AD_____

Award Number: W81XWH-04-1-0278

TITLE: Role of Bone Remodeling in Skeletal Colonization by Prostate Cancer Cells

PRINCIPAL INVESTIGATOR: Robert J. Majeska, Ph.D.
Mitchell B. Schaffler, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
New York, NY 10029-6574

REPORT DATE: August 2005

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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 Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-08-2005	Annual Summary	26 Jan 2004 – 25 Jul 2005

4. TITLE AND SUBTITLE

Role of Bone Remodeling in Skeletal Colonization by Prostate Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0278

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Robert J. Majeska, Ph.D.
Mitchell B. Schaffler, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Mount Sinai School of Medicine
New York, NY 10029-6574

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. **ABSTRACT**
Prostate cancer metastasizes selectively to bone, but the role of host tissue in promoting skeletal metastasis is not fully understood. This project tests whether sites of bone remodeling (resorption and formation) are targets for initial tissue colonization by circulating prostate tumor cells. In this project human prostate cancer cell lines (LNCaP, PC3) expressing high levels of green fluorescent protein (GFP) were selected and found to have similar growth and adhesive properties to cells expressing lower GFP levels. Following injection into the vasculature of nude mice, both LNCaP and PC3 cells were identified in tibial metaphyses and found to localize preferentially to bone surfaces that are resorbing. This finding supports the hypothesis that initial colonization of bone by prostate cancer cells is at least partly targeted to areas of bone remodeling. In addition, inhibition of bone resorption by pre-treatment with a bisphosphonate inhibited colonization by LNCaP and PC3 cells, not only at resorbing sites but to a lesser extent near forming and quiescent bone. This suggests that bone resorption or remodeling may affect the ability of bone to support tumor cell colonization even at sites outside the immediate vicinity of bone turnover.

15. SUBJECT TERMS
metastasis, green fluorescent protein,rememoedeling,histomorphometry Colonization

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	11	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

COVER.....1

SF 298.....2

Introduction.....4

BODY.....4-8

Key Research Accomplishments.....4

Reportable Outcomes.....5

Conclusions.....8

References.....8

Appendices.....9-11

Final Report: Summary of Progress
Role of Bone Remodeling in skeletal colonization by prostate cancer cells.
USAMRMC W81XWH-04-0278

Introduction

Prostate cancer selectively forms bone metastases, and host tissue contributes to this selectivity by providing an environment favorable to metastatic cells (1,2). Metastatic tumor growth in bone may be favored near bone remodeling sites, where tissue resorption and formation produce numerous growth factors (3). However, it is not known whether remodeling sites can target the initial colonization of the tissue by circulating tumor cells. This project tested the hypothesis that colonization of bone by circulating prostate tumor cells occurs preferentially near sites of bone formation or resorption. The tasks in this proposal were 1) to obtain and partially characterize human prostate cancer cell lines highly expressing green fluorescent protein (GFP), 2) to inject those cells into the vasculature of nude mice and determine their early distribution in bone relative to active (forming/resorbing) and quiescent surfaces, and 3) to repeat the second task under circumstances where bone remodeling is experimentally altered. Those tasks were carried out, as described below.

I. Key Accomplishments:

1 Selection of highly fluorescent human prostate carcinoma cell lines (Task 1A).

Human prostate carcinoma cell lines constitutively expressing green fluorescent protein were subjected to fluorescent cell sorting. The most highly fluorescent 20% subpopulations were selected, as were subpopulations with low fluorescence. Both populations were expanded by subculture and partially characterized in vitro (see below). Highly fluorescent populations were used for injections (Tasks 2A, 2B). As proposed, we studied two cell lines (LNCaP-GFP, PC3-GFP).

2 In vitro characterization of GFP-expressing cell lines (Task 1B).

Highly fluorescent and low fluorescent sublines of LNCaP-GFP and PC3-GFP prostate carcinoma cell lines were assayed for growth (Fig 1), substrate adhesion (Fig 2) and invasion through matrigel (Fig 3). These experiments indicated only small differences between cell populations selected for high GFP fluorescence and those with lower fluorescence levels.

3. Relation of spatial and temporal bone colonization patterns of GFP-expressing prostate cancer cells to sites of bone remodeling (Task 2A).

The distributions of LNCaP-GFP and PC3-GFP cells in proximal tibiae were assessed at 24, 48 or 72 hours following injection into the vasculature of nude mice. Each tumor cell was localized with respect to its nearest bone surface, which was identified as forming, resorbing or quiescent. The percentage of cells nearest to each surface type were related to the overall fractions of each bone surface in the same sections. The results are summarized in the extended abstract submitted to ORS (appended). Both cell lines gave similar colonization patterns,, supporting the hypothesis that prostate tumor cells colonize bone near active bone surfaces.

4. Test whether experimentally altering bone remodeling will alter patterns of skeletal colonization (Task 2B).

The distributions of LNCaP-GFP and PC3-GFP cells in bone were assessed as above, in mice that had previously been treated for 6 days with a bisphosphonate (risedronate, RIS) to inhibit bone resorption (REF). RIS treatment reduced colonization by both cell lines. Colonization near resorbing sites was almost completely inhibited, while that near forming and quiescent sites was inhibited to lesser degrees.

II Delays, difficulties and deviations from proposed studies:

1. The time period for evaluation of colonization in vivo was extended from 48 hr to 72 hr post-injection. The initial experiment, showed more flluorescent cells than expected at 24 hr, so we extended the examination period to test if these cell numbers would decline.
2. Both decalcified and undecalcified histologic preparations are being examined rather than undecalcified samples only, as originally proposed. Both techniques are routinely used in this laboratory, but when the project was proposed, we had not yet confirmed that GFP would retain its fluorescence during

decalcification. Paraffin embedding allows us to perform immunohistochemical studies readily, and this can be used for follow-up experiments (e.g. to analyze the location of blood vessels) on the same tissues used for colonization studies.
3. Risedronate was substituted for alendronate to suppress bone resorption (Task 2B). Risedronate, a bisphosphonate of the same generation as alendronate,has been shown to inhibit the development of skeletal breast cancer metastases in animals (REF).
4. We originally proposed (Task 2B) to test colonization when remodeling was stimulated (by PTH) as well as inhibited (by bisphosphonate treatment).Because the young mice in this experiment have high remodeling rates, we may not be able to detect increases easily. For this reason we focused on basal and risedronate-suppressed remodeling states.
5. We experienced a case of microbial contamination following cell sorting selection of high GFP-expressing cells. This necessitated re-growth of cells before repeating the selection.
6. EDTA decalcification has added time to the tissue processing steps. In contrast to acid demineralization, which is rapid but destroys GFP fluorescence, EDTA demineralization of cortical bone may take up to several weeks. Moreover, we allow extra time for EDTA treatment, having observed in unrelated studies that incomplete demineralization leads to poor sections.

III Reportable Outcomes

Preliminary in vivo data with LNCaP-GFP (I.3, above) were accepted for presentation as a Plenary Poster at the American Society for Bone and Mineral Research meeting in September 2005. A copy of the abstract and a file of the complete poster as presented are appended (Appendices 1 and 2, respectively).

Additional findings based on colonization by PC3-GFP cells and the effects of risedronate have been submitted (August, 2005) as an extended abstract for presentation at the Orthopaedic Research Society meeting (March, 2006). A copy is appended (Appendix 3).

Results presented in the ORS abstract are being prepared for publication as a full paper (tentatively to be submitted to Cancer Research), These findings and other data gleaned from tissues prepared as part of these experiments will serve as the basis for further grant submissions (DOD, NIH) in spring 2006.

Data are summarized in the following figures and tables.

IV Figures and Tables

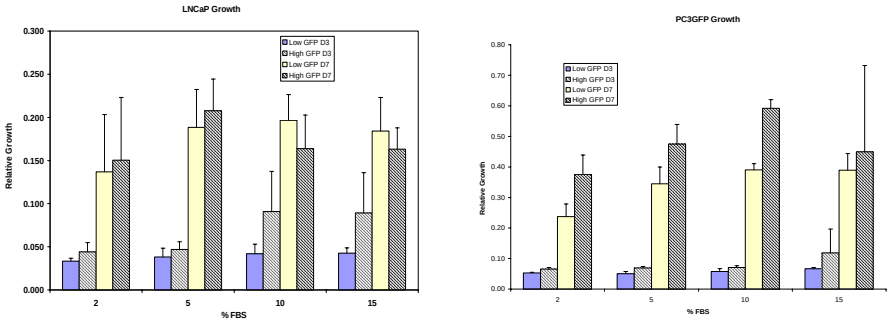


Figure 1 Growth of GFP-expressing prostate cancer cells: dependence on serum concentration.

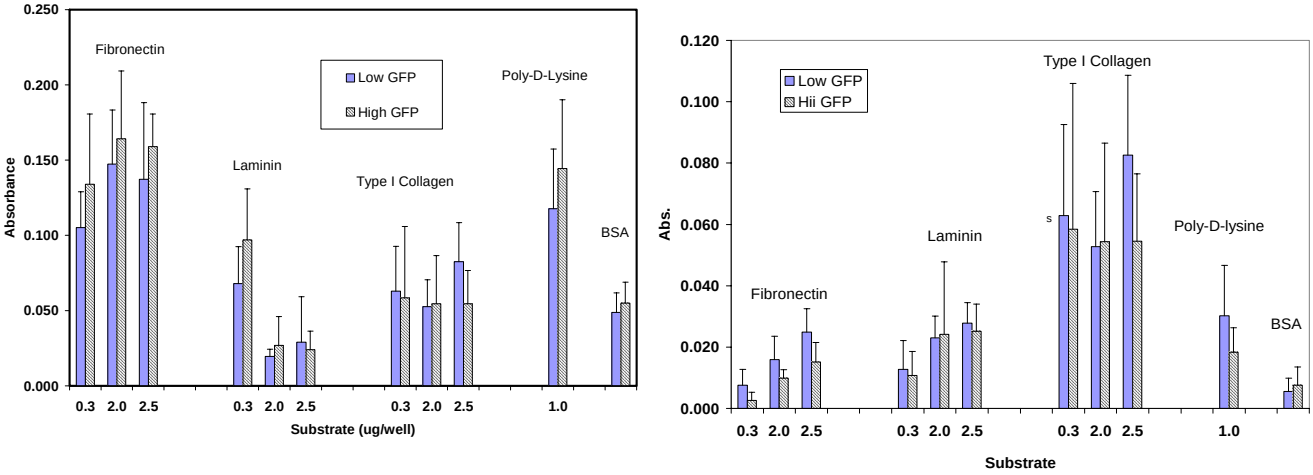
Low-expressing (plain bars) and high-expressing (hatched bars) cells was determined by dye binding on Day 4 and Day 7 after plating. Data show mean ± SD, n = 4.

Left: LNCaP-GFP Right: PC3-GFP

This experiment showed that high-GFP and lo-GFP sublines did not differ significantly in growth over a range of serum concentrations.

Figure 2. Adhesion of GFP-expressing prostate cancer cells.

Microtiter wells were coated with substrates as indicated. Low GFP (plain bars) and high GFP (hatched bars) cells were seeded for 1h at 37C, unattached cells were washed off and the attached cells measured as in Fig 2. Mean ± SD, n = 6. Left: LNCaP-GFP; Right: PC3-GFP



No major differences were seen between high- and low-GFP subpopulations in either line.

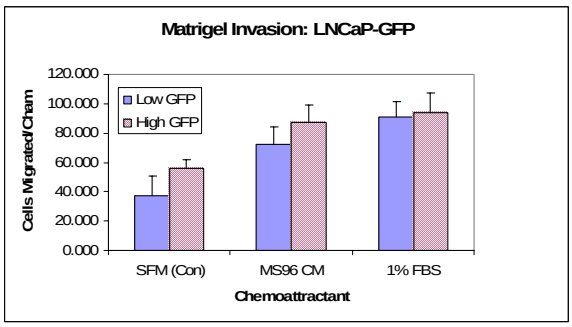
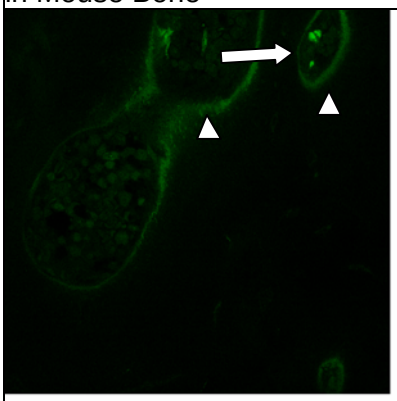
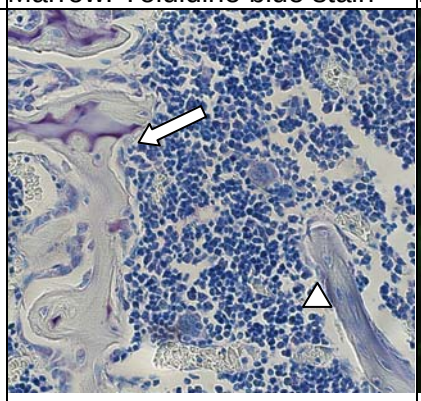
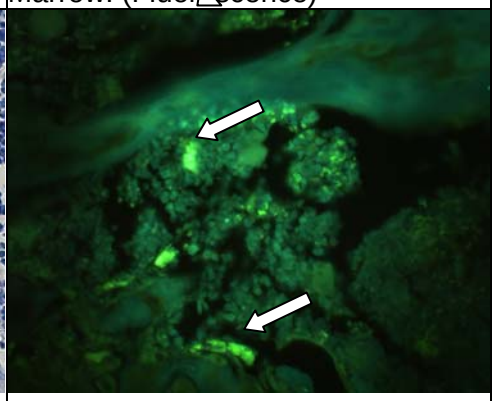


Fig 3. Invasion of LNCaP-GFP cells through matrigel

Filter inserts (8 µ pore size) were coated with Matrigel and placed in 24 well plates containing 0.3 ml of either serum-free medium, medium conditioned by human bone marrow stromal cells (MS96) or 1% FBS.Low-GFP (plain bars) and high-GFP (hatched bars) cellls were seeded onto filters (100,000 cells/well) for 6 hr. The filters were fixed and stained, and cells on the lower surface of the filter were counted. Mean ± SD, n = 3.

This experiment shows that both conditioned medium from bone marrow stromal cells and medium with 1% FBS can stimulate migration of LNCaP-GFP cells through matrigel; however, despite a tendency toward greater migration by high-GFP cells in the absence of a chemoattractive stimulus, there were no differences between high- and low-GFP cells.

Figure 4: Images of mouse tibia 24 hrs following injection of LNCaP-GFP Cells

Fluorescent LNCaP-GFP cells in Mouse Bone	Mouse Trabecular Bone and Marrow: Toluidine blue stain	Mouse Trabecular Bone and Marrow: (Fluorescence)
		
LNCaP cells (arrow); Calcein labeling (Arrowheads) indicates new bone formation. Undecalcified section, confocal image.	Examples of resorbing surface (arrow), quiescent surface (arrowhead). Decalcified section, 40x obj.	Fluorescent LNCaP-GFP cells (arrows); bone surface (arrowhead). Decalcified section, unstained, 40x obj.

LNCaP-GFP cells (100,000 in 0.1 ml PBS) were injected into the tail vein of male nude mice one day after administration of calcein to label newly forming bone. 24 hrs after injection, mice were sacrificed and tissues were prepared for histologic analysis. Tibiae and femora from each animal were ixed in neutral buffered formalin. Tissues were embedded undecalcified in methacrylate or decalcified in EDTA and embedded in paraffin. For undecalcified sections, thick sections (ca 100 µ) were cut, polished and viewed by confocal microscopy. Paraffin sections (5 µ) were mounted unstained to detect fluorescent tumor cells; adjacent sections were stained with toluidine blue for confirmatory histology.

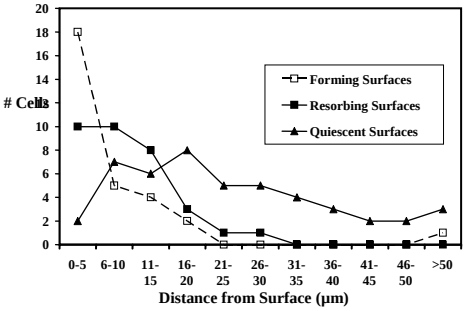


Fig.5. LNCaP Distance From Active and Quiescent Surfaces

Microscopic images of mouse tibial metaphysis were obtained 24 hr after injection of LNCaP-GFP cells were analyzed. The distance of each tumor cell to the nearest bone surface was measured and the bone surfaces were characterized as either forming, resorbing or quiescent based on standard histological criteria. Cells tended to distribute more closely to active rather than quiescent surfaces.

Table 1. Prostate Cancer Cell Colonization of Tibia Metaphysis in Nude Mice: Proximity to Active and Quiescent Bone Surfaces			
Surface Type	*% of Total Bone Surface*	*CaP Cells Nearest Each Surface Type*	
		LNCaP [a]	*PC3* [a]
Resorbing	24.0 ± 5.1	43 (30%)	12 (35%)
Forming	17.6 ± 2.7	34 (23%)	10 (30%)
Quiescent	58.6 ± 5.7	68 (47%)	12 (35%)
TOTAL	100	145 (100%)	34 (100%)
Cell numbers measured at 24h post-injection (LNCaP, n = 8 mice) or 24 and 48h post-injection (PC3, n = 4 mice). Distributions of both cell lines differ from %s based on total surface [a] $p < 0.05$, Chi square			

Colonization of bone by both LNCaP and PC3 cells occurred near to resorbing sites with greater frequency than expected based on the overall percentage of these surfaces in the bone sections analyzed. This finding supports the study's hypothesis that colonization exhibits selectivity for active, rather than quiescent, bone sites.

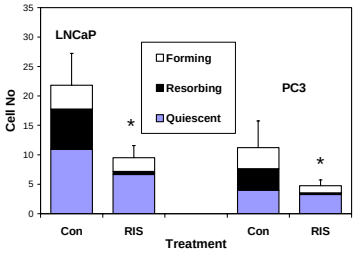


Figure 5. Effect of Risedronate (RIS) on Colonization of Mouse Tibia by Prostate Cancer Cells

Colonization by LNCaP and PC3 cells was assessed at 24-72h post-injection. Data pooled from 16 mice (LNCaP) and 9 mice (PC3). Significant differences ($p<0.05$, ANOVA) between Con and RIS groups at all surfaces. Effect of time post-injection, ns. Inhibition of bone resorption by prior treatment with RIS suppressed overall colonization. Inhibition was almost complete near resorbing sites, but also seen near forming and quiescent sites.

V Conclusions

Expression of high levels of green fluorescent protein (GFP) by human prostate cancer cell lines does not appear to markedly alter growth, adhesion or invasion properties when assayed in vitro. The hypothesis that initial colonization of bone by circulating prostate cancer cells occurs preferentially at sites of bone turnover is supported. Inhibition of bone resorption by risedronate suppresses colonization near forming and quiescent sites as well as resorbing surfaces. Thus, bone resorption may influence the ability of bone to promote tumor cell colonization at sites outside the immediate area of resorption.

VI References

1. Paget S (1889) The distribution of secondary growths in cancer of the breast. Lancet 1:571-573.
2. Mundy GR (2002) Metastasis to bone: causes, consequences and therapeutic opportunities. Nature Reviews/Cancer 2:584-593.
3. Guise TA (2002) The vicious cycle of bone metastases. J Musculoskel Neuronal Interact 2:570-572.

Appended Material

1. ASBMR Abstract (presented Sept 2005, Nashville, TN)
2. ASBMR Presentation (poster)
3. Orthopaedic Research Society abstract (Submitted, Aug, 2005)

Appendix 1 Abstract accepted for presentation at annual meeting of the American Society for Bone and Mineral Research, Nashville, TN, September, 2005.

Bone resorption sites: targets for skeletal colonization by tumor cells?

RJ Majeska[1], LJ Silbert[1], IH Gelman[2], MB Schaffler[1]

[1]Leni and Peter W May Department of Orthopaedics, Mount Sinai School of Medicine, New York, NY, [2]Roswell Park Cancer Institute, Buffalo, NY

Bone is a preferred site for prostate cancer (CaP) metastasis. Once tumor cells enter bone from the circulation, development of metastatic lesions is facilitated locally by growth/survival factors produced or released from matrix during bone resorption. However, it is not clear whether bone resorption or formation may also target the initial tissue colonization of bone by tumor cells. Here we tested the hypothesis that CaP cells in the circulation preferentially colonize bone near sites of ongoing formation and resorption. A human prostate cell line (LNCaP) was transfected to express GFP constitutively (LNCaP-GFP), and highly expressing cells were selected by flow cytometric sorting. Young male nude mice were treated for 6 days with risedronate (RIS, x mg/kg, sc) to inhibit bone resorption or with PBS vehicle. One day later, mice received 105 LNCaP-GFP cells by iv (tail) or intracardiac injection. After 24, 48 or 72h, animals were euthanized and proximal tibiae fixed in fresh 10% buffered formalin, decalcified with EDTA and processed for histology. The distance between each fluorescent tumor cell and the nearest bone surface was measured, and the surface was characterized as either forming, resorbing or quiescent by s5tandard histological criteria. We found that 24h after injection in control mice, 24% of tumor cells in the sections analyzed were closest to a forming surface, while 30% and 46% were nearest resorbing and quiescent surfaces; by contrast, forming, resorbing and quiescent surfaces accounted for 16%, 25% and 58% of total bone surfaces, respectively. This indicates preferential localization of tumor cells near sites of tissue activity. Similar distance distributions were seen at 48 and 72h post-injection. In addition, over 91% of cells near forming and resorbing sites were within 25u of bone surfaces, while only 56% of cells were this close to quiescent surfaces. When bone resorption was suppressed with RIS, almost no tumor cells were seen near active or inactive resorption sites; however, total tumor cells per section also declined from 4.2 ± 0.9 to 2.0 ± 0.6 (SD) in RIS-treated animals. In summary, these data indicate that the initial colonization of skeletal tissues by circulating tumor cells appears to occur preferentially near sites of active bone formation/resorption. The findings also raise the possibility that factors produced locally near sites of resorption may have more widespread effects on the initial stages of tumor cell invasion in bone.

Appendix 2 Poster presented at American Society for Bone and Mineral Research Annual Meeting, Nashville, TN, September, 2005.

Bone Resorption Sites: Targets for Skeletal Colonization by Tumor Cells?

R. J. Majeska[1], L.J. Silbert[1], I.H. Gelman[2], M.B. Schaffler[1]

[1]Leni & Peter W. May Department of Orthopaedics, Mount Sinai School of Medicine, New York, NY USA

[2]Cell and Virus Department, Roswell Park Cancer Institute, Buffalo, NY USA

INTRODUCTION

Bone is a preferred target for metastasis of prostate and other cancers. Why this occurs is still not fully understood; however, a major role for host tissues has long been appreciated (1).

Bone resorption promotes tumor formation by metastatic cells present in bone via production or release of growth factors at resorption sites (2). Whether bone resorption also enhances initial colonization of bone by metastatic tumor cells in the circulation is less clear.

Bone resorption sites are targets for circulating osteoclast progenitors (3). Similar

OBJECTIVES

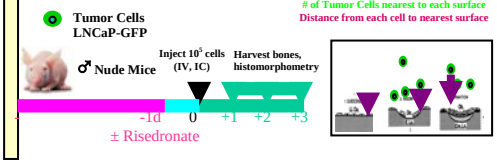
1: Test whether initial colonization of bone by prostate cancer cells is targeted to sites of bone resorption.

Prediction: If colonization is targeted to resorption sites, tumor cells introduced into the circulation will rapidly and preferentially accumulate near resorbing surfaces.

2: Test whether experimentally suppressing bone resorption will inhibit tumor cell colonization in bone, and whether this effect is selective for colonization near resorption sites.

EXPERIMENTAL APPROACH

MEASURE:
% Forming, Resorbing, Quiescent Surfaces
# of Tumor Cells nearest to each surface
Distance from each cell to nearest surface



METHODS

CELLS: LNCaP prostate cancer cells expressing green fluorescent protein (LNCaP-GFP) cultured in RPMI-1640 + 10% FBS and selected by FACS. In vitro growth, adhesion and migration assays showed no effects of high GFP expression.

MICE: Nude mice (6 – 8 wk old) left untreated (Control, n = 12) or injected sc for 6d with risedronate (RIS, 0.2 mg/kg, n = 6) to suppress resorption.
INJECTIONS: Tail vein (iv) or intracardiac (IC), 1 day after last RIS treatment, 10^5 cells in 0.1 ml PBS.
TISSUE PREPARATION: Mice euthanized (CO_2) 1-3d post-injection, tibiae fixed in formalin, EDTA-decalcified and processed for histology. All procedures were carried out under IACUC approval.

ANALYSIS: LNCaP-GFP cells in bone identified by fluorescence microscopy. Bone surfaces classified as resorbing, forming or quiescent by standard histologic criteria. % of all surface types determined in serial toluidine blue-stained using an OsteoMeasure system. Chi-square was used to assess goodness of fit between observed numbers of tumor cells nearest each bone surface and expected values based on the overall proportions of these surface types.

RESULTS

1 Highly fluorescent LNCaP-GFP cells selected by FACS (Fig 1 left panel) were injected into nude mice and detected by fluorescence microscopy in decalcified sections of tibial metaphysis (Fig 1 right panel).

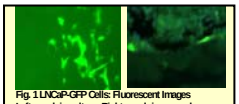


Fig. 1 LNCaP-GFP Cells: Fluorescent Images
Left panel: in culture; Right panel: in mouse bone

- At 1, 2 and 3 d after injection, LNCaP-GFP cells nearest to forming, resorbing and quiescent surfaces were counted. On Day 1, tumor cells nearest to each surface type reflected the relative amounts of those surfaces in metaphyseal sections; however, on Days 2 & 3, cells nearest to resorbing surfaces increased significantly while cells nearest to quiescent surfaces declined (Fig 2).

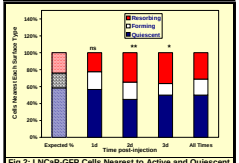


Fig 2: LNCaP-GFP Cells Nearest to Active and Quiescent Bone Surfaces. Expected values (left column) based on overall % surfaces determined morphometrically. Differences (observed vs expected) assessed by chi-square * p < 0.05; ** p < 0.02.

- Over 90% of cells nearest to forming and resorbing surfaces were localized within 25 µm of those surfaces (Fig 3); in contrast, nearly half of the cells nearest to quiescent surfaces were over 25 µm away.

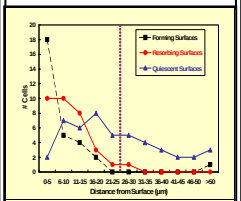


Fig. 3. Distance distributions of LNCaP cells from forming, resorbing and quiescent bone surfaces. Data pooled from control animals at 1, 2, 3d post-injection

- Suppressing bone resorption by treatment with risedronate for 6d prior to injection of LNCaP-GFP cells inhibited colonization by about half (Fig 4). In RIS-treated mice, almost no tumor cells were seen cells nearest to currently or previously resorbing surfaces (identified by scalloped morphology in the absence of detectable osteoclasts). In addition, RIS treatment significantly reduced the number of tumor cells nearest to quiescent bone surfaces.

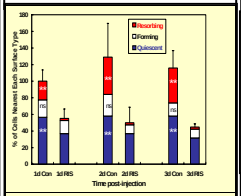


Fig. 4 Risedronate Inhibition of LNCaP-GFP Colonization
% LNCaP-GFP cells nearest to resorbing (red), forming (white) and quiescent (blue) bone surfaces.
Differences between Con and RIS indicated in Control bars (**, p < 0.01; ns, not significant).

DISCUSSION

This study addressed two questions: 1) is colonization of bone by prostate tumor cells targeted to bone resorption sites, and 2) does resorption promote tumor cell colonization of bone.

The decrease of LNCaP-GFP cells, particularly near resorption sites, in bones of animals treated with RIS strongly suggests that resorption promotes tumor cell colonization of bone in this model. This finding agrees with studies showing that inhibitors of bone resorption act as suppressors of metastasis (4, 5), and indicates that resorption can influence the earliest stages of skeletal metastasis (colonization). It is unlikely that this impairment resulted from high levels of RIS acting directly on tumor cells (6); most of the drug would have been cleared before the cells were injected (7).

Impaired colonization in RIS-treated mice was not limited to resorption sites; tumor cells nearest to quiescent surfaces were also significantly reduced. Thus resorption appears to influence colonization beyond the anatomic limits of a resorption site.

If initial colonization of LNCaP-GFP cells were targeted to resorption sites, we expected that the number of cells nearest to resorbing surfaces would be disproportionately high relative to the overall abundance of resorption sites in bone. We observed increased numbers of LNCaP-GFP cells near resorption sites on days 2 and 3, but not on day 1 post-injection. This suggests that resorption may not target initial localization of tumor cells in bone, but may favor the development of more permanent or stable interactions with host tissue (e.g. extravasation). Whether the cells in this study are still within vascular spaces or have entered the tissue matrix is not yet known. In addition, the rate of colonization may also depend on the tumor cells themselves. We observed that PC3 cells colonized bone at higher than expected frequency near resorption sites even at 24h (data not shown). In any event, the mechanisms responsible for tumor cell colonization remain to be clarified.

CONCLUSIONS

- Bone resorption promotes the colonization of mouse bone by circulating prostate cancer cells. This effect may extend to sites not undergoing active bone resorption.
- While the earliest deposition of tumor cells in bone may occur nonspecifically, longer term colonization appears to exhibit some selectivity for sites of bone resorption.

Damien Laudier for invaluable aid in all matters histologic.
Supported by DOD grant W81XWH-04-0278 (RJM)

References: 1. Lancet 1:571-573,1889; 2. Nature Rev. Cancer 2:584-593, 2002; 3. J Bone Mineral Res 18:1404-1418, 2004; 4. Cancer Res 55:2441-3557, 1995; 5. J Clin Inv 107:1235-1244, 2001; 6. BJU Int 94:164-170, 2004; 7. Drugs 61:685-712, 2001.

Appendix 3 Abstract submitted to Orthopaedic Research Society: Meeting, Mar, 2006 (in review)

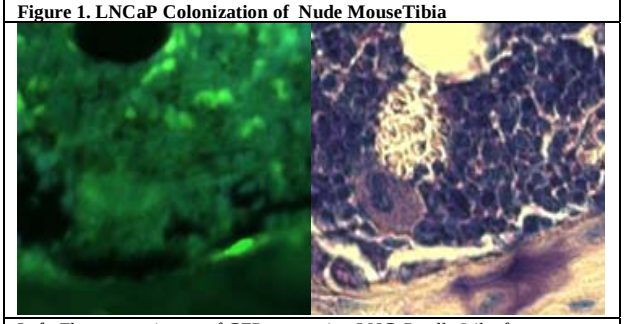
.PREFERENTIAL COLONIZATION OF BONE BY PROSTATE CANCER CELLS NEAR SITES OF RESORPTION

Majeska, R J; Silbert, L J., Gelman, I H., Schaffler, M B
+*Leni and Peter W. May Department of Orthopaedics , Mount Sinai School of Medicine, New York, NY; **Cell and Virus Department, Roswell Park Cancer Institute, Buffalo, NY,

INTRODUCTION: Skeletal metastasis requires a favorable environment for tumor development in host bone[1,2], Bone resorption promotes skeletal metastasis by producing/releasing factors that enhance the growth and survival of tumor cells already in bone[2,3]. However, it is not known whether resorption also promotes the initial colonization of bone by circulating tumor cells, similar to targeting of osteoclast progenitors[3]. If resorption actively targets metastatic colonization of bone, tumor cells would be expected to accumulate in bone near sites of resorption. The purpose of this study was to test that prediction. Specifically we tested whether human prostate cancer cell lines, when injected into the circulation of nude mice, would colonize bone randomly or in association with specific sites in bone. We further examined whether the number and distribution of tumor cells in bone could be altered by experimental suppression of bone resorption.

METHODS: Human prostate cancer cell lines (LNCaP and PC3)[4,5] were transfected to express GFP, then enriched by fluorescent cell sorting and expanded. Nude male mice (6 – 8 wk old, n = 26) were injected with 100,000 tumor cells in 0.1 ml PBS via tail vein (iv) or intracardiac (ic) injection.[6,7] Ten mice also received 4 µg risedronate (RIS, sc) for 6 days[7], ending 1 day before injection of cells to minimize direct RIS effects on tumor cells. Mice were euthanized with CO_2 24 – 72 h after cell injection, tibiae were fixed in buffered formalin, EDTA-decalcified and processed for histology. GFP-expressing tumor cells in proximal tibial metaphyses were visualized under fluorescent light and photographed. Distancefrom each tumor cell to the nearest bone surface was measured from photomicrographs, and each surface was identified as forming, resorbing or quiescent by standard morphologic criteria. In addition, overall proportions of forming, resorbing and quiescent surfaces were determined on serial toluidine blue-stained sections. The null hypothesis that numbers of tumor cells nearest to each surface type would follow the overall proportions of those surfaces in bone was assessed by chi-square test. Differences between cell lines and RIS treatment effects were assessed by ANOVA using SPSS software. All animal procedures received Animal Care and Use Committee approval.

Figure 1. LNCaP Colonization of Nude MouseTibia



Left: Fluorescent image of GFP-expressing LNCaP cells 24h after intracardiac injection. Right: Serial section, toluidine blue stain

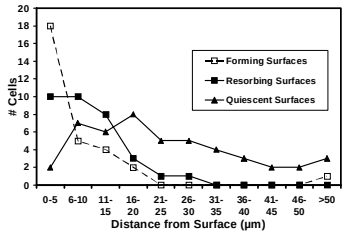
RESULTS: Overall, ca. 24% of bone surfaces in tibial metaphyses of young male nude mice (n = 7) were resorbing, 18% were forming and 58% were quiescent (Table 1). However, more prostate cancer cells were seen near forming and resorbing surfaces, while fewer were near to quiescent surfaces, than predicted based on those values ($p < 0.05$). Both LNCaP and PC3 cell lines showed this preferential distribution, which were also seen 48 and 72h after injection (not shown). The distance between cells and the nearest bone surface also depended on surface activity. As shown for LNCaP cells (Fig 2), >90% were located within 20µm of forming or resorbing surfaces, while only about 50% of cells nearest to quiescent bone surfaces lay within this distance. Pre-treatment of mice with risedronate (RIS) for 6 days to suppress bone resorption reduced the total number of LNCaP and PC3 cells detected in bone by half (Fig 3). RIS completely blocked colonization by cells nearest resorbing surfaces, but also significantly reduced cells nearest to forming (($p< 0.01$) and quiescent ($p<0.05$) surfaces.

Table 1. Prostate Cancer Cell Colonization of Tibia Metaphysis in Nude Mice: Proximity to Active and Quiescent Bone Surfaces

Surface	*% of Total*	*CaP Cells Nearest Each Surface Type*	
		LNCaP [a]	*PC3* [a]
Resorbing	24.0 ± 5.1	43 (30%)	12 (35%)
Forming	17.6 ± 2.7	34 (23%)	10 (30%)
Quiescent	58.6 ± 5.7	68 (47%)	12 (35%)
TOTAL	100	145 (100%)	34 (100%)

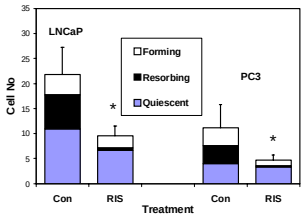
Cell numbers measured at 24h post-injection (LNCaP, n = 8 mice) or 24 and 48h post-injection (PC3, n = 4 mice). Distributions of both cell lines differ from %s based on total surface [a] $p < 0.05$, Chi square

Figure 2. LNCaP Distance From Active and Quiescent Surfaces



Histogram showing distance of LNCaP cells from the nearest bone surface 24 h after injection into vasculature of nude mice. Data pooled from 7 mice and included both iv and ic injections

Figure 3. Effect of Risedronate (RIS) on Colonization of Mouse Tibia by Prostate Cancer Cells



Colonization by LNCaP and PC3 cells was assessed at 24-72h post-injection. Data pooled from 16 mice (LNCaP) and 9 mice (PC3). Significant differences ($p<0.05$, ANOVA) between Con and RIS groups at all surfaces. Effect of time post-injection, ns.

DISCUSSION: This study, showing that initial colonization of mouse bone by circulating tumor cells occurs preferentially near active bone surfaces, supports the concept that bone resorption/remodeling promotes initial stages of skeletal metastasis as well as growth of tumor cells already present in bone. The mechanisms responsible for such targeting remain to be clearly identified, but several host-tumor cellular interactions have already been implicated in metastasis to bone[8,9]. Also of interest was the finding that RIS not only blocked colonization near resorbing surface completely, but also partly inhibited colonization near forming and quiescent surfaces. This suggests that the ongoing bone resorption/remodeling, while occurring at limited, discrete sites, may also influence the behavior of nearby non-resorbing regions of bone.

CONCLUSIONS: Bone resorption and formation sites may be preferred targets for metastatic colonization. In addition, bone resorption may influence colonization by cells at nearby non-resorbing sites.

Acknowledgements: Supported by DOD Grant W81XWH-04-0278

REFERENCES: 1. Lancet, 1:571-573 (1889); 2. Nature Rev/Cancer 2:584-593 (2002); 3. Bone 36:840-853 (2005); 4. Cancer Res 51:3753-61 (1991); 5. Invest Urol 17:16-23 (1979). 6. Clin Exp Metastasis 19:17-24 (2002); 7. Cancer Res 55:3551-3557 (1995). 8. J Bone Mineral Res 20:318-329 (2005). 9. Int J Cancer 110:497-503 (2004).