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13. ABSTRACT ***(Maximum 200 Words)***

Autocrine motility factor (AMF) is expressed by human breast cancer cells, such as MCF7 where its expression is stimulated by heregulin. Tumor cells constitutively secreting mouse AMF caused periosteal new bone formation in two different models of metastasis-an osteoblastic response similar to what is found with about 15% of breast cancers metastatic to bone. Whenever serum AMF concentrations were significantly increased, the animals displayed tumor-associated weight loss (cachexia), a major cause of morbidity and mortality in advanced disease. The effects of AMF on bone were independent of PTHrP, which plays a central role in osteolytic bone metastases. We also have determined the clearance rate of AMF from the mouse circulation. The species-specific effects of AMF remain little understood; so we undertook to clarify the stucture:function relationships involved. We cloned, sequenced and expressed rabbit AMF, for which the x-ray crystallographic data were already partially solved. These results have been published. We also developed a recombinant protein expression system for the mouse and human factors, which we now prepare in 100mg batches. The human factor has been crystallized and the x-ray structure solved to 1.8Å.

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INTRODUCTION

Recent studies show that breast cancer-secreted proteins play a causal role in the initiation and progression of bone metastases by stimulating osteoclasts. A number of tumor-secreted factors enhance invasion and metastasis, but only one to date has been shown to cause specific metastasis to bone: parathyroid hormone-related protein (PTHrP, a potent stimulator of osteoclastic bone resorption), although other factors secreted by breast cancer cells must also act, either independently or in concert with PTHrP, to increase osteolytic metastases. **Autocrine motility factor** (AMF) may also play a important role in metastasis to bone. It is secreted by breast cancer cells and is a known marker of metastasis. We originally found that it is a potent stimulator of osteoclast formation. We tested the role of AMF in the formation of bone metastasis by human breast cancer cells with 3 original hypotheses and experimental aims:

1) **AMF is secreted from breast cancer cells and regulated by factors released from bone matrix**. MCF7 and MDA-MB-231 human breast cancer cell lines make AMF, but neither expression of the factor by breast cancers nor regulation of its secretion has been widely studied. We tested ten standard breast cancer cell lines for AMF expression and for regulation of secretion by known bone matrix factors, including IGFs 1 & 2, TGF-β (which increases PTHrP), FGFs 1 & 2, and BMP2. We have found no factors which regulate the release of the protein from cells. 2) **Secretion of AMF stimulates bone resorption and osteolytic metastases**. Since AMF increases the numbers of osteoclasts, it should enhance bone resorption and therefore metastases to bone. We compared the formation of bone metastasis by cells known to overexpress AMF but not other factors active on bone, relative to control cells, in an animal model of metastasis. We found that in vivo AMF caused periosteal new bone formation in metastases but did not stimulate osteolysis. Thus, the origianl 3rd specific Aim turned out to be incorrect.3) **AMF secretion enhances osteolytic metastasis due to PTHrP**. PTHrP is a well-characterized factor which stimulates bone resorption. We compared in an animal model the formation of bone metastases by breast cancer cells secreting PTHrP alone and cells secreting AMF and PTHrP together. AMF did not increase osteolysis in these experiments. However, we did find that systemic elevation of plasma AMF resulted in cachexia in experimental animals. This important finding has formed the basis of experiments added to the original proposal and which are being carried out during a one year, no-cost extension of our original work

We have presented this progress report as a detailed summary of all the work accomplished relative to the original 16 tasks encompassed by the Statement of Work. These are referenced to the original 3 hypotheses/specific aims, which are given above, in their original form. It is our expectation to provide a supplement next year to this report, which will together, form the final report for this project.

BODY

First, we reproduced the original, approved Statement of Work page and the attendant Specific Aims and follow this with a report on the disposition [as of June 2001] of each of the task proposed:

STATEMENT OF WORK:

Task 1: Grow 10 breast cancer cell lines under 8 different conditions, harvest conditioned media and determine cell number (months 1-12, year 1).

Task 2: Assay AMF and protein concentrations in samples from task 1 (year 2, months 1,2)

Task 3: Determine growth curves of cells for task 4 *in vivo* (10 mice) and *in vitro* (year 1, months 1- 4).

Task 4: Perform animal experiment (20 mice) with CHO & CHO-IC6 cells (year 1, months 5-12)

Task 5: Perform histology and histomorphometry of animals from task 4 (year 2, months 1-12).

Task 6: Transform and select PTHrP-overexpressing clone of CHO-IC6 (year 1 months 6-12).

Task 7: Repeat task 3 (20 mice) for cells from task 6 (year 1, months 1-6)

Task 8: Repeat task 4 (40 mice) for cells from task 6 (year 1, month 7- year 2, month 2).

Task 9: Repeat task 5 for cells from task 6 (year 2, month 3- year 3, month 3).

Task 10: Construct amplifiable AMF expression DNA (year 1, months 1-3).

Task 11: Transfect MDA-MB-231 cells with AMF cDNA and select stable overexpressing cell line (year 1, month 3- year 2, month 6).

Task 12: Repeat task 3 (10 mice)of cells from task 11 (year 2, months 7-12)

Task 13: Repeat task 4 (20 mice) for cells from task 11 (year 3, months 1- 5).

Task 14: Repeat task 5 for cells from task 11 (year 3, months 6-12).

Task 15: Carry out detailed statistical analysis of data from animal experiments (each year months 10-12).

Task 16: Prepare data for publication, including figures, and slides (each year months 10-12).

1) **AMF is secreted from breast cancer cells and regulated by factors released from bone matrix**. MCF7 and MDA-MB-231 human breast cancer cell lines make AMF, but neither expression of the factor by breast cancers nor regulation of its secretion has been widely studied. We screened ten standard breast cancer cell lines for AMF expression and for regulation of its secretion by known bone matrix factors: IGFs 1 & 2, FGFs 1 & 2, PDGF, TGF-β, and BMP2. None controlled secretion.

2) **Secretion of AMF stimulates bone resorption and osteolytic metastases**. Since AMF increases the numbers of osteoclasts, it should enhance bone resorption and therefore metastases to bone. We compared the formation of bone metastasis by cells known to overexpress AMF but not other factors active on bone, relative to control cells, in an animal model in which these cells metastasize to bone. This experiment tested whether AMF alone is sufficient to cause osteolytic metastases. Instead, it added periosteal new bone formation to the underlying osteolytic metastases caused by the parental cells.

3) **AMF secretion enhances osteolytic metastasis due to the well-characterized resorptive factor, PTHrP**. We were unable to compare the formation of bone metastases by MDA-MB-231 cells (which form osteolytic metastasis dependent on their secretion of PTHrP and secrete moderate amounts of AMF) with transfected MDA-MB-231 cells secreting high levels of AMF. PTHrP alone causes osteolytic metastasis, but its effects can be potentiated by other factors which stimulate osteoclasts. This experiment was intended to test whether tumor-secreted AMF can increase osteolytic metastases in the presence of PTHrP. Instead, the experiment was performed with the ovarian cancer cell line, CHO, which we found made PTHrP in amounts equivalent to MDA-MB-231, and equivalently caused osteolytic metastases. The unexpected outcome of this experiment is described in the preceding paragraph.

FINAL SUMMARY OR STATEMENT OF WORK ACCOMPLISHMENTS

Tasks 1&2: *Successfully Completed.* The data were provided as Figure 6 of the 1999 progress report. In addition we tested a large number of conditions, including the peptide growth factors originally listed in the proposal, and in addition a variety of lectins which could have actions to increase secretion of neuroleukin (Gurney et al, 1985). None of these factors had an significant effect on secretion of AMF from breast cancer cells. Since the submission of the original application, no significant progress has been made on the still-unknown mechanism by which a variety of proteins, such as AMF, fibroblast growth factors 1 & 2, and others are post-synthetically secreted from the cytoplasm of cells in the absence of cell lysis. Thus, although activation of the *her2/neu* receptor on breast cancer cells increases mRNA for intracellular AMF (Talukder et al, 2000), we have no knowledge how to manipulate secretion of the active factor from cells, either in vitro or in vivo.We have thus not pursued this intractable issue further.

Tasks 3-5: *Successfully Completed.* The data were provided in the 2000 progress report, Figures

1-9. These data are ready for submission for publication. Original Specific Aim 2, above, predicted that in vivo AMF would function as an osteolytic factor. We found, however, that it stimulated periosteal new bone formation and, in the absence of osteolytic metastases, caused cachexia - a subject which is discussed further below.

Tasks 6-9: *Successfully Completed.* We found, after the completion of tasks 3-5, that basic CHO cells, both ChO-K1 and CHO-1C6, expressed modest levels of PTHrP [data provided in the 2000 progress report, Figure 10.] These levels are comparable to the MDA-MB-231 cell line, the standard model of osteolytic bone metastasis due to breast cancer (Yin et al, 1999; Chirgwin & Guise, 2000). Thus, unintentionally, tasks 6-9 were performed within the experiments for tasks 3-5. Publication of these results will therefore be included within the publication resulting from tasks 3-5. AMF overexpression did not increase osteolytic metastases in the presence of PTHrP, as originally hypothesized [Specific Aim 3, above].

Task 10: *Successfully Completed.* We have constructed a series of amplifiable expression DNAs for AMF and a series of other nonclassically secreted proteins, including FGF2 and clotting factor XIII A chain transglutaminase. These resulted in nonclassical secretion of the expressed proteins from transiently transfected cells.

Task 11: *Unsuccessful to date and Abandoned.* We expended a great deal of effort to establish stable cell lines which would continue to secrete the transfected protein (We tested all 3 described in the previous paragraph). In every case stable cell clones failed to maintain nonclassical secretion. We spent one year carrying out control experiments with step-wise gene amplification (Schimke, 1984; Bendig, 1988) using the mouse dhfr cassette and selection for increasing levels of resistance to methotrexate This method was used by Gurney to create the CHO cell line CHO-1C6 although no data on the amplification or selection have every been published. dhfr$^-$ CHO has been the standard cell line for methotrexate-resistant gene amplification, and it may be that breast cancer cells are not amenable to the standard gene amplification protocols in vitro. an alternate strategy, based on selection of cells transfected with a glutamine synthetase amplifiable cassette and resistance to the anti-metabolite methionine sulfoximine (Cockett et al, 1990) was entirely unsuccessful.

Tasks 12-14: ***Not done***. These task depended on task 11. However, the results of the successful task 3-9 have defined the consequences for bone metastasis of PTHrP +/- AMF and render these tasks no longer essential or of central importance.

Tasks 15-16: *Completed, as well as ongoing*. See previous progress reports as well as Reportable Outcomes, below.

Additional tasks not originally proposed but completed (identified by subject rather than creation of new task numbers):

1) Identification of rabbit AMF. Much of the original work on this protein, under one of its alternate names, phosphoglucose isomerase, was done on material isolated from rabbit muscle, because of the similar properties of rabbit and human proteins [e.g., Le et al, 2000]. However, cloning, sequencing, and expression of the rabbit cDNA protein had not been reported; so we undertook this project. The successful results have been published (Li & Chirgiwn, 2000) and were included with the 2000 progress report.

2) X-ray crystal structure of human AMF. The past year saw the publication of the rabbit crystal structure (Jeffery et al, 2000, 2001), and a t the same time we undertook to solve the structure of the human protein. We cloned and expressed human AMF and purified the protein in sufficient quantity for our collaborator, Christopher Davies, then at University of Sussex, UK, and now at the Medical University of South Carolina, rapidly to crystallize and then solve the structure. The extensive results of this collaboration were recently published (Read et al, 2001) and a copy is appended. Our own data show important biological activities of AMF in cancer in vivo on bone and on cachexia, which are in addition to the numerous roles for the protein demonstrated in vitro by us and others (extensively discussed in the original application and previous progress reports). In addition, data published last week (Funasaka et al, 2001) demonstrate that AMF can stimulate angiogenesis in vitro and in vivo. These data reinforce the physiological importance of AMF in cancer. Future studies of this protein can now be guided by detailed x-ray structures of the protein. In particular the human structure was solved to 1.6Å, permitting very detailed analysis. In particular the high resolution permits identification of glutamic acid [E] 357 as the residue directly responsible for isomerization in the the phosphoglucose isomerase reaction in the glycolytic pathway inside the cell. The identification of E357 has been confirmed by inhibitor studies with the rabbit protein (Jeffery et al, 2001). These data are important because of suggestive results that isomerase activity may be necessary for cytokine activity. Funasaka et al (2001) report that erythrose 4-phosphate, an isomerase competitive inhibitor, decreased AMF-induced angiogenesis. High doses of the sugar phosphate were required (0.5mg/mouse), so non-specific effects are not easily excluded. A more definitive assessment of the relationship of isomerase to AMF activities could be carried out by active site mutagenesis of residue E357, which is now possible from the crystal structures.
We have published the structure of the protein (Read at al, 2001)

3) Role of AMF in cachexia. Many of these data were included in the previous, year 2000, progress report. At this time, we were not fully aware of the importance of these findings. We have developed this important direction and submitted a new Idea proposal to the Army Breast Cancer program. This has been approved for two years of funding (DoD Award # DAMD17-02-1-0586, presently under negotiation for transfer of award to Department of Medicine, Division of Endocrinology, University oif Virginia, Charlottesville, effective 10/01/2002.)

Additional tasks not originally proposed but carried out during no-cost extension year 4 (identified by subject rather than creation of new task numbers):

4) Molecular basis of species-specific binding of AMF to mammalian cells using ligand

chimeras. Our data with biological actions of AMF on bone cells (included in the previous progress reports) identified an approximately 100-fold species preference of mouse cells for mouse AMF and human cells for human AMF. Such data have not been reported by others, who have not had access to the mouse factor, which we originally purified from the CHO-1C6 cell line, but our now expressing in E. coli as we previously described for the rabbit protein (Li & Chirgwin, 2000). The mouse and human AMF sequences are very highly conserved and share at the DNA level a number of conserved, unique restriction sites, which we have used to create a series of mouse:human chimeric protein pairs. The sequence differences map to the surface of the folded protein, rather than the catalytic core of the protein or the intersubunit dimer interface (C. Davies, personal communication); so we anticipated that these chimeric proteins would fold stably and retain near-normal PGI catalytic activity. The restriction sites to be used and the crossover site in the amino acid sequences (which are entirely co-linear and so have the identical numbering system) between the two species follow: Nco I [aa33], Age I [aa 106], Pst I [aa 238], Sac I [aa 448], Bgl II [aa 497]. Each restriction digestion approach yields the complementay pair of mouse:human and human:mouse chimeras. The N-termini show the greatest species divergence; so we think that the first mutant pair may explain the basis for the species-specific receptor interactions. Chimeric His-tagged proteins will be expressed, purified, and assayed for PGI activity as described in section A), above. Each protein will be tested for dose-response effects on the induction of RANK ligand mRNA (by RT-PCR) in the mouse ST2 stromal cell line and in the human HL-60 monocytic maturation and differentiation assay. Purified mutant proteins will be radio-labeled and used in the binding assays described in the next section. Altogether there are 10 of these chimeric ligands, of which we have already constructs 7 of the DNAs. Several of these have been successfully tested at the protein level and give, stable protein with isomerase activity. The chimeric proteins are presently being tested for biologically activity in mouse bone marrow culture assays and are being analyzed structurally by Dr Davies..

5) X-ray crystal structure of mouse AMF. In fashion exactly parallel to that which we used last year to solve the human crystal structure, we have cloned, expressed, purified, and crystallized mouse AMF. The crystal structure has been solved. This protein also has isomerase activity and is active on mouse bone cell cultures. Dr. Christopher Davies is presently drafting a manuscript describing the crystal structure.

6) Is isomerase catalytic activity required for AMF bioactivities? Inside the cell PGI interconverts glucose and fructose 6-phosphates in the glycolytic pathway. It is unclear whether this enzymatic activity is necessary for the extracellular cytokine functions of the protein. We will make 2 mutants of AMF/PGI: E357A, which lacks the side-chain responsible for catalytic proton transfer, and S209T211,214,217/4A, which eliminates the groups needed to bind the phosphate group of the substrate. Experiments will be guided by our 1.6Å x-ray structure of the human enzyme. Mutant proteins will be assayed in vitro for isomerase activity and receptor binding and in vivo for ability to cause cachexia. This subject has very recently been addressed by Tanaka et al (2002). These authors did not mutate catalytic residue E357, so we will continue this experiment to completion. The described mutant has been made, the protein purified and found to be without isomerase activity and is being crystallized by Dr. Davies. Biological activity

assays will be carried out after the end of the funded period (in August 2002).

Long-Term Future Directions. These are beyond the scope of the original proposal or the experimental aims which we have added in progress. However, these represent the outlines of grant proposal(s) which we anticipate having ready for submission within the next 24 months:

Long Term Goals: We have a series of mutants of mouse and human AMF with which to probe the relation between ligand structure and receptor-mediated functions. We have solved the human AMF structure to 1.6Å and collected equivalent data for the mouse and have demonstrated novel biological responses to AMF *in vivo* and *in vitro*. Future progress is absolutely dependent on the availability of the cloned receptor. Ongoing work will enable us to propose the molecular cloning of the correct receptor, to be followed by the characterization of it is functions and signaling in mammalian cells. We have also developed monoclonal antibodies which recognize human AMF with high affinity. These could have therapeutic value.

Significance: AMF is secreted by a variety of breast and other cancer cells. It is a well-established marker of metastatic disease, has a variety of effects on normal cells, and may play a central role in tumor-produced cachexia and other paraneoplastic syndromes. Since AMF is not a normal extracellular protein in adults, its receptor offers an attractive target for therapeutic intervention in metastatic disease. The work proposed will provide the tools to screen for effective drugs to block the pathological effects of AMF secreted by tumor cells.

KEY RESEARCH ACCOMPLISHMENTS

- Pure recombinant AMF produced for 3 mammalian species
- 3-D structure of human protein determined
- New animal model of cancer cachexia developed
- Interspecific mouse:human chimeric proteins developed
- Catalytic site of isomerase identified and inactive mutant E357A made
- 3-D structure of mouse protein solved
- Monoclonal antibodes against human AMF developed
- Role of mouse AMF in bone metastases determined
- Role of AMF in periosteal new bone formation established

REPORTABLE OUTCOMES

Five publications (more in preparation):

Kakonen SM, Selander KS, Chirgwin JM, Yin JJ, Burns S, Rankin WA, Grubbs BG, Dallas M, Cui Y, Guise TA (2002). Transforming Growth Factor-beta Stimulates Parathyroid Hormone-related Protein and Osteolytic Metastases via Smad and Mitogen-activated Protein Kinase Signaling Pathways. *J Biol Chem* **277**:24571-24578.

Read J, Pearce J, Li X, Muirhead H, Chirgwin J, Davies C (2001). The crystal structure of human phosphoglucose isomerase at 1.6 A resolution: implications for catalytic mechanism, cytokine activity and haemolytic anaemia. *J Mol Biol* **309**:447-463.

Chirgwin JM, Guise TA (2000). Molecular mechanisms of tumor-bone interactions in osteolytic metastases. *Crit Rev Eukaryot Gene Expr* **10**:159-178.

Wang F, Duan R, Chirgwin J, Safe SH (2000). Transcriptional activation of cathepsin D gene expression by growth factors. *J Mol Endocrinol* **24**:193-202.

Li X, Chirgwin JM (2000). Rabbit phosphoglucose isomerase/neuroleukin/autocrine motility factor: cloning via interspecies identity. *Biochim Biophys Acta* **1476**:363-367.

New Grant:

Army Breast Cancer Idea grant, BC010307, "Tumor-secreted Autocrine Motility factor (AMF); Causal Role in an Animal Model of Cachexia". Approved for funding for two years as DoD Award # DAMD17-02-1-0586, presently under negotiation for transfer of award to Department of Medicine, Division of Endocrinology, University oif Virginia, Charlottesville, effective 10/01/2002., where the P.I. will move as a Professor of Medicine, at that time.

No patents or inventions were filed or made

No animals were used in the 4th, no-cost continuation year

CONCLUSIONS

The major goals of the original proposal were satisfactorily accomplished. An important role of AMF was established in bone metastases. It was found to be a spatially-limited osteoblastic, rather than an osteolytic, factor. Since osteoblastic responses are found in ~15% of breast cancer bone metastases, this is clinically significant. More importantly, a significant role of AMF in tumor induced cachexia was found, and an animal model of cachexia was established. This work has lead to a second Army breast cancer Idea grant. Cachexia is a major problem for patients with advanced disease but one which has received little scientific attention. Finally, a series of new molecular tools for continuing study of AMF have been developed with the support of this grant: high resolution X-ray crystal structures for human and mouse AMFs, recombinant proteins from 3 species, chimeric mouse-human proteins, and a series of functional mutations in the proteins. A panel of monoclonal antibodies against human AMF was also developed. Work on AMF in cancer of the breast, prostate, and other tumors, as well as papers showing important roles in bone metastases, in rheumatoid arthritis, and in angiogenesis have been published from a number of laboratories in the past 5 years. Continued research into the molecule remains an important and active area, and the funded work has contributed to this in a variety of ways.

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