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Award Number: DAMD17-02-1-0159

TITLE: Hepatocyte Growth Factor and Interleukin-6 in Prostate
Cancer Bone Metastasis

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REPORT DATE: March 2003

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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20030829 042

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

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 this 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**
March 2003**3. REPORT TYPE AND DATES COVERED**
Annual (15 Feb 02 - 14 Feb 03)**4. TITLE AND SUBTITLE**Hepatocyte Growth Factor and Interleukin-6 in Prostate
Cancer Bone Metastasis**5. FUNDING NUMBERS**

DAMD17-02-1-0159

6. AUTHOR(S)

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REPORT NUMBER****9. SPONSORING / MONITORING
AGENCY NAME(S) AND ADDRESS(ES)****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)**

None provided

14. SUBJECT TERMS

No subject terms provided.

15. NUMBER OF PAGES

8

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

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SUBJECT: Annual Report for Award Number DAMD17-02-1-0159

“Hepatocyte Growth Factor and Interleukin-6 in Prostate Cancer Bone Metastasis”

Overview of tasks accomplished in the first year. In the first year of the award, we evaluated the design and progression of experiments of the project. We completed the first task, listed in the statement of work. In addition, I moved from Cornell University to the Fred Hutchinson Cancer Research Center. The environment at FHCRC is conducive to abroad genome wide analysis to determine the entire spectrum of androgen-regulated growth factors in bone, which is listed under task 4. As a first step, we analyzed the expression of hepatocyte growth factor (HGF) and interleukin-6 (IL-6) in mouse bone, which is part of task 1. We also evaluated changes in expression levels as a result of androgen deprivation of bone and bone marrow. The assay development and results are listed in detail in the following section.

• **Development of assays for completion of task 1:**

1. Isolation of RNA from mouse bone. Isolation of undegraded RNA proved to be difficult. We circumvented this problem by generating a fine bone powder in a dry ice bath prior to extraction with TRIZOL®.
2. Semi-quantitative RT-PCR for measurements of HGF, IL-6, Cbfa-1, osteocalcin, osteonectin and actin. After determination of the quality of RNA we determined RNA input for the reverse transcriptase reaction, which is the first step in the RT-PCR. 2 ug of input RNA was in the linear range and found to be the optimal amount of input RNA. 2 ug of RNA were used in all experiments. We tested several primer pairs to find the best for PCR. The PCR fragments for each primer pair were between 300 and 500 bp. Whenever possible, we included a reference primer pair in the PCR, which allowed standardization of each PCR run. However, this was not possible for the HGF PCR, since we encountered primer interference with several reference primers. Therefore, HGF m-RNA expression was measured in a separate PCR-tube and compared to reference primer sets in parallel tubes under identical PCR.

For each primer set, we determined the linear range (shown in figure 1B).

3. Radioactive quantification of PCR products. To obtain better and more direct quantification, we attempted to label PCR products with ³²P-ATP. However, these experiments did not provide the anticipated improvement.
4. Measurements of expression of the Met receptor by Western blotting. For Western blot analysis, powdered bone was solubilized in RIPA buffer and 100ug protein loaded per lane.

• **Results for completion of task 1:**

Changes in bone maturation upon orchietomy in mice. Since the absence of androgen effects the integrity of bone, we measured m-RNA expression of genes that are expressed in a sequential order during bone development. These first included Cbfa-1 and osteonectin. We reasoned that the Cbfa-1 to

osteonectin ratio indicates the maturity of bone, high Cbfa-1 and low osteonectin representing immature bone.

Measurements of HGF/SF in normal and orchietomized mice

To measure HGF m-RNA expression, bone and bone marrow were separated. While bone marrow contains immature osteoblastic cells, the bone harbors mature osteoblasts and osteocytes. Figure 1 shows, that osteonectin m-RNA is decreased in orchietomized bone. This is consistent with features of osteoporosis that we saw histologically in the bone of mice that were orchietomized 1 year prior to analysis. Since it is not certain which cells make HGF/SF, we compared bone and bone marrow from the mice.

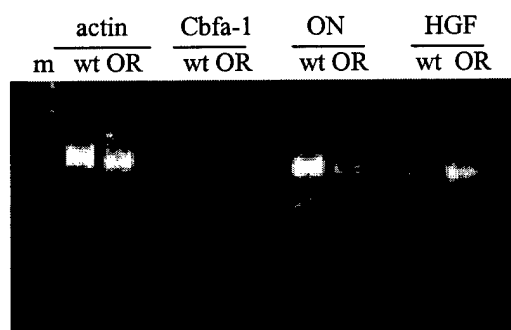


Figure 1: RT-PCR with RNA from bone marrow stromal cell. Bone marrow was separated from bone prior to preparing RNA. Primers to evaluate m-RNAs of HGF, osteonectin (ON), actin and Cbfa-1 were used in the same PCR reaction. Bands were quantified densitometrically to calculate relative expression of HGF.

This study shows that (1) osteonectin m-RNA is decreased in the bone marrow of androgen deprived mice, suggesting that there are fewer osteoblastic cells. In contrast HGF is increased. However, we don't know whether HGF is only synthesized by mesenchymal cells. It is possible that lymphocytes also make HGF and androgen depletion leads to a lymphocytosis, which could account for the increased HGF production (Olsen et al., 2001).

Analyzing HGF m-RNA synthesis in bone did not show any differences between orchietomized and normal bone.

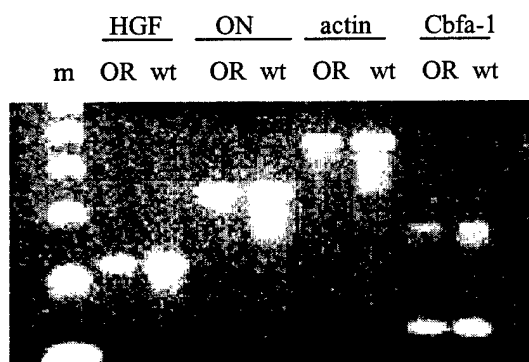
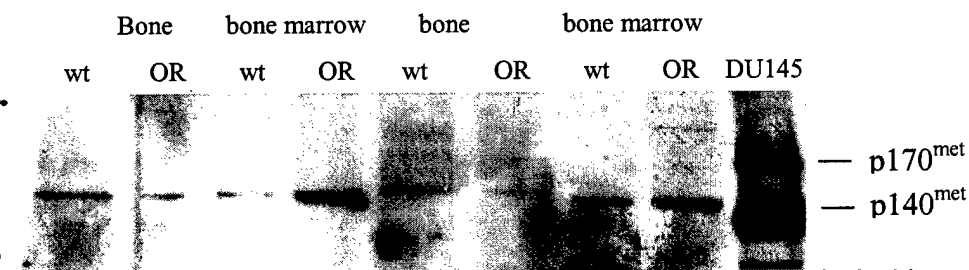


Figure 2: RT-PCR with RNA from bone of orchietomized and wild type mice. Primers to evaluate m-RNAs of HGF, osteonectin (ON), actin and Cbfa-1 were used in the same PCR reaction. Bands were quantified densitometrically to calculate relative expression of HGF.

These experiments show that the effects of androgen withdrawal are most pronounced on mesenchymal bone marrow cells and we will use the bone marrow preparation in addition to bone in subsequent studies.

Measurements of the Met receptor in bone and bone marrow. Expression of Met was analyzed by Western blot (Figure 3).



100 ug whole cell lysate was analyzed in each lane. The blot was probed with a polyclonal anti-met (Santa Cruz).

The HGF receptor, met, is highly expressed in the bone marrow of androgen deprived mice, but not in bone. This is consistent with our results that the bone marrow is most effected by androgen deprivation.

Measurement of IL-6 in normal and orchietomized mice. In contrast to published reports that clearly show the modulation of IL-6 expression by steroid hormones in bone cells (Gornstein et al., 1999; Hierl et al., 1998; Hofbauer et al., 1999; Hurley et al., 1996; Linkhart et al., 1991; Manolagas, 1998; Manolagas et al., 1995; Navarro et al., 1991; Rifas et al., 1995; Ritchie et al., 1997; Sandhu et al., 1999; Swolin-Eide and Ohlsson, 1998; Zhang et al., 1998), we were unable to detect changes of IL-6 at the m-RNA expression level. It is possible, that the C6/BL57 strain is less sensitive the androgenic regulation of IL-6. Furthermore, we did not detect changes in HGF or met expression in the IL-6 knockout versus normal mice.

Therefore we decided to analyze the global changes of gene expression as they occur upon androgen deprivation in mouse bone. Because of the strong c-DNA array community at FHCRC, we expect tat this project will be successful in identifying growth factors that are increased upon androgen ablation. As a next step these can be tested directly in human specimens of prostate cancer metastasis, in a newly established resource at the University of Washington, directed by Dr. Bob Vessella.

During the last year, I moved from Cornell Medical College to the Fred Hutchinson Cancer Research Center. This move has slowed the research during the first year of the award. However, the lab is fully operating at this point and I do not anticipate any problems for the rest of the funding period.

Probable effects of moving the project:

The proposed project has a much greater chance of success at the Fred Hutchinson Cancer Research Center, than at Cornell. Core facilities are more advanced, especially the c-DNA array core. There is another investigator, an associate member of the institute who's laboratory is mainly interested in androgen-regulated gene expression and has identified several androgen regulated genes in the LNCaP cells. This group has also developed the bioinformatics and databases for categorizing the genes from their gene discovery projects.

The c-DNA core facility at Fred Hutchinson has a homemade mouse c-DNA array, which is much cheaper than the commercial array. They also provide a full service of labeling probes, hybridization and data analysis. It will be faster to identify androgen regulated genes in mouse bone, with the expertise of a well established c-DNA array facility.

With the help of the Veterinarian at Fred Hutchinson, I have identified a vendor who can supply castrated mice, thus accelerating the procurement of bone from androgen-deficient mice.

I have recruited a new post-doctoral fellow for the project who will take Dr. Xueke You's place. Chang Xu has spend the last year in a c-DNA facility at the University of Washington and also has a Masters in Biostatitics. He has the perfect technical background for the proposed project and Will rapidly be getting data using the c-DNA approach to identify androgen regulated growth factors and growth factor receptors in bone.

Undoubtedly, the Fred Hutchinson Cancer Research Center will provide a beneficial intellectual and technical environ to achieve the goals of the proposed project.

References:

- Gornstein, R. A., Lapp, C. A., Bustos-Valdes, S. M., and Zamorano, P. (1999). Androgens modulate interleukin-6 production by gingival fibroblasts in vitro. *J Periodontol* 70, 604-609.
- Hierl, T., Borcsok, I., Sommer, U., Ziegler, R., and Kasperk, C. (1998). Regulation of interleukin-6 expression in human osteoblastic cells in vitro. *Exp Clin Endocrinol Diabetes* 106, 324-333.
- Hofbauer, L. C., Ten, R. M., and Khosla, S. (1999). The anti-androgen hydroxyflutamide and androgens inhibit interleukin-6 production by an androgen-responsive human osteoblastic cell line [In Process Citation]. *J Bone Miner Res* 14, 1330-1337.
- Hurley, M. M., Abreu, C., Marcello, K., Kawaguchi, H., Lorenzo, J., Kalinowski, J., Ray, A., and Gronowicz, G. (1996). Regulation of NFIL-6 and IL-6 expression by basic fibroblast growth factor in osteoblasts. *J Bone Miner Res* 11, 760-767.
- Linkhart, T. A., Linkhart, S. G., MacCharles, D. C., Long, D. L., and Strong, D. D. (1991). Interleukin-6 messenger RNA expression and interleukin-6 protein secretion in cells isolated from normal human bone: regulation by interleukin-1. *J Bone Miner Res* 6, 1285-1294.
- Manolagas, S. C. (1998). The role of IL-6 type cytokines and their receptors in bone. *Ann N Y Acad Sci* 840, 194-204.
- Manolagas, S. C., Bellido, T., and Jilka, R. L. (1995). Sex steroids, cytokines and the bone marrow: new concepts on the pathogenesis of osteoporosis. *Ciba Found Symp* 191, 187-196.
- Navarro, S., Debili, N., Le Couedic, J. P., Klein, B., Breton-Gorius, J., Doly, J., and Vainchenker, W. (1991). Interleukin-6 and its receptor are expressed by human megakaryocytes: in vitro effects on proliferation and endoreplication. *Blood* 77, 461-471.
- Olsen, N. J., Olson, G., Viselli, S. M., Gu, X., and Kovacs, W. J. (2001). Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. *Endocrinology* 142, 1278-1283.
- Rifas, L., Kenney, J. S., Marcelli, M., Pacifici, R., Cheng, S. L., Dawson, L. L., and Avioli, L. V. (1995). Production of interleukin-6 in human osteoblasts and human bone marrow stromal cells: evidence that induction by interleukin-1 and tumor necrosis factor-alpha is not regulated by ovarian steroids. *Endocrinology* 136, 4056-4067.
- Ritchie, C. K., Andrews, L. R., Thomas, K. G., Tindall, D. J., and Fitzpatrick, L. A. (1997). The effects of growth factors associated with osteoblasts on prostate carcinoma proliferation and chemotaxis: implications for the development of metastatic disease. *Endocrinology* 138, 1145-1150.
- Sandhu, J. S., Gorczynski, R. M., Waddell, J., Nguyen, H., Squires, J., Boynton, E. L., and Hozumi, N. (1999). Effect of interleukin-6 secreted by engineered human stromal cells on osteoclasts in human bone. *Bone* 24, 217-227.
- Swolin-Eide, D., and Ohlsson, C. (1998). Effects of cortisol on the expression of interleukin-6 and interleukin-1 beta in human osteoblast-like cells. *J Endocrinol* 156, 107-114.
- Zhang, J., Pugh, T. D., Stebler, B., Ershler, W. B., and Keller, E. T. (1998). Orchiectomy increases bone marrow interleukin-6 levels in mice. *Calcif Tissue Int* 62, 219-226.