AD_____

Award Number: DAMD17-98-1-8237

TITLE: Targeting of the Hepatocyte Growth Factor Pathway for the Treatment of Breast Cancer

PRINCIPAL INVESTIGATOR: Jill M. Siegfried, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, Pennsylvania 15260

REPORT DATE: September 2000

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2000	Annual (1 Sep 99 - 31 Aug 00)

4. TITLE AND SUBTITLE
Targeting of the Hepatocyte Growth Factor Pathway for the Treatment of Breast Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8237

6. AUTHOR(S)
Jill M. Siegfried, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pittsburgh
Pittsburgh, Pennsylvania 15260

E-MAIL:
siegfrie@server.pharm.pitt.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 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 Words)*

Hepatocyte growth factor (HCF), also known as Scatter Factor, induces cell growth and cell movement, and is known to promote invasiveness of malignant cells. It also promotes angiogenesis. HGF is known to be produced by fibroblasts within breast tumors, while its receptor, the c-Met protein, is expressed on the breast tumor cells themselves. HGF thus appears to act predominantly as a paracrine factor in breast cancer. High levels of HGF expression within breast tumors correlates with an aggressive tumor phenotype, and HGF has been found to be a powerful negative prognostic indicator for breast cancer. Expression of the c-Met protein by breast tumors in culture also correlates with an estrogen receptor negative phenotype and with loss of estrogen-dependent cell growth. Thus the HGF-c-Met ligand-receptor system may be important in controlling cell growth in breast tumors that have escaped estrogen regulation, a common occurrence in breast cancer patients who have lost responsiveness to anti-estrogen therapy. The hypothesis to be tested in this Idea Grant is that interruption of the HGF-c-Met signaling pathway will inhibit the growth of estrogen-independent human breast cancer cells and could be a useful therapeutic strategy for breast cancer patients who have failed endocrine therapy. We will use two approaches for these studies: (1) an anti-sense strategy that uses vectors constructed in the U6 RNA expression plasmid and delivered by cationic liposomes and (2) a recombinant HGF antagonist molecule (truncated HGF/tHGF) produced in baculovirus and delivered through injection to the peritumoral area.

14. SUBJECT TERMS
Breast Cancer, HGF, c-Met, anti-sense DNA

15. NUMBER OF PAGES
23

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

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

20010305 017

Table of Contents

Introduction

Hepatocyte growth factor (HGF), also known as Scatter Factor, induces cell growth (1) and cell movement (2), and is known to promote invasiveness of malignant cells (3). It also promotes angiogenesis (4). HGF is known to be produced by fibroblasts within breast tumors (5), while its receptor, the c-Met protein, is expressed on the breast tumor cells themselves (6). HGF thus appears to act predominantly as a paracrine factor in breast cancer (7). High levels of HGF expression within breast tumors correlates with an aggressive tumor phenotype (8), and HGF has been found to be a powerful negative prognostic indicator for breast cancer (9). Expression of the c-Met protein by breast tumors in culture also correlates with an estrogen receptor negative phenotype (10,11) and with loss of estrogen-dependent cell growth. Thus the HGF-c-Met ligand-receptor system may be important in controlling cell growth in breast tumors that have escaped estrogen regulation, a common occurrence in breast cancer patients who have lost responsiveness to anti-estrogen therapy. The hypothesis to be tested in this Idea Grant is that interruption of the HGF-c-Met signaling pathway will inhibit the growth of estrogen-independent human breast cancer cells and could be a useful therapeutic strategy for breast cancer patients who have failed endocrine therapy. We will use two approaches for these studies: (1) an antisense strategy that uses vectors constructed in the U6 RNA expression plasmid and delivered by cationic liposomes and (2) a recombinant HGF antagonist molecule (truncated HGF, tHGF) produced in baculovirus and delivered through injection to the peritumoral area. In vitro experiments examining effects of these agents on breast tumor growth will be followed by in vivo experiments using an s.c. model of tumor growth as well as growth in the mammary fat pad.

Body of Report

This research project was scheduled to start September 1, 1998. The month prior to this, the post-doctoral fellow whom we had recruited to work on this project (Ping Liu, Ph.D.), left the laboratory unexpectedly for personal reasons. Thus the first task to be undertaken was to identify another fellow. Unfortunately this took quite some time and a fellow did not join the laboratory until July of 1999. Some work on the project was performed in the meantime by undergraduate students and a technician in the laboratory. However, due to the lack of a dedicated fellow to perform the work, there has been a considerable delay in the progress. Thus, after the second yaer of the project, we are now at the point we hoped to be by the end of the first year. However, as discussed with the Program Officer, we are planning to carry over the salary monies that were not spent in the first year and request a no-cost extension at the end of the grant period. This should allow us to complete the aims by September 1, 2002.

Progress to Date on Statement of Work shown in the next section.

1. Produce purified tHGF from baculovirus-infected insect cells

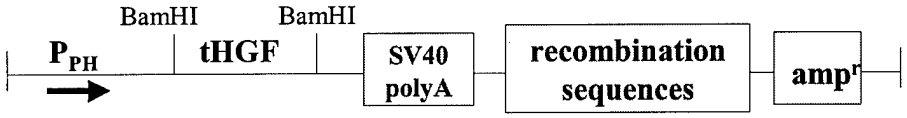
The viral stocks we had previously obtained from our collaborator, Dr. Zarnegar (Ref. 12), were very low titer and we were unable to reconstitute a high-titer viral stock We therefore cloned a new truncated (tHGF) plasmid vector for growth in baculovirus.

Cloning of a truncated HGF (tHGF) plasmid vector: We received a HGF full-length cDNA plasmid (BamHI to DraI) containing nucleotides –26 to +2262 cloned into the pSPORT1 vector (Gibco BRL) from Reza Zarnegar (University of Pittsburgh). To generate the deleted form of HGF, we digested the full-length cDNA with Bgl II which does not cut in the vector but cuts in the cDNA at nucleotides 1204 and 1716. This resulted in two bands, 512 bp and 5788 bp. DNA from the 5788 bp band was gel isolated using the JETSORB Kit (PGC Scientifics). The DNA was then religated back together to generate a truncated form of HGF (tHGF) containing the first 406 amino acids of the chain which includes the hairpin loop, kringles 1-3, and 16 amino acids of kringle 4. The chain is not present due to the creation of a new stop codon at position 1775. The ligated deletion DNA was transformed into XL-1 Blue supercompetent cells (Stratagene) and plated on LB/amp plates. Colonies were grown and DNA was isolated using the Qiagen mini-prep kit. The deletion in this plasmid was verified by restriction analysis.

Construction of a recombinant baculovirus transfer vector containing tHGF: The tHGF plasmid described above was digested with BamHI to remove the HGF sequence from the pSPORT1 backbone vector. This 1.7 kb tHGF fragment was cloned into the BamHI site within the multiple cloning site of pBlueBac4.5 (Invitrogen). pBlueBac4.5 is a baculovirus transfer vector designed to allow expression of a gene of interest, in our case, tHGF, in insect cells. Expression is driven by the late AcMNPV polyhedrin promoter. Since directional cloning was utilized with BamHI sites on each end, proper orientation of tHGF in pBlueBac4.5 was confirmed using restriction enzyme analysis. In addition, the plasmid was sequenced using the polyhedrin forward sequencing primer provided in the MaxBac Kit and an additional primer that we made further 3' in the HGF sequence to ensure that the deletion in HGF was present and that the gene was in the correct orientation for proper expression.

pBlueBac-tHGF

6.6kb



Important features of the pBlueBac-tHGF construct. P_{PH} represents the polyhedrin promoter which allow for efficient, high level expression of the recombinant protein. The BamHI site is part of the multiple cloning site which allowed for insertion of the tHGF gene. The SV40 polyadenylation site allows for increased mRNA stability. ORF1629 and *lac*Z Recombination sequences permits recombination of tHGF with Bac-N-Blue™ linear AcMNPV DNA, restores the essential ORF1629 for production of a viable, recombinant virus, and allows production of blue recombinant plaques for visual selection. The ampicillin resistance gene allows selection in *E. coli.*

Produce purified tHGF from baculovirus-infected insect cells.

Once the tHGF baculovirus plasmid had been constructed, the plasmid was cotransfected into Sf9 insect cells with Bac-N-Blue™ DNA (Invitrogen) by cationic liposome mediated transfection. After 72 hrs, 2mls of transfection supernatant was harvested. This is the transfection viral stock.. Recombinant virus was purified by plaque assay. Sf9 cells were seeded into 100mm dishes and infected with various dilutions of the transfection viral stock for one hour. The cells were overlain with a medium/agarose mix containing X-gal. After nine days, blue plaques began to appear. On day 11, agarose plugs containing blue recombinant plaques were transferred to wells of a 12 well plate seeded with Sf9 cells. On day three after infection, 0.75ml of cells and medium was removed for viral DNA isolation using the EasyDNA™ Kit (Invitrogen). PCR analysis was used to identify pure recombinant plaques from the isolated viral DNA. Six of the 10 plaques picked were identified as pure. Cells were incubated for another nine days before the P-1 viral stocks were harvested.

The next step is to generate a high-titre stock for expression. First, two T25 flasks seeded with Sf9 cells were infected with one of the P-1 viral stocks and incubated for 21 days, which is twice as long as indicated by the protocol. This was how long it took for 80-90% of the cells to be lysed. The medium was isolated and this is called the P2 viral stock, a small-scale, high titre stock. To generate a large scale, high-titre stock, it was necessary to grow up a 500ml suspension

culture of Sf9 cells. To do this, an 80ml starting culture of Sf9 cells at $1x10^6$ cells/ml was initiated in a 250ml spinner flask. As the cells doubled, they were diluted back to $1x10^6$ cells/ml and gradually progressed from a 250ml flask to a 500ml flask, and then to a 1L flask. When the cells were transferred to the 1L flask, they would cease to grow at $1.5x10^6$ cells/ml, hence infection with the P2 viral stock has not been able to be initiated. The procedure was repeated twice with the same result. This growth arrest is probably the result of poor aeration due to the large flask. After consultation with Invitrogen, it was decided to produce the high-titre stock from 2 x 250ml cultures. This is the next step. After the high-titre stock is obtained, a plaque assay will be used to calculate the titre. Once this is done, High 5 insect cells can be infected with the high-titre virus and the truncated protein purified.

2. Produce sense and antisense constructs for c-Met and HGF and verify sequences

This part of the project is completed as described last year.

3. Optimize transfection conditions using breast cancer cells and human fibroblasts using a reporter gene and liposome preparations

As reported last year, we have optimized conditions for transfection of human fibroblasts with the antisense HGF vector. The liposome preparation LipofectACE gave the best results (50-60% transfection efficiency). We have worked during the past year to optimize transfer of the antisense c-Met vector to breast cancer cell lines. Our hypothesis, based on results reported in the literature, is that breast cancers that become estrogen receptor negative will begin expressing the c-Met protein and would be good candidates for therapies targeting c-Met and/or HGF. The figure on the next page shows two ER negative breast cancer cell lines that demonstrate robust expression of the c-Met protein (MDA MB231 and SKBr3). These two cell lines have been used in experiments to optimize transfection efficiency.

Membrane proteins were extracted from four human breast cancer cell lines, two estrogen receptor (-) and two estrogen receptor (+), and one normal lung fibroblast cell line (935NLFB, a negative control). 1 μg of protein was subjected to SDS-PAGE on a 6% Tris-Glycine gel and transferred to nitrocellulose. The transferred proteins were probed using a polyclonal antibody to c-met at a 1:250 dilution. Results were visualized using an anti-rabbit-IgG-HRP antibody at a 1:1000 dilution followed by chemiluminescent detection.

Estrogen Receptor
c-met
+
-
-
+
-
+
+
-
935NLFB
MDA MB231
BT20
MCF7
SkBr3
145 KDa
c-met

We have used the following liposomes to transfer antisense c-Met into the breast cancer cell lines:

To optimize transfection conditions for the two breast cancer cell lines MDA-MB-231 and SkBr-3, several different transfection reagents were chosen and tested at fixed DNA to reagent ratios. The amount of DNA (pEGFP-N1) transfected was kept constant at 2μg/35mm dish. The amount of Lipofectamine and Lipofectace was chosen according to previous optimization experiments. DOTAP and Superfect were also included. The results are shown in Table 1.

Table 1:

Transfection Reagent	DNA:Reagent	SkBr3	MDA-MB-231
Lipofectamine	1:15	40	<10
Lipofectace	1:10	20	40-50
DOTAP	1:06	5	<10
Superfect	1:06	30	60

Superfect gave good results in both cell lines and was chosen for further optimization. Lipofectamine was also included as previous experiments had shown it to give good transfection even though Lipofectamine did not perform very well in MDA-MB-231 in this experiment. Lipofectamine will only be tested at the ratio previously determined to be optimal in these cell lines. For further optimization of Superfect, varying amounts of Superfect was transfected with a fixed amount of DNA (2μg/35mm dish). The results are shown in Table 2.

Table 2:

	MDA-MB-231		**SkBr-3**	
Transfection Reagent	DNA:reagent	% Transfection	DNA:reagent	% Transfection
Lipofectamine	1:15	<10	1:10	30
Superfect	1:03	5	1:03	30
	1:06	10-20	1:06	30-40
	1:09	30	1:09	40-50
	1:15	5	1:15	40
	1:20	<10	1:20	20

Results show that Superfect at a 1:9 ratio of DNA to transfection reagent gave the best efficiency for both cell lines. This ratio was used to transfect 100mm dishes of each cell line with 10μg c-met antisense, c-met sense, and pGEM2U6. Membrane proteins were isolated from the cells 48 hours after transfection and Western Blotting was used to determine whether there was

any down regulation of c-met expression in cells transfected with c-met antisense compared to cells transfected with c-met sense, pGEM2U6 and untransfected cells. The Western results are shown in Table 3. The results are expressed as a percentage of c-met expression in untransfected cells. Results shown a reduction of c-met protein in 128-88T cells transfected with c-met antisense as well as c-met sense. The result from the sense construct was unexpected. Expression of c-met in MDA-MB-231 cells appears to be slightly lower in cells transfected with c-met antisense compared to untransfected cells in two of the experiments, though compared to cells transfected with the backbone plasmid pGEM2U6 there is no reduction. It should also be noted that in the third MDA-MB-231 experiment, 25μg of DNA per transfection was used to see whether increasing the amount of DNA transfected would be more effective in reducing c-met expression. Increasing the amount of DNA transfected had no effect on c-met expression.

Western analysis of the SkBr-3 cell line has proved to be difficult because of very high background. Only one of the SkBr-3 transfection experiments gave interpretable results, this result was more promising than results obtained from MDA-MB-231, with c-met being reduced by 40% with respect to untransfected cells. Transfection of SkBr-3 cells will be repeated to confirm this result.

Table 3:

	c-met Expression			
Cell Line	c-met AS	c-met S	pGEM2U6	No Transfection
128-88T	57	34	90	100
MDA-MB-231	77	104	39	100
	112	84	92	100
	73	65	64	100
SkBr-3	61	101	83	100

Note: DOTAP= N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate
Superfect is an activated dendrimer.

4. Transfer sense and antisense constructs into breast cancer cells and fibroblasts and monitor expression of constructs

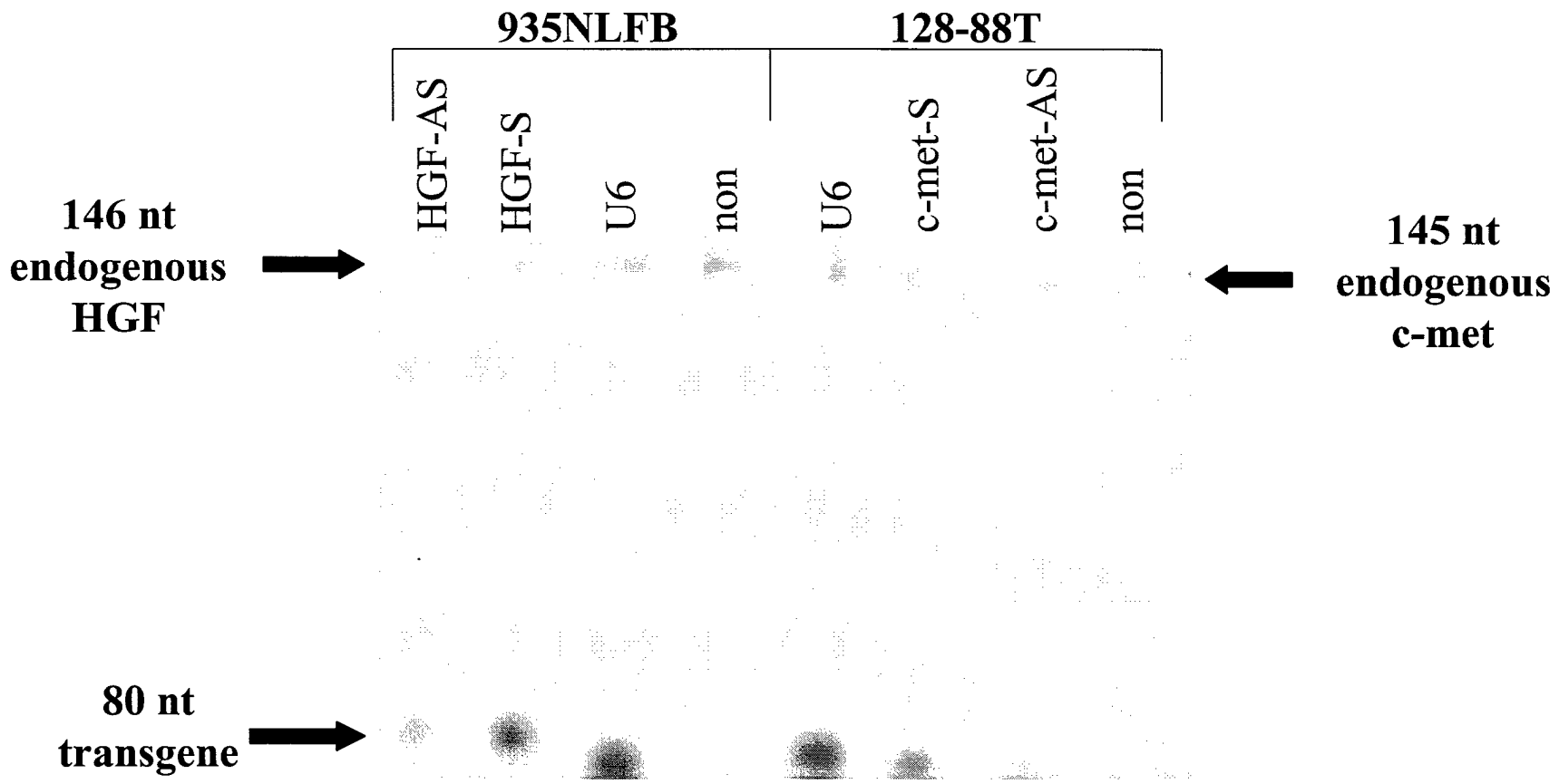
Breast Cancer Cells:

This will be undertaken in the next grant period.

Human Fibroblasts:

RNAse protection was used to distinguish between HGF transgene versus endogenous gene in human fibroblasts. In this assay, we also examined c-Met transgene and endogenous c-Met in a lung tumor cell line, 128-88T. Sense and antisense transgene expression and HGF and c-met endogenous gene expression was demonstrated using a ribonuclease protection assay. 935NLFB and 128-88T cells were transiently transfected with HGF-S, HGF-AS, or U6 and c-met-S, c-met-AS, or U6, respectively. Total RNA was isolated from the cells 48 hr after transfection. 20 μg of total RNA from each treatment was hybridized with 2 x 10^4 cpm of ^{32}P-labeled RNA specific for each treatment. The solution was treated with a mixture of RNase A and T_1. The resulting hybridized products protected from RNase digestion were separated on a 5% denaturing polyacrylamide gel. The probes were designed in such a way that the endogenous probes would not recognize the transgene only the endogenous gene and the transgene probes would only recognize the transgene that contains part of the U6 expression cassette.

The figure on the following page shows endogenous gene expression at 145 or 146 nt and transgene expression at 80 nucleotides. The result demonstrates the vectors are expressed in a robust fashion following transfection.


935NLFB
128-88T
HGF-AS
HGF-S
U6
non
U6
c-met-S
c-met-AS
non
146 nt endogenous HGF
145 nt endogenous c-met
80 nt transgene

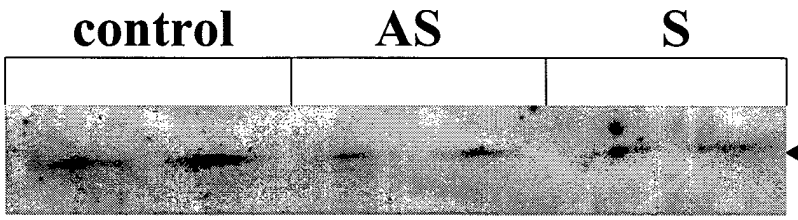
Determine whether down-regulation of protein occurs after transfer of HGF and c-Met antisense vectors

Down-Regulation of HGF protein in Human Fibroblasts

935NLFB cells were plated at 6 X 10^5 cells/100mm plate. 24 hr later the cells were transiently transfected with the HGF-S or HGF-AS constructs or a control plasmid, pGEM2U6. 72 hr after transfection, the conditioned media was isolated which contains secreted HGF. 50 µg of protein from each condition was separated by SDS-PAGE on a 10-20% Tricine gel. The proteins were transferred to nitrocellulose and probed with a monoclonal HGF antibody (500 µg/ml) at a 1:1000 dilution. Detection of HGF was observed by using an anti-mouse-IgG-HRP (1:1000 dilution) followed by chemiluminescent detection. Quantitation of the signals was done using densitometry.

The results of this assay are shown on the following page. HGF protein production was inhibited 45% by the antisense vector and 31% by the sense vector.

Western Analysis of HGF Protein in Normal Lung Fibroblasts

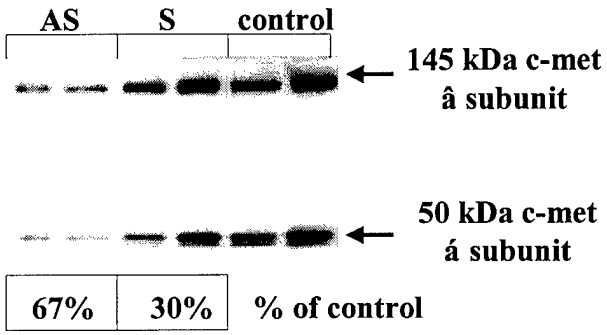


45%	31%

% inhibition of HGF protein

Down-regulation of c-Met protein in 128-88T lung tumor cells using a c-met antisense construct:

We have optimized the transfection protocol in both lung tumor cells and breast cancer cells. Because we experienced technical difficulties carrying out western blots with the breast cancer cells, we used lung cancer cells to demonstrate the effectiveness of transfer of the c-Met antisense plasmid. The following is a representative Western blot of protein isolated from 128-88T cells and detection of the c-met protein. In this experiment, we observed a 30% and 67% decrease in c-met protein levels due to transfection of the c-met sense and antisense constructs, respectively. This experiment was repeated two additional times with similar results.



Western analysis of c-met protein in c-met sense and antisense transfected 128-88T cells. 128-88T cells were transiently transfected in duplicate with either a control plasmid, pGEM2U6, the c-met sense construct or the c-met antisense construct. 72 hours later, membrane proteins were isolated. An equal amount of protein from each sample was loaded on a 6% tricine polyacrylamide gel and separated by size. The proteins were transferred to nitrocellulose and c-met specific protein was detected using a rabbit polyclonal c-met antibody (C-12) followed by a horseradish peroxidase conjugated secondary antibody. The reactive bands were detected using a chemiluminescent substrate and autoradiography. The results were quantitated using densitometry and ImageQuaNT analysis. AS= transfected with the c-met antisense. S= transfected with c-met sense. Control= transfected with pGEM2U6.

Down-Regulation of c-Met in Breast Cancer Cells

This was described on page 11. The effect was much less in breast cancer cells than lung cancer cells, even though the efficiency of transfer was the same. We will be determining the extent of expression of the transgenes in breast cancer cells in the next grant period.

Monitor In vitro Biological Effect of Antisense Plasmid Transfer

Dose response to recombinant HGF in 128-88T cells transiently transfected with the c-met AS construct: We know that we can down-regulate c-Met expression with the c-met antisense construct by more 60%. Next we want to determine if in cells that have been down-regulated by the construct, will they show reduced response to exogenous HGF? In addition, we want to know, in particular, if the cells that took up the antisense construct are the ones that show a

reduced response. To do this, we will use a dual-labeling assay to view green fluorescent protein (GFP) and proliferating cell nuclear antigen (PCNA) at the same time. PCNA is a 37 kDa protein also known as cyclin and is detected in a cell cycle dependent manner. In cells fixed with aldehydes, the staining is diffuse but intense and occurs throughout the cell cycle. The cells will be grown on 18 X 18 mm glass coverslips in 6-well plates to 70% confluency. The cells will be transiently transfected with a GFP plasmid (pEGFP-N1) and the c-met antisense construct. Forty-eight hours after transfection, serum will be removed from the media for at least 8 hours. Various amounts of recombinant HGF (0, 1, 5, 10, 35, and 50 ng) (R&D Systems) will be added to the cells and incubated for an additional 24 hours. The cells will be fixed with 4% paraformaldehyde and membranes were permeabilized using 0.5% Triton X and 0.05% Tween 20. Cells will be incubated with normal rabbit serum to block non-specific binding sites. Primary antibodies for PCNA (Oncogene Research Products) and GFP (I-16, Santa Cruz Biotechnology, Inc.) will be added followed by secondary antibodies (Chemicon) conjugated to Cy3 or Fluorescein for detection of PCNA and GFP, respectively. PCNA positive cells will be red whereas GFP transfected cell will be green. Cell that have been transfected and are also growing, based on PCNA staining, should be yellow. Thus, we will compare the amount of red versus green versus yellow cells in this experiment. We expect to see more yellow cells with higher dosages of HGF in the transfected cells.

First, we have optimized the antibody dilutions, incubation temperatures and times for this assay. The next step in the optimization of this experiment was to perform a dose-response curve with recombinant HGF in 128-88T cells that have not been transfected to see if we see an increase in PCNA staining just in the non-transfected cells alone. Preliminary experiments show a 3-fold increase in PCNA nuclear staining with 50 ng HGF versus no HGF (29% versus 8.9%). The next step is to repeat this experiment in cells that have been transfected with the c-met antisense construct. In this experiment, we will also have control plates where we will isolate membrane proteins to ensure that the c-met protein levels are actually decreased in each experiment due to the antisense construct. This will rule out any questions about whether or not the cells were down-regulated in the first place if we do not see more yellow cells with increasing amounts of HGF. This experiment is currently underway.

Transfection of tumor cells growing on a confluent layer of fibroblasts with GFP: In order to try to mimic the *in vivo* situation we grew 935 normal fibroblasts on 6 well plates to approximately 70% confluency (~6 X 10^5 cells). At this time, 2 X 10^3 128-88T lung tumor cells were added to each well in a 500 l volume. Colonies were allowed to form for 2-4 days. Once visible tumor colonies were formed on top of the fibroblasts, the green fluorescent protein was transiently transfected into the cells using a variety of liposomal formulations. Five liposome formulations were tested: LipofectACE (Gibco BRL), LipofectAMINE (Gibco BRL) , Superfect (Qiagen), DOTAP (Boehringer Mannheim), and DC-Chol (prepared by Leaf Huang's Laboratory at University of Pittsburgh, Ref.13). Transfection efficiency was calculated as percentage of green cells versus non-green cells. The following table shows the results observed with the various liposomes:

Liposome	% Transfection Efficiency
LipofectAMINE	25-30%
LipofectACE	35-40%
Superfect	15-20%
DOTAP	40%
DC-Chol	<5%

DOTAP and LipofectACE showed the highest transfection efficiency. The green fluorescent protein was found mainly in the tumor cell with an occasional fibroblast transfected. This experiment will be repeated using various breast cancer cell lines instead of the lung tumor cell lines to see if the same result is observed.

6. Begin *in vivo* experiments using antisense vectors injected into peritumor area of s.c. tumors

Animal experiments: To extend our *in vitro* observations to an *in vivo* model, we will use the antisense expression plasmids targeting c-met and HGF for direct intratumoral injection with liposomes to try to down-regulate tumor growth. We will optimize the experiment using lung tumor cells and then perform the same experiments using breast cancer cells. Lung tumor cells (201-T, 4 X 10^6) will be implanted into female scid mice (4-6 weeks old, Harlen Sprague Dawley). Once tumors have grown to approximately 2 X 2 mm in diameter (14-21 days post-injection), we will perform intratumoral injections of plasmid DNA complexed with different liposomes. The liposomes which we will initially test include: DC-Chol, LipofectACE (Gibco BRL), LipofectAMINE (Gibco BRL), Superfect (Qiagen), and DOTAP (Boehringer Mannheim). We will do the initial experiment using the green fluorescent protein so that we can easily observe if the DNA entered the tumor cells based on fluorescence. 50 g of pEGFP-N1 (Clontech) complexed with each liposome (50nmol) will be injected intratumorally in a 50 l volume. The mice will be sacrificed 48 hours after a single injection. The tumors will be removed, fixed in formalin and embedded in paraffin. We will observe fluorescence in the tumor sections to determine which liposome gives the highest transfection efficiency. If the fluorescence is quenched for some reason during this procedure we will also perform immunohistochemistry using an antibody for GFP. Once we have determined the optimal liposome formulation and ratios of DNA and liposomes, we will repeat these experiments with the c-met and HGF antisense, sense, and control constructs. We recently have injected the tumors into 10 animals, 2 mice per group, for the first attempt at this experiment and are waiting for the tumors to form.

Key Research Accomplishments

1. The cationic liposome LipofectACE gave optimal gene transfer to human fibroblasts, as monitored by green fluorescent protein expression. For breast cancer cells, Lipofectamine gave the best gene transfer in SkBr3 cells and Superfect in MDA-MB-231 cells. This was in contrast to our positive control lung cancer cells, in which the LipofectACE had given the best transfer efficiency.

2. HGF sense and antisense vectors, as well as c-Met sense and antisense vectors, have been cloned into the U6 RNA expression system. The sequences have been verified, and the vectors target the first 40 bp of the HGF and cMet mRNA, starting at the ATG transcription start site. Plasmid DNA has been purified for optimal gene transfer.

3. An RNAse protection assay strategy has been developed and reagents synthesized to carry out the assay for endogenous and transgene expression in cells transfected with vector DNA encalsulated into cationic liposomes. Expression of the U6 constructs as monitored by a ribonuclease protection assay method shows that the transgenes are being transcribed to RNA in human fibroblasts and contorl lung cancer cells. Work with breast cancer cells will continue in the next grant period.

4. Western analysis shows that several estrogen receptor negative human breast cancer cell lines express high levels of the c-met protein, whereas estrogen receptor positive human breast cancer cell lines are c-met negative. Therefore, the HGF-c-met system may be important in controlling cell growth in estrogen-independent breast cancer cells.

5. The HGF and c-met sense and antisense sequences have transferred to target cells. A 45% down-regulation of HGF protein levels in normal fibroblasts using the antisense construct was observed by Western analysis. c-Met was down-regulated up to 40% in one breast cancer cell line and 30% in another, but the result was not always different from transfer of empty vector.

6. Methods have been developed to test these constructs in vitro and in vivo for effects on tumor growth, and to test whether there is selective uptake by tumor cells in vitro and in vivo.

Reportable Outcomes

We presented an abstract of our work on use of these vectors in gene transfer at the DOD Breast Cancer Research Program Era of Hope Meeting held in Atlanta, Georgia in June of 2000. A copy of the abstract, which appeared in the Proceedings from the meeting, is enclosed as an Appendix.

Conclusions

Progress on this project was initially delayed due to loss of personnel from the laboratory. Transfer of DNA vectors into human fibroblasts is feasible using LipofectACE cationic liposomes. Sense and antisense constructs in theU6 RNA have been produced to target the HGF gene in human fibroblasts and the c-Met gene in human breast cancer cells. These vectors are taken up by cells at about 40-50% efficiency and are expressed robustly. Down-regulation of the target protein occurs with varying degrees of efficiency. Methods have been developed to test these reagents for growth effects in vitro and in vivo. This novel approach may provide a new treatment option for patients with estrogen-independent breast tumors.

REFERENCES

(1) Sanders EJ: The roles of epithelial-mesenchymal cell interactions in developmental processes. Biochem. Cell Biol. 66: 530-540, 1988

(2) Stoker M, Gherardi E, Perryman M, Gray J: Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature 327: 239-242, 1987

(3) Rosen EM, Meromsky L, Setter E, Vinter DW, Goldberg ID: Purified scatter factor stimulates epithelial and vascular endothelial cell migration. Proc. Soc. Exp. Biol. Med. 195: 34-43, 1990

(4) Bussolino F, DiRenzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffer A, Comoglio PM: Hepoatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J. Cell Biol. 119: 629-641, 1992

(5) Grey AM, Schor AM, Rushton G, Ellis E, Schor SL: Purification of the migration stimulating factor produced by fetal and breast cancer patient fibroblasts. Proc. Natl. Acad. Sci. USA 86: 2438-2442, 1989

(7) Tamashita, J.-i., Ogawa, M., and Beppu, T. Immunoreactive hepatocyte growth factor is present in tissue extracts from human breast cancer but not in conditioned medium of human breast cancer cell lines. Res. Commun. Chem. Pathol. Pharmacol. 82: 249-252, 1993.

(8) Yao, Y., Fuchs, A., Joseph, A., Hastings, H.M., Goldberg, I.D., and Rosen, E.M. Scatter factor protein levels in human breast cancers: clinicopathological and biological correlations. Am. J. Pathol. 149: 1707-1717, 1996.

(9) Yamashita, J.-i., Ogawa, M., Yamashita, S.-i., Nomura, K., Kuramot, M., Saishoji, T., and Shin, S. Immunoreactive hepatocyte growth factor is a strong an independent predictor of recurrence and survival in human breast cancer. Cancer Res. 54: 1630-1633, 1994.

(10) Byers, S., Park, M., Somers, C., and Seslar, S. Breast carcinoma: a collective disorder. Breast Cancer Res. Treatment 31: 203-215, 1994,

(11) Gherardi, E., and Stoker, M. Hepatocyte growth factor-scatter factor: mitogen, motogen, and met. Cancer Cells 3: 227-232, 1991.

(12) Yee, C.J., DeFrances, M.C., Bell, A., Bowen, W., Petersen, B., Michalopoulos, G.K., and Zarnegar, R. Expression and characterization of biologically active human hepatocyte growth factor (HGF) by insect cells infected with HGF-recombinant baculovirus. Biochemistry 32: 7922-7931, 1993.

(13) Gao. X., and Huang, L. A novel cationic liposome regent for efficient transfection of mammalian cells. Biochem. Biophys. Res. Commmen. 179: 280-285, 1991.

LIPOSOME – MEDIATED DELIVERY OF AN ANTISENSE HGF VECTOR TO HUMAN FIBROBLASTS

L.P. Stabile and J.M. Siegfried

University of Pittsburgh – School of Medicine – Department of Pharmacology
Pittsburgh, Pennsylvania 15261

E-mail: Siegfrie@server.pharm.pitt.edu

Hepatocyte growth factor (HGF) induces cell growth and movement and is known to promote invasiveness of malignant cells and angiogensis. HGF is produced by stromal cells within breast tumors while its receptor, c-met, is found on the breast tumor cells themselves. Increased HGF expression in tumor stroma is correlated with increased tumor aggression. We have shown by Western analysis that several estrogen receptor negative human breast cancer cell lines express high levels of the c-met protein, whereas estrogen receptor positive human breast cancer cell lines are c-met negative. Therefore, the HGF-c-met system may be important in controlling cell growth in estrogen-independent breast cancer cells. Our hypothesis is that disrupting the HGF/c-met pathway in estrogen independent breast cancer cells will inhibit their ability to grow *in vitro* and *in vivo*. The approach which we are using to study this hypothesis is to inhibit the action of HGF through the use of antisense (AS) constructs to both HGF and c-met. The HGF and c-met sense (S) and AS sequences have been cloned into a U6 expression cassette which allows for high expression of these sequences in target cells. Transfection conditions have been optimized in human fibroblasts and several breast cancer cell lines using green fluorescent protein and different liposome reagents. Use of lipofectACE and lipofectAMINE gave 60-70% transfection efficiencies in normal fibroblasts and breast cancer cells, respectively. Since HGF is a paracrine factor, we targeted human fibroblasts in culture with the HGF AS construct and c-met positive human breast cancer cells with the c-met AS construct. Expression of the U6 constructs as monitored by a rionuclease protection assay shows that the transgenes are being transcribed to RNA. A 50% down-regulation of HGF protein levels in normal fibroblasts using the AS versus the S construct was observed by Western analysis. This novel approach may provide a new treatment option for patients with estrogen-independent breast tumors.

The U.S. Army Medical Research and Material Command under DAMD17-98-1-8237 supported this work.