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Androgen, Estrogen, and the Bone Marrow Microenvironment

PRINCIPAL INVESTIGATOR:
Beatrice Knudsen, MD, PhD

CONTRACTING ORGANIZATION:
Fred Hutchinson Cancer Research Center
Seattle, WA 98108-1024

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14. ABSTRACT In this project we plan to analyze androgen- and estrogen-responsive gene expression in the bone marrow. We plan to work to: determine if castration-induced gene expression changes in mouse bone marrow are caused by the deficiency of testosterone or estrogen; analyze androgen- and estrogen-sensitive cytokine and gene expression changes in human bone marrow transplanted into NOD/SCID mice, and; examine androgen- and estrogen-sensitive gene expression in the bone marrow of patients with low and high circulating testosterone levels. This research project was delayed as a result of second-level review required by the US Army Medical Research Materiel Command's Human Subjects Research Review Board (HSRRB). Final approval from the HSRRB was received on November 21, 2006. We are now organizing the animal work and obtaining human samples of bone marrow from patients with prostate cancer for analysis.					
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SUBJECT: Annual Report for Contract Number W81XWH-06-1-0171

“Androgen, Estrogen, and the Bone Marrow Microenvironment”

INTRODUCTION:

In this project we proposed to analyze androgen- and estrogen-responsive gene expression in the bone marrow. In males, the main source of estrogen is through conversion of androgen by aromatase. We postulate that gene and protein expression in the bone marrow microenvironment is subject to regulation by androgen and estrogen and could affect the growth and progression of micrometastatic prostate cancer cells. When prostate cancer cells leave the circulation through fenestrations in the bone vasculature, they lodge in the fertile soil of the bone marrow. Interactions between prostate cancer cells and the bone marrow regulate the early steps of metastasis formation. This environment differs from the environment of established prostate cancer metastasis, in which a fibrotic bone marrow stroma surrounds the cancer cells and cancer cells stimulate an osteoblastic response in adjacent bone. Almost nothing is known about the initial interactions of micrometastatic prostate cancer with the bone marrow microenvironment (BM-ME). During this period critical decisions in the fate of micrometastatic cancer cells occur that determine their latency, survival and proliferation. Most likely, factors in the BM-ME play a major role in regulating the progression of micrometastatic disease. While model systems exist for several steps in metastasis formation, including interactions of prostate cancer cells with endothelial cells and osteoblastic and osteoclastic bone cells, there is no *in-vivo* system to investigate the interactions between prostate cancer cells and the BM-ME. Therefore, there are many unanswered questions related to events that will ultimately determine who develops lethal prostate cancer metastases. In this grant application we will begin to investigate mechanisms that control the fate of prostate cancer cells when they first enter the BM.

Early androgen ablation has a significant survival benefit in patients at risk for prostate cancer recurrence or with increasing PSA levels after surgery or radiation therapy. At the initiation of androgen ablative therapy, the disease is often not apparent by conventional radiographic methods. However, the majority of patients will have micrometastatic disease outside the prostate. Therefore it is important to understand if and how androgen ablative therapy affects the bone marrow cells that surround the micrometastatic cancer cells. In this study, we plan to work to: determine if castration-induced gene expression changes in mouse bone marrow are caused by the deficiency of testosterone or estrogen; analyze androgen- and estrogen-sensitive cytokine and gene expression changes in human bone marrow transplanted into NOD/SCID mice, and; examine androgen- and estrogen-sensitive gene expression in the bone marrow of patients with low and high circulating testosterone levels.

BODY:

This research project was subject to second-level review by the U.S. Army Medical Research Material Command's Human Subjects Research Review Board (HSRRB). Because of this review, study implementation was delayed until we met specific requirements for compliance with human subjects protection and received approval from our local IRB and then the HSRRB. We received final approval from the HSRRB on November 21, 2006.

Task 1: To characterize androgen and estrogen regulated gene and protein expression in mouse bone marrow

- Determine if specific genes are regulated by androgen or estrogen in bone marrow
- Measure global gene regulation by androgen and estrogen in mouse bone marrow
- Correlate gene and protein expression for genes of interest

We completed the experiments for this task and published the data in Xu, et al. (2007) (this grant [W81XWH-06-1-0171] was referenced in the publication, **see attached manuscript**). To summarize, castration of mice increases expression of 159 genes (including 4 secreted cytokines) and suppresses expression of 84 genes. IGFBP5 is most consistently increased and the increase in expression is reversed by testosterone administration. IGFBP5 protein expression was analyzed by immunohistochemistry and is

consistent with the results from the RNA expression analysis. IGFBP5 protein is detected in vivo in osteoblasts, BM stromal cells, and endothelial cells. Primary human bone marrow stromal cell cultures secrete IGFBP5. In vitro, treatment of immortalized human marrow stromal cells with charcoal stripped serum increases IGFBP5 mRNA expression, which is reversed by androgen supplementation. IGFBP5 is incorporated into the extracellular matrix. Further, IGFBP5 immobilized on extracellular matrices of stromal cells enhances the growth of immortalized prostate epithelial cells.

The third sub-task is to determine whether the effects of androgen on the BM stroma are mediated through the conversion of androgen to estrogen. In this case, the genes that we found regulated through androgen ablation would be a consequence of the low estrogen levels. This information is important to know because patients could receive estrogen supplementation. To analyze the effects of estrogen on the mouse bone/bone marrow, we treated mice with DES. DES treatment caused massive production of bone and the array data demonstrated a large increase in the expression of genes driven by connective tissue derived growth factor (CTGF). Based on these data, we realized that androgen and estrogen exert their primary activity on the mesenchymal cells in the bone marrow. This observation causes a problem for the mouse model in aim 2 (see below).

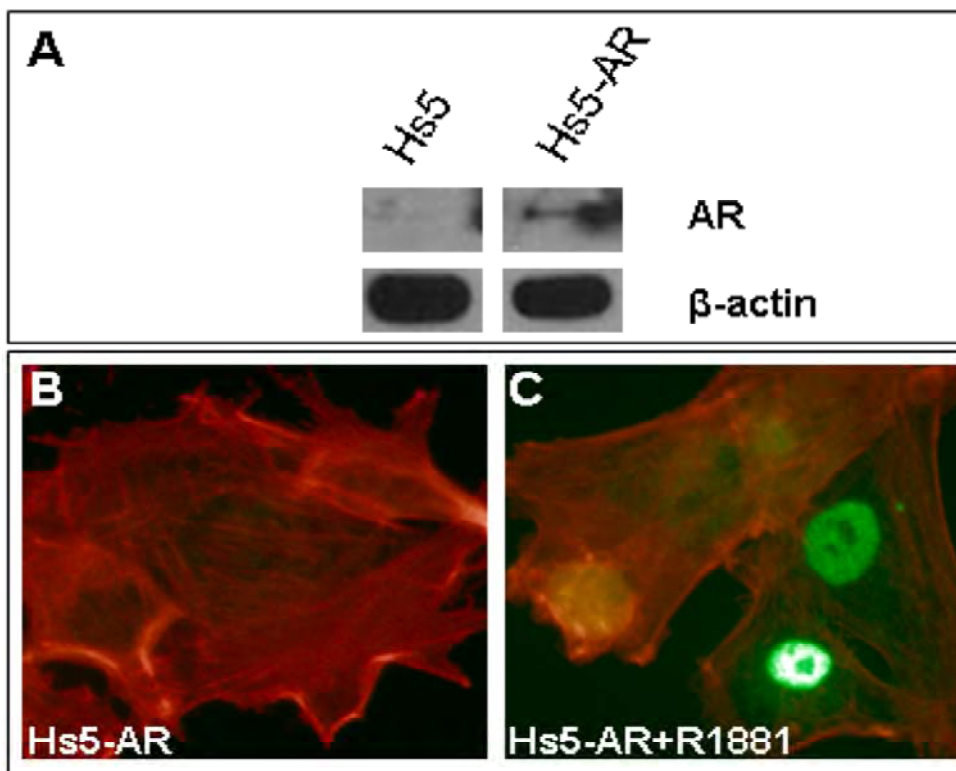
Via experimentation we have observed that, using our proposed panel, mice generally respond differentially to hormonal manipulation compared to people. Although mouse models have been previously established using aromatase inhibitors (reviewed in Brodie, et al. 2005), our experience has been such that we are not able to appropriately adjust doses of androgen inhibitory drugs (i.e. we did not see a difference between treated and untreated animals on DNA arrays). In contrast, mice appeared to be extremely sensitive

to DES and in our pilot study to determine the dose and using concentrations that are proportional to human doses, we could not find a dose that avoids the massive response in the bone. We concluded that the hormonal system in mice is significantly different from human. We decided to pursue human systems that we build with our primary cell models.

Task 2: To characterize androgen or estrogen regulated cytokines and gene expression in human bone marrow

Proposed predicted outcomes, products and deliverables:

- ⇒ Establishment of mouse model for studies of prostate cancer metastases using human cells



- ⇒ Androgen and estrogen sensitive gene expression changes in human bone marrow
- ⇒ Androgen and estrogen sensitive cytokine expression changes in human bone marrow

In the grant application, we proposed to generate a mouse model of prostate cancer metastasis that consists of human bone marrow (BM) in immunodeficient mice. SCID/hu model by transplanting human CD34+ cells into severely immunodeficient mice. The methods for this task involve reconstituting human bone marrow in the NOD-SCID mouse model. Based on our data we realized that the model system in Aim 2 will unlikely generate conclusive results for the following reasons:

- The transplantation of CD34+ cells reconstitutes the BM, but does not the BM stroma, in which we observed the greatest effect of androgen regulation. CD34+ replenish the hematopoietic BM, but cannot differentiate into mesenchymal cells. Therefore, the transplantation model would determine effects of androgen ablation on the mesenchymal mouse BM stroma, which we already know. If the mouse factors interact with human cells, there might be a secondary effect on human hematopoietic cells.
- The analysis of human cytokines would reflect a secondary effect from the mouse stroma and the indirect regulation by androgen. We would not be able to distinguish between expansion of cell lineages secreting normal amounts of a cytokine per cell and the hypersecretion of cytokines by a lesser cell number.
- The mouse cells that are the androgen responsive cell type in the reconstituted mice are heavily pretreated with radiation. We don't know whether the radiation effect interferes with the response to androgen.

Based on data that we generated after submitting the grant, we realized that the CD34+ transplantation model is not to be the best way to address the effects of androgen on human BM, since we expect the major effect on human BM stromal cells, which are not transplanted with the CD34+ cells. We therefore decided to continue with the immortalized human BM stromal cells (Hs5 and Hs27a) to build an ex vivo system of the human BM environment. As a first step, we increased the expression of AR in the Hs5 BM stromal cells. We generated an androgen responsive human bone marrow stromal cell line by expressing the human Androgen Receptor (AR) in the Hs5 human bone marrow stromal cell line (**Figure 1**).

Figure 1. Exogenously expressed AR in Hs5 BM stromal cells becomes concentrated in the nucleus when stimulated by exposure to the synthetic testosterone (R1881). A. Western blot demonstrating increased AR expression in AR transfected cells. B. Immunofluorescence showing the dramatic increase in nuclear translocation of AR in transfected cells treated with the androgen precursor R1881.[anti-AR (green) and F-actin (red)]

Although we have generated a stable Hs5 cell line expression human AR, the expression of AR is not stable and decrease over time. We will therefore are attempting to express the AR in mesenchymal precursors,

because these cells may be able to tolerate higher and more stable AR expression. In addition we plan to alter culture methods to retain AR expression. This is accomplished by charcoal-stripped fetal calf serum. In the last year, more effective methods of using NOD/SCID mouse reconstitution have been developed. We are currently considering establishing one of these models in collaboration with the bone marrow transplantation group at the Fred Hutchinson Cancer Center (D. Beverly Torok-Storb is a collaborator on the grant application).

Task 3: To test the association between serum and bone marrow testosterone levels and expression of androgen or estrogen sensitive genes in bone marrow aspirates from patients

- Androgen-sensitive gene expression changes in human bone marrow
- Correlation between serum and bone marrow testosterone

To pursue this task, we obtained human samples of bone marrow from twenty patients with prostate cancer from Dr. Vessella, a collaborator on the grant. To evaluate the association between testosterone (T) and dihydrotestosterone (DHT) levels in serum and bone marrow, we obtained supernatants of BM aspirates. However, we quickly realized that there is an unknown dilution factor of BM aspirates when during their collection from the patients. This prohibited the accurate determination of T and DHT concentration. We have not been able to find a suitable normalization strategy to correct for the dilution factor. Normalizing to total protein did not work, because of variable amount of red cell lysis in the samples.

The androgen-responsive cells in the BM are believed to be the stromal cells (BMSC). Therefore, we used samples that were concentrated for BMSC. The Vessella lab isolates disseminated cancer cells from BM aspirates through serial depletion of CD45+ and BerH4+ cells. The remaining cells in BM aspirates should be

enriched in BMSC since BMSC are negative for CD45 and BerH4 expression. Thus the remaining samples were used for isolation of mRNA.

The regulation of T and DHT concentration in the BM is complicated. Levels are regulated by (1) diffusion from blood (2) local synthesis and (3) catabolism. To determine whether T levels could be locally decreased by an increase in T catabolism, we analyzed gene expression levels of enzymes that regulate the derivatization and inactivation of DHT. The genes are: AKR1C1, AKR1C2, AKR1C3, RODH-4, RODH-5, and RL-HSD).

Testosterone levels for twenty samples from bone marrow of patients with prostate cancer were analyzed and subdivided into the top and bottom quartiles (see table).

Table 1. Testosterone levels in patients with prostate cancer

	sample i.d.	BM T - ng/ml
1	23316K	0.2
2	23343R	0.56
3	23334D	0.68
4	23290M	0.69
5	23289C	0.72
6	23333L	0.82
7	23349C	0.82
8	23283K	1.03
9	23329L	1.03
10	23331K	1.03
11	23319B	1.08
12	23300B	1.14
13	23340K	1.25
14	23301H	1.26
15	23285C	1.28
16	23253G	1.31
17	23293M	1.4
18	23291C	1.41
19	23320H	1.66
20	23328J	1.94

**Top and bottom quartiles
from Table 1**

Bone Marrow Testosterone levels		BM T - ng/ml
1	23316K	0.2
2	23343R	0.56
3	23334D	0.68
4	23290M	0.69
5	23289C	0.72
6	23253G	1.31
7	23293M	1.4
8	23291C	1.41
9	23320H	1.66
10	23328J	1.94
low Testosterone Levels		
High Testosterone Levels		

B) Two bone marrow samples were selected from the top and bottom quartiles for evaluation of five androgen metabolising genes (Table 2).

Table 2. Bone Marrow gene expression levels for selected androgen metabolising genes

Bones Marrow Sample	Testosterone (ng/ml)	AKR1C1	AKR1C2	AKR1C3	RL-HSD	RODH-4
23334D	0.680	0.160	0.062	3.204	0.002	0.006
23343R	0.560	10.056	0.010	0.505	0.026	0.002
23328J	1.940	0.003	0.004	0.039	0.002	0.018
23253G	1.310	7.499	0.317	0.201	0.005	0.034
PEC control	n/a	0.027	0.054	0.867	0.000	0.000

All gene expression values are de-logged Ct's normalized to ACTB

low testosterone high testosterone

We did not observe an association between expression of DHT catabolizing genes and T levels, suggesting that T levels are not regulated through catabolism of DHT.

KEY RESEARCH ACCOMPLISHMENTS:

1) Characterized androgen responsive genes in mouse bone marrow (BM) via castration (androgen ablation) and published the results in a publication:

Regulation of global gene expression in the bone marrow microenvironment by androgen: androgen ablation increases insulin-like growth factor binding protein-5 expression. Xu C, Graf LF, Fazli L, Coleman IM, Mauldin DE, Li D, Nelson PS, Gleave M, Plymate SR, Cox ME, Torok-Storb BJ, Knudsen BS. Prostate. 2007 Nov

1;67(15):1621-9.

2) Measurements of testosterone, dihydrotestosterone and of genes that regulate the local androgen concentration in samples of human bone marrow.

3) Generating a human model system for studies of androgen regulation of the bone marrow stroma and its effects on cancer cells.

4) established a 3D system of bone marrow stromal cells and DU145 cancer cells

Below we report the outcomes that are relevant to each of the tasks listed in the grant: We are organizing the animal work for the human bone marrow xenograft studies in mice by generating the appropriate stromal cell line/androgen response system and determining the most effective controls to address the complexity of the mouse:human heterologous system.

REPORTABLE OUTCOMES:

Publication:

- Xu C., et al., Prostate, 67:1621-1629. (2007)
- Invited to submit a review by Journal of Cellular Biochemistry on androgen regulation of bone marrow stroma.

Meeting presentations:

- Epithelial to Mesenchymal Transitions (Cold Spring Harbor Laboratories, March 2008, poster)
- Annual Meeting (Society for Basic Urologic Research, November 2008, poster)
- Cancer Biology Seminar (Fred Hutchinson Cancer Research Center, talk)

Summer students working on project:

- Brian Schulte (Duke University, Durham, NC)
- Michael Hwang (Lakeside High School, Seattle, WA)

Project continuation: preliminary data for RO1 grant application

CONCLUSIONS:

We have generated a panel of androgen responsive genes, including cytokines, which are increased/decreased in the absence of androgen in a mouse bone marrow (Xu, et al., 2007). We focused on IGFBP5, which is the most consistently increased gene in the panel and show that its response is reversed by administration of androgen.

Preliminary results show that with the androgen metabolizing genes examined in the bone marrow samples from patients with prostate cancer there is little evidence for a positive correlation between androgen (testosterone) and androgen metabolizing gene expression levels. It is expected that testing the remaining six samples in the two quartiles will either verify the lack of a relationship between androgen and androgen catabolizing gene expression in metastatic prostate cancer of the bone marrow of these samples or reveal a technical problem with the analysis.

Lastly, the complexities involved in the mouse NOD-SCID/human bone marrow reconstitution model have forced us to find interim solutions for studies of prostate cancer/bone interactions in a human system. Hs5 cells transfected with AR are more suitable to investigate the effects of androgen on cancer cell/bone marrow stroma interactions.

The funding has generated a publication and established a project in the laboratory in which we will continue with studies on the interactions between prostate cancer cells and the environment and the effects of androgen in this system.

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Brodie A., et. al., Clinical Cancer Research, Jan 15;11(2 Pt 2):884s-8s. Review. (2005)

Xu C., et al., Prostate, 67:1621-1629. (2007)