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Bob L. Anderson July 11, 2000

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INTRODUCTION

Research into mechanisms of breast cancer metastasis to bone has been hampered by the lack of animal models that mimic the pattern of spread in humans. Most of the current models use immunocompromised mice and abnormal routes of introduction of the tumor cells to bone. In addition, until now, there has been no model that allows a comparison of primary tumors and secondary metastases in bone with the same primary tumor metastasising to another site. The aim of this project is to determine the factors that are important in the development of breast cancer metastases in bone. To achieve this aim, we have developed the first murine model of spontaneous metastasis of breast cancer cells to bone. During the first year of this grant, we completed a detailed characterisation of this model. The purpose of this was to enable us to use selected clones with defined metastatic patterns to seek genes that control metastasis to bone. We are taking two approaches to achieve this aim. The first approach is to analyse in our improved model, the function of genes already implicated from other studies in bone metastasis. The other approach is to complete a genome wide screen for differentially expressed genes between clones that can or cannot metastasize to bone. When such factors are identified, it should be possible to develop specific therapies that target these factors and thereby reduce the incidence and morbidity associated with bone metastases.

BODY

The proposed specific aims of this project are as follows:

Task 1. To complete the characterization of the tumor model.

Task 2. To determine the association between the early detection of tumor cells in bone marrow with the subsequent development of overt metastases.

Task 3. To identify the factors that are required for metastasis to bone:

- by analysis of candidate genes listed in Table 1 of the proposal.
- by genome wide screening for novel genes required for metastasis to bone.

Task 1: Characterisation of the tumor model

This was completed in the first year of the grant and presented in the 1999 annual report. The results have been published in a paper in *Clinical & Experimental Metastasis* in 1999. The only incomplete aspect of the characterisation was a method whereby tumor burden in different tissues could be quantitated. Our initial plan was to use the fluorescence released by green fluorescent protein (GFP) to detect and measure the extent of tumor burden. However, we found that expression of high levels of GFP resulted in slower tumor growth and reduced metastasis, possibly due to immunological rejection of tumor cells presenting GFP peptides on class 1 MHC. However, a poorly expressing line containing the GFP construct grew and metastasized with kinetics indistinguishable from the parental line and enabled us to develop a quantitative PCR assay based on the ratio of GFP DNA to vimentin DNA. We use real time quantitative PCR (RTQ-PCR) based on the Taqman chemistry and can reliably detect small numbers of tumor cells in an organ. We have since developed a similar assay based on the presence of the *neo* gene in the tumour cells. This is useful for our gain or loss of function experiments where the levels of the gene of interest are manipulated by transducing the cells with expression constructs for the gene and for *neo*, which is used to select successfully transduced cells. Briefly, tissue samples, including bone, are snap frozen in liquid nitrogen and pulverised at this temperature in a steel homogeniser. Aliquots of the powder are weighed and DNA extracted. A multiplexed PCR measures the level of the reporter gene (GFP or *neo*) and vimentin in the same sample using RTQ-PCR. By spiking a homogenate of

bone from a non-tumor bearing mouse with tumor cells, we have established that we can detect 100 tumor cells in 20 µg DNA which contains about 3×10^6 cells.

Task 2. Determination of the association between the early detection of tumor cells in bone marrow with the subsequent development of overt metastases.

The kinetics of metastatic spread to bone of three sublines, 67NR, 66cl4 and 4T1, and the clonal line 4T1.2 derived from the original spontaneous carcinoma, were assessed in female BALB/c mice of 6-8 weeks of age following varying routes of inoculation. This was reported in the 1999 annual report. However, now that we have the RTQ-PCR technique established, we are in the process of repeating the analysis in 4T1.2 cells using the more sensitive PCR approach to confirm the kinetics of metastatic spread of this tumor.

Task 3. Identification of the factors that are required for metastasis to bone, both by analysis of candidate genes listed in Table 1 of the proposal and by genome wide screening for novel genes required for metastasis to bone.

We are using two approaches to identify genes differentially expressed between 66cl4 and 4T1.2, i.e. between the two clones that are fully metastatic but with only one capable of metastasizing to bone. The two approaches are (a) cDNA microarray analysis and (b) candidate gene analysis.

(a) cDNA microarrays

An array containing 5000 human genes was used to screen for differences in gene expression between 66cl4 and 4T1.2. The array was competitively hybridized with Cy3 (green) labelled 66cl4 and Cy5 (red) labelled 4T1.2 cDNA. Genes over-expressed in 66cl4 appear green and those over-expressed in 4T1.2 appear red. Genes with equivalent expression in the two lines appear yellow (Figure 1). Approximately 40 differentially expressed genes were identified. Differential expression has been further tested by northern analysis for nine of these genes and confirmed in four (Figure 2). This indicates the importance of confirming the microarray results using an independent technique. The four genes for which differential expression has been confirmed are osteopontin (OPN), caveolin-1 (Cav-1), bone morphogenic protein-4 (BMP-4) and protease nexin-1 (PN-1). Differential expression of the remaining 31 genes identified in the initial array screen is currently being tested by northern and/or RT-PCR analysis.

Interestingly, northern blot analysis of RNA from tissue culture cells shows little difference in caveolin-1 expression between 4T1.2 and 66cl4 (data not shown). Thus, caveolin-1 expression by 66cl4 tumor cells is induced *in vivo* by factors not present *in vitro*. This result illustrates the importance of the microenvironment on gene expression and therefore the advantages of using an *in vivo* model system. Differential expression of caveolin-1 in tumor derived material was also confirmed by western blot analysis (Figure 3) and immunohistochemistry (Figure 4) and of both caveolin-1 and protease nexin-1 by *in situ* hybridization (Figure 5).

Only one of these four genes, the bone matrix protein osteopontin, has previously been implicated in tumor metastasis, although not in metastasis to a specific organ (Schwirzke *et al.*, 1999). Interestingly, we find decreased osteopontin mRNA levels in the more aggressively metastatic 4T1.2 line compared to 66cl4. This observation will be investigated further. While the other proteins have not been linked to the metastatic process, the current state of knowledge about their function suggests that they could potentially be important in metastasis and that further study is warranted. For example, caveolin-1, the major structural component of membrane invaginations called caveolae

(Okamoto *et al.*, 1998), interacts with a number of signalling molecules including H-ras, Src family tyrosine kinases, eNOS and EGFR and appears to down-regulate signalling via the p42/44 MAPK pathway (Galbiati *et al.*, 1998). Down-regulation of caveolin-1 expression has been shown to cause transformation of cells *in vitro*, leading to the suggestion that caveolin-1 may function as a tumor suppressor (Lee *et al.*, 1998). In our model of metastasis, caveolin-1 is down-regulated in the lymph node, lung and bone metastasizing clone, 4T1.2, compared to 66cl4 that metastasizes only to lymph nodes and lung, leading us to hypothesize that is a bone metastasis suppressor gene.

Protease nexin I (PN-I) is a soluble, broad spectrum protease inhibitor (Akaaboune *et al.*, 1998) expressed in various cell types including bone cells and has been implicated in glioma tumorigenesis (Rao *et al.*, 1990). PN-1 is expressed at higher levels in the bone metastasizing clone. BMP-4 regulates development of skeletal structures by inducing differentiation of osteoblasts from mesenchymal precursors and by mediating apoptosis of the neural crest (Ahrens *et al.*, 1993). BMP-4 is expressed at lower levels in the bone metastasizing clone.

Caveolin-1 and PN-1 gain or loss of function experiments are underway. Both genes have been cloned into the mammalian expression vector pCI-neo in both sense and anti-sense orientations. The 4T1.2 and 66cl4 tumor lines have been transfected and single cell clones are being selected. In addition, we have obtained a dominant negative form of caveolin-3 from Dr. J. Hancock (Roy *et al.*, 1999) as an alternative way of blocking caveolin-1 function. This construct blocks caveolin-1 function (J. Hancock: personal communication).

(b) Candidate gene approach.

Since our cDNA microarrays are not yet complete (but will be expanded to 40,000 genes for the human array and 11,000 genes for the mouse array by the end of 2000), we have surveyed the literature for genes already implicated in breast cancer metastasis. So far, we have tested for expression of parathyroid hormone related protein (PTHrP), for expression and activity of several proteinases, and expression of cell adhesion molecules such as the integrins $\beta 1$, $\beta 3$, α_v and $\alpha 6$ and the adhesion molecule VCAM-1.

Parathyroid hormone related protein.

PTHrP shares structural and functional homology with parathyroid hormone, including the mobilization of calcium via increased bone resorption (Evely *et al.*, 1991) (Suva *et al.*, 1987) and has been implicated in the tendency of breast tumor cells to grow in bone (Guise *et al.*, 1996) (Mundy, 1991) (Southby *et al.*, 1990). However, recent clinical studies indicate that PTHrP expression in primary breast tumors predicts a more favorable prognosis and a reduced tendency to metastasize to bone (Henderson *et al.*, manuscript submitted for publication). We therefore investigated the role of PTHrP in our model of breast cancer metastasis to bone. *In vitro* secretion of PTHrP varies considerably between cell lines, but does not correlate completely with bone metastatic capacity. However, *in situ* PTHrP expression by tumors derived from these cell lines also does not correlate with *in vitro* secretion levels, suggesting that tumour cell PTHrP expression is regulated by the microenvironment (data not shown).

We are in the process of establishing the relevance of PTHrP in bone metastasis using gain or loss of function experiments. PTHrP over-expression was insufficient to produce overt bone metastases in the 66cl4 tumor that normally metastasizes to lymph nodes and lung but not bone (Table 1). However, PTHrP over-expression did increase the extent of bone metastasis detected in mice injected with the 4T1.13 tumor cells that normally metastasize to lung and bone, in a similar way to 4T1.2. Conversely, inhibition of PTHrP expression in this cell line resulted in loss of bone metastases. Therefore, PTHrP alone is insufficient to direct cells to metastasize to bone, but is able to modulate the growth of cells already capable of homing to bone. These data are being prepared for publication.

Cell line	In Vitro PTHrP Secretion (pmol/l/mg protein)	Lung Metastases	Bone Metastases
67NR	1.8	No	No
66cl4	2.0	Few	No
66cl4PTHrP	160	Few	No
4T1.13	2.0	Yes	Small
4T1.13PTHrP	15.0	Yes	More extensive
4T1.13PTHrP antisense	2.0	Yes	No

Table 1. Effect of PTHrP expression on metastasis to lung and bone.

Proteinases

The matrix metalloproteinase (MMP) and plasminogen activator (PA) families have been linked to the ability of breast tumor cells to metastasize (Andreasen *et al.*, 1997) (Ornstein *et al.*, 1999) (Pulyaeva *et al.*, 1997). In collaboration with Drs. Rik Thompson and E. Allan at St. Vincent's Institute of Medical Research, we have determined the activity profiles of MMP2, MMP9, uPA and tPA for the three lines, 67NR, 66cl4 and 4T1.2 (Table 2). The 4T1.2 line has high levels of uPA and MMP9, moderate levels of tPA and the ability to activate MMP2. The 66cl4 line exhibits low to moderate levels of these activities and low to negligible amounts are detected in 67NR. These activities are consistent with their metastatic capacities and with their invasive potential using *in vitro* invasion assays. Interestingly, tPA activity was similar in all three lines. These data are being prepared for publication.

Protease/ Activity	Cell Line		
	67NR	66cl4	4T1.2
MMP2 activation	+/-	+	+++
MMP9	-	+	+++
uPA	-	+	+++
tPA	+	+	+

Table 2. Proteinase activity

Adhesion molecules

Adhesion molecules play an important role in tumor metastasis by specifying sites for vascular endothelial cell adhesion. Particular combinations of integrin α and β subunits have been implicated in bone metastases, for example $\alpha_v\beta_3$, while other combinations specify binding to different cell types (Rajotte *et al.*, 1998). Integrin $\alpha_v\beta_3$ binds fibronectin, vitronectin, von Willibrand factor and osteopontin. Mice null for β_3 expression have non-functional osteoclasts and an osteopetrotic phenotype (McHugh *et al.*, 2000). Vascular cell adhesion molecule-1 (VCAM-1) expression by the endothelium is required for homing of hemopoietic progenitor cells to the bone marrow after transplantation (Frenette *et al.*, 1998). To investigate whether these adhesion molecules play a role in specifying the ability to metastasize to bone, the expression of integrins β_1 , β_3 , α_v and α_6 and the adhesion molecule VCAM-1 has been measured in 67NR, 66cl4 and 4T1.2. Differential expression was only seen for β_3 and VCAM-1 (Figure 6), suggesting that these adhesion molecules may be important factors in enabling circulating 4T1.2 tumor cells to attach to the vascular endothelium of bone. The significance of these observations is being investigated by gain or loss of function experiments.

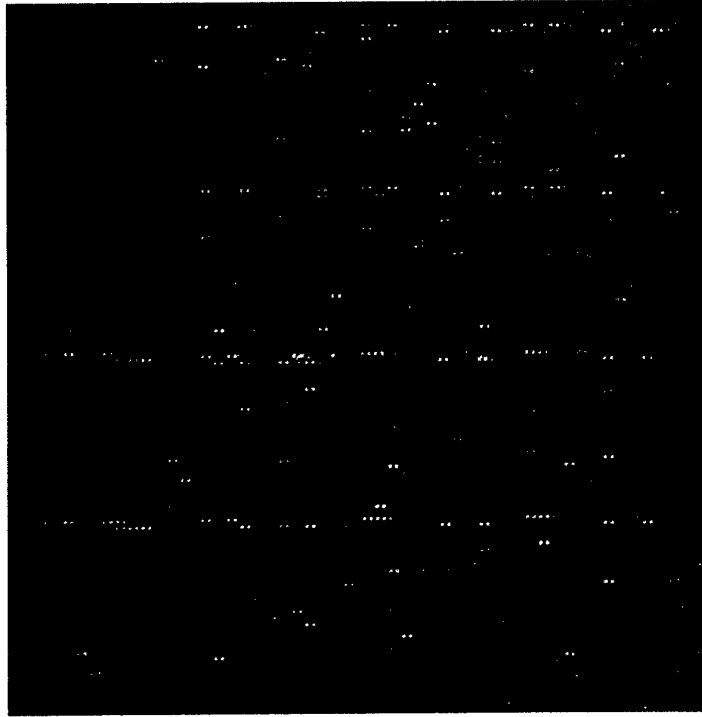


Figure 1: Differential expression using a human cDNA microarray. RNA from 4T1.2 was labelled with Cy5 (red) and RNA from 66cl4 with Cy3 (green), followed by competitive hybridization.

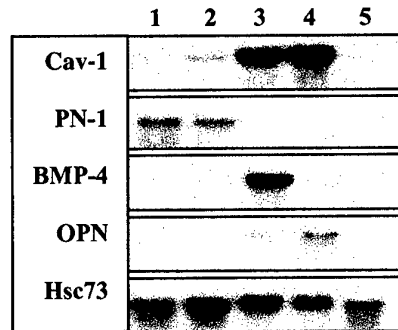


Figure 2: Confirmation of differential gene expression. Total RNA was extracted from primary tumors 35 days post imfp injection of 10^5 cells of (1) 4T1.13, (2) 4T1.2, (3) 66cl4, (4) 67NR, and (5) normal mammary gland. Northern blot analysis was performed using cDNA probes for the indicated genes. The mouse cytosolic cognate Hsp70 (Hsc73) was used to control for loading differences. 4T1.13 is a second bone metastasizing clone.



4T1.2 66cl4 67NR

Figure 3: Western blot analysis of *in vivo* caveolin-1 expression. Protein was extracted from the indicated primary tumors, run on a 10% denaturing SDS polyacrylamide gel and transferred to nitrocellulose. The membrane was then probed with rabbit anti-caveolin-1 polyclonal antibody and HRP-conjugated anti-rabbit secondary antibody.

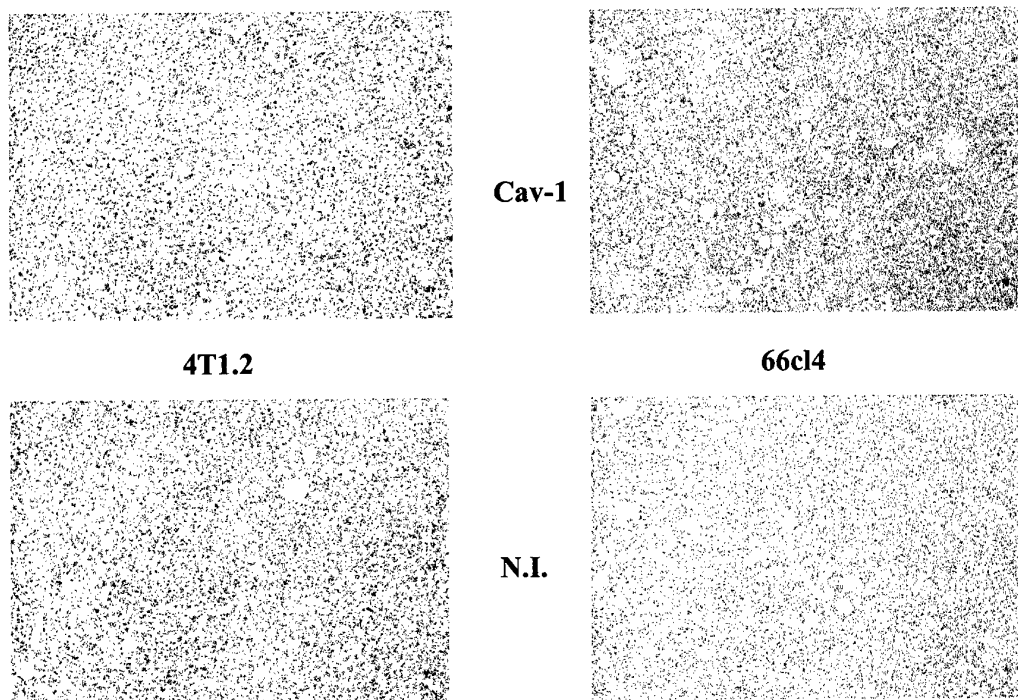
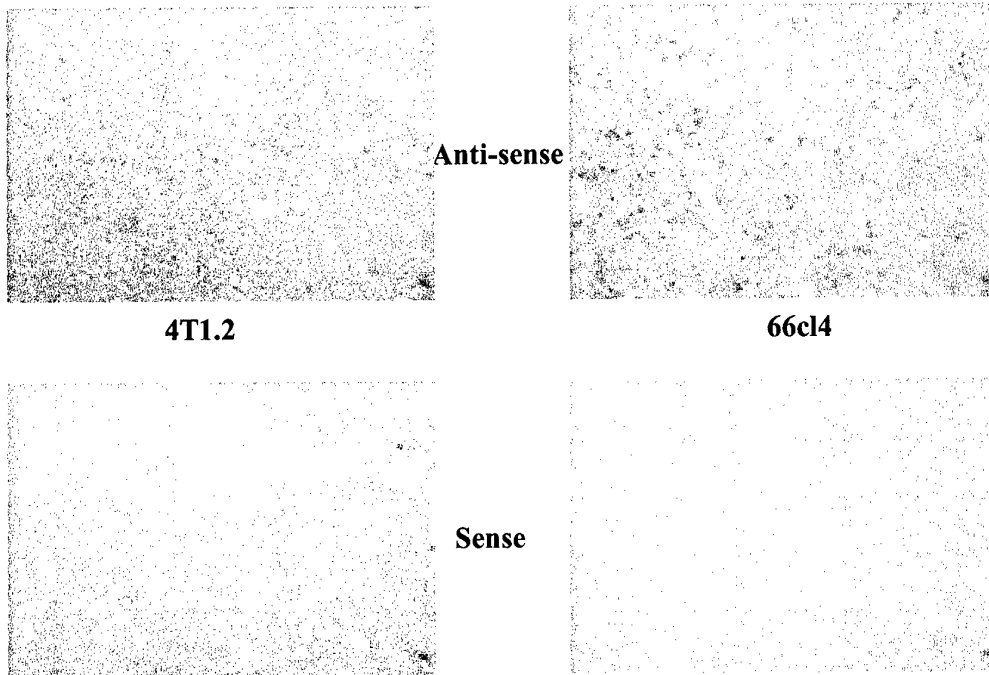


Figure 4: Immunohistochemical detection of caveolin-1 expression in primary tumors. Anti-caveolin-1 (Cav-1) polyclonal antibody was hybridized to sections from 4T1.2 and 66cl4 primary tumors. A biotin-conjugated secondary antibody was used to detect the primary antibody and streptavidin-peroxidase used for color reactions. Non-immune (N.I.) rabbit antiserum was used as a control.

A. Caveolin-1



B. Protease nexin-1

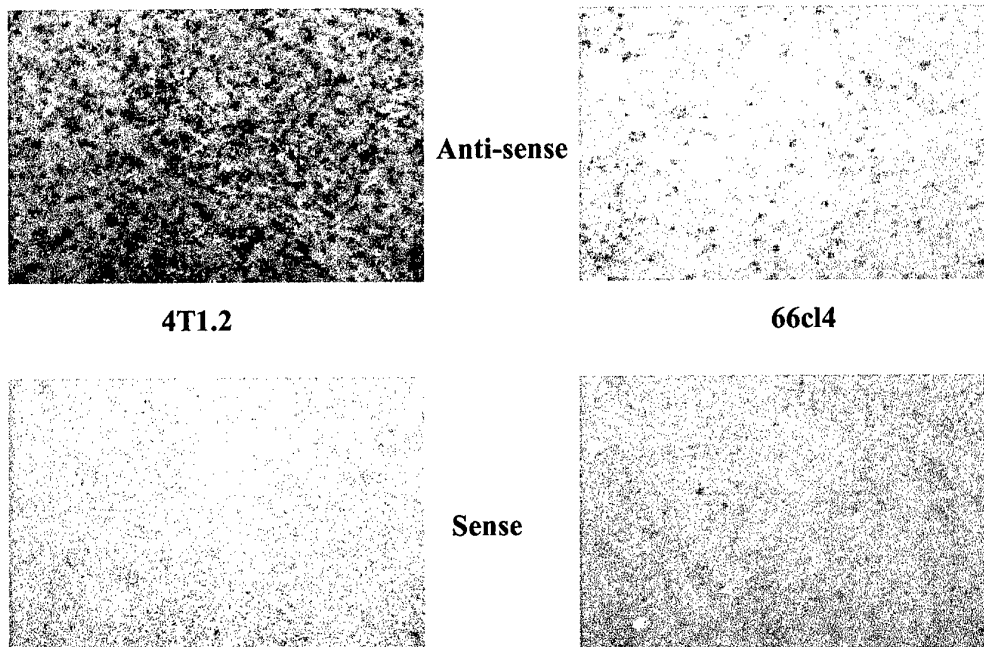


Figure 5: *In situ* hybridization of caveolin-1 and protease nexin-1 expression in primary tumors. Anti-sense and sense DIG labelled riboprobes to (A) Caveolin-1 and (B) Protease nexin-1 were hybridized to sections cut from primary tumors of 4T1.2 and 66cl4. An alkaline phosphatase conjugated anti-DIG antibody was used to detect the riboprobes.

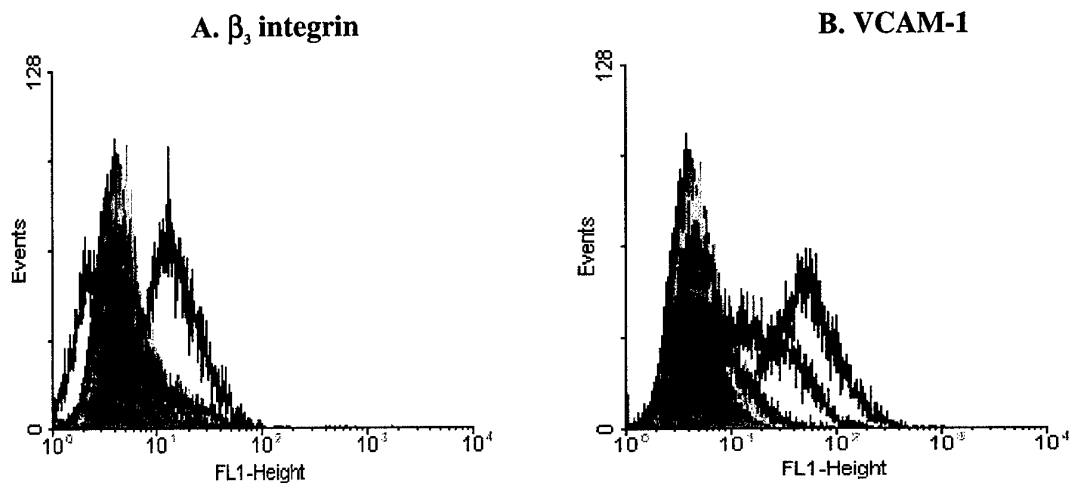


Figure 6. FACS profiles of β_3 integrin (A) and VCAM-1 (B) expression in the tumor lines. Tissue culture cells were harvested and incubated with primary antibody for 20 minutes, on ice. After washing, cells were incubated with FITC-conjugated secondary antibody for 20 minutes and then analyzed by FACS.

A. — 4T1.2, unlabelled
 — 4T1.2, IgG control
 — 4T1.2, β_3 monoclonal antibody
 — 66cl4, β_3 monoclonal antibody
 — 67NR, β_3 monoclonal antibody

B. — 4T1.2, unlabelled
 — 4T1.2, IgG control
 — 4T1.2, VCAM-1 monoclonal antibody
 — 66cl4, VCAM-1 monoclonal antibody
 — 67NR, VCAM-1 monoclonal antibody

KEY RESEARCH ACCOMPLISHMENTS

- Discovery of a murine breast carcinoma line that metastasizes from the mammary gland to bone.
- Characterization of clones derived from the original carcinoma, which have differing metastatic potentials.
- Development of a quantitative PCR technique to measure tumor burden in different tissues.
- Demonstration that the appearance of micrometastases in bone marrow correlates with the subsequent development of overt bone tumors.
- The discovery of four genes that have the potential to regulate metastasis from breast to bone, using cDNA microarray analysis.
- Demonstration that PTHrP does not dictate metastasis to bone but does enhance tumor growth within bone environment.
- Demonstration that increased levels of uPA activity, MMP9 activity and MMP2 activation correlate with the ability to metastasize to bone.
- Demonstration that $\beta 3$ integrin and VCAM-1 levels are elevated in the bone metastasising clone.

REPORTABLE OUTCOMES

- The development and characterization of the only murine model of spontaneous metastasis from the mammary gland to bone. This model offers great potential in testing novel therapeutics targeted at bone metastases.
- Manuscript published in *Clinical and Experimental Metastasis*.
Lelekakis, M., Moseley, J.M., Martin, T.J., Hards, D., Williams, E., Ho, P., Lowen, D., Javni, J., Miller, F.R., Slavin, J., Anderson, R.L. (1999) A novel orthotopic model of breast cancer metastasis to bone. *Clin. Exp. Metastasis* 17: 163-170.
- Abstract and poster presentation to the 7th International Congress of the Metastasis Research Society, 1998.
A Model of spontaneous breast cancer metastasis to bone. Anderson, R.L., Lelekakis, M., Miller, F., Williams, E.D., Hards, D., Martin, T.J. and Moseley, J.M.
- Abstract, poster and platform presentation to the Keystone Symposium entitled Molecular Pathogenesis of Bone Disease, California, 1999.
Characterization of a novel murine model of breast cancer metastasis to bone. Tavarria, M., Sloan, E., Lelekakis, M., Ho, P., Hards, D., Williams, E., Martin, T.J., Moseley, J. and Anderson, R.L.
- Keystone travel grant awarded to M. Tavarria for the best short platform presentation.

- B. Med. Sc. degree awarded to Mr. Marcus Foo based on his thesis that investigated the relationship between the presence of metastatic cells in bone marrow and the development of overt bone tumours.
- Platform presentation to the DOD Era of Hope Meeting, 2000.
Mechanisms of breast cancer metastasis to bone. Anderson, R.L., Lelekakis, M., Tavarria, M., Sloan, E., Martin, T.J., Hards, D., Ho, P., Moseley, J.M.
- Komen Dissertation Award to E. Sloan for salary support whilst investigating the function of some of the genes found by cDNA microarray analysis to be differentially expressed in the bone metastasising clone.

CONCLUSIONS

The major achievement in the first year of this grant was the characterization of the murine breast cancer model of metastasis from the mammary gland to bone. To our knowledge, this is the only model that mimics the human disease and it provides a unique opportunity to search for the genes that control metastasis to bone. We have demonstrated that only in the bone metastasising clone do we find metastatic cells in the bone marrow, implying that the other clones cannot home to bone. By the use of intracardiac injections, we have shown that the non-metastatic clone can proliferate in bone when forced there by arterial blood flow, but the lung metastasising clone cannot proliferate in bone marrow.

In the second year, we have focussed on the characterisation of genes that are differentially expressed between the bone metastasising clone and another fully metastatic line that cannot metastasize to bone.

Our **candidate gene approach**, as outlined in the proposal, has focussed on genes implicated by others in breast cancer metastasis in general or to bone in particular. For PTHrP, we have demonstrated that, contrary to some published data, PTHrP does not drive metastasis to bone. However, cells that express more PTHrP do grow more rapidly in the bone environment. This finding is backed up by new clinical data (Henderson et al, submitted for publication) that show that patients with high PTHrP expression in their primary tumors have longer survival and fewer bone metastases. For a number of other candidate genes, we have demonstrated a correlation between increased expression and the ability to metastasize to bone. The relevance of these correlations awaits the outcome of gain or loss of function experiments, as have been largely completed for PTHrP.

Our **genome wide screening** has revealed differential expression of a few genes not previously implicated in metastasis. Functional studies are underway with these genes to determine their importance in bone metastasis.

Ultimately, we will need to examine the expression of genes that appear in the mouse model to be important in bone metastasis, in primary human breast tumors from patients for whom clinical outcome is known. It is our belief that we will be able to identify genes whose expression is prognostic of subsequent metastatic behaviour of the tumor. For example, if the expression of a particular gene is linked to subsequent metastasis to bone, it will be possible to treat that subset of patients more aggressively to prevent bone metastasis or with drugs such as bisphosphonates that may reduce or eliminate bone disease. Further, a gene that directly regulates metastasis to bone may become the target for a new therapeutic strategy.

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APPENDIX:

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A novel orthotopic model of breast cancer metastasis to bone

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Abstract

Breast cancer affects approximately one woman in twelve and kills more women than any other cancer. If detected early, patients have a five year survival rate of 66%, but once metastatic disease has developed, there is no effective treatment. About 70% of patients with metastatic disease have bone involvement, while lungs and liver are the other common targets. Bone metastases cause severe pain, pathological fractures and hypercalcaemia and thus are a significant clinical problem. The development of new therapies for metastatic breast carcinoma depends on a better understanding of the mechanism of homing of the tumour cells to bone, liver and lungs and the factors required for their growth in these organs. Research on mechanisms of breast cancer metastasis, particularly to bone, has relied on *in vitro* studies or on tumour models in which the inoculation route is designed to promote delivery of tumour cells to a specific organ. Metastases in bone are achieved by inoculation into the right ventricle of the heart. To our knowledge there has been no report of a model of metastatic spread from the mammary gland to distant sites which reliably includes bone. In this paper, we describe our recent development of a novel murine model of metastatic breast carcinoma. The new model is unique in that the pattern of metastatic spread closely resembles that observed in human breast cancer. In particular, these murine breast tumours metastasise to bone from the primary breast site and cause hypercalcaemia, characteristics not normally found in murine tumours, but common in human disease. Furthermore, in a preliminary characterisation of this model, we show that secretion of parathyroid hormone-related protein, a role for which has been implicated in breast cancer spread to bone, correlates with metastasis to bone. This model therefore provides an excellent experimental system in which to investigate the factors that control metastatic spread of breast cancer to specific sites, particularly bone. The special advantage of this system is that it involves the whole metastasis process, beginning from the primary site. Existing models consider mechanisms that pertain to growth of tumour once the site has been reached. An understanding of the regulation of these factors by potential therapeutic agents could lead to improvement in therapies designed to combat metastatic disease. For the first time, this development will allow exploration of the molecular basis of site-specific metastasis of breast cancer to bone in a clinically relevant model.

Introduction

Bone metastases are a major cause of morbidity in breast cancer and occur in 70% of patients who develop metastatic disease. Hypercalcaemia is a common complication due to excessive bone resorption which is caused by increased numbers of active osteoclasts. This leads to the breast cancer patient suffering severe pain and bone fractures [1]. The preference of breast tumour cells for growth in bone is not fully understood, but is undoubtedly facilitated by their ability to stimulate adjacent osteoclasts to resorb bone. This process is promoted by the ability of these tumour cells to express bone compatible matrix proteins and adhesion

molecules and by tumour cell interactions with local growth factors in bone. Several factors that have normal roles in bone turnover and metabolism have been detected in primary breast cancers. These include parathyroid hormone-related protein (PTHrP), tumour necrosis factor- α (TNF α), transforming growth factor- β (TGF β) and prostaglandins [1] [2], interleukin 6 (IL6) [3], interleukin 11 (IL11) [4], osteopontin [5] [6] and bone sialoprotein [6]. The ability of these factors to alter bone turnover and to be regulated in the bone environment may facilitate establishment of tumours in bone.

The development of animal models of human tumour cells injected into immunodeficient mice for the study of breast cancer spread to bone has provided strong evidence for the importance of PTHrP in the establishment of bone metastases [7]. However, these models are dependent upon

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the injection of tumour cells into the left ventricle of the heart in order to promote tumour cell delivery to bone and therefore can only evaluate factors important for invasion of bone and interaction of tumour cells with the bone environment. Until now, no models of spontaneous metastasis of breast tumour cells from the primary site to bone have been reported, although orthotopic inoculation has been shown to result in development of secondary tumours in lymph nodes and lungs [8]. Breast cancer cells can be forced to the site of interest, for example by intravenous injection to promote growth in lungs, into the portal vein to produce liver tumours, and into the carotid artery to induce brain tumours [8]. To induce tumour cell growth in bone, cells can be injected directly into the tibia [9] or into the arterial system [10]. However, all of these routes result in a bolus of cells arriving in bone or other organ of interest, rather than the situation that occurs in clinical disease, in which cells escape gradually over time from the primary tumour and circulate through the vascular system or lymphatics before colonizing a distant organ.

We report here the development of a novel model of metastasis from the mammary gland to bone using a clonal tumour line derived from a spontaneously arising mammary tumour in a BALB/cfC3H mouse [11]. The isolation of sublines that are either non-metastatic, or that metastasise to lungs or that metastasise to lungs and liver has been described previously [12]. Here we report that one of the subpopulations, 4T1, also metastasises to bone. We have further characterised a single cell clone derived from 4T1 (4T1.2) that metastasises to bone and lung. The model is unique in that the pattern of metastatic spread closely resembles that observed in human breast cancer, causing osteolytic lesions in bone. Given the documented role of PTHrP in bone lesions of human breast cancer, we have examined the expression patterns of PTHrP both *in vitro* and *in vivo* and have found that both primary tumours and bone lesions of the bone metastasising clone express this growth factor.

Materials and methods

Cell culture

Four sublines of breast cancer cells, 67NR, 66cl4, 4T1 and 4T1.2, derived from a spontaneous carcinoma in a Balb/cfC3H mouse were used. The derivation of the first three of these sublines has been described previously [12]. Briefly, 67NR is a geneticin resistant variant derived from subpopulation 67, 66cl4 is a thioguanine/ouabain resistant variant of subpopulation 66 and 4T1 is a thioguanine resistant variant of subpopulation 410.4. Clone 4T1.2 was derived by single cell cloning of 4T1 (Figure 1).

The tumour lines were maintained in a 5% CO₂ incubator in MEM (alpha modification) containing 10% FCS and antibiotics (penicillin and streptomycin). They were checked regularly for mycoplasma contamination using the Gene-Probe kit. To minimise genetic drift, the cells were maintained in culture for no more than four to six weeks.

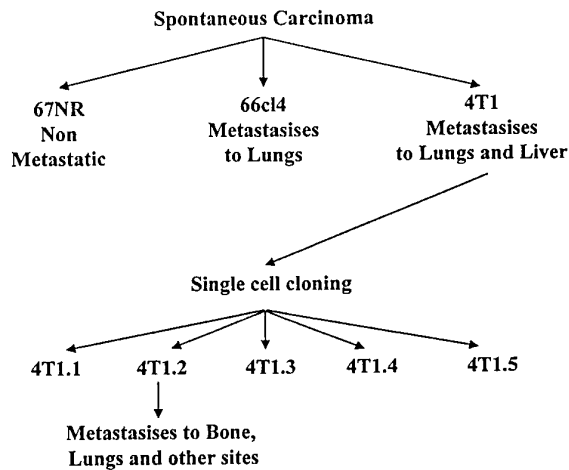


Figure 1. Flow chart showing the derivation of the breast tumour lines used in this study.

Prior to inoculation, cells were checked for viability using trypan blue.

In vivo studies

Female BALB/c mice aged 6–8 weeks were injected into the fourth mammary fat pad with 1×10^5 viable cells in a 10 μ l volume, or into the lateral tail vein with 5×10^5 cells in a 0.2 ml volume, or into the left ventricle with 2×10^5 cells in a 0.2 ml volume.

Spontaneous metastasis from the mammary fat pad

Tumours were palpable 7–10 days after injection into the mammary fat pad. Primary tumour size was measured three times a week using callipers and the final tumour weight was measured after sacrificing the mice and excising tumours. Tumour burden in the lungs was quantitated by counting nodules after inflation of the lungs with India ink. Metastases in other organs were observed visually or by histological examination of formalin fixed samples. Blood was collected by cardiac puncture under anaesthetic at the time of sacrifice to obtain plasma for PTHrP and calcium assays. In some experiments, occult metastatic cells were quantitated by recovery of clonogenic cells from disaggregated tissues and growth in selective media as described previously [12]. By sacrificing groups of 5–6 animals at different times after initiation of the primary tumour, the kinetics of metastasis to the lung and bone were determined. Lungs were digested as previously described [12]. The spinal column was removed, individual vertebrae split open with scalpels, agitated, and stirred briefly in media containing 60 μ M 6-thioguanine. The crushed spinal column was removed and the released cells were incubated for 7–10 days at 37°C before colonies were fixed, stained, and counted.

Kinetics of bone colonisation following intravenous injections

Groups of 3–6 mice were sacrificed 7, 12, 17 and 19 days after intravenous injection of 5×10^5 tumour cells. Lungs were

enzymatically digested and vertebral columns were crushed and released cells plated in selective media as described above.

Bone metastasis following intracardiac injections

Tumour cells were injected into the left ventricle and hence directly into the arterial system. Mice were culled when they first showed signs of distress. The number of lung metastases was assessed by India ink inflation and the presence of tumour deposits in the bone was detected by examination of histological sections of the spine and femur.

Histology

Primary tumours, lungs, liver, femora and spines from tumour-bearing and control mice were fixed in 10% buffered formalin. Bones were decalcified in EDTA [13] and embedded in paraffin. Standard haematoxylin and eosin (H&E) staining procedures were employed for assessment of morphology. For detection of bone metastases, the paraffin embedded samples were cut into 5 μ m sections and every twentieth section was stained and examined by microscopy.

Immunohistochemistry

PTHrP. PTHrP immunostaining was carried out as previously described [14] using a sheep polyclonal antibody raised against synthetic human PTHrP (50–69) at dilutions of 1/200 and 1/300 overnight at 4 °C. Normal sheep serum was used for non-immunecontrols and normal mouse skin was used as a positive control in all assays. Other controls included deletion of each of the reagents.

Keratin. A rabbit polyclonal anti-keratin (wide spectrum 2622 from Dako Corp.) was used overnight at dilutions of 1/500 and 1/750 at 4 °C. Antigen retrieval was achieved by microwave treatment in 0.05 M tris buffer pH10 and detection was by biotin-streptavidin-peroxidase.

Estrogen receptor: A monoclonal antibody against the human estrogen receptor (ER) (Novocastra NCL-ER-6F11), which cross-reacts with the mouse protein, was used overnight at dilutions of 1/250 and 1/500 at 4 °C. Antigen retrieval was achieved by microwave exposure in 0.01M citrate buffer, pH6 and detected with biotin-streptavidin-peroxidase.

In all cases, specific reaction is indicated by brown staining in the sections.

Radioimmunoassay for PTHrP

PTHrP was measured with an N-terminal directed antibody using a radioimmunoassay described previously [15]. To assay release of PTHrP from cultures, cells in an exponential phase of growth were incubated in serum-free medium containing 0.1% BSA for 1 h. The medium was then replaced with fresh serum-free medium containing 0.1% BSA for 20 h. PTHrP was measured on aliquots of the medium

cleared of cell debris by centrifugation. To correct for cell numbers, the remaining monolayer of cells was rinsed with PBS, drained, dissolved in 1M sodium hydroxide overnight at 37 °C and the protein content measured using a modified Lowry assay.

Plasma calcium assay

Total calcium was measured using the Arsenazo III assay (Trace Scientific, Australia), following the recommended protocol.

Results

The kinetics and patterns of metastatic spread of three sublines, 67NR, 66cl4 and 4T1, derived from the original spontaneous carcinoma (Figure 1), were assessed in female BALB/c mice of 6–8 weeks of age following varying routes of inoculation. In addition, we have characterised a single cell clone of 4T1, called 4T1.2, that shows a strong propensity to metastasise to bone. In a previous analysis, 67NR was characterised as being non-metastatic, 66cl4 as metastasising to lung and 4T1 as metastasising to lung and liver [12].

Kinetics of 4T1 metastasis

After inoculation into mammary gland, clonogenic 4T1 cells were always recovered from the lungs prior to recovery from the bone. Clonogenic cells were recovered by day 15 (first day assessed) from the lungs but not from bone at a time when the median weight of the primary tumours was 216 mg. Clonogenic 4T1 cells were not recovered from bone at day 19 (6 mice tested) when the median weight of the primary tumours was 343 mg, but were recovered from vertebrae of 5 of 6 mice at day 22 when the median weight of the primary tumours was 546 mg. In similar experiments, clonogenic cells were not recovered from vertebrae of mice bearing 67NR or 66cl4 primary tumours. After intravenous injection, clonogenic 4T1 cells were recovered from lungs at all times. Lung metastases become grossly visible by day 19. Vertebrae were uniformly negative at days 7 and 12, but became uniformly positive by day 17 following injection. An attempt to isolate a clone of 4T1 for which clonogenic cells were not detected in the vertebrae was unsuccessful. Twenty clones were randomly isolated and injected intravenously. Although some clones appeared to be quantitatively more metastatic to bone than others, clonogenic cells were recovered from spines by day 20 in all cases. Subsequent analyses of overt tumour growth were performed using clone 2 of 4T1 (4T1.2).

Analysis of growth patterns of the breast carcinoma sublines

The pattern of metastatic spread of 67NR, 66cl4 and 4T1.2 was assessed following inoculation into the mammary gland. Mammary tumours were palpable within 7–10 days. The

tumours grew rapidly and the mice were killed when the tumours reached a weight of 1–2 g after 30–44 days. Some of the 4T1.2 tumour-bearing mice exhibited signs of partial paralysis of the hind limbs by this time. An analysis of primary tumour size, lung metastases and bone metastases is shown in Table 1, with data from two independent experiments (Experiments 1 and 2). The tumour lines exhibited different growth rates *in vivo* that did not mirror their growth rates *in vitro*. 67NR, which has a slower growth rate *in vitro*, grew more rapidly *in vivo* than the other two lines. 4T1.2, which has a similar growth rate *in vitro* to 66cl4, grew more slowly *in vivo* than 66cl4. The 4T1.2 line showed a greater capacity to metastasise to the lungs from the mammary gland compared to 66cl4, whilst almost no tumour nodules were detected in the lungs of mice inoculated with 67NR (one mouse was found to have one lung tumour nodule in Experiment 1). In other experiments, all mice were culled on the same day after tumour inoculation and despite 4T1.2 primary tumours being smaller, the mice bearing 4T1.2 tumours still exhibited more lung nodules (data not shown). From visual examination of the mice, it is evident that 66cl4 tumours do not colonize other organs apart from the lung, whilst mice bearing 4T1.2 tumours often had visible nodules on the diaphragm and rib cage and enlarged lymph nodes. By histological examination of the spine and femur, only the 4T1.2 clone exhibited tumour growth in the bone, confirming the assays for clonogenic tumour cells mentioned above.

Intracardiac injection of tumour cells

To investigate further whether the 67NR and 66cl4 cells are unable to reach bone or whether they can reach, but not grow in the bone environment, tumour cells were injected into the left ventricle and hence into the arterial system leading to bone. Mice were assessed 11–17 days later when they first showed signs of distress (Table 1, Experiment 3). Whilst all three tumour lines showed an ability to colonize the lung, no histological evidence of tumour growth was found in the spine or femurs of four mice examined after inoculation of 66cl4 tumour cells. One of four mice examined had 67NR tumour growing in bone while two out of three mice carrying 4T1.2 tumour cells had tumour masses in the bones (Table 1).

Histological analysis of primary and secondary tumours

Primary tumours that were 10–15 mm in diameter showed areas of necrosis towards the central regions. All comprised sheets and cords of large undifferentiated cells typical of carcinoma. Large numbers of mitotic figures were apparent. There were no obvious differences in morphology of tumours generated from the different cell lines (see Figure 2A for the 4T1.2 primary tumour). Pleural, subpleural and perivascular tumour nodules were evident in the lungs of mice bearing 66cl4 and 4T1.2 tumours (not shown). No tumour masses were detected in the livers of any animals although extramedullary hemopoiesis developed in livers of 4T1.2 tumour-bearing mice (not shown).

In the femora of 4T1.2 tumour-bearing mice, a single metastasis generally developed in the distal diaphysis, close to the growth plate and in some cases occupied large areas of the marrow space by 5 weeks post tumour inoculation (Figures 2E, F). Occasionally, attached muscle adjacent to the bone metastasis became involved. Tumours grew in the spines of 4T1.2 animals in multiple vertebrae and often involved surrounding muscle (Figure 2G). Tumour growth in the spine led to complete replacement of bone marrow by tumour cells and invasion progressed into the spinal canal. Areas of active osteolysis could be seen adjacent to trabecular bone surfaces in both the spine (Figure 2H) and femur (not shown) of 4T1.2 bearing animals.

PTHrP, keratin and ER expression in primary and secondary tumours

Primary tumours generated by all three cell lines stained positively for PTHrP (see Figure 2C for 4T1.2). In tumours from 66cl4 and 4T1.2 cells, staining was usually most intense in dividing cells with mitotic figures, and at the edges of the tumours where normal tissue invasion was occurring. Tumours from 67NR cells tended to stain more weakly, with mitotic cells weakly positive or negative for PTHrP (not shown). Metastases in lungs also stained weakly positive for PTHrP. In both the femur and spine, 4T1.2 tumours were positive for PTHrP but there was no apparent difference in intensity from that seen in the tumour at the primary site (Figures 2C and E). For comparison, the PTHrP staining of a 4T1.2 tumour in femur and a non-immune control are shown (Figures 2E and F, respectively). Strong staining for keratin and weaker staining for the ER was evident for all three primary tumour types (see Figure 2B and D for keratin and ER immunostaining in 4T1.2 tumours, respectively). 4T1.2 tumours in bone also stained positively for keratin (not shown).

PTHrP secretion

PTHrP levels were measured both in the medium of cultured cells and in the plasma of tumour-bearing mice. PTHrP secretion into the medium of the tumour sublines grown in culture was measured in exponentially growing cells placed in serum-free medium for 20 h. As shown in Figure 3, PTHrP levels were highest in the 4T1.2 line, intermediate in 66cl4 cells and negligible in 67NR cells. Plasma PTHrP levels in tumour-bearing mice are shown in Table 2. Compared to the value in non-tumour-bearing mice, plasma PTHrP was elevated approximately 1.7 fold in mice bearing 4T1.2 tumours. This increase was significant ($P < 0.04$) when analysed using a Student *t* test.

Plasma calcium levels

Circulating calcium levels are presented in Table 2 along with the PTHrP data. In parallel with the elevation of plasma PTHrP levels in mice bearing 4T1.2 tumours, there was a significant increase in plasma calcium. In mice bearing 67NR tumours, calcium levels were also significantly elevated but to a lesser degree. No hypercalcaemia was evident in mice bearing 66cl4 tumours.

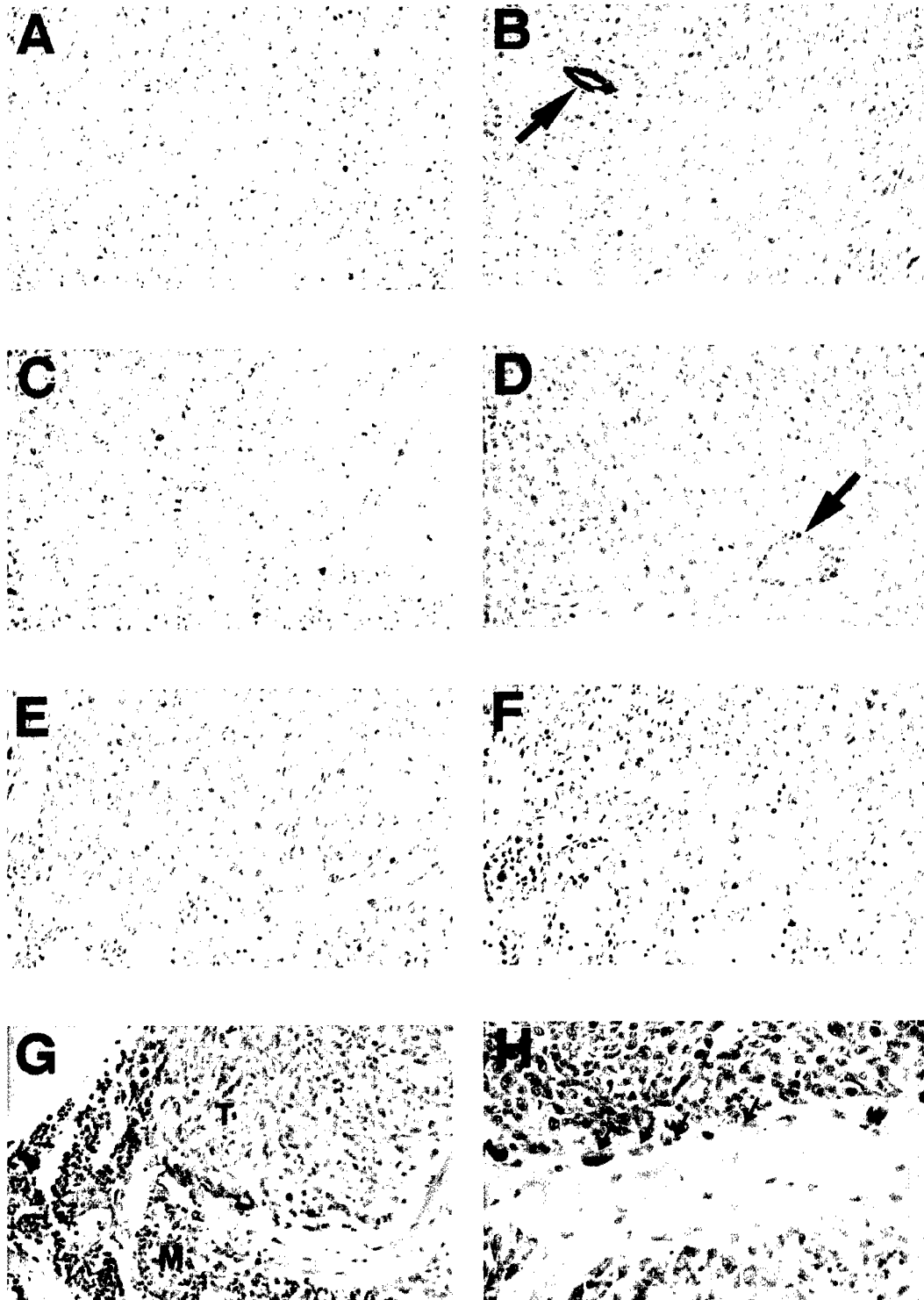


Figure 2. Morphological and immunohistochemical analysis of the 4T1.2 tumour. Section of the primary tumour showing morphological appearance by H&E staining (A) and immunostaining of the primary tumour for keratin (B), PTHrP (C) and estrogen receptor (D). Brown staining indicates regions of specific immunostaining. The arrows in **B** and **D** indicate strong immunostaining in epithelial cells lining the ducts. PTHrP immunostaining of a section through the femur, showing the growth plate and PTHrP positive tumour cells adjacent to it (E). Non-immune control of adjacent section of the femur to E (F). An H&E stained section through the spine showing a region of tumour (T) and a region of normal marrow (M) (G). A–G were photographed at $\times 80$ magnification to show distribution of specific proteins throughout the tumour. A higher power image ($\times 100$) of a section of the spine stained with H&E showing the large multinucleated osteoclasts (indicated with arrows) adjacent to the trabecular bone, causing osteolysis of the bone (H).

Table 1. Tumour growth characteristics after injection of cells into the mammary fat pad or into the left ventricle.

Tumour subline	No. Mice	Time of growth (days, mean±SE)	Tumour weight (g) (mean±SE)	Lung metastases (mean±SE)	Bone metastases (No. +ve mice)	
					Femur	Spine
Experiment 1						
67NR	7	32.6±0.4	2.3±0.8	0.2±0.4	0/3	0/3
66cl4	6	32.8±0.4	1.3±0.2	2.3±2.6	0/3	0/3
4T1.2	6	34.0±0	0.9±0.3	22.5±8.5	3/3	1/3
Experiment 2						
67NR	8	44.1±1.8	2.1±0.2	0	0/4	0/4
66cl4	14	30.6±1.4	1.2±0.1	15.7±1.6	0/4	0/4
4T1.2	15	36.6±0.9	1.0±0.1	34.3±2.7	4/4	3/4
Experiment 3						
67NR	5	17.0±0	–	12.2±6.0	0/4	1/4
66cl4	5	11.0±0	–	213.0±64.1	0/4	0/4
4T1.2	5	14.8±0.2	–	82.4±16.0	0/3	2/3

For the spontaneous metastasis assays shown in Experiments 1 and 2, 1×10^5 tumour cells were inoculated into the fourth mammary fat pad. Mice were culled when the primary tumour reached a size of approximately 1g or when the mouse first showed signs of distress. For the intra-cardiac experiment shown in Experiment 3, 2×10^5 tumour cells were inoculated into the left ventricle of the heart and mice were culled when they first showed signs of distress. Where appropriate, the primary tumour was excised and weighed. Lung metastases were scored after India ink inflation of the lungs. Tumour deposits in bone were detected by examination of H&E sections of the spine and femur.

Table 2. Plasma concentrations of PTHrP and calcium in mice bearing 67NR, 66cl4 and 4T1.2 tumours.

Cell Line	Plasma PTHrP (pM)	Plasma Ca (mM)
	Mean ± SE	Mean ± SE
67NR	8.7±0.7 (n = 15)	2.33±0.03*
66cl4	4.7±0.5 (n = 21)	2.24±0.05
4T1.2	12.1±1.5* (n = 19)	2.37±0.04**
Control mice	7.6±1 (n = 13)	2.21±0.02 (n = 13)

The samples were obtained from plasma taken when the mice were killed at the end of the experiment (Day 32–36 post inoculation). The 'n' values indicate the number of mice analysed in each group. The P values were calculated using a two sample Student *t* test assuming unequal variances. Data with a single asterisk showed a *P* value < 0.05 whilst those with a double asterisk had a *P* value < 0.005 compared to values obtained from control mice. The other data were not significantly different to values obtained in control mice. The controls were age matched, non-tumour bearing mice.

Discussion

Although much has been learned about the process of metastasis, less is understood of the mechanisms that dictate why tumours spread successfully to one particular site and not another. The establishment of a tumour in a tissue depends both on factors that it expresses and those released locally [16]. The concept that the microenvironment of an organ

PTHrP secretion

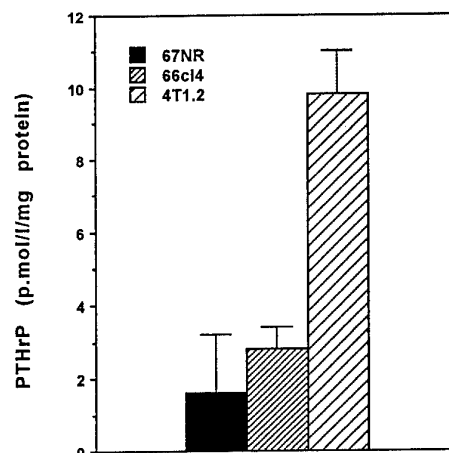


Figure 3. PTHrP secretion by the tumour sublines. PTHrP in the medium of cells in exponential phase of growth was measured by RIA and standardised to the protein content of the cells in the sample.

provides the fertile 'soil' for growth of a tumour cell was first proposed by Paget over one hundred years ago [17]. Bone is the favoured site of metastases of breast, prostate and lung cancers and thus the bone microenvironment must provide an appropriate fertile 'soil' for these tumours [16]. Once present in bone, tumour cells cause disruption of normal bone remodeling, with promotion of osteoclast formation and enhanced osteolysis usually occurring in the case of breast tumours. We have described here a clinically relevant model of breast cancer metastasis in which primary tumour cells spread from the mammary gland to bone and other distant sites. This model, with its use of subpopulations of cells that metastasise from the primary site in the mammary

gland to different organs, now provides the means to address the mechanisms of site-specific metastasis in a clinically relevant setting.

The 4T1.2 clonal cell line that we describe here metastasises to lung and bone following orthotopic inoculation into the mammary fat pad. The parental line 4T1 was shown previously to spread to lungs and liver [12] and here we show that clonogenic cells can be detected in spines of 4T1 bearing mice. Kinetic studies revealed that clonogenic cells could be detected in the lung prior to detection in the spine. As shown previously, the sister populations, 66cl4 and 67NR metastasised to the lung (66cl4) or did not metastasise at all (67NR). 4T1.2 cells generated tumours in both femur and spine and these were accompanied by local destruction of bone with active osteoclasts visible along bone surfaces. In none of our experiments with 4T1.2 did we observe tumours in the liver, although extramedullary hemopoiesis was observed.

Intracardiac inoculation confirmed that only the 4T1.2 cells were consistently metastatic to bone. This route of inoculation also led to an increased incidence of lung metastases in all three groups, including the normally non-metastatic 67NR tumours. No bone metastases were seen in mice inoculated with 66cl4 cells but spine involvement was observed in one of three mice inoculated with 67NR cells. This suggests that the 67NR cells may have some ability to grow in bone but are deficient in the mechanism required to detach from the primary site.

Our preliminary characterisation of this model has included use of keratin immunohistochemical staining to confirm the epithelial nature of the tumour cells and identification of ER in primary tumours generated from all three cell lines. Keratin staining was evident in all tumour sublines and was used as a guide to the identification of tumour cells at metastatic sites. The presence of the ER in the primary tumours, as evidenced by immunological staining, indicates an opportunity for the evaluation of anti-estrogen therapies in the modulation of site specific metastatic spread.

Since there is strong evidence implicating PTHrP in the metastatic spread of breast cancer to bone [18], we examined whether its expression correlated with bone metastasis in this model. PTHrP is the major mediator of hypercalcaemia in cancer patients including those with lung and breast cancers [19]. The primary tumours of all three cell lines investigated here stained positively for PTHrP, as did the bone metastases of 4T1.2 tumours. There were no major differences in the intensity of staining of this protein in the tumours generated from each of the cell lines indicating that, at the primary site, the presence of PTHrP in the tumour alone is insufficient to direct spread and growth specifically in bone. Nevertheless, levels of PTHrP secreted by the 4T1.2 cells *in vitro* were higher than those secreted by the 66cl4 or 67NR. Thus, it would seem that the higher level of cellular PTHrP secretion *in vitro* may be associated with the ability to generate bone metastases *in vivo*. Furthermore, levels of circulating PTHrP were raised significantly in mice bearing 4T1.2 tumours relative to controls and were higher than in 67NR and 66cl4 tumour-bearing animals. This may reflect

increased release of PTHrP from tumour both at the primary site and in bone. Consistent with this and the known activity of PTHrP on bone was the observation that circulating calcium was slightly raised in animals inoculated with 4T1.2 cells.

The increased calcium levels observed in the 67NR tumour bearing mice, in which PTHrP levels were not raised, may reflect an alternative mechanism of osteolysis that could have facilitated tumour growth in the spine of one mouse inoculated via the intracardiac route with 67NR cells. Assessment of the expression and regulation of other bone resorbing cytokines in these cells will be of interest.

Whilst evidence for the role of PTHrP in the establishment of breast tumours in bone is convincing, it remains to be established whether other bone resorbing factors may be produced by the tumours to provide alternative or complementary mechanisms. Manipulation of the level of PTHrP expression by transfection or by clonal selection of the 4T1, 66cl4 and 67NR sublines will aid in addressing the importance of PTHrP secretion in directing site specific spread from the primary tumour, and its ability to be regulated in bone. We have derived clones of 4T1 with varying levels of PTHrP secretion and clones in which no PTHrP secretion can be detected. In experiments underway, the dependence on PTHrP expression for metastasis to bone and other sites is being tested using these clones. By analysing and comparing the gene expression profiles of the different sublines, we will be able to determine the contribution of other factors to the processes of homing and growth in bone. While this approach may endorse a role for PTHrP in promoting bone metastasis, it is likely to show that additional factors also have a critical function in homing to bone and in the development of bone metastases. In particular, it is likely that other bone resorbing cytokines, such as interleukin 6 [3] and interleukin 11 [4] may be as effective as PTHrP in enhancing bone metastasis formation.

This is the first natural model of mammary metastasis to bone to be reported. The model provides an excellent experimental system in which to investigate the factors that control metastatic spread of breast cancer to specific sites, particularly bone, using genetically matched clones that do or do not metastasise to bone. An advantage of this unique system is that it uses subpopulations of a spontaneous metastatic tumour and represents the entire metastatic process from the primary site. The model is ideal for definition of the molecular mechanisms of site-specific metastasis and for the evaluation of therapeutic strategies such as anti-estrogens, and thus will contribute significantly to the clinical diagnosis and management of breast cancer patients.

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Genes involved in breast cancer metastasis to bone

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Bone metastases occur in 90% of women who eventually die from breast cancer. Many of these patients suffer severe pain, fractures, spinal cord compression and hypercalcaemia. Current therapies for bone disease include surgery, radiation therapy and the use of bisphosphonates. These treatments assist palliation but generally do not improve overall survival. The development of new therapies will rely on a better understanding of the mechanisms responsible for selective metastasis of breast cancer to bone. Using a unique orthotopic mouse model of breast cancer metastasis¹, we have identified several genes that may be involved in metastasis to bone.

By cDNA microarray analysis and by using a candidate gene approach we have identified several genes that are differentially expressed in genetically matched tumour sublines that do, or do not, metastasise to bone. These include caveolin-1, protease nexin I, beta 3 integrin and PTHrP. We have confirmed differential expression by northern and/or western analysis, as well as by *in situ* hybridisation. By altering expression of these genes in tumours and examining the metastatic phenotype, we will be able to identify their role in metastasis to bone. For example, our data suggests that PTHrP is not a primary determinant of metastasis to bone, but can modulate the extent of tumour burden. If these genes are shown to be important in metastasis to bone, they will become attractive targets for new therapeutic strategies to treat bone metastasis.

1. Lelekakis *et al.* (1999) *Clin. Exp. Met.* 17: 163-170

CHARACTERISATION OF AN ORTHOTOPIC MOUSE MODEL OF BREAST CANCER METASTASIS TO BONE

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We are interested in defining the genetic events that mediate breast cancer metastasis to bone. Breast cancer preferentially metastasises to several organs including lymph nodes, lung, liver and bone and these metastases result in much of morbidity and mortality associated with this disease. The study of breast cancer metastasis to bone, in particular, has been hampered by the lack of animal models that mimic the human disease.

We have developed an orthotopic mouse model of breast cancer metastasis to bone that enables, for the first time, analysis of the complete metastatic process (1). The model consists of genetically matched breast tumour cell lines that have different metastatic capacities. One line metastasises to lymph nodes and lung, another to lymph nodes, lung and bone. We are using two different approaches, cDNA microarray screening and candidate gene analysis, to investigate mechanisms of breast cancer metastasis to bone in this model.

cDNA microarray analysis has identified several genes, including caveolin-1, protease nexin I, osteopontin and BMP-4, that are differentially expressed in cells that metastasise to bone compared to those that do not. Candidate genes being investigated include PTHrP and the proteases uPA, MMP2 and MMP9. Gain or loss of function experiments are being used to investigate the importance of these genes. Interestingly, PTHrP appears to play only a minor role in bone metastasis in this model. This model provides a unique system with which to study mechanisms of breast cancer metastasis to bone.

(1) Lelekakis et al (1999) *Clin. Exp. Met.* **17**: 163-170