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Earl. [Signature] LtC, MS
1406.01
13. Abstract (Maximum 200 Words) To molecularly define the different steps in prostate-bone metastasis, we developed a new human xenograft model for osteosclerotic bone metastasis after intracardiac inoculation in SCID mice. Individual clones of BAG-tagged C4-2 cells show a range of bone colonization capacities, however C4-2-3H5 generates overt osteosclerotic lesions after 4 months. Subcutaneous xenograft material from the 3H5-BAC, one other subline showing molecular metastases, and two sublines with very low bone-metastasising capabilities were subjected to gene array analysis and a series of candidate genes either up- or down-regulated have been identified. Intra-tibial inoculation of bones showed that each of these sublines has similar capacity to grow in bones, emphasizing bone homing / extravasation events as being the cause of differential activity. Further funding is required for real-time quantitative PCR (TaqMan) of prostate biopsies and radical prostatectomies which have been accrued, and in situ hybridization and/or immunohistochemistry analysis on tissue arrays. Continued attempts to refine the animal model and dissect the morphologic and molecular etiology of prostate bone metastasis have been limited by the low frequency of bone metastasis in this model, and the long latency period. Attempts to develop an alternative model using xenografted primary prostatic material have been initiated.
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INTRODUCTION:

Prostate cancer commonly spreads to bone resulting in bone forming lesions that are extremely painful and difficult to treat. In 80% patients at diagnosis metastasis will have already occurred. Unlike breast cancer, which has attracted considerable research effort over the years, work directed towards understanding the etiology and genetic basis of prostate cancer is in its infancy. Although prostate and breast tumors both demonstrate a predilection for metastasis to bone, metastases to bone from prostate are predominantly osteoblastic while those from breast tumors are predominantly osteolytic. The molecular basis for this difference is not known, and may be exploited in controlling this very painful manifestation of prostate cancer. The combination of early diagnosis and new treatments for the treatment of osteolytic disease- a feature of breast cancer- has resulted in a vastly improved prognosis for breast cancer patients. Also, it is likely that osteolytic activity, which often is present in prostate bone metastases, is actually required for the osteoblastic nature of prostate bone metastases. If so, inhibition of this process may be palliative in prostate tumors also. While prostate specific antigen is a very useful marker of prostate disease and its recurrence, it is not completely reliable. Thus there is an urgent need to define the disease and its progression in order to identify early markers that are of diagnostic value and also potential therapeutic targets, particularly with regard to the treatment of the painful, debilitating bone lesions.

The purpose of our work has been aimed towards defining the genes responsible for prostate cancer and its spread to bone. Of paramount importance has been the development of an animal model of prostate cancer which gives rise to osteoblastic bone lesions in which the molecular ontogeny of disease could be defined, thus allowing also the identification of prognostic markers and the genes responsible for disease progression. Such a model would also have value for testing new treatment strategies. The specific aims of Phase I were to develop a series of genetically tagged clonal sub-lines from a prostate cancer cell line that would show different metastatic potential in an animal model. PTHrP levels were to be compared to metastatic potential and RNA derived for gene-array analysis. Collection of human prostate cancers which were metastatic to bone or were not metastatic would a) provide material in which to validate the genes discovered using the animal model and in rare cases when enough material could be obtained b) would provide a broad basis for comparison of gene profiles in order that those relevant to human disease also could be easily identified.

We hypothesized that since the LNCaP C4-2 cell line can support a low level of osteosclerotic metastasis in SCID mice (1, 2), heterogeneity within that cell line would enable the derivation of subclones which either performed well or poorly in this regard. These would in turn enable the determination of gene expression profiles which associate with (i) bone metastatic potential and (ii) different stages of prostate-bone metastasis.

Our approach was to develop a genetically tagged series of clonal sublines from a well characterized prostate cancer cell line which would show differential bone metastatic potential in our intra-cardiac model. We then sought to generate a molecular profile of these sublines in order to identify genes associated with each stage of the bone metastasis process. These will be validated in clinical material which we are accruing.
REPORT BODY:

The rationale for this project dictated the development of clonal prostate cancer cell lines which would either give rise to osteoblastic bone lesions in mice or not metastasise to bone, to provide genetically matched material for comparison of gene profiles in order to identify the responsible genes. Material derived from the animal model during the course of disease progression would provide the means to identify genes whose expression is altered as the metastatic process occurs and detailed histological examination and localisation of known regulators of bone formation provide information about the etiology of interactions of tumor cells with bone which ultimately give rise to the osteoblastic lesions.

Progress

Task 1: To genetically tag the LNCaP-C4-2 cells, generate individual subclones, and determine the time course for bone metastasis. As previously reported (12 and 18 month reports), we originally planned to use the TSU-prl cell line. However, before laboratory work had commenced we became aware of osteosclerotic metastasis in a LNCaP-derived subline. The LNCaP cell line (3, 4) is a well-accepted model for androgen-dependent prostate cancer (PrCa). Co-inoculation with bone marrow stromal cells led to LNCaP tumors in both intact and castrated SCID mice, and a series of sublines with different levels of androgen-dependence were derived (2). In particular, the C4-2 sub-line causes paraplegia in 20-50% of animals, and gives rise to a low level of osteosclerotic lesions characteristic of those seen in the majority of prostate cancer patients (1, 2).

Consistent with our original proposal, we have generated 81 C4-2-BAG subclones during the genetic tagging process, which we have subsequently examined for metastatic potential in SCID mice. We chose C4-2 since it leads to a low level of bone metastasis which authentically resemble the human, such that derivation and comparison of both highly competent and poorly competent (for bone metastasis) clones would be possible. Their known capacity to form osteosclerotic lesions made them the model of choice.

1.1 Lac-Z (BAG) Transduction and cloning: Eighty-one individual clonal sublines with BAG (bacterial beta-galactosidase) tag were derived, and the remaining G418-resistant cells were pooled to generate the C4-2-BAG-pool population. Each of the clones was expanded and frozen for storage. Fourteen were analyzed in vivo at the time of the 18 month report, and an additional 15 have been analysed since. Due to the fact that no additional sublines showing bone metastasis were obtained in the second set of 15 analysed, and the long latency period seen with the sublines, further testing of additional sublines was not performed.

1.2 Pilot Study for Chronology of Bone Metastasis Ontogeny: This task had to be modified because of the lower frequency and longer duration required for osteosclerotic lesions from the C4-2 cells (1). Attempts to use the C42-3H5 clone, which shows overt osteosclerotic lesions after 4 months (as seen in Figure 1, 2) were thwarted by the low rate of metastasis (~20% hit rate), such that many of the mice scheduled for this experiment would not show any evidence of bone lesions, making it impossible. Instead, we tried to address this task using intra-tibial inoculation, knowing that this could also be addressed by the gene array analysis, as described below. Direct intra-tibial injection of the non-competent clones compared to 3H5-BAC (see below) showed no difference in the onset and growth of the bone-metastatic lesions, indicating
that differences did indeed exist in the capacity of each subline to home to, arrest, and/or extravasate into the extravascular space in the bone. However, without a reliable frequency of lesion formation after intra-cardiac injection, we are unable to sample the ontogeny of this. We have also been able to examine the 3H5-BAC subline chronologically, with high resolution x-ray analysis throughout the onset and maturation of the lesion, and have seen evidence of an early lytic phase followed by a predominantly blastic, but somewhat mixed lesion. Histologically (Figure 2), new bone formation was observed. In keeping with the Faxitron images, this new bone extended out from the existing bone (such as the tibia shown in Figure 2) into the surrounding muscle. It was contiguous with existing mouse bone.

Task 2: To determine inter-relationships between organ- and bone-metastatic potential and PTHrP expression among the TSU-pr1-BAG clones. Ultimately, we have reached the conclusion that this aim is not possible, and it has been replaced with earlier gene array analysis of five different sublines, which was not otherwise scheduled until Stage II. The reasons for this are detailed below.

2.1 PTHrP Analysis of Clones: PTHrP was analyzed by RIA measuring an N-terminal PTHrP epitope for the first 14 sublines tested in vivo, but levels of PTHrP secreted in culture or PTHrP levels in the subcutaneous xenograft, determined by immunohistochemistry (IHC), did not associate with bone metastasis potential. The possibility that loss of N-terminal activity and processing to C-terminal fragments could potentially promote osteosclerotic lesions was assessed. No change was seen between clones on Western analysis with region specific antibodies. In general, the levels of PTHrP recorded for all sublines was relatively low.

2.2 In Vivo Analysis of BAG clones: 14 C4-2-BAG clones and the C4-2-BAG-pool have been tested for growth analysis in vitro and in vivo (subcutaneous). In addition, these, and an additional 15 sublines, have been inoculated intracardially into male SCID mice to assess competence for bone metastasis. Of the original 14 tested, 13 showed strong subcutaneous growth, 2 showed strong molecular-bone metastasis, 4 medium level of molecular-bone metastasis, 5 low levels of molecular-bone metastasis, and 3 were distinctly negative for bone
metastasis, despite showing good subcutaneous growth (see 12 month report). Three non-
metastatic (1G2B, 2B6, 3A6) and the two most metastatic (3F9 and 3H5) sublines were subjected
to further analysis using quantitative PCR analysis (genomic Taqman for the b-galactosidase tag
(5) as well as Faxitron analysis. On repeat analysis, only the C42-3H5 subline was found to
reliably generate osteosclerotic lesions after 4 months similar to that seen one month after direct
intratibial injection of 10,000 C4-2 parental cells (see 18 month report). This was not evident
at the 2 or 3 month time point, but became evident in part of the group at 4 months. The 3F9
subline showed molecular metastasis only, with no evidence of histological burden, and no
radiographic alteration in the limbs which carried these cells. In addition, each of these 5 sublines
was analysed by intra-tibial inoculation, and found to generate similar lesions over similar time
frames.

Of the additional 15 sublines analysed over the past year for bone metastatic activity after intra-
cardiac injection, none were found to show any bone metastatic activity over a 5 month period
or longer. These observations indicated that the bone metastatic potential of the C4-2-3H5
subline is rare amongst these sublines, and further testing was discontinued. This, combined with
the long duration of onset (4-5 months) of the C4-2-3H5 subline, was a major shortcoming in our
request for Stage II funding.

2.3 Further Analysis of PTHrP in clonal tumours: It was initially hypothesised that we would
obtain bone metastases from different sublines with altered expression of PTHrP, and that such
differences in PTHrP would enable dissection of factors in addition to PTHrP which may have
a major influence on the lesion. Since we were unable to generate more than 1 subline with bone
metastatic capacity, we were forced to modify the approach. Instead, the one clonal subline C4-2-
3H5 which shows such metastatic capacity was compared to the other non-metastasising subline
using gene array analysis, as described below.

Task 3: To discover and identify additional molecules influencing prostate-bone
metastasis:

3.1 Examination of known bone-turnover markers in “outlier” clonal metastases: Although the
various osteoclast (OCL) and osteoblast (OBL) factors can be detected by either
immunohistochemistry or in situ hybridization, since C4-2-3H5 cells were the only subline to
produce any bone metastatic activity, there is no basis for comparison with other bone-
melanostasising lines with different PTHrP status. Instead, the production of these factors by the
sublines themselves, may be instructive, and may provide insights into why the C4-2-3H5
subline was more capable of bone metastasis than all other sublines. An economical approach
to comparisons between each subline is the use of the relatively new “gene array technology”.
This allows the simultaneous analysis of many different gene products and we felt this was the
best approach to this aspect of the work. Accordingly, each of the five major sublines described
above was analysed as described below. We are also in the process of developing a more
specialised “bone factor” low-density, high sensitivity filter array, which will allow further
analysis of this aspect of the work.

3.2 Stage-specific LCM and RNA preparation: RNA isolation from the subcutaneous tumours
has been achieved, and analysed by gene array as described below. LCM analysis from bony
lesions has proved very problematic, since the decalcification process does not preserve RNA
well, and the material cannot be sectioned without decalcification. We have tested a number of
tissue processing protocols (different fixation and embedding regimens) without success, and
have had ongoing liaison with Arcturus regarding the LCM from calcified tissues. A number of
groups with similar problems have contacted us, through Arcturus, and this would appear to be
a universal problem that other groups are also faced with. This has hindered progress in this
aspect of the work, and instead, further analysis, as designed for stage II, will require \textit{in situ}
hybridisation analysis rather than LCM and gene array analysis until additional technological
developments allow isolation from bony tissues.

3.3 LCM and RNA preparation from tumors with "outlying" bone behavior (months 19-30): as
per 3.1 above, we were not able to perform this task, because only one subline with appreciable
bone metastasis (C4-2-3H5) was obtained out of
30 tested. Again, our only recourse was to compare
the C4-2-3H5 cells with others that did not have bone-metastatic capacity,
as described below using
genearay analysis.

3.4 Comparative Gene Expression: Subcutaneous
xenograft material from the
C4-2-3H5 and C4-2-3F9
sublines and three non
bone-metastasising clones
was subjected to gene array analysis using Research
Genetics Prostate Filters
(GF221). Figure 3 shows
the fold change in gene
expression of each
designated clone compared
to 3H5 level, as an example
of the effects we are
starting to measure. As
indicated, 13 candidate
genes have emerged which
were differentially
regulated in all 1G2B and
2B6 samples compared to
3H5.

One of these, the 67 kDa
laminin receptor has been
associated with progression
in a wide variety of tumors, suggesting a role in invasiveness, metastasis and tumor growth (6).
In our model down-regulation of this receptor has been associated with ability of 3H5 to form
bone metastases. Further studies will be required to determine the relevance of this ectopic
xenograft system, and whether laminin receptor plays a functional role in this model system. The

\begin{figure}
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\includegraphics[width=\textwidth]{fold_expression_change}
\caption{Total RNA was extracted from subcutaneous xenografts using Trizol followed by RNaseq clean-up. Radioactively labelled (\textsuperscript{35}P) cDNA was generated by reverse transcription, and hybridized to Research Genetics GF221filter arrays. RNA was harvested from xenografts from two different animals from two different inoculation regimes, for each clone, and these samples were each hybridized to three different GF221 filters. Figure 3 shows genes >2x under- and over- expressed in 3H5 xenografts compared to both 1G2B and 2B6 xenografts. Standard Deviations shown.}
\end{figure}
other candidates identified in this study have not been extensively studied in cancer, and it remains to be determined whether they are involved in the formation of osteosclerotic prostate-bone metastasis.

These candidate genes await additional funding to be verified by real-time quantitative PCR (TaqMan) of LCM-derived prostatic cancer cells taken from PrBx and radical prostatectomies RPx which we have accrued (see below), and ultimately using \textit{in situ} hybridization (ISH) and/or IHC on PrCa tissue arrays.

\textbf{Task 4: Accrual and Extended analysis of clinical material}

\textit{4.1 Identification and scheduling of candidate cases:} We still continue the monthly meetings between Drs. Costello (urologist; primary prostatectomy), Choong (orthopedic surgeon, bone metastases) and Slavin (Pathologist) and this has proven very effective in facilitating accrual, as detailed below.

\textit{4.2 LCM of clinical material at different prostate cancer stages (Months 1-30):} To date, specimens have been harvested by Dr Slavin in Pathology directly into liquid nitrogen, and are catalogued and stored at -70°C. As shown in Table 3, current accrual has generated are these current (from stage II) 71 RPx including seminal vesicles, 162 PrBx of which 19 match the RPx, 2 prostate cancer lymph nodes, 2 bone metastases, and one bone biopsy. We have developed LCM conditions followed by RNA extraction, and have applied these to one of our frozen prostate carcinoma specimens, as shown in Figure 2, with good results. We have decided to accumulate these specimens until needed, so as to take best advantage of any new developments in the LCM technology. However, we have determined that our methodology is adequate to generate viable RNA from the frozen tumor specimens after LCM. We have applications pending for the development of low density, high sensitivity gene expression subarrays which we believe will be suitable for gene expression profiling of biopsy-derived material and provide a rapid post-array validation procedure for genes identified with the larger format high density arrays. Since our Stage II application was unsuccessful, we will continue to store these samples until we have sufficient support to continue the experiments as proposed.

\textbf{Table 1: Summary of tissue accrual to date.}

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<th>Biopsy</th>
<th>Sem Ves.</th>
<th>LN</th>
<th>Biopsy &amp; Primary (matched)</th>
<th>Bone metastasis</th>
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<td>Accrued before study</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Accrued in first year</td>
<td>32</td>
<td>2</td>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Current totals</td>
<td>188</td>
<td>91</td>
<td>2</td>
<td>26</td>
<td>3 plus 1 biopsy</td>
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\textbf{KEY RESEARCH ACCOMPLISHMENTS:}

- Established successful genetic tagging of a human prostatic cell line with proven osteoblastic bone metastasis capacity
- Screened sufficient clones for bone metastasis to generate a subset for
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comparative analysis of gene expression

- Tentatively determined a lack of relationship between PTHrP and bone metastatic potential of these clones
- Established a prostatic tumour tissue repository amenable to laser capture microscopy (LCM) and RNA extraction
- Effected successful LCM and RNA extraction for accrued frozen prostatic carcinoma tissue
- Performed preliminary gene array analysis and identified 13 gene products with potential relevance to prostate-bone metastasis

3. REPORTABLE OUTCOMES:

Manuscripts:

A manuscript describing the C4-2-3H5 model, and other subclones, is in preparation. In addition, training of Dr. Williams for gene array analysis, led to one collaborative paper:


Abstracts:


- Williams, E.D., Moseley, J.M., Slavin, J., Crowe, H., Costello, A.J. and Thompson, E.W. Prostate cancer under the micro (array) scope. Genito-Urinary Oncology Group Symposium, Annual Scientific meeting, Sydney, March 23-23, 2000 (podium presentation by Dr. Williams)


Also, during the course of these studies, we became aware that the epithelio-mesenchymal transition previously documented in human breast cancer cell lines by the PI, is also evident in human prostate cancer cell lines which we had obtained for bone metastasis analysis. A Manuscript describing this is in preparation, and the work has been reported preliminarily to a number of conferences:


Dr. Williams undertook a UICC-supported training fellowship with the Advanced Technologies Center, NIH to learn gene array technology, resulting in the following publication:


Presentations


Dr. Williams: Friday November 3, 2000: Prostate Cancer Symposium: Novel Strategies in Prostate Cancer Treatment and Diagnosis, Royal Melbourne Hospital, Urology: Gene array and prostate cancer.

Cell Lines: We have already made our LNCaP-C4-2-BAG subclones available to other scientists. Each subline is a valuable new resource, and in particular, we have detailed gene expression profile data on 4 specific cell lines as subcutaneous xenografts.

Tissue Repository: We have accrued a significant repository which could be made available on a collaborative basis after human ethics committee approval.

Funding applied for based on work supported by this award: We have unsuccessfully applied for
Stage II finding for PCRP 1997. We are refining the work scope and will make further applications. We are also looking for a more rapid and reliable model.

Employment or research opportunities applied for and/or received based on experience/training supported by this award: Dr. Williams has become a dedicated prostate cancer researcher and is well underway in developing an independent research career in this area. Generation of the model has been challenging, but she has shown persistence. The award has been invaluable in providing for the establishment of such a model.

CONCLUSIONS:

The LNCaP-C4-2 subline is the only cell line reported to generate osteoblastic lesions in any model anywhere. The strike rate reported was 20%, and we have already generated clonal, genetically tagged sublines which showed up to 100% metastasis to bone, however, this was not reproducible. The resultant cells, LNCap-C4-2-3H5 nonetheless represent a valuable resource for ourselves and others to characterize the sequence of molecular events leading prostate bone metastasis, at least radiologically. Our goal, which is the molecular profiling in prostate cancer of different stages of bone metastasis, may still be possible from analysis of the C42-3H5 cells compared to sibling sublines which are incompetent at different stages of metastasis, apparently homing and/or extravasation. Molecular understanding of these mechanisms will enable better characterization of prostate tumours at the time of diagnosis, and may lead to palliative therapies which would limit bone colonization and the accompanying morbidity, in prostate cancer sufferers.

REFERENCES:

APPENDICES:
Conference reports:


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FOR THE COMMANDER:

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PHYLIS M. PINEHART
Deputy Chief of Staff for Information Management

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