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13. ABSTRACT (Maximum 200 Words) Hepatocyte growth factor (HGF) induces cell growth and cell movement and promotes tumor invasiveness. HGF is produced by fibroblasts within lung tumors, while its receptor, the c-Met protein, is expressed on the breast tumor cells themselves. High levels of HGF expression correlate with an aggressive tumor phenotype. Expression of the c-Met protein by breast cancer cells in culture also correlates with an estrogen 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 fail anti-estrogen therapy. The hypothesis to be tested in this Idea Grant is that interruption of the HGF-c-Met signaling pathway will inhibit growth of estrogen-independent human breast cancer cells and could be a useful therapeutic strategy for breast cancer patients who fail 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.				
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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 began September 1, 1998. We experienced some personnel changes that delayed some of the work under this award. However, as discussed with the Program Officer, we received a no-cost extension, which has allowed us to complete the aims as of September 1, 2002. This is a final report of our work under this award.

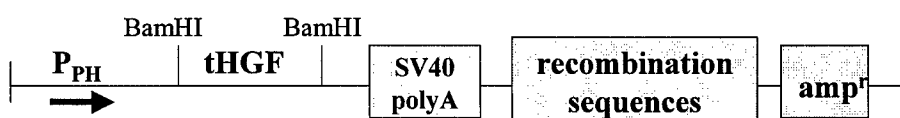
1. Produce purified tHGF (an HGF antagonist) from baculovirus-infected insect cells

This task is now completed.

The vector below was cloned. It has been used to produce purified tHGF.

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 *lacZ* 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*.

Previously, a high titer virus was produced for the expression of a truncated HGF protein. The optimal time for exposure was determined to be 4 days, and the optimal Multiplicity Of Infection is 8. This high-titer virus was used to infect 250 ml suspension cultures of High 5 insect cells and the protein-containing supernatant was harvested after incubation for 4 days. The resultant protein was purified by column chromatography and concentrated using Centicon Plus-80 Filter Devices (Amicon). Approximately 120µg of truncated HGF protein has been synthesized and purified to date. This is sufficient for both in vitro and in vivo testing.

The protein was examined by western blot at all steps of synthesis and purification. Media was removed prior to infection to show that the High 5 cells do not produce HGF protein. Media was removed prior to column purification to indicate total yield and loss due to column purification. Aliquots were removed during every step of the column purification to show which fraction contained the tHGF protein. tHGF is optimally eluted at 1.5M NaCl.



Figure 1: Western blot showing the different stages of synthesis and purification of tHGF, a 48 kD protein. (Full-length HGF is 97 kD). 15 μ l of each fraction was run in each well. A. Media from uninfected High 5 cells, B. Unpurified media 5 days post-infection, C. Media after passing through column, D and E. 0.3M NaCl fraction, F. 0.5M NaCl fraction, G. 1.0M NaCl fraction, H. 1.5M NaCl fraction, I. 2M NaCl fraction.

Effect of truncated HGF protein on activation of the MAP kinase pathway by HGF.

Previous studies in the lab have shown that HGF stimulates activation of the MAP kinase pathway. To see the effect of the truncated HGF protein synthesized in our lab on the activation of the MAP kinase pathway by HGF, 201T tumor cells were deprived of growth factors overnight and then exposed to 10ng/ml HGF with increasing concentrations of tHGF (0ng/ml-200ng/ml) as well as tHGF alone (100ng/ml) for 10 minutes. Protein from the cells was then isolated and quantified and 10 μ g of protein run on 10% tricine gels. Western blot analysis was performed to examine the levels of Phospho-P44/P42 MAPK activity. Loading was checked by stripping the blots and re-probing with β -actin. The results show that tHGF is able to inhibit the activation of the MAPK pathway by HGF at concentrations as low as 5ng/ml. High levels of tHGF in combination with HGF shows some slight partial agonist activity.

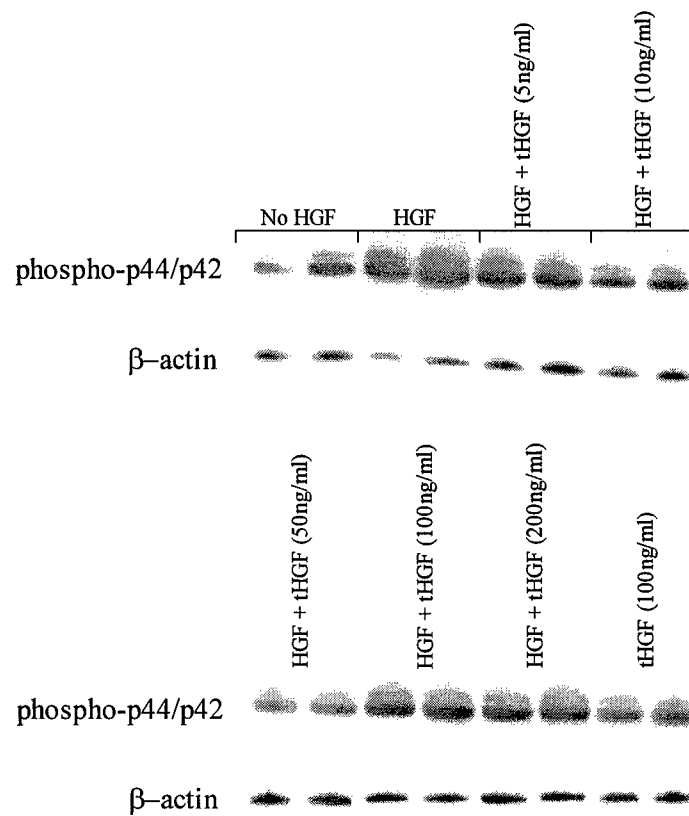


Figure 2. Inhibition of HGF activated Phospho-MAPK pathway by tHGF protein.

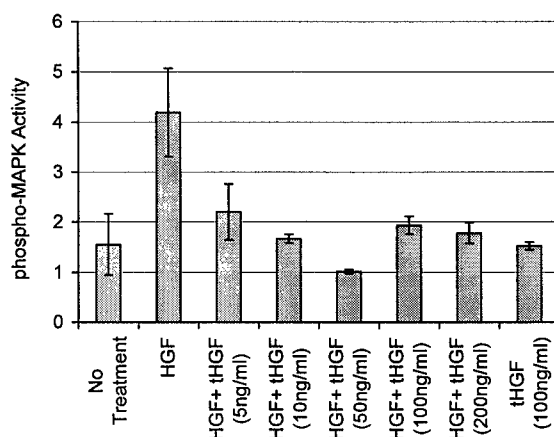


Figure 3. Phospho-MAPK activity in 201T cells treated with HGF and truncated HGF proteins. Cells were exposed to 10ng/ml HGF with increasing concentrations of tHGF (0ng/ml-200ng/ml) as well as tHGF alone (100ng/ml). Results of densitometric scan of gels shown above.

Scatter Assay.

The biological activity of HGF has been previously examined using a scatter assay. HGF was shown to produce scattering in 201T and MDCK cells. In this study, MDCK cells were serum deprived overnight and then exposed to increasing concentrations of HGF(10ng/ml-100ng/ml) or tHGF (50ng/ml-200ng/ml). Cells were also exposed to both HGF (100ng/ml) and tHGF (200ng/ml). Cells were observed and photographed the next day. Scattering was observed at all concentrations of HGF and tHGF. Scattering was also observed in some areas of the untreated cells. This is possibly because of a high level of sensitivity by MDCK cells.

A second experiment was performed using 201T cells. Again, 201T cells were serum deprived overnight and then exposed to increasing concentrations of HGF (10ng/ml- 100ng/ml) or tHGF (10ng/ml-200ng/ml). Cells were also exposed to both HGF and tHGF at concentrations of a.10ng/ml each, and b. 10ng/ml and 50ml/ml respectively. As with the MDCK cells, HGF produced scattering in cells at 10ng/ml. tHGF showed slight scattering at high concentrations. Some inhibition of scattering was observed when cells were exposed to both HGF and tHGF. This results suggests that tHGF may not be a potent inhibitor of scattering, which is mediated through the protein Rho, while it does inhibit the activation of MAPK, which is mediated through Ras.

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

The constructs for the human genes were completed previously. Last year, we also produced a murine antisense HGF vector that can be used in *in vivo* studies in the mouse. This goal is now completed.

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

This part of the project was completed as described last year. Conditions for vector transfer were optimized and used in all subsequent experiments (see previous annual reports).

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

This part of the project was completed last year. We identified SkBr3 breast cancer cells and MDA-MB-231 cells as the best cell lines to use because the transfer of vector was high. We could easily detect vector expression in SkBr3 cells but not in MDA-MB-231 cells.

Expression of HGF AS vector was also high in human lung fibroblasts. See previous report.

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

Down-Regulation of HGF protein in Human Fibroblasts

Completed as described in last year's report. Up to 50% downregulation was observed at the protein level. See previous report.

Down-Regulation of HGF protein in Murine Fibroblasts

This was also completed last year. The AS HGF vector against both the human and murine genes did not produce downregulation of HGF in the murine fibroblasts. We were unable

to resolve why this was the case, when they both work in human fibroblasts. We were therefore unable in the timeframe of this grant to test the HGF AS *in vivo*, since *in vivo* the fibroblasts being targeted are of murine origin. In concept, this approach will work in humans but we were unable to test this *in vivo* using our present models.

Down-Regulation of c-Met in Breast Cancer Cells

As reported last year, a 30% decrease in c-Met protein levels were observed in SkBr3 cells after AS vector transfer. This compared with no decrease in MDA-MB-231 cells, even though vector transfer was equivalent. As stated above, vector expression was not detected in the MDA-MB-231 cells. [See previous report.] We proceeded with *in vivo* experiments only with SkBr3 cells.

Monitor *in vitro* Biological Effect of Antisense Plasmid Transfer

We showed last year that phosphorylation of c-Met declined when conditioned medium was used to stimulate c-Met from fibroblasts that had been transfected with the HGF AS vector, compared to the activity an equivalent amount of recombinant HGF. This strongly suggests that the decreases in protein we observed will have biological meaning [see previous report].

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

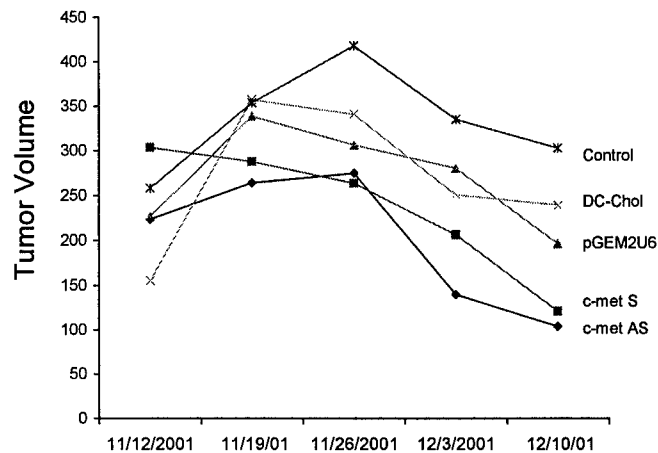
Liposome optimization for *in vivo* experiments

We completed this study last year. DC Chol liposomes were the best choice for *in vivo* transfer of the AS vector into breast cancer cells [see previous report].

***In vivo* testing of c-Met antisense construct. [We did not use the HGF AS *in vivo* because we could not demonstrate that it had a biological effect in murine fibroblasts. Fibroblasts in the SC tumors of mice will be murine in origin.]**

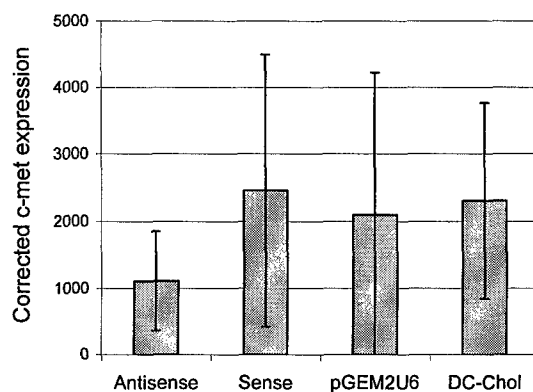
A large scale *in vivo* experiment was then performed where SkBr3 cells were injected s.c. at two sites in 26 female SCID mice. After 5 weeks, mice were divided into five treatment groups and received either c-met antisense plasmid, c-met sense plasmid, vector or DC-Chol alone. The fifth group received no treatment. Injections were repeated three times a week for four weeks. Tumor volume was measured each week. After the fourth week, mice were sacrificed and the tumors harvested. Each tumor was divided into thirds. One third was harvested for protein, one-third was RNA analysis and the final third was fixed in formalin for immunohistochemical analysis. Analysis of tumor volume shows that tumors injected with either

c-met antisense or c-met sense have a reduction in tumor volume of approximately 60%, compared to vector alone. In this experiment, the SkBr3 cells showed a regression in tumor size one month after beginning injections. This may have been due to apoptosis occurring due to lack of blood supply. However, the AS cMet treatment group had the smallest tumor volume. We are currently repeating this experiment. We will also be carrying out in vivo experiments with tHGF after we purify our protein preparation of contaminating endotoxin. We also plan a dual treatment with AS c-Met and tHGF.



Total RNA from each tumor from this experiment was isolated and examined for expression of the c-met antisense and sense. This was performed by radiolabelled primer extension using a primer specific for the U6 expression plasmid that the antisense and sense sequences were cloned into. While expression of endogenous U6 is shown in all tumor samples, expression of the c-met antisense or sense was not detected. This may have been because we examined the tumors too late after vector transfer to see the expression

Western analysis of c-met expression shows that tumors that were treated with the c-met antisense plasmid have an approximate 50 % reduction in c-met expression compared to sense, vector and vehicle. This suggests that the antitumor effect of AS vector was due to down-regulation of c-Met. The effect of S c-Met vector on tumor growth is not explainable at this time. However, in other studies, investigators have often reported a paradoxical effect of sense vectors, which could not be explained by changes in the target gene.



Future Plans

Our results to date support the concept that the HGF-c-Met pathway is a good target for therapy of ER negative breast cancer. We will repeat *in vivo* experiments to confirm the anti-tumor effect of AS c-Met, and perform *in vivo* experiments with tHGF alone and in combination with AS c-Met . We plan to apply for future funding to continue preclinical development of therapies that target this pathway, either these particular molecules or siRNAs against c-Met and HGF.

Key Research Accomplishments

1. The cationic liposome LipofectACE gave optimal gene transfer to human fibroblasts *in vitro*, as monitored by green fluorescent protein expression. For breast cancer cells, Lipofectamine gave the best gene transfer in standard in vitro cell culture to SkBr3 cells and Superfect to MDA-MB-231 cells. This was in contrast to our positive control lung cancer cells, in which the LipofectACE had given the best in vitro transfer efficiency. In co-culture with human fibroblast monolayers, LipofectACE gave the best transfer to SkBr3 cells. In the animals studies, however, we found that DC Chol liposomes gave the best transfer to s.c. SkBr3 tumors. Thus depending upon the system to be used for testing, the liposome must be optimized. For pre-clinical animal studies, the DC-Chol liposomes are superior.
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. A murine HGF antisense vector was also produced and sequenced for in vivo studies, since the stromal cells in the tumors will be murine in origin.
3. An RNA protection assay and a primer extension strategy have been developed and reagents synthesized to carry out the assay for endogenous and transgene expression in cells transfected with vector DNA encapsulated into cationic liposomes. Expression of the U6 constructs as monitored by a ribonuclease protection assay and the primer extension method show that the transgenes are being transcribed to RNA in human fibroblasts and control lung cancer cells. Breast cancer cell line SkBr3 also shows expression of the antisense vector, but MDA-MB-231 cells do not.
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 been transferred to target cells. A 50% down-regulation of HGF protein levels in normal human fibroblasts using the antisense construct was observed by Western analysis. c-Met was down-regulated up to 30% in vitro in one breast cancer cell line and not at all in another.
6. tHGF, a truncated HGF that acts as a competitive inhibitor for c-Met, has been produced from a baculovirus vector. This protein shows ability to antagonize HGF activation of MAPK in an approximate 1:1 ratio. By itself, it does not show agonist activity. At high levels, in combination with high levels of HGF, it does show some partial agonist activity. tHGF may thus be a useful antineoplastic agent in c-Met positive breast cancer.

7. Preliminary *in vivo* results show that the AS c-Met vector can decrease breast tumor growth. The c-Met protein is down-regulated about 50% in tumors that also show decreased tumor volume.

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. We are preparing a publication of our results to be submitted in Fall, 2002, following completion of additional *in vivo* experiments.

Conclusions

Sense and antisense constructs in the U6 expression vector have been produced to target the HGF gene in human and murine fibroblasts and the c-Met gene in human breast cancer cells. 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*. The antisense c-Met vector is a promising antineoplastic agent for breast tumors that express c-Met (ER negative tumors). The tHGF molecule has activity as an HGF antagonist and may also be useful as an antineoplastic agent. This novel approach may provide a new treatment option for patients with estrogen-independent breast tumors.

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

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Hepatocyte growth factor (HGF) induces cell growth and movement and is known to promote invasiveness of malignant cells and angiogenesis. 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 ribonuclease 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.

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