line metastasized to the axillary lymph node in seven out of ten animals and to the lungs in six out of ten (median number = 13). No metastases were seen in the MTLn2 parental line. The MTLn2-Tf10 cells showed intermediate properties compared with the MTLn2 and MTLn2-Tf20 cells. The results indicate that variant cells with a high response to transferrin may be more metastatic than the bulk cells in a poorly metastatic population. The selection of cells with high levels of TfR and a higher proliferative response to transferrin results in sublines with greater potentials for spontaneous metastasis. **J. Cell. Physiol. 174:48–57, 1998.** © 1998 Wiley-Liss, Inc.

An important property of metastatic cells is thought to be their ability to respond to paracrine growth factors found in target organs for metastasis formation (Nicolson, 1988a,b). In previous studies we explored the possibility that the metastasis of tumor cells to a target organ was enhanced, in part, by the ability of the malignant cells to respond to growth factors encountered in the micro-environment of that organ. For example, we examined cell lines derived from the rat 13762NF mammary adenocarcinoma for their proliferation in response to medium conditioned by lung fragments (Nicolson, 1988c; Cavanaugh and Nicolson, 1989). A growth response was seen only in those sublines that had a high propensity for lung metastasis formation. A tumor cell mitogen purified from such medium and partially sequenced was found to be the iron transport protein transferrin (Cavanaugh and Nicolson, 1990, 1991). Subsequent studies showed a correlation between tumor cell response to transferrin and metastatic capability in five out of six tumor model systems in animals and humans (Nicolson, 1988c; Cavanaugh and Nicolson, 1990, 1991; Inoue et al., 1993).

Transferrin binds to a specific cell surface receptor, the transferrin receptor (TfR)[3], a $M_r \sim 180{,}000$ homodimeric integral membrane glycoprotein (Testa et al., 1993), that can bind two iron-saturated transferrin molecules and is responsible for the delivery of iron into cells either through internalization of iron-transferrin (Laskey et al., 1988) or activation of a NADH-dependent oxidoreductase associated with the plasma membrane that mediates the trans-plasma membrane

Contract grant sponsor: USAMRMC; Contract grant number: DAMD17-94-J-41089; Contract grant sponsor: American Cancer Society; Contract grant number: CB-164; Contract grant sponsor: Susan G. Komen Foundation.

Garth L. Nicolson is now at The Institute for Molecular Medicine, 1761 Kaiser Ave., Irvine, CA 92614.

*Correspondence to: P.G. Cavanaugh, Department of Tumor Biology, Box 108, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

Received 6 February 1997; Accepted 17 June 1997

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transport of iron from transferrin (Thorstensen and Romslo, 1988). Rapidly dividing cells, including various tumor cells (Testa et al.; 1993; Neckers and Trepel, 1986; Shindelman et al., 1981), usually express high levels of TfR. The expression of TfR in human tumor cells, as determined by histochemical analysis, correlates with tumor grade, stage, progression, and metastasis in breast carcinomas (Wrba et al., 1986), bladder transitional cell carcinomas (Seymour et al., 1987), and malignant melanoma (Van Muijen et al., 1990). In addition, high levels of TfR have been observed in a metastatic lesion of a maxillary neoplasm, but not in the parental tumor (Yoda et al., 1994). The expression of TfR was higher in a human melanoma line selected for metastatic capability in nude mice than in the poorly metastatic tumor cells of the parental population (Van Muijen et al., 1991). Transferrin receptor expression on non-small cell lung cancers has been reported to be an indicator of poor prognosis in certain groups of patients (Whitney et al., 1995), and transferrin was found to be the major mitogen in bone marrow for prostatic carcinoma cells with a high affinity for metastasis in bone marrow in humans (Rossi and Zetter, 1992). In animal tumors, the proliferative response to transferrin is associated with malignant progression in a series of murine B16 melanoma sublines (Nicolson et al., 1990, Stackpole et al., 1995).

The proliferative effect of transferrin on cells is thought to be due primarily to its ability to transport iron into the cell, thus maintaining the activity of key enzymes required for proliferation, such as ribonucleotide reductase (Testa et al., 1993). However, some studies have suggested that iron transport alone cannot explain the growth-stimulating activity of transferrin (Kovar and Franek, 1989; Sanders, 1986; Sirbasku et al., 1991). Also, iron delivered by transferrin mediates processes that soluble iron cannot (Alacantra et al., 1991), and stimulation of TfR through transferrin-independent means induces several activities in T cells (Salmeron et al., 1995).

We modulated tumor cell response to transferrin using a selection technique that allowed the survival of only those rat mammary adenocarcinoma cells that responded to low levels of transferrin. We found that the metastatic capability was markedly greater in the transferrin-selected cell populations than that in the parental line.

MATERIALS AND METHODS

Cells and cell culture

The rat 13762NF mammary adenocarcinoma was originally developed in Fischer 344 rats by dietary administration of 7,12-dimethlybenz[a]-anthracene; the MTLn2 line was originally cloned from a lung metastasis arising from a 13762NF tumor growing in the mammary fat pad (Neri et al., 1982). MTLn2 cells displayed little or no metastatic ability upon injection into syngeneic F344 rats (Neri et al., 1982; Welch et al., 1983). The MTLn2 line was maintained at 37°C in a humidified 95% air-5% CO_2 atmosphere in minimum essential media, alpha modification (α-MEM) containing 5% (vol/vol) fetal bovine serum (FBS). The line was routinely tested for mycoplasma contamination and found to be uncontaminated.

Rat holo-transferrin preparation

Rat transferrin (apo-form; Sigma, St. Louis, MO) was dissolved at 1 mg/ml in 25 mM sodium bicarbonate, pH 8.0, containing 1 mg/ml ferric ammonium citrate. After incubation for 1 h at 25°C, the solution was dialyzed extensively (2 × 4 liters) against 25 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.5.

Selection procedure

Cells were removed from stock plates with 0.25% trypsin, 2 mM EDTA (in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline [PBS]), and seeded onto 100-mm cell culture plates at a density of 20,000 cells/plate in 10 ml of α-MEM containing 1% FBS. One day later, the medium was changed to 10 ml of α-MEM containing 0.3% FBS, and rat holo-transferrin was added to selection plates at a final concentration of 0.5 μg/ml. The control plates received only the corresponding amount of Tf solvent (25 mM HEPES-buffered Dulbecco's modified Eagle's medium [DMEM], pH 7.5, containing 10 mg/ml bovine serum albumin [BSA]). Seven days later, the cells were removed from the plates with 0.25% trypsin, 2 mM EDTA, and re-seeded onto 100-mm cell culture plates at a density of 20,000 cells/plate. The selection process was then repeated 10 times. For selection rounds 11–20, FBS was eliminated from the medium.

Cell growth assays.

The cells were removed from stock plates and seeded in 96-well plates in 100 μl of α-MEM containing 1% FBS at a density of 2,000 cells/well. One day later, the medium was changed to 100 μl of α-MEM containing 0.3% FBS, and increasing amounts of rat holo-transferrin was added to the test wells. Five days later, the cells were quantitated using a crystal violet stain assay as follows: The cells were washed with PBS, and then fixed at 25°C with PBS containing 5% (vol/vol) glutaraldehyde. After 30 min, the cells were washed with water, allowed to dry, and then stained for 30 min at 25°C with 50 μl 0.1% crystal violet in 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 9.5. The stained cells were washed with water and then solubilized with 50 μl of 10% acetic acid. Cell numbers were determined by measuring absorbance at 590 nm on a Dynatech model MR5000 plate reader (Chantilly, VA). Absorbance in this system directly correlates with cell numbers up to ≈ 50,000 cells/well (Cavanaugh and Nicolson, 1990; Keung et al., 1989).

Immunofluorescent detection of cell surface TfR

Cells grown on LabTek slides (Nunc, Naperville, IL) were washed three times with PBS and equilibrated to 4°C. The primary antibody (anti-rat TfR, clone OX-26, BPS, Indianapolis, IN) or normal mouse IgG were each diluted 1:100 at 4°C in PBS containing 10 mg/ml of BSA (PBS-BSA). The antibody or IgG solution was added to the cells, and the slides were incubated on ice for 2 h. The cells were washed three times with PBS-BSA and then incubated on ice with a 1:100 dilution (in PBS-BSA) of an Fab′ fragment of phycoerythrin-conjugated rabbit anti-mouse IgG (Zymed, San Francisco, CA). The cells were washed three times with PBS-BSA and ex-

amined for fluorescence using a Nikon Diaphot phase-contrast microscope (Melville, NY).

Fluorescent-activated cell sorting (FACS) analyses

All cell washes and antibody incubations were performed using 25 mM HEPES-buffered DMEM (pH 7.5) containing 10 mg/ml BSA (DMEM-BSA). The cells were removed from culture plates using 0.25% trypsin, 2 mM EDTA (in Ca^{2+}, Mg^{2+}-free PBS). As soon as detachment was complete, FBS was added to 1% (vol/vol), to neutralize the trypsin. The cells were washed twice by centrifugation, resuspended in DMEM-BSA, and counted. The cell density was adjusted to 1×10^6/ml, and the cell suspensions were equilibrated to 4°C. Normal mouse IgG or anti-rat-TfR (final dilution = 1:100) was added to the cell suspensions, and the cells were then incubated at 4°C for 1 h, washed twice with DMEM-BSA, and incubated at 4°C with a 1:100 dilution of an Fab′ fragment of phycoerythrin-conjugated rabbit anti-mouse IgG. The cells were washed twice with DMEM-BSA and then analyzed for fluorescence using a Becton-Dickinson FACScan instrument (San Jose, CA).

Spontaneous metastasis assays

All experimentation involving animals was approved by an Institutional Animal Care and Use Committee, and animals were treated and housed under conditions specified by the NIH, the Department of Health and Human Services, and the Department of Agriculture. Cultured cells were removed from culture plates with calcium, magnesium-free Hanks' basic salt solution (CMFH) containing 1 mM EDTA, and 0.25% trypsin. The cells were washed three times with CMFH, and cell density was measured using a model ZM Coulter Counter (Coulter, Hialeah, FL) and adjusted to 5×10^6/ml with CMFH. The cells were kept at room temperature and were injected in a 0.2-ml volume into the left mammary fat pad (2 cm anterior to the hind leg) of Metofane (Methoxyflurane; Pittman-Moore, Washington Crossing, NJ) anesthetized syngeneic female F344 rats (age range: 6–8 weeks). Six weeks later, the animals were killed with an overdose of Metofane and examined for gross metastatic lesions. In addition, lungs of certain animals were fixed in buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin-eosin, and examined for micro-metastases.

Affinity isolation of TfR using immobilized Tf

Cyanogen bromide-activated agarose was washed with 10 volumes of 1 mM HCl and equilibrated in a coupling buffer consisting of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3. Apo-transferrin was added to the agarose in coupling buffer at a ratio of 10 mg of protein per ml of packed gel. The mixture was incubated at 4°C overnight in an end-over-end mixer. The gel was treated with 1 M ethanolamine, pH 8.0, for 2 h at 25°C and was washed with coupling buffer, then with 0.1 M acetate, pH 4.0, containing 0.5 M NaCl, and again with coupling buffer. The immobilized Tf was iron saturated by exposure to 1 mg/ml ferric ammonium citrate in 0.1 M NH_4HCO_3. The gel was washed with PBS containing 0.5% (vol/vol) Triton X-100 (Van Driel et al., 1984; Turkewitz et al., 1988). To reduce inherent bound Tf, cells were incubated in two changes of α-MEM only (2 h each) before the analysis. The cells (70–80% confluent in 100-mm dishes) were then washed three times with 5 ml of PBS (4°C), and 3 ml of PBS containing 1.0 mg of Biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma, initially dissolved in 0.2 ml DMSO) was added. The dishes were incubated at 4°C for 90 min while being shaken. The cells were then washed five times with 5 ml of PBS and lysed in 3 ml/dish of PBS containing 2.0% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM N-α-*p*-tosyl-L-lysine chloromethyl ketone, 0.1 mM iodoacetamide, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, at 4°C for 2 h. The lysate was centrifuged at 13,000*g* for 10 min. Lysate supernatant protein was determined using the detergent compatible BCA assay (Pierce, Rockford, IL). The lysate supernatant (1–2 mg of total protein) was combined with an excess of transferrin-agarose (0.4 ml of packed gel) and incubated for 2 h at 37°C. The gel was harvested by centrifugation at 2,000*g* and washed three times by suspension in PBS containing 2% Triton X-100. The centrifugation step was then repeated. Bound-cell-lysate proteins were released by exposure of the gel to 0.4 ml of nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample treatment solution at 95°C for 10 min. The samples were separated on a 7.5% SDS-PAGE gel according to Laemmli (1970) and blotted onto Immobilon membranes (Millipore, Bedford, MA) using a 10 mM CAPS, pH 10.0, transfer buffer. The membrane was blocked for 2 h at 25°C with PBS containing 10% nonfat dry milk and 0.5% Tween 20 and then incubated with blocking solution containing a 1:2,000 dilution of streptavidin-horseradish peroxidase (streptavidin-HRP; Boehringer Mannheim, Indianapolis, IN) for 1 h at 25°C. The membrane was washed four times with 40 ml of PBS containing 0.5% Tween 20, and HRP-enhanced chemiluminescence (ECL) substrate (DuPont, Wilmington, DE) was applied. Light-emitting bands were detected by autoradiography and quantitated using a Hoeffer (model GS300 San Francisco, CA) scanning densitometer.

Scatchard analysis of cell surface TfR

To 1 ml of a 1 mg/ml solution of Rat holo-transferrin (in 25 mM HEPES, pH 7.5) was added 0.5 MCi of $Na^{125}I$ (ICN, Cleveland, OH) and 10 μl of a 10 mg/ml solution of chloramine-T. This mixture was incubated at 25°C for 1 h, and 10 μl of a 20 mg/ml sodium bisulfite solution was added. The sample was passed through a PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated and run with PBS. One-milliliter fractions were collected, and aliquots of each fraction were examined for radioactivity using a gamma counter. Radioactive fractions corresponding to the void volume were pooled. Specific activity was ≈ 300,000 cpm/μg protein. Tumor cells were grown in 12-well plates to 70–80% confluence. To reduce inherent bound transferrin, the cells were incubated in two changes of α-MEM only (2 h each) before the analysis. Cells were washed twice with PBS, and 1 ml PBS containing 1% (vol/vol) liquid gelatin (Sigma) was added as a non-specific blocking agent. The plates were equilibrated to 4°C, and increasing levels of ^{125}I-rat holo-transferrin (from 0.33 to 6.0 μg/well) were added (in multiples of five) to wells. Immediately prior to the addition of transferrin, two wells at

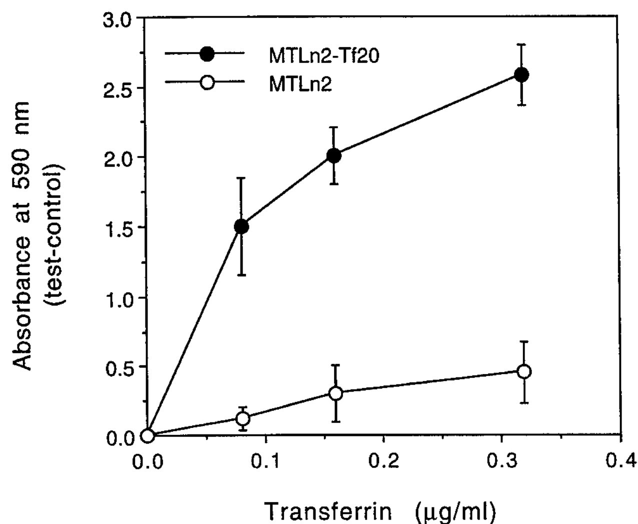


Fig. 1. Proliferative response of MTLn2 and MTLn2-Tf20 cells to transferrin. Cells were plated in α-MEM containing 1% FBS at a density of 2,000 cells/well in 96-well plates. One day later, the medium was changed to medium containing 0.3% FBS, and increasing amounts of rat holo-transferrin were added. Five days later, cell numbers were determined using a crystal violet stain procedure. Points represent the mean ± SD of quadruplicate samples.

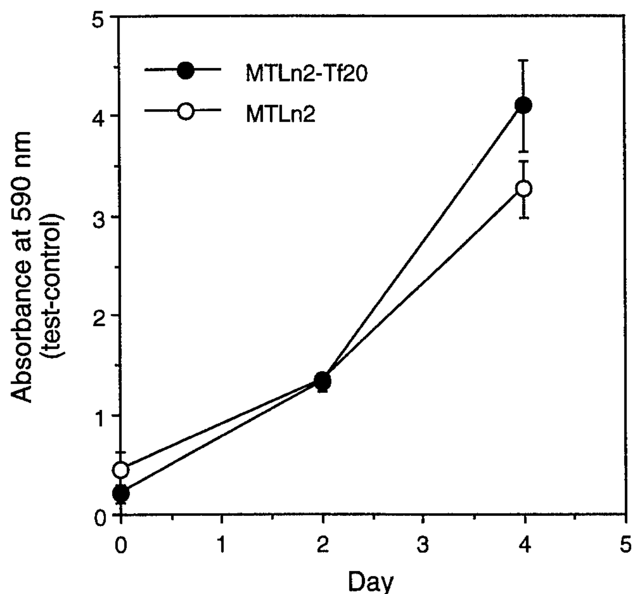


Fig. 2. Growth rate of MTLn2 and MTLn2-Tf20 cells under usual culture conditions (α-MEM containing 5% FBS). Cells were plated in α-MEM containing 5% FBS at a density of 2,000 cells/well in 96-well plates. Two and 4 days later, cell numbers were determined using a crystal violet stain procedure. Points represent the mean ± SD of quadruplicate samples.

each dose received a 200× excess of unlabeled transferrin. The wells were incubated for 2 h at 4°C and then washed five times with PBS. The cells were lysed with PBS containing 2% Triton X-100, lysates were placed into scintillation vials, and cell-bound counts were determined using a Packard A5550 gamma counter (Packard, Meriden, CT).

RESULTS

When poorly metastatic rat MTLn2 mammary adenocarcinoma cells were cultured at 20,000 cells per 10-cm dish in α-MEM containing 0.3% FBS, none of the cells survived. However, if a similar culture was supplemented with 0.5 µg/ml of rat transferrin, two to ten colonies of surviving cells appeared. When these cells were harvested and re-exposed to the same transferrin selection conditions, ten to 30 colonies appeared. As the selection cycles continued, increasing numbers of surviving cells were found in the cultures. After 18 to 20 cycles of selection, the selected cell subpopulation produced a nearly confluent culture. Furthermore, during the last ten cycles of selection for transferrin-responsive cells, FBS could be eliminated from the system. With each cycle of selection, however, all cells failed to survive in transferrin-free medium. After 20 cycles of selection, an MTLn2 cell population was obtained (MTLn2-Tf20) that demonstrated an enhanced ability to proliferate in response to transferrin when compared with the parental MTLn2 cells (Fig. 1). Both lines grew at the same rate in the standard culture conditions using α-MEM supplemented with 5% FBS (Fig. 2).

We next determined if the amount of cell surface TfR was increased in the MTLn2-Tf20 cells. Immunofluorescent microscopy analysis of cell surface TfR using anti-rat TfR (clone OX-26) followed by phycoerythrin-conjugated anti-mouse IgG revealed visibly greater cell surface fluorescence in the selected MTLn2-Tf20 subpopulation than in the parental MTLn2 cells (Fig. 3). We also analyzed the expression of cell surface TfR by FACS. The cells were rapidly removed from plates with trypsin/EDTA, stained for TfR at 4°C, and analyzed for fluorescence. This analysis indicated that there was 6.8 times more TfR on the MTLn2-Tf20 line than on the MTLn2 line (Fig. 4). In addition, this procedure revealed that the MTLn2-Tf10 line expressed a level of TfR between that of the MTLn2 and MTLn2-Tf20 lines, indicating that TfR per cell increased gradually during the selection protocol.

An additional procedure based on affinity isolation of TfR after biotinylation was used to quantitate cell-surface TfR in the cell lines. This procedure was performed after we found that the anti-rat TfR antibody used for immunofluorescent studies was unable to immunoprecipitate biotinylated TfR (data not shown). The cell surfaces were biotinylated, cells were lysed with PBS containing Triton X-100, and the resulting solubilized cell material was exposed to immobilized transferrin. The agarose-transferrin preferentially bound to biotin-TfR in the lysate, which was released with SDS-PAGE sample buffer. The released TfR could be measured after SDS-PAGE separation followed by electrotransfer and detection of biotinylated bands by incubation of the blot with streptavidin-HRP followed by ECL. This method indicated that MTLn2-Tf20 cells had five- to sixfold more TfR per cell than did the parental MTLn2 cells (Fig. 5).

To verify the results of biotin-TfR affinity isolation and FACS and to assess any changes in the affinity for Tf in the two lines, conventional Scatchard analy-

MTLn2:

MTLn2-Tf20:

Fig. 3. Immunofluorescent analysis of cell surface TfR on MTLn2 and MTLn2-Tf20 cells. The cells were grown on LAB-TEK chamber slides and stained at 4°C with the OX-26 anti-rat TfR antibody followed by a phycoerythrin-conjugated Fab′ fragment of anti-mouse IgG. The cells were then examined for fluorescence using a Nikon Diaphot microscope. Phase-contrast microscopy indicated that the cells were of approximately equal density. Cells initially treated with an irrelevant mouse IgG produced no fluorescence (data not shown).

sis using ^{125}I-Tf was performed. Liquid gelatin was found to be an ideal blocking agent for this, and nonspecific binding was reduced to near-background levels. In agreement with other assays, Scatchard analysis revealed that the MTLn2-Tf20 cells possessed five- to sixfold more surface TfR than did the MTLn2 cells. No significant differences in affinity for Tf were seen (Fig. 6).

To ascertain the metastatic behavior of both cell lines, spontaneous metastasis assays were performed. When 1×10^6 cells of either cell line were injected into the left mammary fat pad of syngeneic Fisher 344 rats, primary tumors formed at equal (70–80%) frequencies, and the tumors grew to a similar size. After 6 weeks of tumor growth, seven out of ten rats receiving the MTLn2-Tf20 cells had palpable left axillary lymph node metastases (Table 1). Necropsy revealed that six out of ten rats receiving MTLn2-Tf20 had lung metastases, but metastases were not evident in any of the animals injected with the parental MTLn2 line (Table 1). Representative photographs of lungs from animals injected with MTLn2 or MTLn2-Tf20 cells are shown in Figure 7.

Another experiment was performed to compare the spontaneous metastatic capability of MTLn2 and MTLn2-Tf10 cells. The procedure was identical with that for the previous study, with the exception that six rats were used in each group. In this experiment, the MTLn2-Tf10 cells formed lung metastases in one out of six rats and left axillary lymph node metastases in three out of six rats. The only metastasis seen with the MTLn2 cells was an axillary lymph node metastasis in one animal (Table 1). Thus, it appeared that the metastatic capability of the MTLn2-Tf10 line was between that of the MTLn2 and MTLn2-Tf20 lines. As the expression of cell surface TfR in the MTLn2-Tf10 line was also shown to be intermediate to that of the MTLn2 and MTLn2-Tf20 lines (Fig. 4), these results provided further proof that TfR expression correlated with metastatic capability in this system. Examination of lung sections for micro-metastases revealed that animals injected with MTLn2 cells possessed occasional diffuse sub-pleural micro-metastases. However, lungs from rats receiving MTLn2-Tf20 cells displayed extensive nodular metastases with large areas of lung replaced by tumor cells.

Cultures were established from the lung metastases from one animal injected with the MTLn2-Tf20 cells. When all cultured cells were re-analyzed for rat TfR expression by FACs, the results indicated that a maintenance of hierarchy of TfR expression was maintained, with mean fluorescence for MTLn2 being 65.7, that for

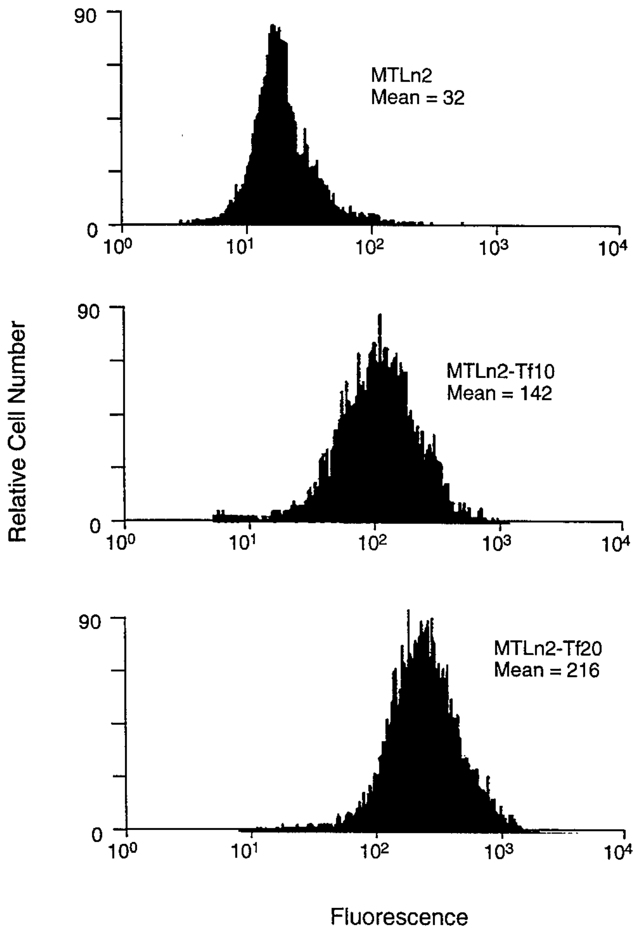


Fig. 4. Analysis of cell surface TfR using FACS. The cells were removed from the culture plates with trypsin/EDTA, stained at 4°C with the OX-26 anti-rat TfR antibody followed by a phycoerythrin-conjugated Fab′ fragment of anti-mouse IgG, and analyzed for fluorescence using a Becton-Dickinson FACstar instrument. An isotype-control fluorescence of 4–8 was obtained when any of these cell lines were initially incubated with normal mouse IgG (data not shown).

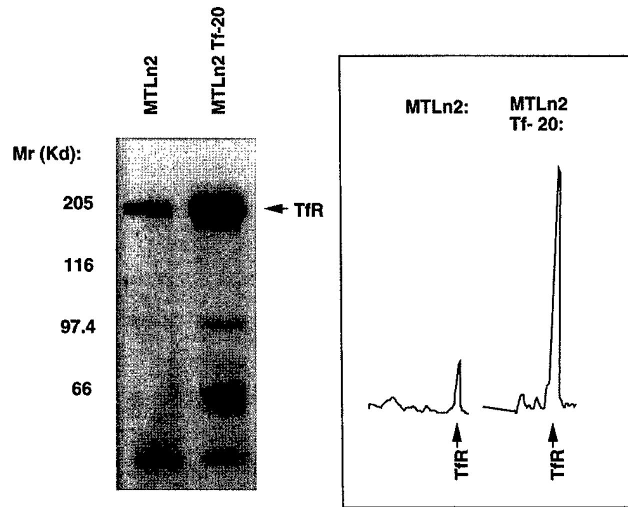


Fig. 5. Results of cell surface TfR measurement in MTLn2 and MTLn2-Tf20 cells by affinity isolation of biotinylated TfR. The cell surfaces were biotinylated at 4°C, the cells were lysed, and equal cell equivalents of cell lysates were exposed to an excess of Tf-agarose. The agarose was collected, washed, and bound lysate proteins were removed and separated by SDS-PAGE. After Western blotting of bound proteins, the biotin groups were detected by exposure of the blot to streptavidin-HRP followed by ECL. **Left:** results of the ECL; **right:** densitometric scans of the lanes shown on the left. The ratio of the areas under the TfR peaks was 5.8:1 (MTLn2-Tf20 : MTLn2).

MTLn2-Tf20 being 176.4, and MTLn2-Tf20 (metastases, second passage) equaling 199.8. No loss and perhaps even a slight gain of TfR expression was seen as the MTLn2-Tf20 cells metastasized. As lungs from rats receiving MTLn2 cells displayed no gross metastases, these samples were not cultured.

DISCUSSION

Our previous results (Cavanaugh and Nicolson, 1989, 1990, 1991; Inoue et al., 1993; Nicolson et al., 1990) and those from other laboratories (Seymour et al., 1987; Van Muijen et al., 1990, 1991; Yoda et al., 1994; Rossi and Zetter, 1992; Stackpole et al., 1995) have indicated that in many malignant cell systems, the proliferative response of tumor cells to transferrin and expression of TfR correlates with metastatic capability. The observation of a cellular property associated with and perhaps thought to be responsible in part for metastatic properties has frequently resulted in the use of in vitro selection techniques to isolate tumor cells with high or low levels of a metastasis-associated

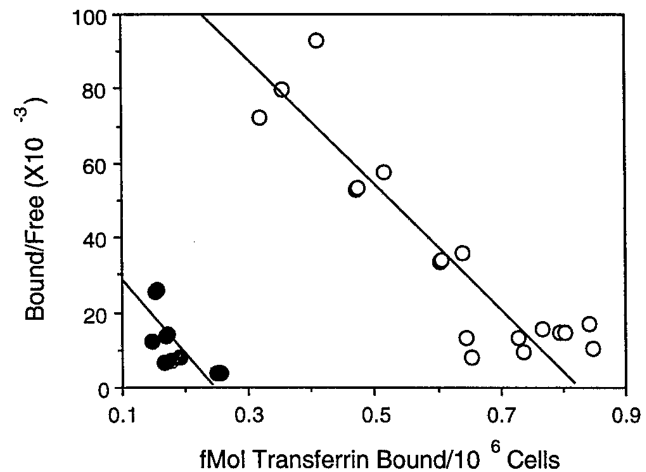


Fig. 6. Scatchard analysis of transferrin binding to MTLn2 and MTLn2-Tf20 cells. The cells were grown to 70–80% confluence on 12-well plates, washed twice with serum-free medium, and equilibrated at 4°C. Increasing amounts of ^{125}I-transferrin were added to wells in replicates of five. Two hours later, the cells were washed and lysed, and cell bound (lysate) radioactivity was determined using a gamma counter. To control for nonspecific binding, two wells of each dose received a 200-fold excess of unlabeled transferrin. Nonspecific bound radioactivity obtained from these wells was then subtracted from the data. Results are representative of two experiments.

TABLE 1. Results of spontaneous metastasis assays[1]

Cell line	Primary tumor median diameter	No. of animals with axillary node metatases/ total no. injected	Median diameter of lymph node metastases	No. animals with lung metastases/ no. injected	Median no. of lung metastases	Range of lung metastases
Experiment No. 1						
MTLn2	13	0/9	0	0/9	0	0
MTLn2-Tf20	16.5	6/10	5	6/10	18	0->100
Experiment No. 2						
MTLn2	14	1/6	0	0/6	0	0
MTLn2-Tf10	10	3/6	3	1/6	0	0->100

[1]Syngeneic female F344 rats were injected with 1×10^6 MTLn2, MTLn2-Tf10, or MTLn2-Tf20 cells into the left mammary fat pad. Six weeks later, rats were sacrificed and examined for gross inguinal, axillary, and lung metastases. All measurements are in millimeters.

marker or activity. The influence on metastatic behavior can then be determined by assessing the metastatic capability of the selected cells in vivo. These types of experiments have resulted in the identification of a number of tumor cell properties thought to be associated with the ability to form metastases (Tulberg and Burger, 1985; Kalebic et al., 1988; Ishikawa et al., 1988; Brodt, 1989; Katz and Witz, 1993; Tressler and Nicolson, 1989; LaBiche et al., 1993).

The differential expression of TfR and response to transferrin seen in metastatic cells naturally raised the question as to whether the metastatic behavior of tumor cells could be altered by artificially manipulating the transferrin/TfR system. A number of processes were considered in which transferrin responsiveness and TfR expression could be enhanced in a poorly metastatic cell line to create a high metastatic line or reduced in a highly metastatic cell line to decrease its metastatic capability. We had previously performed Tf growth-response assays (Cavanaugh and Nicolson, 1989, 1990), in which highly metastatic rat mammary adenocarcinoma cells proliferated in response to transferrin under low-serum conditions, whereas poorly metastatic cells proliferated to a much lesser degree or not at all. A logical method to select transferrin-responsive cells from a low metastatic population, then, was to scale-up the transferrin growth assays into an in vitro selection process whereby those few cells from the poorly metastatic population that responded to Tf could be isolated, expanded, and re-selected.

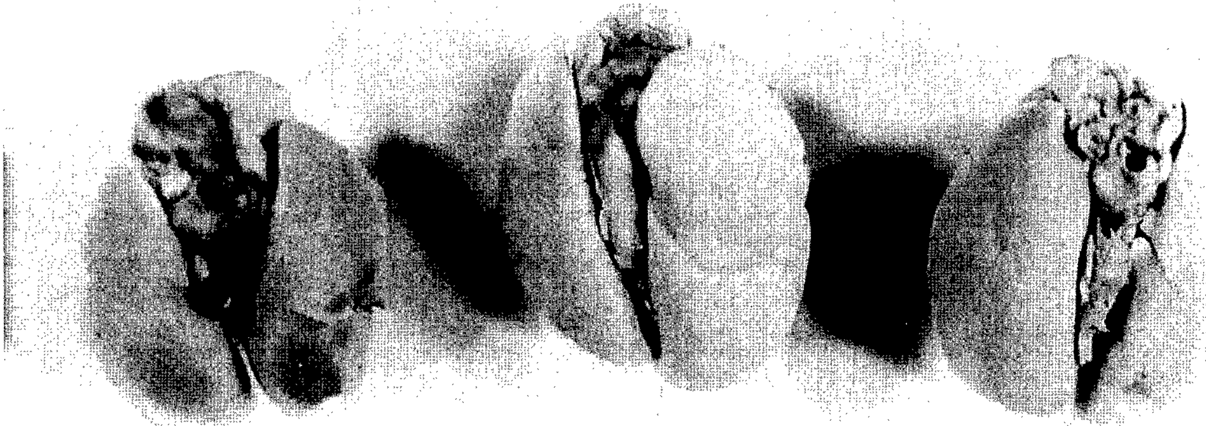
The poorly metastatic MTLn2 line (originally isolated from a lung metastasis; Neri et al., 1982) was the ideal choice for these studies because its growth rate under the usual culture conditions (minimum essential media, alpha modification [αMEM] containing 5% FBS) was equal to the more highly metastatic MTLn3 line (Neri et al., 1982). Also, the MTLn2 line is totally unable to survive in low-serum or serum-free conditions. Other poorly metastatic lines from the 13762NF series (such as MTPa) were less well suited to the selection process because their basal growth rate was much lower than that of the high metastatic lines (Neri et al., 1982), and they demonstrated greater survival properties in low serum conditions. This last characteristic contributed background during the selection process. Finally, the rat 13762NF mammary adenocarcinoma was an ideal choice for this experiment because it is a syngeneic tumor system, and the various sublines generated from it were originally selected based on their different spontaneous metastatic capabilities (Neri et al., 1982). Also, the metastatic spread of cells in the 13762NF series mimics the pathogenesis of mammary adenocarcinoma cells in humans, with an initial metastatic migration to the regional lymph node(s) followed by metastasis to other organs. Selection and test results with the MTLn2 line were obtained rapidly, because these cells replicate and form large primary tumors in a short time. Other poorly metastatic animal tumor cell lines from other tumor systems are currently being selected for high transferrin-responding cells in this same manner; however, those selections are currently in their early stages.

We have found that a series of metastatic rat MTLn2 mammary adenocarcinoma cells that have high numbers of TfR and the ability to proliferate in response to low concentrations of transferrin can be selected from a low TfR-expressing, poorly transferrin-responsive parental cell population. The selected cells demonstrated a much greater ability to form spontaneous axillary lymph node and lung metastases in syngeneic rats than did the parental line. Our results confirmed previous observations and indicate that a tumor cell's ability to respond to transferrin can be an important characteristic for metastasis formation, at least in some tumor cell systems.

The existence of micro-metastases in the lungs of animals injected with MTLn2 cells indicates that some of these cells possessed the ability to initiate the metastatic process. The further progression of MTLn2-Tf20 cells to gross metastatic lesions in the animals receiving these may indicate that the enhanced ability to respond to transferrin may not be responsible for the initial formation of metastases, but for the rapid growth of these into larger lesions. Nevertheless, the results indicate a more aggressive metastatic behavior for the MTLn2-Tf20 cells.

Our results raise the question as to whether the amount of transferrin present in a target organ influences metastatic cell growth. Significant levels of transferrin can be thought to be circulating through any tissue, since a relatively high level of transferrin is present in blood. Whether other localized pools of transferrin exist in a given tissue that can exert an effect other than, or in addition to serum transferrin is questionable. The non-lactating mammary gland synthesizes some, but low levels of transferrin (Lee et al., 1987; Grigor et al., 1990), and its status as a site of transferrin localization is uncertain. As tumor growth in the mammary fat pad for both lines was equal, this might indicate that low levels of transferrin were local-

MTLn2:



MTLn2-Tf20:

Fig. 7. Photographs of representative lungs from rats injected with the MTLn2 parental cell line (**top**) or the MTLn2-Tf20 cell line (**bottom**).

ized there, or that tumor growth in the fat pad was a result of interaction with many other factors. Stimulated lymph nodes can be thought of as containing high levels of transferrin, since macrophages situated in these synthesize transferrin needed for optimum lymphocyte growth (Djeha et al., 1993). Assuming tumor cell–containing lymph nodes are stimulated, then this mechanism may play a role in high-TfR expressing tumor cell growth in the lymph node. This may explain why we found gross lymph node metastases in many rats injected with the MTLn2-Tf20 line, but not in those injected with the MTLn2 line. Previously, we found no evidence that perfused adult rat lung synthesizes transferrin (Cavanaugh and Nicolson, 1991). However, more transferrin seems to be found in the adult perfused lung than in many other organs. Rossi and Zetter (1992) report that extensively washed human adult lung contains more immuno-recognizable transferrin than does kidney, skin, bone marrow, or liver; and Meek and Adamson (1985) find more transferrin localized to adult perfused mouse lung than in any other tissue examined. This may explain why tumor cells with high TfR expression and ability to proliferate in response to transferrin form larger, more visibly apparent lung metastases.

Heightened proliferative response to transferrin does

not necessarily explain the ability of these cells to preferentially metastasize to, or target the lung. Other unknown tumor cell factors such as those which might mediate increased adhesiveness to lung tissue or motility in response to lung components may also be involved. However, the possibility that the lung acts as a transferrin storehouse indicates that a transferrin/TfR-mediated mechanism for lung targeting by tumor cells might exist.

We have not ruled out the possibility that increased expression of TfR and response to Tf may have other roles in metastasis formation. Other functions of transferrin have been described, such as its ability to promote angiogenesis by stimulating endothelial cell invasion and migration (Carlevaro et al., 1997) and to affect the adhesion and migration of chicken mesoderm explants (Sanders, 1986).

The results further suggest that continued exploration of therapeutic strategies that interfere with transferrin binding or iron uptake by tumor cells (Elliot et al., 1988; Kemp et al., 1995) are warranted.

ACKNOWLEDGMENTS

The authors thank Dr. Nguyen Van and Kim Steck for their assistance in performing FACs analysis and Dr. Jae Ro for his assistance in evaluating lungs for micrometastases. The early phase of these experiments was supported in part by a grant from the Susan G. Komen Foundation (Dallas, TX) to P.G.C. This work was supported by grant DAMD17-94-J-41089 from the USAMRMC to P.G.C. and American Cancer Society grant CB-164 to G.L.N.

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Breast Cancer Research and Treatment (Accepted with Revision)

Transferrin receptor overexpression enhances transferrin responsiveness and the metastatic capability of a rat mammary adenocarcinoma cell line.

Philip G. Cavanaugh[1] , LiBin Jia, and Garth L. Nicolson

Running Title: Tumor cell transferrin responsiveness in metastasis.

The Department of Tumor Biology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030 (P.G.C.); National Human Genome Research Institute. NIH, MD 20892 (L.J.); and The Institute for Molecular Medicine, 15162 Triton Lane, Huntington Beach, CA, CA 92649 (G.L.N.).

[1]To whom requests for reprints and correspondence should be addressed at: The Department of Tumor Biology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Phone: (713) 792-8965, Fax: (713) 794-0209.

Summary
We previously found that breast cancer cell transferrin receptor expression and proliferative response to transferrin often correlated with metastatic capability. To further explore this, we transfected mammary tumor cells with a cDNA coding for the transferrin receptor and examined the effects of its overexpression on various cellular properties. A human transferrin receptor expression plasmid made by excising the cDNA for the receptor from pcDTR1 and ligating it into the multiple cloning site of pcDNA1Neo. The resulting construct was transfected into the poorly metastatic rat MTLn2 line that expresses low endogenous levels of rat transferrin receptor and transfection-induced receptor expression was ascertained using antibodies specific for the human protein. Approximately 50% of the initial Geneticin-resistant transfected MTLn2 cells overexpressed human transferrin receptor protein. High expressors were further isolated by four sequential FACs sorts. The final cell population expressed ≈ 3 to 7 times more cell surface transferrin receptor than did vector transfected controls. Both lines proliferated at the same rate in normal (media plus 5% FBS) culture conditions. However, in serum-free conditions, the transferrin receptor overexpressor cells displayed a pronounced proliferative response to transferrin whereas the control line did not. When injected into the mammary fat pads of female nude mice, cells from both lines formed micrometastases to the lung that were specifically visualized by immunohistochemical staining of rat cytokeratin 17. This revealed that the transferrin receptor transfected line formed larger lesions of this nature more frequently than did cells from the vector transfected controls. Overexpression of cell surface human transferrin receptor on MTLn2 cells appeared to affect their *in vitro* growth response to transferrin and their ability to form metastases *in vivo.*
Key Words: Transferrin, Transferrin Receptor, Metastasis, Breast neoplasms.

Introduction
Tumor cell responses to factors that regulate growth (1-3), motility (2-5), adhesion (2, 3, 6) , and other cellular responses (2, 3) in the normal organ environment have been shown to be involved in successful metastasis to particular organ sites. In exploring one aspect of target-organ environmental effect on tumor cells, we examined the ability of media conditioned by viable organ fragments to influence the *in vitro* proliferation of cells that either possess or lack a propensity to metastasize to that organ site (7-10). In this regard, we found that lung-conditioned medium caused preferential growth of certain lung-metastasizing tumor cells when compared to cells that metastasize poorly to that location (7-10). In particular, the lung-metastasizing MTLn3 derivative of the 13762NF rat mammary adenocarcinoma responded by proliferating upon exposure to rat lung-conditioned-medium, whereas nonmetastatic MTPa and MTC cells derived from the same tumor system did not (9, 10). Traditional biochemical means were utilized to purify the MTLn3 mitogen from the lung-conditioned media, and this proliferation-enhancing factor was eventually identified as the iron transport protein transferrin (9-11). Others have also observed that tumor cell proliferative response to transferrin correlates with metastatic capability and that transferrin responsiveness may be involved in secondary site-specific tumor cell growth (12-14).

Transferrin exerts a proliferative effect on cells in culture by supplying iron for key synthesis processes required for cell growth. Transferrin binds two atoms of iron, one at each of two sites located at the amino terminal and carboxy terminal domains of the protein. Iron replete transferrin interacts with a cell surface transferrin receptor (TfR), a homodimeric disulfide-linked glycoprotein of $M_r \approx 190,000$. The receptor-ligand complex is internalized in structures that mature into acidic endosomes; the low pH of which causes the release of the bound iron from the complex (15 ,16). Iron is then translocated to needed areas such as sites of synthesis of mitochondrial electron transport proteins and ribonucleotide reductase (15, 16). The latter enzyme consists of two non-identical R1 and R2 subunits; the iron saturated state of R2 is required for activity. Maintenance of the function of this enzyme is essential for the synthesis of DNA and the proliferation of cells (15-17).

Observation of a preferential response to transferrin in certain cell lines of the rat mammary adenocarcinoma system led us to study the TfR expression of those same cells. Scatchard analysis of transferrin binding by various 13762NF sublines revealed that TfR expression also correlated with metastatic capability (18). Others found also that TfR expression in neoplasms associates with tumor stage, progression, or predicted survival. In a series of immunohistological studies, a correlation of this nature was seen in melanomas (19, 20), breast carcinomas (21, 22), bladder cell transitional cell carcinomas (23), a maxillary cancer (24), and in nonsmall-cell lung cancer (25). The indicated connection between metastatic capability, transferrin responsiveness, and TfR expression in certain tumor cells led us to examine whether or not we could affect the metastatic capability of one of these cell lines by altering these characteristics. In this regard, we found that a highly transferrin-responsive subline culled from the minimally transferrin-responsive, poorly metastatic rat MTLn2 13762NF mammary adenocarcinoma subline did indeed express higher TfR numbers and exhibited an enhanced metastatic capability in syngeneic Fischer 344 rats (26, 27). These observations influenced us to pursue a more direct approach to altering tumor cell TfR expression, one which would not involve prolonged *in vitro* selection processes wherein other cell properties might lso be selected. The transfection of a cell line with a plasmid encoding the sense TfR cDNA was a logical approach to this; it was our hope that the only cell trait altered would be TfR expression. A human system was the model of choice because the only complete TfR cDNA available was that encoding the human transferrin receptor (hTfR). A system that displayed clear-cut evidence of successful plasmid-induced hTfR expression was desired. Using a non human cell line as a recipient for transfection, specific detection of expressed protein could be performed using antibodies particular for the human receptor. In our hands, the MTLn2 cell line expressed rather low amounts of rat TfR protein, exhibited a meager proliferative response to transferrin, and displayed a poor metastatic capability in syngeneic rats. Thus, the line was a convenient indicator of plasmid effectiveness and one which was advantageous for examining the effects of TfR over expression.

In this study, we report the construction of a eukaryotic expression plasmid containing the human hTfR cDNA. We found that transfection of human MTLn2 mammary adenocarcinoma cells with this construct was able to increase cell surface hTfR expression. When compared with vector-transfected controls, the hTfR-transfected cells were observed to proliferate more rapidly in response to transferrin serum-free conditions. This observation led us to perform spontaneous metastases assays in nude mice, and we found that the hTfR-transfected line was also able to form greater numbers of metastatic lesions than were vector-transfected control cells.

Materials and methods:

Cells and Cell Culture

The rat 13762NF mammary adenocarcinoma tumor was originally induced in Fischer 344 rats by dietary administration of 7,12-dimethlybenz[a]-anthracene; the MTLn2 line was originally derived from a lung metastasis arising from a 13762NF tumor growing in the mammary fat pad (28). The line displayed little spontaneous or experimental metastatic ability in syngeneic F344 rats (28, 29). The MTLn2 line was maintained at 37°C in a humidified 95% air and 5% CO_2 atmosphere in α-MEM containing 5% (v/v) fetal bovine serum (FBS, Hyclone, Logan, UT). The line was passaged by removal from plates using phosphate buffered saline (PBS) containing 0.25% trypsin (Gibco/BRL, Bethesda, MD) and 2 mM EDTA and was routinely examined and found to be free of mycoplasma.

hTfR expression vector construction

The pcDTR1 plasmid containing the entire coding region of human transferrin receptor (hTfR) was obtained from Dr. Lukas Kuhn of the Swiss Institute for Experimental Cancer Research (Epalinges, Switzerland). The pcDTR1 plasmid was cut with Eco*RV* and Xba*I*, and the 2.3-kb fragment containing the hTfR coding region (minus the 3' untranslated region) was isolated by agarose gel electrophoresis and electroelution. The pcDNA1Neo eukaryotic expression plasmid (Invitrogen, San Diego, CA) was treated with the same two enzymes and the 7.4-kb linearized plasmid likewise isolated. The hTfR fragment was ligated into pcDNA1Neo by conventional procedures by incubation with T4 DNA ligase for 12 h at 16°C. Bacteria were transformed with the ligation product, and plasmid DNA minipreps obtained from ampicillin resistant colonies were analyzed for their ability to release a 2.3 kb fragment upon digestion with Eco*RV* and Xba*I*. One colony expressing this plasmid was expanded, and plasmid DNA for use in transfection procedures was isolated using a Qiagen maxi-kit (Qiagen, Chatsworth, CA).

Transfection procedure

MTLn2 cells were grown to 40 to 50 % confluence in six-well culture plates. Cells were transfected for 8 h using 0.2 to 0.5 ng of plasmid DNA per well in serum-free media containing 5% v/v Lipofectamine (Gibco/BRL). Lipofectamine-media-DNA mixtures were made following the manufacturer's suggested protocol. Media was replaced with that containing 5% FBS for 24 h and then with media containing 5% FBS and 400 μg/ml G418 (Geneticin, Gibco/BRL). Cells resistant to G418 were passaged and maintained in media containing 5% FBS and 400 μg/ml G418.

Immunofluorescent detection of cell surface TfR

Cells grown on LabTek (Nunc, Naperville, IL) slides were washed three times with 25 mM HEPES (pH 7.5) buffered Dulbecco's modified minimal essential medium containing 1 mg/ml liquid gelatin (DMEM-lg, Sigma, St. Louis, MO) and equilibrated to 4° C. Phycoerythrin-conjugated anti-human TfR (clone T56/14; Biodesign International, Kennebunkport, ME) or phycoerythrin-conjugated normal mouse IgG (Biodesign International) were diluted 1:100 in 4°C DMEM-lg, added to the cells, and slides were incubated on ice for 2 h. The cells were washed three times with DMEM-lg and examined for fluorescence using a Nikon Diaphot phase-contrast microscope (Melville, NY). The procedure utilized for staining for the rat TfR was identical except that the cells were initially incubated with mouse anti-rat TfR (clone OX-26, BPS, Indianapolis, IN), or normal mouse IgG_1, then with an Fab' fragment of PE-conjugated anti-mouse IgG (Zymed, San Diego, CA).

FACS analyses and sorting

All washes and antibody incubations were performed using DMEM-lg. Cells were removed from culture plates using 0.25% trypsin and 2 mM EDTA (in Ca^{2+}, Mg^{2+}-free PBS). As soon as detachment was complete, FBS was added to 1% (v/v) to neutralize the trypsin. Cells were washed twice by centrifugation and resuspension in DMEM-lg and counted, cell density was adjusted to 1×10^6/ml, and the cell suspensions were equilibrated to 4°C. Phycoerythrin-conjugated normal mouse IgG or anti-hTfR was added to the cell suspensions to a final dilution of 1:100. The mixture was incubated at 4°C for 2 h, washed twice with DMEM-lg, and analyzed for fluorescence using a Becton-Dickinson FACscan instrument (San Jose, CA). Identical procedures were used for sorting experiments with the exception that cells were analyzed, and those cells exhibiting the highest 5% fluorescence in the anti-TfR group were sorted using a Becton-Dickinson FACstar instrument.

Affinity Isolation of TfR using Immobilized transferrin

This method was adapted from TfR isolation procedures reported by others (30, 31) and was performed as previously described (27). Human transferrin (apo form) was immobilized onto cyanogen-bromide activated agarose, and iron saturated by exposure to ferric ammonium citrate. Cells were surface biotinylated at 4°C using NHS-LC-biotin, and lysed, and equal amounts of lysate protein were added to an excess of the transferrin-agarose. The suspensions were mixed for 2 h at 4°C. The agarose was collected, and washed, and bound material was removed with SDS-PAGE treatment solution. Released components were separated by SDS-PAGE, and blotted onto a polyvinylidene difluoride (PVDF) membrane, and biotinylated bands were detected by incubation with streptavidin-conjugated horseradish peroxidase (HRP) followed by an enhanced chemiluminescence

(ECL) HRP substrate (DuPont, Wilmington, DE). Bands were quantitated using a Hoeffer model GS300 scanning densitometer (San Francisco, CA).

Scatchard analysis of cell surface TfR
This was performed as previously described (27). Human holo-transferrin was radioiodinated using the chloramine-T method. Cells at 50 to 60% confluency growing in 12-well plates were rendered transferrin deficient, equilibrated to 4°C, and treated with increasing concentrations (five wells per concentration) of ^{125}I-transferrin. Two wells of each dose also received a 200-fold excess of unlabeled transferrin. Cells were incubated at 4°C for 2 h, washed, and lysed, and lysate radioactivity was determined. Specific bound radioactivity was calculated and data was plotted.

Rhodamine-transferrin uptake
Cells grown on multichambered LabTek slides to 40 to 50 % confluence were washed twice for 1 h each in α-MEM only. Media was changed to DMEM-lg and rhodamine-conjugated human transferrin was added to a final concentration of 0.5 µg/ml. Negative-control wells also received un-conjugated transferrin at a concentration of 20 µg/ml. Two hours later, wells were washed three times with DMEM-lg, and equilibrated to 4°C, and cells were examined for fluorescence.

Cell growth assays
Cells were made transferrin deficient by placement in two 24h changes of serum-free α-MEM. Cells were removed from stock plates and seeded in 100 µL of α-MEM containing 2 mg/ml bovine serum albumin (BSA) at a density of 2,000 cells/well in 96-well plates. One day later, cells were washed twice with and the medium was changed to 100 µL DME:F12 (1:1, v:v)containing no FBS, and increasing amounts of holo-human transferrin were added into test wells. Five days later, the cells were quantitated using a crystal violet stain assay (27): Cells were washed with PBS, fixed at 25°C for 30 min with PBS containing 5% v/v glutaraldehyde, washed with water, allowed to dry, and stained for 30 min at 25°C with 50 µL of 0.1% crystal violet in 50 mM 3-[cyclohexylamino]-1-propansulfonic acid, pH 9.5. Stained cells were washed with water and solubilized with 50 µL of 10% acetic acid. Cell numbers were determined by measuring absorbance at 590 nm on a Dynatech (Chantilly, VA) model MR5000 plate reader. Absorbance in this system directly correlates with cell number up to ≈ 50,000 cells/well (10, 11, 32).

Spontaneous metastasis assays
All experimentation involving animals has been approved by the Institutional Animal Care and Use Committee. All animals are treated and housed under conditions specified by the NIH, the Department of Health and Human Services, and the Department of Agriculture. Cells at 50 to 70% confluence were removed from culture plates with Ca^{2+} and Mg^{2+}- free Hank's basic salt solution (CMFH) containing 1 mM EDTA and 0.25% trypsin. Cells were washed three times with CMFH, cell density was determined using a model ZM Coulter Counter and adjusted to 5 X 10^6/ml with CMFH. Female nu/nu mice (6 to 8 weeks old) were anesthetized with Metofane (Methoxyflurane; Pittman-Moore, Washington Crossing, NJ). A small incision was made ≈ 1.0 cm posterior to the left foreleg, and 0.1 ml of the cell suspension was injected into the left mammary fat pad lateral to the rib cage ≈ 0.5 cm posterior to the fore eg (33, 34). The incision was closed with a wound clip. Four to six weeks later, the mice were killed with an overdose of Metofane and examined for the presence of metastatic lesions.

Immunohistochemical staining for cytokeratin-17
Lungs were removed from nude mice, fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Blocks were sectioned at 5 µM thickness and the resulting slides stored at 25°C. Sections were deparaffinized in two changes of xylene, equilibrated in two changes of absolute ethanol, treated with 10% H_2O_2 (in methanol) for 30 min, placed in 10 mM citric acid (pH 6.0), and heated for 5 min in a microwave oven. Sections were washed in PBS and incubated in a block solution (PBS containing 5% v/v goat serum and 2 mg/ml liquid gelatin) for 30 min. All subsequent antibody incubations were performed in block solution, and washes in PBS occurred between all steps. Slides were incubated overnight at 4°C in 1:100 normal mouse IgG_1 or anti-cytokeratin-17 (Chemicon, Temecula, CA), for 2 h at 25°C in 1:100 biotin-conjugated anti-mouse IgG (Sigma), and for 1 h at 25°C in 1:100 streptavidin-HRP (Sigma). Slides were incubated in 3,3'-diaminobenzidine substrate (Sigma) for 5 min, washed, equilibrated in absolute ethanol followed by xylene, coverslipped, and

examined by phase contrast microscopy. The sizes and frequencies of metastases seen in each line were compared using the Mann-Whitney test.

Results

MTLn2 cells transfected with either the pcDNA1Neo vector (MTLn2/Neo cells) or the pcDNA1Neo/hTfR plasmid (MTLn2/hTfR cells) formed confluent wells of G418- resistant cells. Therefore, no initial cloning or colony selection was required; analysis of hTfR expression and subsequent FACS sorting was performed on uncloned initial transfected populations. Immunofluorescent analysis of these by FACS indicated that the vector transfected cells expressed no detectable hTfR protein (Figure 1). In contrast, approximately 50% of the hTfR transfected cell population displayed fluorescence over the isotype-stained control, indicating hTfR expression and successful transfection (Figure 1). Those MTLn2/hTfR cells exhibiting the highest 5% fluorescence were sorted, recultured, likewise stained, and sorted again. This process was repeated four times, and during the sorting process, the mean hTfR expression was seen to increase slightly (Figure 1). Upon re-culturing, a downward drift of hTfR levels from those seen at sorting as well as a return to heterogenous TfR expression occurred, thus a focused population of high expressors could not be maintained. However, the slight increase in mean hTfR expression obtained with sorting could be maintained over many culture passages. The heterogeneity in hTfR expression observed in MTLn2/hTfR cells was not caused by analyzing cells in various phases of the cell cycle, as synchronization of the cells in the G1-S phase by aphidicolin had no effect on the FACS signal seen (data not shown). Cells from the fourth sort were used for subsequent experiments; these cells were not cloned and represented a mixed population of high hTfR expressors culled by sorting only from the original transfected population.

Fluorescent microscopic examination of unfixed MTLn2/hTfR cells stained at 4°C with PE-anti-hTfR revealed cell surface fluorescence of varying intensity in nearly every cell, with no visible fluorescence observed in like-treated MTLn2/Neo cells (Figure 2). As with FACS analysis, this difference in hTfR expression was stable over many (5-10) passages in cultures maintained in G418 containing media (data not shown). Both lines stained positive for and maintained low levels of rat TfR (Figure 2).

Analysis of cell surface TfR content in these cell lines by affinity isolation of biotinylated TfR revealed that MTLn2/hTfR cells expressed six fold more total TfR than did MTLn2/Neo cells (Figure 3A). Using this method, no difference in the apparent M_r of rat and recombinant hTfR was seen. Using a similar procedure, Van Driel *et al.* (30) have reported a slightly higher M_r for the Rat TfR than for that of human. Our results may have been due to altered glycosylation of human TfR in a rat cell line, and/or by increased M_r caused by biotinylation. This analysis was performed using reducing SDS-PAGE as we have observed greater signal using these conditions. In addition to TfR, two biotinylated transferrin binding proteins (marked by an * , Figure 3A) of unknown nature were seen.

Scatchard analysis of ^{125}I-transferrin binding to MTLn2/Neo or MTLn2/hTfR cultured cells at 4°C showed a three fold greater specific binding for the MTLn2/hTfR line, again indicating greater numbers of TfRs on these cells (Figure 3B). The reported K_d for transferrin binding to the hTfR is 1-40 nM (18,35). Here, a lower affinity than this was observed for human transferrin binding to the rat TfR on MTLn2/Neo cells (Figure 3B), however, this may have been caused by inherent decreased affinity between the rat receptor and human ligand. An even lower affinity yet was observed human Tf binding to the recombinant human TfR on MTLn2/hTfR cells. The precise cause for this is unknown, although differences in behavior between transfected and native protein may have, in addition to its being placed in a foreign environment, played a role in decreased affinity. Affinity changes of this type have been reported by others when dealing with transfected TfR (35). Causes for the differences in the magnitude of change in cell surface TfR expression between MTLn2/Neo and MTLn2/hTfR measured by the two assay procedures are unknown. However, lack of absolute precision in quantitation of bands seen by ECL may render the affinity isolation assay less accurate.

Visual fluorescence examination of MTLn2/Neo and MTLn2/TfR cells that were allowed to take up rhodamine conjugated transferrin showed a much stronger fluorescent signal for the MTLn2/hTfR line (Figure 4). This indicated that the plasmid produced TfR in this line was functional and that over the 24h incubation time, more transferrin was internalized by the MTLn2/hTfR line than by the MTLn2/Neo line.

Once demonstration of a remarkable, stable increase in functional TfR expression in the MTLn2/hTfR transfected cells was ensured, attempts were made to measure an increase in that line's ability to proliferate in response to transferrin were made.
In these assays, cells are plated overnight at low density in media containing 1% FBS, media is replaced with that containing no serum, increasing amounts of human holo-transferrin are added, and cell numbers are determined four days after transferrin addition (9, 10, 27). Here , we found significant background growth (in the 0 µg/ml transferrin wells) with the MTLn2/hTfR line. It was thought that this line had become extremely sensitive to transferrin and was proliferating in the serum-free/transferrin-free environment in response to residual transferrin remaining from the 1% FBS plating conditions. Therefore, the assay was repeated with an effort to more thoroughly eliminate serum from the cells. Stock cultures were exposed to two 24 h rounds of serum-free incubations, then subcultured into the assay plate, also in serum-free media. These severe conditions eliminated background proliferation in the MTLn2/hTfR cells, and when human or murine transferrin were added into this system, a proliferative effect was seen for this line, but not for the MTLn2/Neo cells (Figure 5). The results indicated that the MTLn2/hTfR cells had become particularly responsive to transferrin, and that the addition of this protein permitted the proliferation of this line in serum-free conditions. Both lines grew at similar rates in media containing 5% (V/V) FBS (data not shown), therefore, the ability of either to respond to a mixed growth factor milieu as is present in FBS was the same and no particular difference in either line's ability to respond to a complex mixture of those factors was seen.

The finding of an increased proliferative rate in response to transferrin for the MTLn2/hTfR cells in serum- deprived conditions led us to investigate as to whether or not the metastatic properties of the line had changed. Nude mice were chosen as recipient animals since the transfected gene expressed was human. We performed spontaneous metastasis assays, because these are more rigorous than experimental metastasis assays and necessitate complete metastatic competence in the cells being studied. To ensure injection into the proper primary site, the cut-down procedure of Price *et al* .(34) was used. Using this method, cells are injected into a mammary fat pad adjacent to the rib cage, alleviating the frequent formation of questionable intra-peritoneal metastases found when cells were injected into an abdominal mammary fat pad. After either line was injected, it was found that primary tumors of approximately the same size were formed at approximately the same rate (Table 1). The spontaneous metastasis assay was terminated at five weeks as some of the primary tumors approached the maximum size allowable. Necropsy examination revealed no gross metastases in the nude mice injected with either line; therefore, lungs from recipient animals were fixed in formalin, embedded in paraffin, stained with hematoxylin and eosin, and examined for micrometastases by light microscopy. Serial sections from two separate areas of lung tissue were examined. This revealed two small (100 to 200 µm in diameter) micrometastases in MTLn2/Neo injected mice and six larger (2000 to 5000 µm in diameter) like lesions in MTLn2/hTfR treated animals. Immunohistochemical staining of the sections with anti-rat cytokeratin 17 enabled more definitive and sensitive micrometastasis detection. Use of this procedure revealed additional lung tumors; these were enumerated and are shown in table 1. Clusters of three or more cells were counted as micrometastases and micrometastasis diameters were estimated based on a cell diameter of ≈15 µM. The data reveals that the MTLn2/hTfR line was the more metastatic of the two, forming increased numbers ($P < 0.05$) of metastatic lesions of increased size ($P < 0.05$) and resulting in a total lung tumor burden of approximately seven times that of the MTLn2/Neo line. Representative micrographs of lung micrometastases seen are shown in Figure 6.

Cultures of MTLn2/hTfR micrometastases were established from lung fragments taken from two of the mice injected with this cell line. When analyzed for hTfR expression by immunofluorescence, these cells stained positive with an intensity equal to that of the original injected MTLn2/hTfR cells (data not shown), indicating that hTfR expression was maintained throughout *in vivo* growth and metastasis formation.

Discussion

We previously found a correlation between tumor cell TfR-dependent proliferative response to transferrin and the metastatic behavior of certain cell lines. Others have reported on the augmentation of metastatic behavior of minimally metastatic cells by the transfection of those cells

with a plasmid carrying the cDNA for a purported metastasis-associated factor (36-43). Likewise, we wished to add credence to our previous observations by modifying and increasing the metastatic capability of one or more poorly metastatic tumor cell types by transfecting them with a plasmid carrying the gene encoding the TfR.

The initial step in assessing the performance of the hTfR plasmid construct we made was to examine its ability to cause increased human TfR expression in a test line. The MTLn2 line was chosen for this, because we found it to express no immuno-detectable human TfR in typical culture conditions. Therefore, it afforded a system where the presence of transfection-dependent protein could be made in the absence of a background signal. Also, since this was previously defined as a low-transferrin-responsive, poorly metastatic rat breast cancer line (26, 27), we viewed it as one where the effects of TfR over-expression might be more clearly elucidated. We were surprised to find that the MTLn2/hTfR transfected cells had acquired the ability to proliferate in response to transferrin in severely serum-starved conditions. This gave us the incentive to perform metastasis studies in nude mice, where we found that the MTLn2/hTfR transfectants appeared to gain competence in forming lung micrometastases.

That the cells used in the present study targeted the lungs was probably due to the previously established behavior of this line. Increased TfR expression most likely did not bring about lung targeting but may have caused increased growth of the metastatic lesions at that site, as high levels of Tf are reported to occur in perfused adult lung tissue (12, 44). Presumably, the cells were responding to Tf via the TfR and proliferating through typical iron transport function mechanisms. However, a number of studies have indicated that Tf or signals elicited through the TfR can evoke specific cellular responses which are not associated with traditional iron transport activities. Some of these responses include: the ability to induce tyrosine phosphorylation and activate protein kinase C in T cells (45, 46), to stimulate endothelial cell migration (47), to stimulate the adhesion and migration of chick mesoderm explants (48), and to facilitate the *in vitro* adhesion of prostate cancer cells (49). There is a possibility that increased TfR expression and Tf binding capability may have contributed one or more of these types of activities to our transfected cells, which may be partially responsible for their increased metastatic behavior.

For these studies, a procedure to accurately assess micrometastases presence using a technique more sensitive than hematoxylin-and-eosin staining was researched, as the MTLn2/Neo line formed so few of these lesions. The obvious choice to immunohistochemically stain lung sections for rat and human TfR was considered, but this would have entailed the use of frozen sections and antibodies of unknown cross-reactivity to mouse TfR. An anti-rat and human cytokeratin-17 antibody was found that could specifically stain the rat tumor cells used, with minimal or no staining of murine lung cells. The capability of this antibody to perform in formalin-fixed, paraffin-embedded tissues further supported its use as the reagent of choice for micrometastasis detection. Metastases enumerated with this reagent clearly indicated an increased metastatic ability for the MTLn2/hTfR line.

As mentioned earlier, a proposed role for transferrin responsiveness and transferrin receptor expression in metastasis has been made by many other investigators (12-14, 19 - 24). However, to our knowledge, no others have reported on the manipulation of TfR expression or transferrin response in an attempt to influence the metastatic performance of a certain tumor cell type. Sasaki *et al.* (50) have reported that the transfection of a human hepatoma cell line with an antisense TfR construct can reduce cell surface TfR numbers; however, no mention was made of any effect this had on tumorigenicity or metastatic capability of those cells. The TfR construct we used lacks the 3' untranslated region, which contains sites through which TfR expression is regulated via recognition by iron regulatory site binding proteins (51,52). Others have observed that transfection with a similar plasmid produces cells with increased sensitivity towards H_2O_2 mediated DNA damage (51). However, we did not observe any obvious increase in the fragility of our transfected cells, which indicates that this type response was not apparent.

The assessment of the metastatic capability of a rat tumor line in nude mice is somewhat unusual, but the experiment's design was necessitated by the presence of a human protein on the cell surfaces. However, this type of experiment has been performed occasionally, an example being the testing in

nude mice of the metastatic capability of a rat mammary adenocarcinoma line transfected with the human epidermal growth factor receptor (53).

One aspect of these findings that requires further study is the mechanism of tumor cell response to transferrin; We would like to discover whether or not this is through traditional iron delivery-dependent mechanisms or through more exotic TfR-mediated signal transduction events similar to those seen in T cells (45). A clearer understanding of this response should increase our knowledge of metastatic behavior. These results confirmed previous observations and indicated that breast tumor cell expression of TfR may be an important characteristic for metastasis formation, at least in some tumor-cell systems. The results suggest that continued exploration of therapeutic strategies that interfere with transferrin binding or iron uptake by tumor cells (54,55) are warranted.

Acknowledgments
Supported by grant DAMD17-94-J-41089 from the USAMRMC to P.G.C. and grant R35-CA44352 from the NCI-NIH to G.L.N. The authors would like to thank Dr. Nguyen T. Van of the M. D. Anderson FACS core facility for his assistance in performing FACS analysis and sorting; to Jacqueline Furr of the M. D. Anderson histopathology core facility for her assistance in preparing tissue sections, and to Dr. Jae Ro of the of the M. D. Anderson department of Pathology for his assistance in examining slides for micrometastases.

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Table 1. Results of spontaneous metastases assays.

Mouse #	***1° Tumor Volume (cm³):***	***Total # of Micrometastases***	***Diameter of Micrometastases (μM)***
MTLn2/Neo			
1	1.52	0	
2	0.48	3	200, 200, 300
3	1.26	2	40, 300
4	1.26	1	100
5	2.85	1	100
6	2.45	2	300, 300
7	0.76	2	400, 400
8	0.10	0	
9	1.02	0	
(total)		11	2940
average	0.77	1.22	327
MTLn2/TfR			
1	0.34	1	200
2	0.34	1	100
3	1.13	1	40
4	0.72	2	2500, 4500
5	0.12	5	20, 400, 1600, 2500,2500
6	0.53	0	
7	0.94	3	200, 200, 400
8	0.53	2	120, 1200
9	0.53	3	200, 400, 600
10	1.41	2	1200, 2500
(total)		20	21380
average	0.66	2.00	2138

The left mammary fat pads of female nude mice were injected with 5 X 10^5 MTLn2/Neo or MTLn2/TfR cells. Five weeks later, mice were killed and the lungs were fixed in formalin, embedded in paraffin, and sectioned. Lung sections were stained immunohistochemically using an anti-rat cytokeratin-17 antibody and specifically stained rat tumor micrometastases were enumerated. The results indicate relatively few metastases for either group but that larger, more frequent micrometastases were formed by the MTLn2/TfR cells than by the MTLn2/Neo cells.

Figure Legends

Figure 1. Immunofluorescent analysis of TfR expression on MTLn2/Neo and MTLn2/hTfR cells by FACS. Cells were removed from plates, stained at 4°C with either a PE-conjugated anti-human TfR or PE-conjugated mouse IgG_1, and analyzed. **A:** MTLn2/Neo cells treated with PE-conjugated mouse IgG_1 (MTLn2/hTfR cells displayed an identical isotype control signal; data not shown); **B:** MTLn2/Neo cells stained with PE- conjugated anti-human TfR; **C:** MTLn2/hTfR cells stained with PE-conjugated anti-human TfR. Approximately 50% of the MTLn2/hTfR transfected population displayed immunodetectable human TfR. **D:** An increase in TfR expression was evident as sorting progressed from the first sort (MTLn2/hTfR FACS1) to the fourth sort (MTLn2/hTfR FACS4).

Figure 2. Representative fluorescent micrographs from MTLn2/Neo and MTLn2/hTfR cells stained unfixed at 4°C with PE-conjugated anti-human TfR or with anti-rat TfR followed by PE-conjugated anti-mouse IgG. **A:** MTLn2/Neo cells as seen with visible light; **B:** UV-light-illuminated photograph of cells in A stained with anti-rat TfR (MTLn2/hTfR cells appeared identical to A and B when like-treated); **C:** UV-light-illuminated photograph of MTLn2/Neo cells stained with anti-human TfR; **D:** UV-light-illuminated photograph of MTLn2/hTfR cells stained with anti-human TfR. When either cell line was treated with PE-conjugated normal mouse IgG_1, no fluorescent signal was obtained (data not shown).

Figure 3. **A:** Results from measurement of cell surface TfR on MTLn2/Neo or MTLn2/hTfR cells by affinity isolation of biotinylated TfR. Cells were made transferrin deficient, surface biotinylated at 4°C, lysed, and equal lysate cell equivalents were incubated with an excess of transferrin-agarose. The agarose was washed, bound material was removed, separated by SDS-PAGE, blotted, and biotinylated bands were detected by incubation with streptavidin-HRP and ECL. The ratio of MTLn2/hTfR : MTLn2/Neo TfR was 6.3:1. **B:** Scatchard analysis of ^{125}I-transferrin binding to MTLn2/Neo and MTLn2/hTfR cells. Cells were grown on 12-well plates, made transferrin deficient, and incubated at 4°C with increasing levels of ^{125}I-transferrin. Negative control wells also received a 200-fold excess of unlabeled transferrin. Cells were incubated for 2 h at 4° C, washed, and lysed, and radioactivity was determined. Points represent the mean ± SD of three wells corrected for nonspecific binding.

Figure 4. Representative fluorescent micrographs of MTLn2/Neo and MTLn2/TfR cells allowed to internalize rhodamine-conjugated transferrin. Cells were grown on multichambered slides, made transferrin -deficient, incubated at 37°C for 24 h with 0.5 µg/ml rhodamine-conjugated transferrin, washed, and analyzed by fluorescent microscopy. **A:** Results from the MTLn2/Neo line; **B:** Results from the MTLn2/hTfR line. No fluorescence was seen when a 50-fold excess of unconjugated transferrin was included (data not shown).

Figure 5. Results of the analysis of the proliferative response of MTLn2/Neo and MTLn2/hTfR cells to transferrin. Cells were plated at 2,000 cells/well in 96-well plates in serum-free media. One day later, cells were washed, and serum-free media with increasing levels of either human or murine transferrin was placed into wells. Five days later, cells were quantitated using a crystal violet stain assay. Points represent the mean ± SD from four wells.

Figure 6. Representative micrographs of MTLn2/Neo and MTLn2/hTfR tumors growing in nude mice. **A:** Section of an MTLn2/Neo primary mammary fat pad tumor, the center of which is indicated by the arrow. **B:** A typical MTLn2/Neo lung micrometastasis, indicated by the arrow; **C:** A typical MTLn2/hTfR lung micrometastasis; arrows indicate the borders of the tumor. **D:** An MTLn2/Neo lung micrometastasis stained with anti-cytokeratin-17, indicated by the arrow.

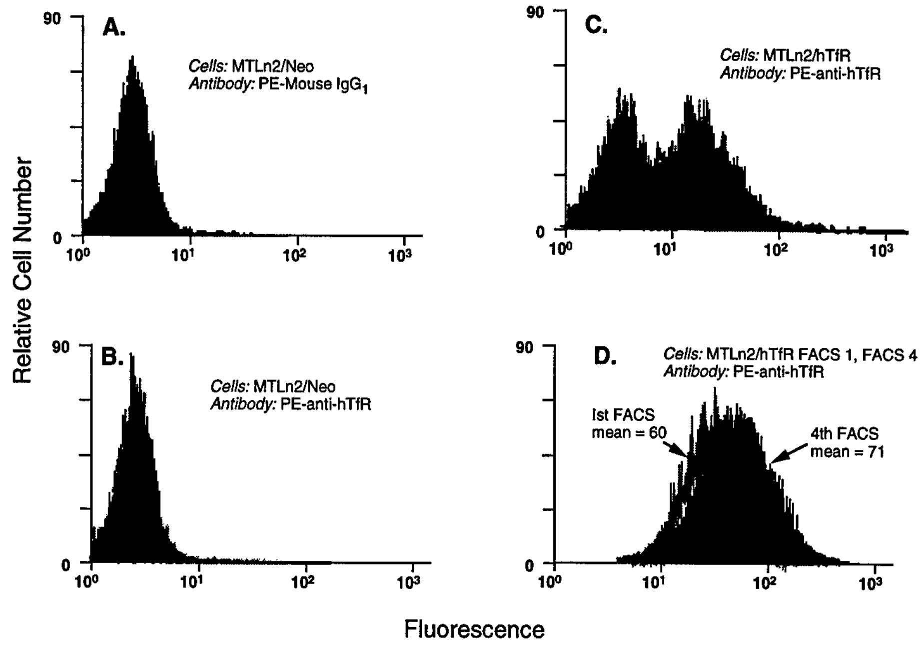

Figure 1

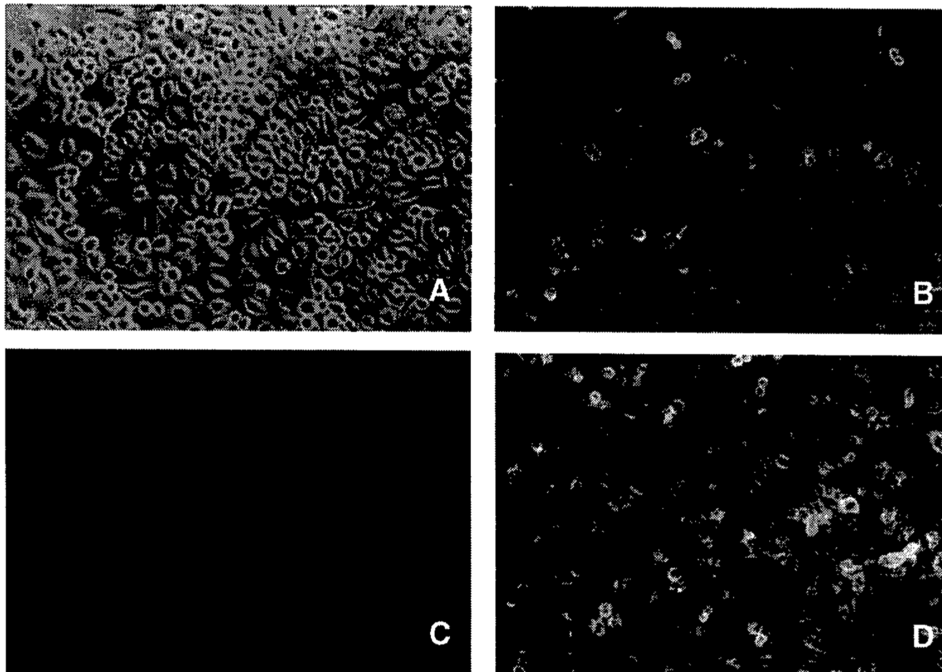

Figure 2

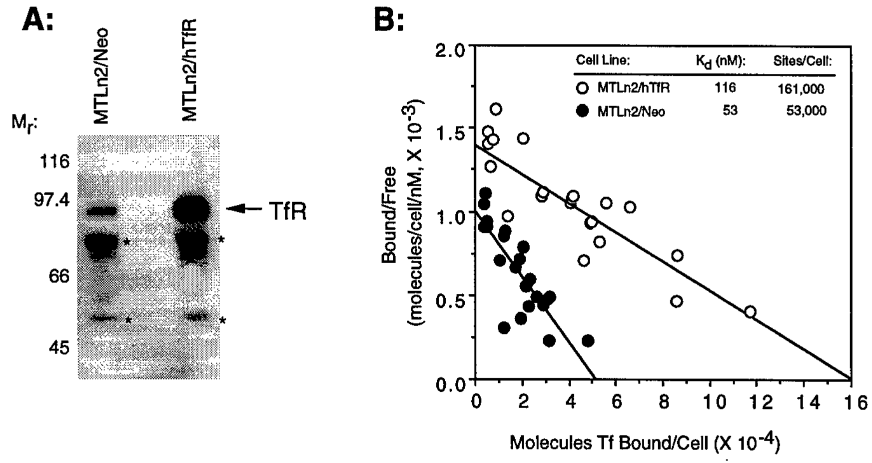

Figure 3

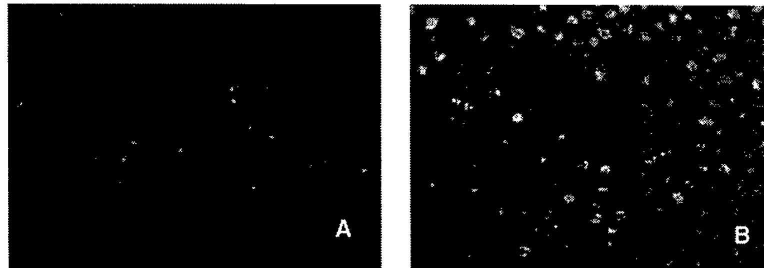

Figure 4

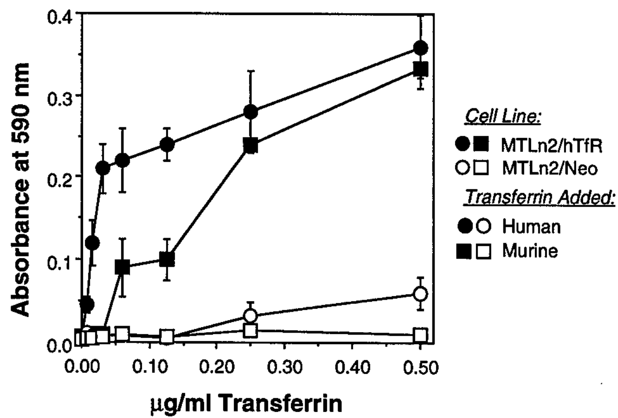

Figure 5

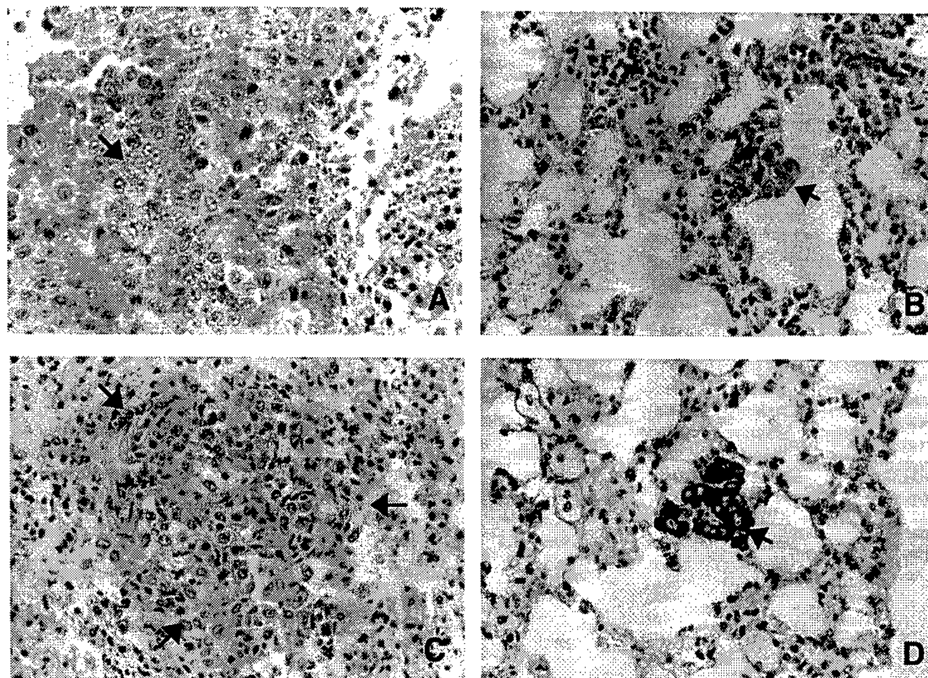

Figure 6