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TITLE: PSA Converts Parathyroid Hormone-Related Protein (PTHrP) from an Osteolytic to an Osteoblastic Factor: Role in Bone Metastasis

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13. ABSTRACT (Maximum 200 Words)

Prostate cancer metastases cause disorganized new bone formation, despite expressing the bone-destructive factor, PTHrP. We report a molecular basis for this paradox: PTHrP is cleaved at residues 22 and 23 by the serine protease, prostate-specific antigen (PSA). The fragments generated by PSA are too short to activate the PTH1 receptor but stimulate new bone by activating the ETA receptor by an unknown, indirect mechanism. PTHrP peptides in neonatal mouse calvariae caused a 2.5-fold stimulation of new bone area ($p<0.001$). Osteoblast numbers were correspondingly increased. The response was equivalent to ET-1. The actions were blocked by atrasentan (0.01mM), a selective ETAR antagonist. PTHrP(1-23) gave a strong bone anabolic response at 1nM ($p<0.01$ vs control), while PTHrP(1-34) instead caused extensive osteolysis. We found that PTHrP1-23 does not bind to endothelin or PTH receptors but the peptide increases cellular expression of both muscle- and bone-specific genes.

An animal model has been developed in which a human prostate cell line makes PSA-derived PTHrP fragments but not intact PTHrP or endothelin-1. In 2005 this model will be used for preclinical testing of atresantan and of PTHrP-neutralizing antibody. These treatments should be effective against PTHrP fragments, in treating osteoblastic bone metastases due to prostate cancer.

14. SUBJECT TERMS
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PSA converts parathyroid hormone related protein (PTHrP) from an osteolytic to an osteoblastic factor: role in bone metastasis

Annual progress report

John M. Chirgwin, Ph.D., University of Virginia

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INTRODUCTION:

Bone metastases are a major source of morbidity and mortality in patients with advanced cancers of the breast and prostate (Mundy, 2002). Parathyroid hormone-related protein, PTHrP, is established as the major known causal agent in osteolytic bone metastases (Guise et al, 1996; Guise, 1997). These metastases are characterized by net destruction of bone in the vicinity of tumor cells and are commonly caused by breast cancers (Guise & Chirgwin, 2003a). By contrast, prostate cancers, which share with breast cancers a propensity to metastasize to skeletal sites, usually cause osteoblastic metastases, lesions that are characterized by net formation of disorganized new bone of poor mechanical quality. In these metastases it has been observed clinically that bone resorption is increased even more than in breast cancer osteolytic metastases. It is also known that prostate cancers usually express the same osteolytic factor, PTHrP (Guise & Chirgwin, 2003b). However, an understanding has been lacking of why prostate cancer metastases are usually osteoblastic rather than osteolytic, despite their expression of PTHrP. Our DoD Idea proposal offered a novel molecular basis to explain this paradox. PTHrP fragments are released by the action of prostate-specific antigen, PSA, which is a chymotrypsin-like serine protease. When these observations were published by Cramer et al and Iwamura et al in 1996, it was thought that the 23-amino acid fragment was inactive, since 1-34 is the minimum fragment length required to activate the PTH type 1 receptor efficiently. In 2001, however, Schluter et al observed that a short fragment of PTHrP stimulated cardiomyocyte contraction, apparently by activating endothelin type A receptor [ET_AR] on the cells, possibly via a short peptide homology between ET-1 residues 6-9 [LMDK] and PTHrP residues 8-11 [LHDK. In light of this unexpected observation, we hypothesized that in prostate cancers, PSA could cleave PTHrP into novel bone-stimulatory ligands and that these might act through the ET_AR (**Figure 1**). We recently established an animal model of osteoblastic metastasis that identifies ET_AR ligands as causal agents of skeletal pathology (Yin et al, 2003).

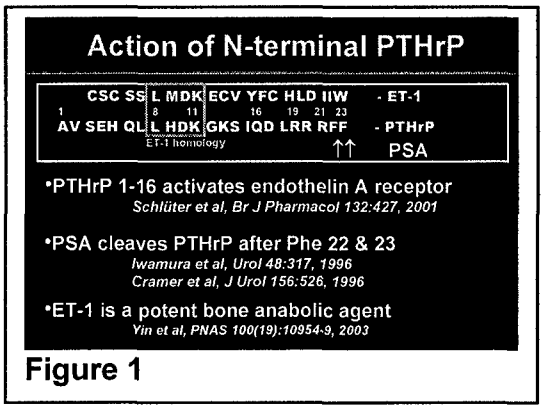

Figure 1

In 2003 we established that PTHrP 1-23 is a potent bone anabolic agent, with maximal bone-stimulatory activity at 1 nM. The effects of this fragment was blocked by the selective ET_AR agonist, ABT-627, atrasentan. This compound is effective in an animal model (Yin et al, 2003) and has completed Phase II clinical trials, in which it was effective at decreasing skeletal-related events in men with advanced, hormone-refractory prostate cancer (Carducci et al, 2003; Nelson et al, 2003). This drug has now been taken to the FDA for approval for the treatment of men with bone metastases due to advanced prostate cancer. In addition, PTHrP-neutralizing antibodies are now in clinical trials in cancer patients (Sato et al, 2003; Onuma et al, 2004). This humanized antibody recognizes the N-terminus of the molecule and was originally shown, prior to its humanization, to be effective in decreasing cancer bone metastasis by our Co-investigator (Guise et al, 19996).

In October 2003 the P.I. moved, along with his colleagues Drs. K.S. Mohammad and T.A. Guise, from the University of Texas Health Science Center in San Antonio, to the University of Virginia in Charlottesville. The UVa Cancer Center has been extremely supportive. UVa is a major center of prostate cancer research and has many world-class researchers in basic signaling mechanisms in prostate cancer cells. The previous progress report covered 04/01/03 – 12/01/03, rather than a full 12 month period. There was a 6-month gap in which research was not supported by the DoD award during the transition between institutions. A revised statement of work (SOW) was submitted in 02/2003 and appended here.

Summary of scientific findings in 2003:

1) PTHrP1-23 stimulated strong osteoblastic responses at 1nM
2) Effects of PTHrP1-23 on bone were blocked by the ETa receptor antagonist atrasentan

New scientific findings in 2004:

3) PTHrP1-23 did not bind to or activate overexpressed ETa or ETb receptors

4) PTHrP1-23 did not bind to the type 1 PTH receptor

5) Binding of PTHrP1-23 to any of these three G protein-coupled seven-transmembrane domain receptors was not altered by co-expression of any of the receptor activity modifying proteins, RAMPs 1-3.

6) PTHrP1-23 did not induce detectable secretion by calvarial organ cultures of endogenous endothelin-1 (which would have explained the efficacy of the ETa-selective receptor antagonist atrasentan).

7) We recently found that treatment of primary mouse osteoblasts with endothelin-1 strongly induced the expression of a series of genes at 1 hour: interleukin-6, connective tissue growth factor (CCN2), and the related CCN1 protein, Cyr61. In a parallel experiment with cells treated for 1 hr with PTHrP1-23, there was *no change* in the mRNAs for these three genes by real-time PCR.

We **conclude** that, contrary to the model of Schluter et al (2001), **PTHrP1-23 does not stimulate osteoblastic responses by binding to the endothelin receptor.** The effectiveness of atrasentan to block new bone formation due to PTHrP1-23 is thus without a mechanistic basis. Atrasentan does not block new bone formation due to BMP2, adrenomedullin, or IGF receptor activation

8) We have established an animal model in which the human prostate cancer cell line C4-2B causes a mixed bone response to inoculated tumor cells. In the third year of the proposed work we will use this model to test the efficacy of two treatments: a) the ETa receptor antagonist atrasentan; and b) a monoclonal neutralizing antibody directed against the N-terminal sequence of PTHrP.

9) Since our finding that PTHrP1-23 did not bind to known receptors for endothelin or PTHrP, we have begun experiments to identify the targets of PTHrP1-23 in bone. Gene array experiments with calvariae treated for 24hrs with either ET-1 or PTHrP1-23 showed that both factors had strong activities to stimulate myogenic differentiation. PTHrP1-23 in particular stimulated increases in mRNAs for skeletal muscle-specific transcription factors (such as MyoD and Myf5) as well as a panoply of muscle-specific structural proteins. ET-1 was found also to act directly on primary osteoblasts to alter expression of IL-6, Cyr61, CTGF and Dkk1. Our preliminary results suggest that PTHrP1-23 may stimulate new bone formation by upregulating TIMP-1 and the microphthalmia transcription factor Mitf1 (which is associated with a bone phenotype). Other major responses to PTHrP1-23 include a dramatic increase in mRNA for CopZ1 (coatomer subunit zeta1) and dramatic decreases in the mRNAs for Sirt2 (involved in longevity, NAD/NADH sensing and chromatin deacetylation) and CD36 (anti-angiogenic scavenger receptor b2 for type 1 collagen and for thrombospondin). At this point changes in expression of these genes in osteoblasts (as opposed to cells in the myogenic lineage) have not been confirmed.

Overall Progress according to Specific Aims and SOW Tasks:

Nearly all of the important aspects of Aims 1 and 2 have been completed. Task 4 has so far been unsuccessful due to limitations of the available monoclonal antibody. Task 4 will be attempted again in 2005 with a new anti-PTHrP N-terminal antibody. The technical details of how each of the tasks has been carried out have changed substantially since the original submission. However, the overall Aims are unchanged; so we do not believe it necessary to submit a revised SOW for the third year. We will be happy to do so should the reviewers thing differently. The discovery during 2004 that PTHrP1-23 does not interact with the endothelin receptors, although its action is blocked by an endothelin receptor antagonist, was entirely unexpected, but it does not affect the rest of the work proposed. The important parts of the proposal are Aims 1 and 3. Aim 1 is complete and establishes the important new insight that, as predicted, PTHrP1-23 is a potent osteoblastic factor, with actions very different from the osteolytic actions of PTHrP1-34. Specific Aim 3 will establish the importance of PTHrP1-23 in a preclinical animal model. We are on-track to complete this Aim in 2005. The experiments will use two agents that are already in advanced clinical trials in cancer patients.

BODY of Report:

Work is reported according to the **revised (2/2003) SOW**, which is included in the Appendix.

For **Specific Aim 1** most of the 5 tasks are now completed

Tasks 1 & 2 previously completed.

Tasks 3 & 4: Overall goal was to identify or modify a prostate cancer cell line expressing PTHrP and active PSA, thus secreting cleaved N-terminal fragments of PTHrP. A necessary part of this task was to assure that the cell line caused appropriate bone metastases in an animal model. Since the PSA gene is absent from the mouse genome (Borgono et al, 2004), the cell lines for this experiment are limited to established human prostate lines. This task was previously modified and is now **almost complete.**

A modified Task 3a was described in the 2003 progress report. Five human prostate cancer cell lines and several derivatives were screened for expression of PTHrP and PSA. The results are shown in Table 1 (from the previous report).

TABLE 1

Prostate Cancer Cell Line	PSA	N-term PTHrP	Intact PTHrP	Ratio N-terminal to Intact PTHrP
PC3, wt	0	10.7	9,1	1.2
LNCaP, wt	193	32,3	0.57	57
LNCaP, dox-reg	185	61.7	1.54	40
LAPC4	trace	138.6	1.88	73
C4-2B	165	34.8	0.91	38
PC3, AR+ v1	0	0	0.37	0
PC3, AR+ v2	61	0	8.96	0

PSA expressed as pg/ml/10^5cells/48hrs. N-terminal PTHrP expressed as pM/10^5cells/48hrs (Phoenix single-site competition RIA). Intact PTHrP expressed as pM/10^5cells/48hrs (Nichols two-site IRMA). Media collected serum-free from subconfluent cultures. Dox-reg are LNCaPs stably expressing dox-inducible regulatory system. Data from cells treated with doxycycline. PC3 AR+ are PC3s stably transfected with wt androgen receptor. V1 and v2 are independent clones. Dox-reg, LAPC4, and PC3 AR+ cells grown in presence of synthetic androgen R1881.

Table 1 suggested that the androgen independent derivative C4-2B (Thalmann et al, 1994; Wu et al, 1994) was the best candidates cell line to be used in the animal experiments, since it express PSA and shows a high ratio of N-terminal to intact PTHrP, suggesting substantial cleavage of PTHrP. Our previous experience with LNCaP cells has been that they cause very little bone responses. We have now established an animal model with the C4-2B cells inoculated intratibially into male nude mice. Importantly, *C4-2B cells do not produced detectable endothelin-1.* Results of the pilot animal experiment are shown as x-rays of affected tibiae in **Figures** (below).

Task 5: Immuno-affinity purify PTHrP fragments from media of cell lines shown in Table 1 and analyze fragments by HPLC/mass spectrometry (months 9-15, 25-30). We coupled the N-terminal PTHrP neutralizing mAb available to us, 3F5, to agarose resin by two different standard chemistries (Pierce Chemical Co kits), according to the manufacturer's directions. Coupling was successful but in both cases, elution of bound ligands with low pH resulted in activation of the mAb. We thus have no success to date with this task.
The 3F5 mAb neutralized PTHrP bioactivity in vivo (Kakonen et al, 2002), but much larger amounts were required than a previously used antibody (Guise et al, 1996). We have now obtained a supply of this previous and more potent antibody through an MTA negotiated with Chugai Pharamceuticals. The mouse mAb has been humanized by Chugai and in this form is in extensive clinical trails (Onuma et al 2004). We will repeat the experiments outlined in the previous paragraph with this newly-available mAb during 2005. It remains a distinct possibility that this task will not be successfully completed if the antibody is not suitable for immunoaffinity purification.

For **Specific Aim 2**):

Original Tasks 6-9 are completed. An additional set of experiments under **task 8a** was added, to look at the effects of PTHrP1-23 on gene expression by the mouse pre-osteoblastic cell line MC3T3-E1. We have compared the response of a variety of subclones of this cell line to that of primary mouse osteoblasts when treated with endothelin-1 and we conclude that the MC3T3-E1cell line variants are of little value. Gene array data (although not proposed in the SOW) are provided here, since they address the scientific questions asked by the previous version of this task. We have examined extensively the effects of PTHrP1-23 on standard cell lines overexpressing endothelin (A or B) or PTH type1 receptors, all plus or minus co-expression of the three RAMPs (Udawela et al, 2004), which have been reported to interact with the PTH1R () but not ETa or Etb.

New and unexpected data from modified task 8a

During the previous work period we initiated two new collaborations to accelerate the work proposed. The laboratory of Masashi Yanagiswa (the discoverer of endothelin), Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, tested PTHrP peptides for binding to and activation of the cloned endothelin A and B receptors. These are technically very difficult experiments, since ET-1 binding to its receptor is essentially irreversible and the assays require highly specialized expertise (Kedzierski & Yanagisawa, 2001). We have also collaborated with the laboratory of Thomas Gardella at Massachusetts General Hospital to determine peptide-binding parameters to the PTH type 1 receptor. Again this is a highly specialized technique, in which Dr Gardella is a world expert (Gensure et al, 2003.

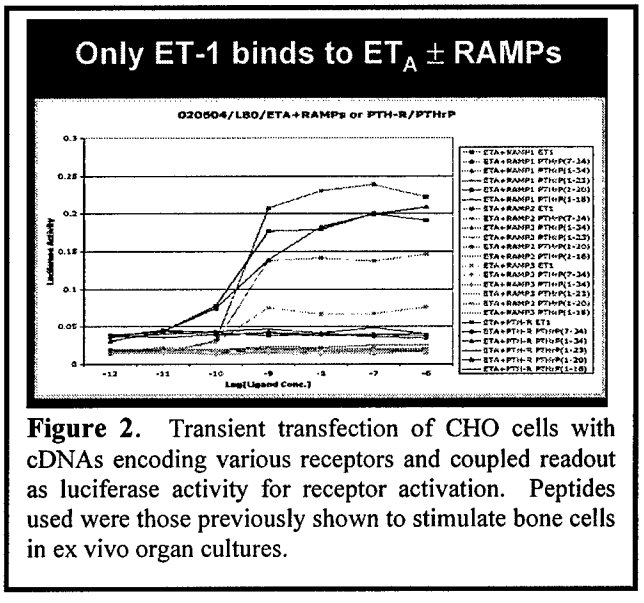

Figure 2. Transient transfection of CHO cells with cDNAs encoding various receptors and coupled readout as luciferase activity for receptor activation. Peptides used were those previously shown to stimulate bone cells in ex vivo organ cultures.

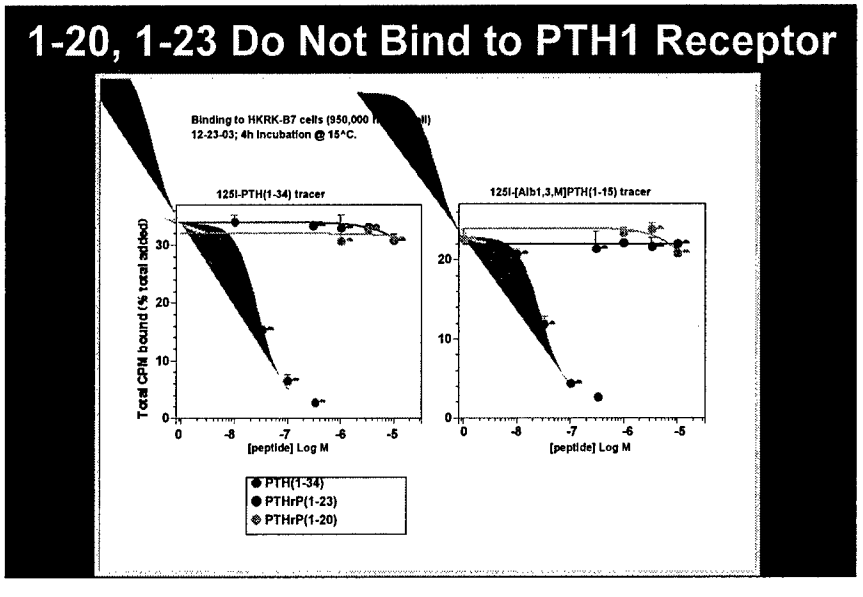

Figure 3: PTHrP1-34, but not 1-20 or 1-23, displaced two different radiolabeled PTH1R ligands from cells stably overexpressing the type 1 PTH receptor.

In collaboration with Drs. Masashi Yanagisawa and Hirokazu Usui at UT Southwestern and Dr. Tom Gardella at Harvard, we have completed testing the binding of PTHrP N-terminal fragments to cells overexpressing cloned ETa ETb or PTH1 receptors, plus or minus co-expression of the cDNAs for RAMPs 1, 2, or 3.

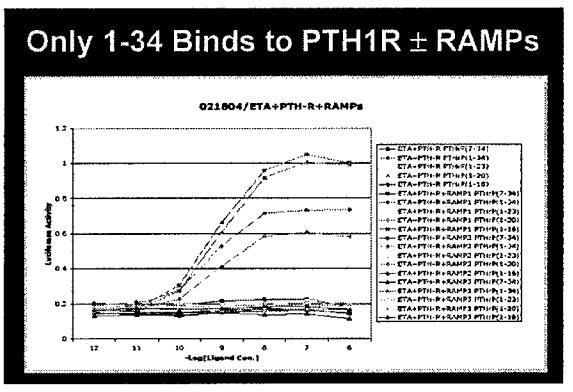

Figure 4. Co-expression of RAMP 1,2 or 3 subunit proteins did not confer binding of PTHrP1-23 to receptor-overexpressing cells.

Figures 2-4 show that there is no detectable binding of PTHrP N-terminal fragments to any of these combinations of receptor subunits.

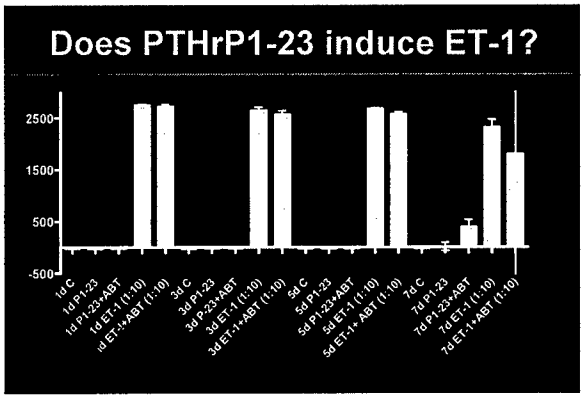

Figure 5. PTHrP1-23 treatment of neonatal mouse calvarial organ cultures did not increase endogenous ET-1 production. Scale is in pg/ml immunoreactive ET-1 (R&D Systems assay, limit of detection of assay = 0.5pg/ml). Media changed and assayed at 48 hr intervals. ET-1 was increased only at 7 days, but abundant new bone formation is visible at 5 days, when no ET-1 was detected.

These experiments eliminate any simple explanation of how the ETa-selective receptor antagonist atrasentan blocks the new bone formation induced by 1 nM PTHrP1-23. A possible explanation is that PTHrP1-23 induces endogenous expression of ET-1 in bone cells, which then serves as a downstream mediator of the bone-anabolic effects of PTHrP1-23. We tested this possibility by assaying ET-1 concentrations in media of neonatal calvarial organ cultures treated with PTHrP1-23. **Figure 5** shows that PTHrP does not increase free ET-1 concentrations in these cultures. The strongly positive bars are the ET-1 in control cultures to which synthetic 21 amino acid ET-1 has been added. In work described in greater detail below, we have used Affymetrix gene array analyses to identify some of the pathways in osteoblasts which are activated by anabolic treatments such as endothelin-1. These experiments, carried out by Dr. Greg Clines, a senior endocrine fellow training with Dr. Theresa Guise, showed that treating bone cells with ET-1 strongly increased IL-6, Cyr61 and CTGF mRNAs at 1 hr and decreased the mRNA for Dkk1 at 24 hrs. We tested these 4 primer sets with real time PCR. None of these genes responded to treatment with PTHrP1-23 for 1 or 24 hrs. **Figure 6** shows the results for IL-6, the most highly ET-1 responsive mRNA in primary mouse osteoblasts.

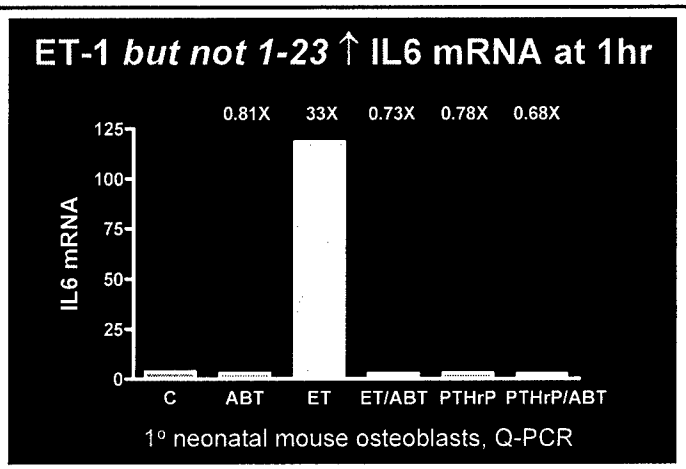

Figure 6. Real-time PCR quantitation of mouse IL-6 mrNA in primary neonatal calvarial osteoblasts treated for 1 hr. Positive control is 100nM ET-1. ABT=10-5M atrasentan. PTHrP1-23 tested at 100nM. Only ET-1 increased IL-6.

How does PTHrP1-23 stimulate new bone formation? Neonatal mouse calvariae were cultured ex vivo plus or minus ET-1, adrenomedullin, or PTHrP1-23 at 100nM. RNA was collected at 24hrs for Affymetrix gene array analysis, while parallel cultures were carried on for 7 days to assure that the cultures responded to the factors by forming new bone. Results of the gene arrays are preliminary and have not been validated by real-time PCR of primary mouse osteoblasts. Thus the changes in gene expression may be occurring in cells not of the osteoblast lineage, such a s fibroblasts, endothelial cells and myogenic cells. **Figure 7** summarizes the results. A number of ubiquitin /proteasome pathway components were increased 10X or more by PTHrP1-23.COP-Z expression was increased 12X. Dlx3 and TIMP-1 were increased 3X. The former is a transcriptional regulator that has an established role in osteoblastic bone formation (Haldeman et al, 2004; Hassan et al, 2004)

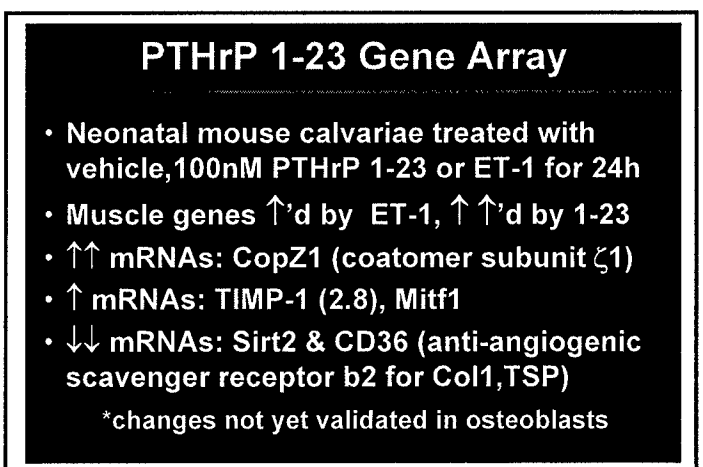

Figure 7. Preliminary calvarial gene array results. Latest Affymetrix gene chip (12/04) represents ~35k sequences. Many highly changed mRNAs are known only as ESTs.

and may be involved in transducing osteoblastic responses to ET-1 and BMP-2. TIMP-1 appears to be more than a simple inhibitor of metalloproteinases. Increased TIMP-1 expression is correlated with poor prognosis and metastasis in several cancers, including prostate (Isisag et al, 2003), where TIMP-1 may be regulated by NFkappaB transcriptional activity (Andela et al, 2003). Transgenic overexpression of TIMP-1 targeted to the osteoblast lineage results in increased bone formation (Geoffroy et al, 2004). Expression of Sirt2, the Zipf537 transcription factor, and the multifunctional scavenger receptor CD36 were decreased 10X. CD36 binds type I collagen and thrombospondin. The interactions between CD36 and TSP can alter bone remodeling (Carron et al, 2000).

In the final year of the proposed work (2005), the expression of these identified genes will be validated by Q-PCR with mRNAs from cells treated for 1, 24 and 72 hrs +/- 100nM PTHrP1-23. PCR will be carried out with mRNAs from primary neonatal mouse calvarial osteoblasts and with the mouse myogenic cell line C2C12. These experiments should indicate whether PTHrP1-23 activates bone formation by direct action on osteoblasts or through an epistatic mechanism via skeletal muscle differentiation (which seems unlikely). If a direct response of osteoblasts to the peptide is established, then Scatchard binding analysis of [^{125}I-Tyr22]-labeled PTHrP1-23 binding to primary osteoblasts will be carried out as a first step to identifying the apparently novel receptor for this peptide.

For **Specific Aim 3**):

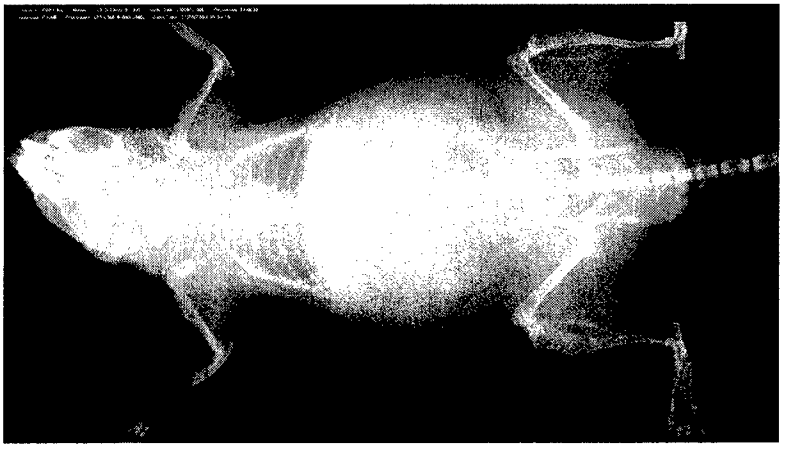

Figure 8: Faxitron digital X-ray at 24 wks. Injected limb is at lower right; unaffected limb is at upper right of X-ray.

Task 10: The proposed pilot animal experiment for a prostate cancer cell line secreting PSA-generated PTHrP fragments has now been carried out with the C4-2B cell line. Detailed histology and histo-morphometry of the bone lesions in these animals will be carried out in early 2005. Results from this experiment will be used to optimize the central animal experiment of the project, which is Task 11. Male nude mice are inoculated intratibially with $2x10^5$ cells and develop bone lesions detectable by digital X-ray at 10+/-2 weeks. Progress of the tumor in bone is indolent and the animals do not develop cachexia, even at 6 months. **Figure 8** shows a whole animal X-ray of a tumor-bearing mouse at 24 weeks post inoculation. A characteristic bone lesion is seen at the lower right, where the tumor has caused a characteristic but unusual honeycombed appearance of the bone. We have not observed fractures in these animals and the lesions are distinctly different than those caused by the osteolytic cell line PC3 or the strongly osteoblastic xenograft LuCAP23.1. The C42B bone lesions develop over the course of 4-6 weeks and then show little change over further periods. **Figure 9**, left *vs* right panels, shows such changes in two (upper *vs* lower panels) tumor-bearing animals. The bones from these animals are now being processed for histology and histomoprhometry. On the basis of these data, we plan to treat mice (next task) for 4-6 weeks with atrasentan, anti-PTHrP mAb, or vehicle, from the time lesions become visible by X-ray (approximately 10 weeks), as determined by biweekly Faxitron x-rays.

Task 11: Carry out full animal experiment with a prostate cancer cell model identified from Aim 1 in an in vivo model of bone metastsis (n=12 mice/group) and intramuscular tumor growth rate model (n=6 mice/group) with 72 mice total (in year 2005). Previously, this experiment was proposed using a series of cell lines derived from PC3, overexpressing hK2 and or PSA. Instead we will use C4-2B cells which form intratibial bone lesions. The selected prostate cell line (PSA-positive and producing high concentrations of PTHrP fragments shorter than 1-34 and low concentrations of endothelin-1 and longer PTHrP molecules) will be tested in the intra-tibial model, with which he have experience. Animals will be treated with artrasentan (ABT-627) as described (Yin et al, 2003) or PTHrP-neutralizing mouse monoclonal antibody as described (Guise et al, 1996). We have drug and purified mAB available. We will follow progress of tumor in bone by X-ray, as described in the preceding task. C42Bs are PSA+ and tumor burden will also be followed by PSA assay of serum samples. Efficacy of the treatments will be analyzed from the detailed histomorphometric analysis of the tumor and bone parameters in the following task. It is not practical to quantify the x-ray images of the mixed lesions such as those shown in **Figure 9**.

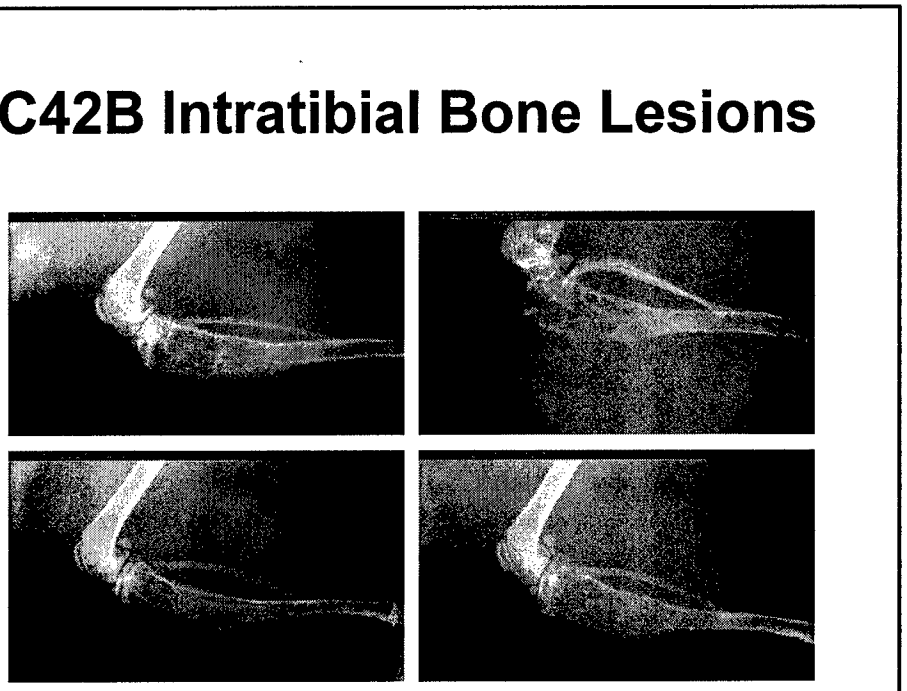

Figure 9: Faxitron digital X-ray images (4X) of left tibiae of male nude mice inoculated with $2x10^5$ C42B cells. Upper panels are of same mouse at 8 wks (left) and 14 wks (right). Lower panels are of same mouse at 12 wks (left) and 16 wks (right).

Task 12: Carry out detailed histomorphometric analysis of the bones and soft tissues of the 8 mice from task 10 (bones only, already underway) and the 48 metastasis model mice (48+288 bone histology blocks) from the previous task.

Task 13: Perform statistical analysis of data from animal experiments and prepare manuscripts for publication.

KEY RESEARCH ACCOMPLISHMENTS:

- PTHrP fragments shown to stimulate new bone formation (2003)

- Effects of PTHrP fragments shown to be **indirect**, via activation of the endothelin axis
- Mechanism of action of PTHrP1-23 **not** via ETa, ETb, or PTH1 receptors
- PTHrP1-23 does **not** induced endothelin-1 synthesis
- PTHrP1-23 does not activate ET-1 responsive genes in osteoblasts, such as IL-6
- PTHrP1-23 activates expression of myogenic genes and potential osteoblastic genes such as TIMP-1, Mitf1, and Dlx3
- Animal model developed with human prostate cancer cell line, which a) causes mixed osteoblast/osteolytic bone lesions; b) secretes PTHrP N-terminal fragments; c) does not produced substantial intact PTHrP; d) does not secrete endothelin-1 or adrenomedullin, two potent, potentially confounding, osteoblastic factors; secretes PSA and hK2.
- Reagents for critical preclinical testing of animal model (atrasentan and PTHrP-neutralizing monoclonal antibody) obtained

REPORTABLE OUTCOMES:

Relevant publications:

Chirgwin JM, Mohammad KS, Guise TA. Tumor-bone cellular interactions in skeletal metastases. J Musculoskeletal Neuronal Interact, 4:308-318, 2004.

Clines GR, Chirgwin JM, Guise TA. Skeletal complications of malignancy: central role of the osteoclast. Chapter for Topics in Bone Biology, Vol 2, accepted for publication, 2004

Indirectly related publications:

Guise TA, Chirgwin JM. Biology of bone metastases. Chapter in Diseases of the Breast, 3rd edition. Harris, Lippman, Morrow, and Osborne (eds). Lippincott Williams & Wilkins, pp 1285-1296, 2004.

Graham Solomons JT, Burns S, Wessner L, Krishnamurthy N, Zimmerly E, Swan MK, Krings S, Muirhead H, Chirgwin J, Davies C. The crystal structure of mouse phosphoglucose isomerase* at 1.6 Å resolution and its complex with glucose 6-phosphate reveals the mechanism of sugar ring opening. J Mol Biol, 342:847-860, 2004.

*Phosphoglucose isomerase is identical to tumor autocrine motility factor, which plays an established role in tumor progression, including prostate cancer.

Guise TA, Kozlow WM, Heras-Herzig A, Padalecki SS, Yin JJ, Chirgwin JM. Molecular mechanisms of breast cancer metastases to bone. Clin Breast Cancer, accepted for publication, 12/2004.

Invited lectures in which project data were presented:

22 January 2004
Cell & Cancer Biology Branch
National Cancer Institute
National Institutes of Health
Roles of osteoblast-stimulating factors in bone metastases.

2 October 2004
American Society for Bone and Mineral Research
Annual Meeting,
Seattle, Washington
Working group on G protein-coupled receptor signaling
Endothelin Axis in Osteoblasts: Molecular Mimicry by PTHrP Fragments

17 December 2004
Department of Orthopaedics,
Center for Tissue Regeneration and Repair
University of California Davis School of Medicine,
Stimulation of bone metastases by PSA-generated fragments of PTHrP and by adrenomedullin

CONCLUSIONS:

The project continues to be successful. Our experiments *ex vivo* establish that PTHrP 1-23 is a potent bone-anabolic agent, while our parallel experiments establish that tumor-produced ligands for the ET A receptor are causal agents in osteoblastic bone metastases (Yin et al, 2003), **Figure 10.**

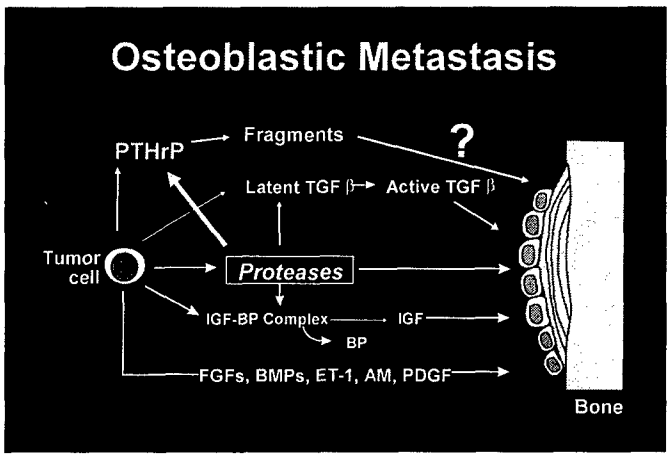

Figure 10. Summary of roles of both ET-1 and PTHrP N-terminal fragments as osteoblastic factors in prostate cancer bone metastases. Question mark indicates uncertainty about the role of the endothelin A receptor in PTHrP fragment action.

We have found a human prostate cancer cell line that makes PTHrP N-terminal fragments by the proteolytic action of PSA on intact PTHrP (139 aa and larger), but does cause osteoblastic responses in bone, while not express ednothelin-1 or large amounts of other osteoblastic factors such as adrenomedullin. This turned out to be a challenge, but it has been accomplished. We are now on track to complete the crucial third specific Aim during the final year of the project. This will establish a preclinical model for therapy aimed against bone responses caused by N-terminal fragments of PTHrP produced by tumor cells.

Detailed ligand binding experiments were not proposed in the original grant, but these have been carried out with expert collaborators in the fields of ET receptors and PTHrP receptors. These results substantially strengthen the molecular basis of our observations. However, they raise a substantial quandary about the role of endothelin in the observed phenomena. We currently have a clinically important biological response and potential drugs for treatment, but no clear molecular basis for the action of PTHrP1-23. Thus we have pursued mechanistic studies beyond those originally proposed, including gene arrays. The results identify new, testable targets for the actions of PTHrP1-23 on bone. These will be investigated in 2005. We have also prepared Phe to Tyr22-substituted PTHrP1-23. This will be radiolabeled with ^{125}-I and used to test for receptor-mediated binding to primary mouse osteoblasts and to mouse C2C12 myoblasts and myotubes to further define the mechanisms by which PTHrP stimulates osteoblastic responses.

Although the role of ET-1 in PTHrP1-23 stimulation of bone formation is mechanistically murky, the anti-ET-1 drug atrasentan has been taken by Abbott in late 2004 to the FDA for approval to treat men with advanced prostate cancer and bone metastases (Fisher, 2002; Lee, 2003; Ryan et al, 2004; Jimeno & Carducci, 2004). The experiments to be carried out in 2005 for Specific Aim 3 will test whether atrasentan is effective against tumor cells that make PTHrP fragments but not ET-1 In addition, our results provide insights into a physiological role for PSA (and hK2) in prostate cancer bone metastases. They suggest that inhibitors of PSA would not benefit patients with PTHrP+ PSA+ bone metastases, but would rather change the bone response from osteoblastic to osteolytic. Neprilysin, another protease secreted by prostate cancers, also cleaves PTHrP at residue 23 (Ruchon et al, 2000) and may play an endogenous role in normal bone. Neprilysin inactivates endothelin-1 and appears to be decreased with prostate cancer progression (Nelson & Carducci, 2000). It may be that changes in protease expression (PSA, hK2, neprilysin, et al) alter the processing of a set of bone active factors such as PTHrP and ET-1 and contribute to the variable phenotype of bone metastasis characteristic of prostate cancer (Roudier et al, 2003).

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APPENDIX:

STATEMENT OF WORK, revised February 2003:

The actual numbered tasks remain unchanged, as do the **Specific Aims**.

Additions to, changes in, or completions of individual tasks are indicated in **bold**. The time frame in Virginia is now:

03/01/2003-12/31/2003 = *months 1-9* (in *italics*)
01/01/2004-12/31/2004 = *months 10-21* (in *italics*)
01/01/2005-12/31/2005 = *months 22-33* (in *italics*)

Months not italicized are the months from the original SOW.

Please note that **Proposed Animal Use Is Unchanged**

For Specific Aim 1):

Task 1: Reclone prohK2 & proPSA cDNAs into pcDNA vectors, test DNAs by transient transfections into 293 cells and assay as in task 4, below (months 1-3). **COMPLETE.**

Task 2: Isolate stable single cell lines with DNAs from task 1 (months 4-12). **Complete for proPSA.** Cell lines for hK2 remain to be isolated (*months 1-9*).

Task 3: Isolate stable double (hK2+PSA) cell line with DNAs from task 1 and hK2 stable clone from task 2 (months 13-24; now *months 6-21*).

An additional section to task 3 has been added. The Isaacs' laboratory has just published (Denmeade et al, March 2003) detailed characterization of a series of prostate cancer cell lines for their expression of both hK2 and PSA. These lines are LAPC-4, CWR22Rv1, MDA PCA-2b, LNCaP, and C4-2B (derived from LNCaP), all of which are available to us through the resources of the NCI-supported UVa Cancer Center. We propose to assay these cell lines for PTHrP expression and secretion by three assays. First RNAs will be prepared from the 5 cell lines or xenografts and analyzed by standard RT-PCR for PTHrP gene expression. Second, conditioned media from the cells will be assayed for content of intact PTHrP with the Nichols Institute two-site IRMA. Third, the same media samples will be assayed with a single-site competition RIA, which employs a high-affinity antibody that recognizes the amino-terminus of PTHrP (residues 1-7), from Phoenix, Inc, which has been used successfully by our UVa colleague, Dr. S.J. Parsons. This assay will detect all cleaved forms of PTHrP. We expect that at least some of these cell lines, which make high amounts of active PSA and hK2, may cleave PTHrP efficiently. Media from these lines will be negative with the Nichols ELISA and positive with the Phoenix RIA.

Task 3a: Screen 5 cell lines for PTHrP expression (*months 1-9*). If none are positive, we will transfect a PTHrP expression DNA (already available and tested) into one of the hK2+, PSA+ lines. Resultant clones would then be screened with the three assays for PTHrP.

Task 3b: Isolate PTHrP+ clones for a prostate cancer hK2+, PSA+ cell line (*months 10-21*).

Task 4: Screen clones from tasks 2&3 for enzymatic activity and protein expression (months 6-12, 18-24, now *months 1-21*)

Task 5: Immuno-affinity purify PTHrP fragments from media of cell lines from tasks 2&3 and analyze fragments by HPLC/mass spectrometry (months 13-18, 25-30, *now months 9-15, 25-30*). **Analysis of fragments from cell lines newly identified from additional subtasks 3a & 3b now included.**

For Specific Aim 2):

Task 6: Prepare synthetic peptides (months 1-3). **COMPLETED.**

Task 7 Test synthetic peptides on osteoclast formation in marrow cultures (months 4-9). **COMPLETED.**

Task 8: Test synthetic peptides on new bone formation in calvarial cultures (months 6-24). **Substantially completed.** Final experiments as originally proposed (*months 1-6*).

Additional section to task 8. Neonatal mouse calvarial organ cultures are slow and expensive. Now that we have demonstrated the essential correctness of the original hypothesis in organ culture, we will obtain more accurate dose response data using a well-characterized mouse pro-osteoblastic cell line, MC3T3-E1. Twenty-three published papers have tested the ET-1 receptor responsiveness of these cells , and others have shown PTHrP responsiveness McCauley et al, 2001). Thus, the cells express receptors for both peptides and respond to them. The cells will differentiate into mature osteoblastic cells in culture when treated with ascorbate and form mineralized nodules, as well as express late markers, such as osteocalcin. We will carry out detailed dose responses of the peptides using these cells. We are presently determining the most convenient marker of differentiated response to assay (such as VEGF promoter-luciferase activity (Wang et al, 2002), alkaline phosphatase secretion, or osteocalcin production.)

Task 8a: Test peptides on MC3T3-E1 cells (*months 10-24*).

Task 9: Perform tasks 7, 8, and 8a on samples from task 5 (months 19-20, 31-32, now *months 9-15 & 37-30*).

For Specific Aim 3):

Task 10: Carry out pilot animal experiment for PC3/ev and PC3/hK2 cell lines from Aim 1) in metastasis model with 8 mice (months 15-20, now *months 10-15*).

Task 11: Carry out full animal experiment for the final four cell lines from Aim 1in metastasis model (n=12 mice/group) and intramuscular tumor growth rate model (n=6 mice/group) with 72 mice total (months 25-30, now *months 25-30*). **Alternatively, this task could be carried out with one of the cell lines identified in task 3a or 3b.**

Task 12: Carry out detailed histomorphometric analysis of the bones and soft tissues of the 8 mice from task 10 (bones only, months 21-24, now *months 16-21*) and the 48 metastasis model mice (48+288 bone histology blocks) from the previous task (months 31-34, now *months 30-33*).

Task 13: Perform statistical analysis of data from animal experiments and prepare manuscripts for publication (months 11-12, 23-24; 35-36, now *months 3-6, 20-21 and 32-33*).

Summary of the original 01 year progress report,

submitted during the relocation to University of Virginia effective 10/01/02:

The aims, tasks, and the schedule remain essentially unchanged from those originally proposed:

Task 1: complete

Task 2: underway and expected to be completed by 6/30/03

Task 3: to be initiated after transfer to Virginia

Task 4: assay procedures already established. Experiments to be initiated after transfer to Virginia

Task 5: affinity columns of immobilized anti-PTHrP N-terminal 3F5 monoclonal antibody already made. They are presently being tested and will be used as proposed after transfer to Virginia

Task 6: completed for PTHrP peptides 1-16, 1-20, 1-23, and 1-34

Tasks 7 & 8: completed for PTHrP peptides 1-16 and 1-34, underway for 1-20 and 1-23. Task 7 now uses an assay that combines the cultures of task 8, but is otherwise unchanged

Tasks 9-13: will be carried out according to the originally proposed schedule following transfer to Virginia

At this time of the transfer from UTx to UVa, all of the proposed tasks were on schedule. We anticipate being able to keep to the original tasks and timetable despite the inevitable dislocations and delays associated with a cross-country move of laboratory and personnel.

References added for February 2003 revision:

Denmeade SR, Sokoll LJ, Dalrymple S, Rosen DM, Gady AM, Bruzek D, Ricklis RM, Isaacs JT. Dissociation between androgen responsiveness for malignant growth vs. expression of prostate specific differentiation markers PSA, hK2, and PSMA in human prostate cancer models. *Prostate* **54**:249-257, 2003.

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Wang X, Tokuda H, Hirade K, Kozawa O. Stress-activated protein kinase/c-Jun N-terminal kinase (JNK) plays a part in endothelin-1-induced vascular endothelial growth factor synthesis in osteoblasts. *J Cell Biochem* **87**:417-423, 2002.