AD_____

GRANT NUMBER DAMD17-96-1-6224

TITLE: The Role of Integrins in Breast Cancer Metastasis

PRINCIPAL INVESTIGATOR: Janet Price, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
M. D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, 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

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Sep 96 - 31 Aug 97)

4. TITLE AND SUBTITLE
The Role of Integrins in Breast Cancer Metastasis

5. FUNDING NUMBERS
DAMD17-96-1-6224

6. AUTHOR(S)
Janet Price, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas
M. D. Anderson Cancer Center
Houston, Texas 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

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

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Our objective is to investigate the role of integrins in metastasis in human breast cancer. Integrins play an important role in cell adhesions and interactions with extracellular matrix proteins and the maintenance of normal differentiation. The scope of the study is to use variants of human breast cancer cell lines that differ in integrin expression to examine the relationship between integrin expression and metastasis to different organs. Variants of the human breast cancer cell line MDA-MB-435 with elevated expression expression of αv and α6 integrins are highly metastatic to the lungs of nude mice. Differences in expression of alternate isoforms of the α6 integrin are not associated with metastatic potential. Injection of breast cancer cells into the left heart of nude mice can target cells to the bone marrow, although ovariectomy of the mice does not affect the survival and growth of cells in the bone and bone marrow. A bone metastasis-derived variant of the MDA-MB-231 breast cancer line was found to express higher levels of the vitronectin receptor, αvβ3 and have high binding affinity to the bone matrix protein osteopontin. The cells selected *in vitro* and *in vivo* are being used for further studies of the potential role of integrins in breast cancer metastasis.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer Metastasis Integrins	19
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

JP In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

JP In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

JP In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Janet Price 9/29/97

PI - Signature Date

TABLE OF CONTENTS

	page number
Front cover	1
Standard form	2
Foreword	3
Table of contents	4
Introduction	5
Body	6 - 13
Conclusions	13
References	14-15
Appendices	16 - 19

Annual Report for Grant Number DAMD17-96-1-6224

The role of integrins in breast cancer metastasis

Introduction

The subject of this proposal is metastasis, the most common cause of death of women with breast cancer. At the time of diagnosis of breast cancer, prognosis is related to the disease stage and the presence of cancer cells in the axillary lymph nodes. Twenty percent of women with early stage node-negative breast cancer may subsequently develop metastatic disease, while as many as 90% of women with locally advanced breast cancer or with extensive lymph node involvement will have further or more extensive metastasis recurrence. In addition to the axillary lymph nodes, the other common sites of breast cancer metastasis are brain, liver and lungs. However, bone is the most common site of breast cancer metastasis, leading to pain, pathologic fractures, central nervous system compromise and hypercalcemia. The factors determining the pattern of metastasis of breast cancer cells to different organs, and especially the bone have not been determined.

The objective of this proposal is to investigate the role of cell surface integrins in the metastasis of human breast cancer. Cell surface integrins play an important role in cell adhesions and interactions with extracellular matrix proteins and the maintenance of normal patterns of differentiation. Immunohistochemical analysis of breast cancer specimens has shown that alterations in integrin expression are commonly seen, with either increased, decreased or *de novo* expression of various integrins, or loss of polarization when compared with benign or normal samples. One clinical study showed that increased expression of $\alpha 6$ in primary breast cancers was related to shorter survival (1). In a nude mouse model of breast cancer metastasis developed in our laboratory (2), the cells with highest metastatic capability had higher surface expression of $\alpha 6$ and αv integrin sub-units than poorly metastatic cells. A study using the MDA-MB-435 cell line transfected with a dominant negative variant of the $\beta 4$ integrin subunit, that effectively reduced expression of the $\alpha 6\beta 1$ integrin on the cell surface showed that these cells had reduced invasive capability (3). These results complement our initial observation that the more metastatic cells had higher levels of the integrin expression, and provide further evidence for an association with malignant progression. Breast cancer cells isolated from a nude mouse bone metastasis showed elevated expression of the $\alpha v\beta 3$ integrin. This integrin is also expressed by osteoclasts and mediates binding to the bone matrix protein osteopontin (4). The bone-metastasis derived breast cancer cells bound avidly to the osteopontin, and we propose that elevated expression of the $\alpha v\beta 3$ integrin may mediate events that are critical for the development of bone metastasis.

The scope of our study is to use variants of established human breast cancer cell lines that differ in integrin expression to examine further the relationship between integrin expression and metastasis to different organs. The experimental model is the implantation of the breast cancer cells into immunodeficient nude mice. The experiments are designed to provide new information on the cancer-cell matrix interactions that are part of the metastatic progression of breast cancer. This knowledge could potentially identify new approaches for therapeutic intervention.

Body of the Proposal

Task 1: Further development of an animal model for human breast cancer metastasis.

Experimental methods:
a) Left-heart injection to target cells to the bone and bone marrow.

Injection of cells into the left ventricle of the heart of nude mice is a recognized method of inducing bone metastasis, using various types of cancer cell lines (5 – 7). In our preliminary studies, we showed that human breast cancer cells could be recovered from cultures of bone marrow flushed from the femurs of nude mice at intervals after injection of 5×10^5 cells into the left-heart. The marrow is collected by flushing 2 – 3 ml of PBS through the femurs removed at necropsy of the mice. The cells are then plated in 100 mm tissue culture plates in 10 ml of medium with 5%-FBS (the normal growth medium for the breast cancer cells). After 1 – 2 weeks of culture, the human breast cancer cells are distinguishable by their morphology. In addition, the mouse bone marrow cells do not proliferate in these growth conditions. The presence of breast cancer metastases in other organs of the mice was determined at autopsy, and from histological sectioning of selected organs (brain and adrenal glands).

Results: Human breast cancer cells can be recovered from the bone marrow of nude mice injected into the left heart (**Table 1**). The results are presented as the number of bone marrow cultures with human breast cancer cells/ number of mice injected. In contrast, the bone marrow cultures from mice that were injected i.v. did not contain detectable numbers of human breast cancer cells (data not shown), showing that the route of injection dictates whether or not the breast cancer cells will arrest and proliferate in the bone marrow. In contrast to other reports with the MDA-MB-231 cell line (7), symptoms of osteolytic metastases in the nude mice were not seen. Histology of randomly sampled tissues from mice injected with any of the breast cancer cell lines failed to show evidence of bone metastases. However, no radiographic studies were performed (the necessary equipment is not currently available to this study), that might reveal lesions not yet clinically apparent. Repeat experiments are in progress in which the mice will be left as long as possible, based on the assumption that more time is needed for the bone metastases to develop, and to become symptomatic.

Table 1 Detection of breast cancer cells in the bone marrow of mice injected into the left-ventricle of the heart

Cell line	Breast cancer cells in marrow	Metastases in other organs
MDA-MB-435	6/8	lungs, adrenals, brain
MDA-435-BM	6/6	lungs, adrenals, brain, mammary fatpad, ovary
MDA-MB-231	10/12	lungs
MDA-MB-468	0/6	adrenals

The time course studies that are part of this Task have not yet been completed, as the *lacZ*-expressing variant of the MDA-MB-435 cell line was not made available by Dr. Brunner, as had been expected at the time of grant submission. An alternate approach that is in progress is to introduce the pCMVβ vector (purchased from Clontech) into selected breast cancer cell lines. The marked cells will then be used in time course studies, which will address questions of the kinetics of breast cancer cell arrest and growth in the bone marrow environment, and the potential development of bone metastases. We have transfected the original cell lines, and cells that have been selected from the bone marrow or other organs in mice, as these may be a more aggressive population with which to perform the studies. Since the grant submission, a study has been published using the method we proposed, showing that the *lacZ* gene product can be used to quantify breast cancer metastasis to the long bones of nude mice (8).

b) Effect of ovariectomy on bone marrow and bone metastasis following left-heart injection of breast cancer cells.

Ovariectomy in rodents has been shown to lead to bone loss (9) and increased IL-6, a cytokine that can activate osteoclast activity (10). We proposed to use ovariectomy to alter the bone turnover in the mice, based on the assumption that this would alter the incidence of breast cancer growth in the bone and bone marrow. Two groups of 6 week old female nude mice had their ovaries removed, and two groups of mice had laparotomy as the control surgical procedure. In one arm of the experiment, MDA-MB-435 breast cancer cells were injected 3 weeks after surgery, and in the second group, cells were injected 6 weeks after surgery. Six weeks after tumor cell injection, the mice were killed and examined for metastases. The femurs were removed, and the bone marrow collected for culture.

Result and discussion: No differences were seen in the metastasis incidence to the brain, lungs and adrenals, and the incidence of bone marrow cultures with breast cancer cells between the groups of ovariectomized or sham surgery mice. Either the procedure of ovariectomy in nude mice does not affect the rate of bone turnover, or this does not affect the growth and survival of breast cancer cells in the bone marrow. One possible drawback of using nude mice for this study is the possibility that these animals do not undergo the same degree of bone resoption as seen in immunocompetent mice. It has been reported previously that nude mice have an impaired hypothalamo-pituitary-ovarian axis, and do not respond normally to removal of ovaries (11). Furthermore, the human breast cancer cells may be poorly responsive to the murine cytokines released in the environment of resorbing bone. An alternative that will be tested is to use the 66.3 mouse mammary tumor cell line (12), syngeneic to BALB/c mice, made available by Dr. Fred Miller , Karmanos Cancer Institute. In recent studies in this laboratory, we have found that the cells are highly tumorigenic and metastatic from mammary fatpad tumors, and that one mouse in the initial study had bone metastasis.

c) Selection of new variants from metastases in nude mice.

One limitation of studying breast cancer metastasis in nude mouse models is that there

are few reproducibly metastatic cell lines (9). The MDA-MB-435 is one line that does metastasize from tumors in the mammary fatpad to different organs (2). In the experiments in part a) we noted that the MDA-MB-231 cell line formed metastases in the lungs of the mice injected via the left-heart. Therefore, an additional experiment was performed to test the lung colonizing capability of the MDA-MB-231 cells, by injection of 10^6 cells into the lateral tail veins of female nude mice. Up to eight weeks after injection, the mice were killed, and examined for experimental metastases. The numbers of macroscopic metastases in the lungs were counted with the aid of a dissecting microscope. Some metastases were dissected from the lungs, and established in tissue culture.

Results: When the MDA-MB-231 cells were injected i.v., lung metastases were found consistently. New variants were isolated from the metastases (231-LC1, 231-LC2), and upon re-injection i.v., these cell lines formed more experimental lung metastases than the original cell line (**Table 2**). The cells recovered from the lungs may represent a selected population of cells capable of arrest and growth in this organ. These new variants will be used in further studies in this proposal, and will be a valuable resource for other studies, for example, in pre-clinical studies testing agents that either block cell arrest, or target early micro-metastases. The advantage of an experimental metastasis model over a spontaneous metastasis model, is that in the former there is less variability of the time of initial arrest of the metastatic cells in the target organ.

Table 2 Experimental metastatic potential of MDA-MB-231 breast cancer cells

Cell line	Incidence of lung metastases	Median number (range)
MDA-MB-231	3/5	22 (0 – 55)
231-LC1	4/5	100 (0 - >150)
231-LC2	5/5	75 (13 - >150)

Task 1 progress and recommendations: The experiments to accomplish Task 1 are still in progress, with a modification to the initial plan in that new *lacZ* expressing variants of breast cancer cells will be isolated in the laboratory for use in the kinetic study. As appropriate, one or more of these will be tested in the ovariectomy study. An alternative approach for this aspect of the project is to use a mouse mammary tumor cell line, that is being currently characterized in the laboratory.

Task 2: Phenotypic characterization of breast cancer cells with different levels of integrin expression.

a) Selection of MDA-MB-435 cells expressing different levels of a6 integrin

Experimental methods: In order to test the relationship between α6 integrin expression and metastatic potential of human breast cancer cells, clonal populations were isolated based on high or low levels of α6. A suspension of MDA-MB-435 human breast cancer cells was prepared by incubation with 0.02% EDTA in PBS. The cells were washed to remove excess EDTA, then suspended in PBS with 2% fetal bovine serum. Monoclonal antibody raised against human α6 integrin (Mab 1972, Centricon) was added at a 1:500 dilution and the cells were incubated on ice for 30 mins. After washing to remove unbound antibody, the cells were incubated with FITC-conjugated goat anti-rat IgG (Sigma Chemical Co., St Louis,MO) diluted 1:40 in PBS with 2% FBS, for 30 min on ice and in the dark. The cells were then washed with PBS and suspended at a concentration of 10^6 cells/ml. Cells expressing high α6 expression and low α6 expression were separated by analysis with an EpicsElite analyzer (Coulter, Hileah, FL), that sorted cells into the top 5% and lowest 5% on the basis of fluorescence intensity. Individual cells from the high and low expressing populations were plated in wells on 96-well microtiter plates. Clones that grew in the wells were expanded in culture and sample frozen for subsequent analyses.

The expression of α6 and αvβ3 integrins on the isolated clones was determined in the same manner as above, by incubation with specific antibodies and FITC-conjugated secondary antibodies, followed by fixation in PBS with 1% paraformaldehyde. FACS analysis was performed on an EPICS Profile Cell Sorter (Coulter) with a 525-nm band pass filter to detect FITC and gated on forward *versus* side scatter to exclude debris, dead cells and cell clumps. Analysis was based upon cursors set at 2% for isotype-matched negative controls. The cells of the selected clones were plated in 0.3% agarose with 10% FBS and the numbers of colonies of diameter greater than 50 μ counted after 2 – 3 weeks incubation (as described previously, 12). Colony-forming efficiency is a possible indicator of metastatic potential for the MDA-MB-435 cell line (13), and was used as an initial screen for clones with altered malignant phenotypes. Two clones were selected based on surface expression of α6, and injected into the mammary fatpad of nude mice, to measure local tumor growth and metastasis formation. 10^6 cells in 0.1 ml PBS were injected into the mammary fatpad. Tumor growth was monitored weekly, and the tumors were surgically removed at 1.5 cm mean diameter and weighed. All mice were killed at day 120 after injection, the weights of remaining tumors recorded, and the numbers of metastases in the lungs counted

Results and Discussion: Fifteen individual clones from the α6-high population and 7 from the α6-low population grew from the single cells seeded into microtiter plates. Upon measuring integrin expression of the selected cells by fluorescence activated cell sorting (FACS), the differential in α6 expression was found to be stable. There were no changes seen in the levels of the αv integrin sub-units. The agarose colony forming efficiencies of the six clones were compared with the original MDA-MB-435 cell line, and also the metastasis derived variants, MDA-435 Lung2 (high metastatic) and MDA-435 Br1 (low-metastatic, ref.2) (**Table 3**). In contrast to the initial assumption that high

α6 expression would correspond with high colony forming efficiency, this was not the result found in the 6 clones tested. The 3 α6-high clones displayed low colony forming efficiency compared to the parental cell line and the α6-low clones. Thus higher α6-expression gives no advantage for cells growing in anchorage independent conditions.

Table 3 Integrin expression and anchorage independent growth of MDA-MB-435 clone and variants

Cell line	α6-expression %[a]	α6-expression MFU[b]	αvβ3-expression %	αvβ3-expression MFU	colony forming efficiency %[c]
MDA-MB-435	98	8.2	99.4	8.2	21.6
435-Lung2	98	16.1	80	10.2	26.9
435-Br1	68	6.5	23.4	4.05	10.6
435α6HD3	99.8	23.4	99	5.8	5.2
435α6HG6	99.9	31.9	99.5	5.9	5.05
435α6HF8	99.3	12.9	90	5.8	1.5
435α6LF9	75.3	3.3	80.1	4.24	16.5
435α6LF7	80.6	4.15	91.9	6.3	15.5
435α6LE2	85.4	4.5	57.4	5.4	4.6

Legend: a, percent positive staining cells; b, mean fluorescence units, a measure of relative fluorescence intensity of the positively stained cells; c, number of colonies per 35mm-well of 0.3% agarose/number of cells plated x 100.

The growth curves of the mammary fatpad tumors of the two selected clones 435-α6LF9 and 435α6-HG6 (low and high expressing clones respectively) are shown in **Fig.1** (see appendix). The initial tumor incidence and growth rates of the clones were indistinguishable from those of the parent cell line. However, by day 50 half of the 435-α6HG6 tumors began to regress, and at the end of the study only 4 of the 8 animals had tumors in the mammary fatpad, (**Table 4**).

Table 4 Tumorigenicity and metastasis of α-selected MDA-MB-435 cells

Cell line	Tumor incidence[a] median(range)	Tumor weight[b]	Lung metastasis Incidence[c]		
MDA-MB-435	7/7	0.8 ± 0.3	3/7	(43%)	0 (0 - >150)
435-αLF9	8/8	1.1 ± 0.4	3/8	(37.5%)	0 (0 – 45)
435-αHG6	4/8	1.05 ± 0.6	¾	(75%)	85 (0 - >150)

Legend : a: Number of mice with tumors at day 100/ number of mice injected; b, mean tumor weight ; c, number of mice with lung metastases / number of mice with tumors.

When all mice were killed, at 120 days after tumor cell injection, the incidence

and numbers of lung metastases was higher in the mice injected with α6-high clone, than the other groups (although the number of mice in the α6-HG6 group was small). It was noted however that the incidence of metastasis was lower than that found previously with the MDA-MB-435 cells. One explanation for this may be that this batch of nude mice had a higher than usual incidence of *Staphylococcus aureus* infections, and a number of mice had to be killed before the experiment was complete. This study is being repeated, with the addition of injection of cells into the left-heart, to see if distribution of metastases by this route of injection is affected by the level of α6-integrin expression.

If the *in vivo* study results are confirmed in the repeat experiment, these may suggest that α6-expression does not confer an advantage on the breast cancer cells when growing in the mammary fatpad. Why the higher expression of the integrin sub-unit might promote regression of the tumor is currently unknown. In the repeat studies, histological analyses of the tumors at different phases of growth (active and regressing) will be performed to determine if this is a result of induction of apoptosis. To test whether the residual host cell activity in the nude mice is involved, some parallel studies may be performed using SCID mice. However, higher α6 expression was found to be associated with metastatic growth in the lungs of nude mice. Other investigators have shown that interfering with α6-integrin expression in the MDA-MB-435 cells with a dominant negative β-sub unit (3) diminishes the invasive ability of the cells. The results of our studies complement these and suggest that invasion and distant metastasis of the breast cancer cells is promoted by the presence of higher levels of α6-integrins.

b) Characterization of MDA-MB-231 cells selected from a nude mouse bone metastasis

Experimental methods: An in vitro binding assay was used to compare the binding affinity of the MDA-MB-231 cells and the MDA-231BM cells, that were isolated from a bone metastasis in a mouse injected with the original cell line (14). Microtiter plate wells were coated with osteopontin (OPN), 10μg/ml, then washed with PBS-with 1% bovine serum albumin (BSA) to block non-specific binding. The control wells were also incubated with the PBS-BSA. Cells were plated onto the coated and control wells in medium with 1% BSA. At intervals of 15 min., non-attached cells were aspirated and the wells washed with PBS-BSA. The proportions of cells bound to the wells were determined using the MTT assay. FACS analysis and immunoprecipitation of biotin-labeled cells was performed to determine relative levels of αvβ3 integrin expression.

Results and Discussion: The MDA-2321BM cells displayed a significantly increased affinity to bind to purified OPN compared with the MDA-MB-231 cells (60% to 80% compared with less than 20% of MDA-MB-231). Binding to OPN was calcium dependent, and was blocked in the presence of RGD peptides (>90% of specific binding with 25 μg/ml of GRGDS peptide). Binding to OPN was also reduced in the presence of monoclonal antibodies directed against the αv integrin subunit and the αvβ3 integrin heterodimer **(Fig. 2)**. This integrin is also known as the vitronectin receptor. Osteoclast binding to OPN is thought to be mediated through this integrin, and may thus play a role in osteolysis (4). Analyzing the expression of integrins on the cells by FACS we found an increase in the αvβ3 surface expression on the MDA-231BM cells. These results were confirmed by immunoprecipitation of proteins of the appropriate molecular weights (130 kDa and 110 kDa for the αv and β3 subunits, respectively) from lysates of biotin labeled

cells. (These results will be presented in a poster at the Era of Hope meeting in November, 1997).

The increased expression of the αvβ3 integrin on the MDA-231BM cells apparently leads to increased binding to OPN, although additional surface components are probably also involved. Increased expression of this integrin heterodimer in the selected line has been maintained *in vitro*, suggesting that this is a stable property of the cells, and that the bone-metastasis derived cells are a selected sub-population of the original cell line. A recent report showed that increased OPN in plasma is associated with increased tumor burden and reduced survival of women with metastatic breast cancer (15). As αvβ3 interacts with OPN, this may suggest a potential paracrine/autocrine loop that could regulate metastatic properties of breast cancer cells. In preliminary studies in this laboratory we have determined that OPN mRNA expression is elevated in the metastasis-derived variants of MDA-MB-231 (from bone and lungs of nude mice). These cell lines will be used for continuing studies to address how the αvβ3 integrin and OPN expression may be involved with breast cancer metastasis.

Similar studies to those in Task 2 part a) will be performed using the MDA-MB-231 cell line, selected *in vitro* for high and low expression of αvβ3 integrin. Recent experiments using the antibody for this integrin LM609 (from Centricon) have given unsatisfactory results, with low specific staining (see Table 4). A fresh batch has been ordered, and will be used for selection of the clones if satisfactory initial results are found. These *in vitro* selected clones will be compared with the *in vivo* selected variants (from bone, bone marrow and lung) for anchorage independent growth, binding to matrix, and integrin expression, as well as tumorigenicity and metastasis in nude mice.

Task 2 progress and recommendations: The experimental studies are progressing, and completion of the tasks is anticipated by the second year.

Task 3: Characterization of integrin profiles of human breast cancer cells

[Note: The initial timing of the experiments in this task was in months 18 – 48. The length of the project was reduced from 4 to 3 years before funding, and in consequence the sequence and timing of some of the tasks has been altered.]

Experimental methods. The expression of α6 alternate isoforms in human breast cancer cell lines was detected using RT-PCR. The panel of breast cancer cell lines included lines that have been tested for tumorigenic and metastatic properties in nude mice (16). Polyadenylated mRNA was isolated from sub-confluent cultures, and cDNA reverse transcribed using the Promega cDNA kit. The primers were those reported by Hogervorst et al (17), for a sequence in the 3' region of the carboxyl terminus of the protein. Sense, 5'-CTAACGGAGTCTCACAACTC-3', and antisense, 5'-ACTCTGAAATCAGTCCTCAG-3'. As a positive control the reaction was optimized using cDNA from the immortalized human mammary epithelial cell line HBL100. The amplification conditions used were identical to those reported (17). The reaction products were separated on 1.4% agarose gel, stained with ethidium bromide and viewed under UV illumination. The band intensity of the two products, isoform A (844 bp) and isoform B (714 bp) were determined by densitometry . All reactions were run in parallel

with amplification reactions using primers for β-actin. The levels of surface expression of α6-integrin protein on the same panel of breast cancer cell lines were measured by FACS (see previous task).

Results and discussion: All of the human breast cancer cell lines expressed both isoforms of the α6 integrin, and there was no association between the proportion of isoforms expressed and the malignant behavior as assessed by implantation into nude mice (Fig. 3a). The ratio of α6A/α6B isoform abundance ranged from 0.7 – 2.6, with the exception of the MCF-7 cell line (ratio 3.5) which expressed relatively little mRNA. Analysis of the α6 isoform expression in the α6-selected clones is in progress. However, there does not appear to be any relationship between relative expression of the two isoforms and malignant behavior of the breast cancer cell lines.

The α6 surface expression on the breast cancer cell lines, measured by FACS analysis, showed a trend toward higher surface expression in the cell lines with a higher degree of tumorigenicity and metastasis in nude mice (16). The cell lines with lowest expression were two estrogen receptor-positive lines, MDA-MB-134 and MCF-7. Both of these lines are dependent on the presence of supplemental estrogen to grow in nude mice, and neither form distant metastases (18, and unpublished data). Further comparisons of α6 expression in the different breast cancer cell lines will use cDNA probes in northern blot analyses. We hope to confirm the different levels of expression of α6, especially since there appear to be some inconsistencies between abundance of transcripts and protein in two of the lines, MDA-MB-134 and MDA-MB-361. The different cell lines may be used to determine if there is significant post-translational modification of α6 in breast cancer cells. In addition, this panel of cells will be used in the continuing experiments that will measure any differences in which β sub-units co-precipitate with α6 (predominantly β1 in the metastatic cell lines), and expression and phosphorylation of $p125^{FAK}$.

Task 3 progress and recommendation: The experimental studies are progressing, and completion of all the tasks is anticipated by the third year.

Conclusions

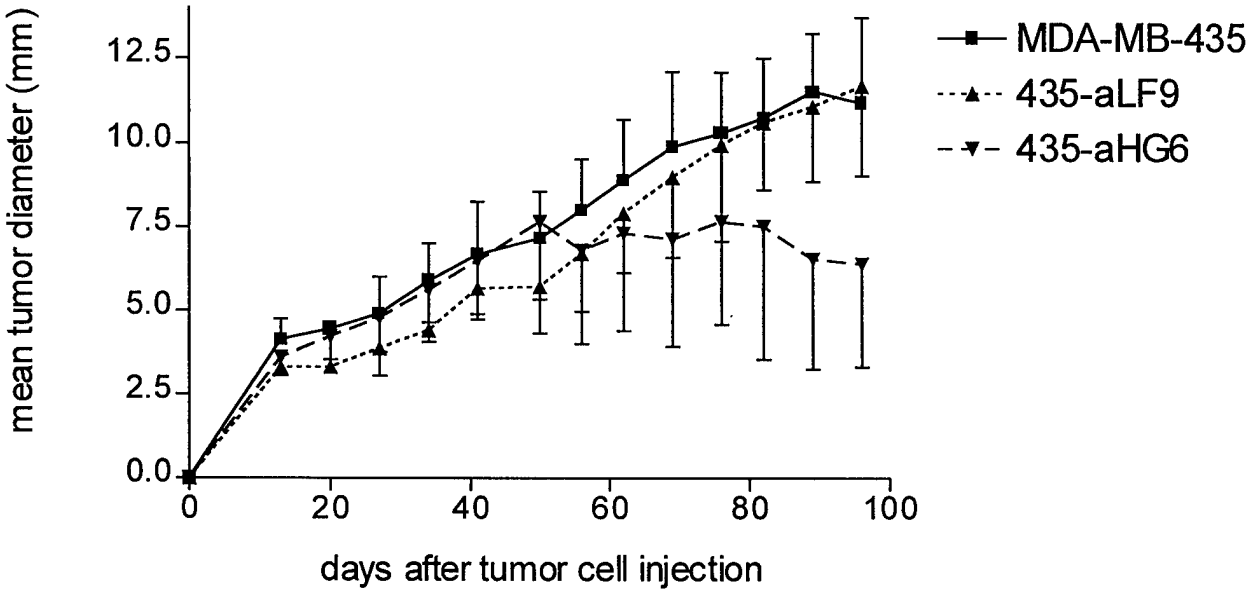
The conclusions from the first year of the study are that the nude mouse can be used for studies of the metastatic capability of human breast cancer cells to different organs, including the lungs and bone marrow. Ovariectomy of the nude mice before intra-cardiac injection of cells did not alter the outcome, suggesting that an alternate experimental approach is needed for this part of the study, which aims to determine whether the environment of resorbing bone promotes breast cancer metastasis. Breast cancer cells can be targeted to different organs by different routes of injection in the nude mice. Variants of the breast cancer cell lines have been isolated that will be used for further studies of potential role of integrins in metastasis. The initial results from the selection of cells with high α6 expression appear to confirm the hypothesis that high levels of this integrin promote distant metastasis of breast cancer cells.

References

1. Friedrichs K, Ruiz P, Franke F, Gille I, Terpe HJ, and Imhof BA. High expression of α6 integrin in human breast carcinoma is correlated with reduced survival. Cancer Res. **55**: 901-906, 1995.
2. Price JE, Polyzos A, Zhang RD, and Daniels LM. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. Cancer Res. **50**: 717-721, 1990.
3. Shaw LM, Chao C, Wewer UM, and Mercurio AM. Function of the integrin α6β1 integrin in metastatic breast carcinoma cells assessed by expression of a dominant-negative receptor. Cancer Res. **56**: 959-963, 1996.
4. Ross FP, Chappel J, Alvarez JI, *et al.* Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin αvβ3 potentiate bone resorption. J. Biol. Chem. **268**: 9901-9907, 1993.
5. Arguello F, Baggs RB, and Frantz CN. A murine model of experimental metastases to bone and bone marrow. Cancer Res. **48**: 6879-6881, 1988.
6. Kjonniksen I, Nesland JM, Pihl A, and Fodstad O. Nude rat model for studying metastasis of human tumor cells to bone and bone marrow. J. Natl. Cancer Inst. **82**: 408-412, 1990.
7. Mbalaviele G, Dunstan CR, Sasaki A, Williams PJ, Mundy GR, and Yoneda T. E-cadherin expression in human breast cancer cells suppresses the development of osteolytic bone metastases in an experimental model. Cancer Res.**56**: 4063-4070, 1996.
8. Sung V, Cattell DA, Bueno JM, Murray A, Zwiebel JA, Aaron AD, and Thompson EW. Human breast cancer cell metastasis to long bone and soft organs of nude mice: a quantitative assay. Clin. Expl. Metastasis **15**: 173-182, 1997.
9. Finkelman RD, Bell NH, Strong DD, Demers LM, and Baylink DJ. Ovariectomy selectively reduces the concentration of transforming growth factor β in rat bone: implications for estrogen deficiency associated bone loss. Proc. Natl. Acad. Sci USA. **89**: 12190-12193, 1992.
10. Jilka RL, Hangoc G, Girasole G, *et al.* Increased osteoclast develoopment after estrogen loss: mediation by interleukin-6. Science **257**: 88-91, 1992.
11. Weinstein Y. Impairment of the hypothalamo-pituitary-ovarian axis of the athymic "nude" mouse. Mech. Ageing Devel. **8**: 63-68, 1978.
12. Miller FR, Miller BE, and Heppner GH. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. Invasion Metastasis **3**: 22-31, 1983.
13. Zhang RD, Fidler, IJ, and Price JE. Relative malignant potential of human breast carcinoma cell lines established from pleural effusions and a brain metastasis. Invasion Metastasis **11**: 204-215, 1991.
14. Verschraegen CF, Kozielski T, and Mendoza J. Development of a bone metastasis (M) model from a human breast carcinoma in the nude mouse. Proc. AACR. **34**: 70 (A418), 1993.
15. Singhal H, Bautista DS, Tonkin KS, O'Malley FP, Tuck AB, Chambers AF and Harris, JF. Elevated plasma osteopontin in metastatic breast cancer associated with increased tumor burden and decreased survival. Clin. Cancer Res.**3**: 605-611, 1997.

16. Price, JE. Metastasis from human breast cancer cell lines. Breast Cancer Res. Treat. **39**: 93-102, 1996.
17. Hogervorst F, Kuikman I, van Kessel AG, and Sonnenberg, A. Molecular cloning of the human α6 integrin subunit. Alternative splicing of the α6 mRNA and chromosomal localization of the α6 and β4 genes. Eur. J. Biochem. **199**: 425-433, 1991.
18. McLeskey SW, Zhang L, Kharbanda S, Kurebayashi J, Lippman ME, Dickson RB, and Kern FG. Fibroblast growth factor overexpressing breast carcinoma cells as models of angiogenesis and metastasis. Breast Cancer Res. Treat. **39**: 103-117, 1996.

Fig. 1 Growth of α6-selected clones of MDA-MB-435 in nude mice



Mice were injected with 10^6 cells into the mammary fatpad, and tumor growth followed for up to 120 days. The tumors on half of the mice injected with the 435α6HG6 (high expressing) cells regressed after growing to a mean diameter of 6 – 7 mm.

Figure 2

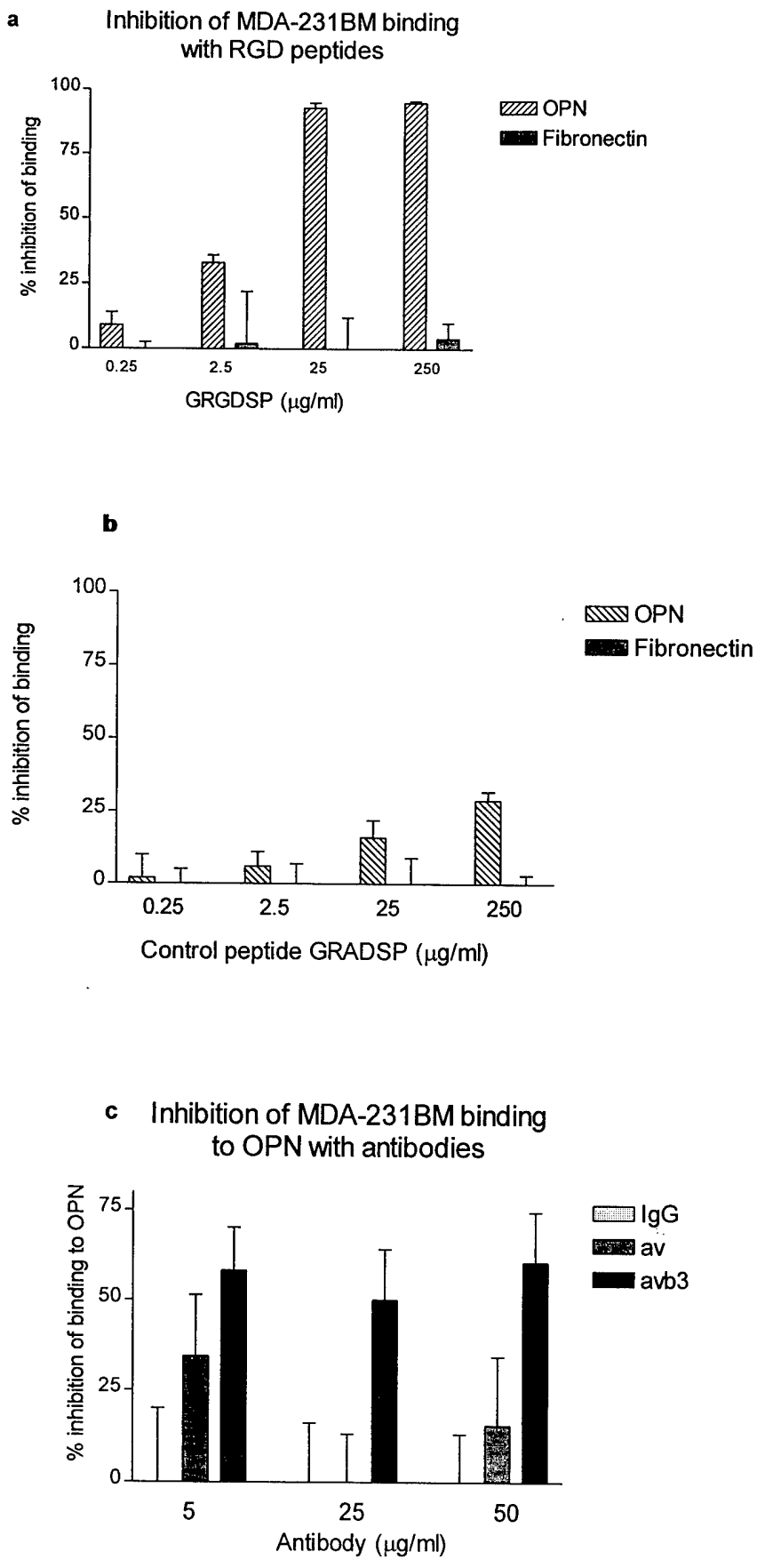

a
Inhibition of MDA-231BM binding with RGD peptides
% inhibition of binding
100
75
50
25
0
0.25
2.5
25
250
GRGDSP (μg/ml)
OPN
Fibronectin
b
% inhibition of binding
100
75
50
25
0
0.25
2.5
25
250
Control peptide GRADSP (μg/ml)
OPN
Fibronectin
c
Inhibition of MDA-231BM binding to OPN with antibodies
% inhibition of binding to OPN
75
50
25
0
5
25
50
Antibody (μg/ml)
IgG
av
avb3

Figure 2 legend:

2a: Inhibition of binding of MDA-231BM cells to OPN in the presence of GRGDSP. Cells were incubated with or without the peptide for 30 min., then plated onto OPN-coated microtiter plates. After 60 minutes incubation, non-attached cells were washed off, and relative numbers of bound cells determined with the MTT assay. The results are expressed as % inhibition of binding, compared with binding to OPN in the absence of peptide.

2b: Identical experiment, using a control peptide, GRADSP, that lacks the RGD sequence.

2c: Inhibition of binding of MDA-231BM cells to OPN, following incubation of cells with monoclonal antibody to αv or $\alpha v\beta 3$ integrin or mouse IgG, at the concentrations shown. Binding was compared with binding to OPN in the absence of antibody or IgG.

Figure 3 legend: The cell lines shown in the figures are 1) HBL100, an immortalized mammary epithelial cell line;2) MDA-MB-134; 3) MCF-7; 4) BT-474; 5) BT-20; 6) MDA-MB-361; 7) MDA-MB-468; 8) MDA-MB-435; 9) MDA-435Lung 2; 10) MDA-MB-231

Fig. 3a

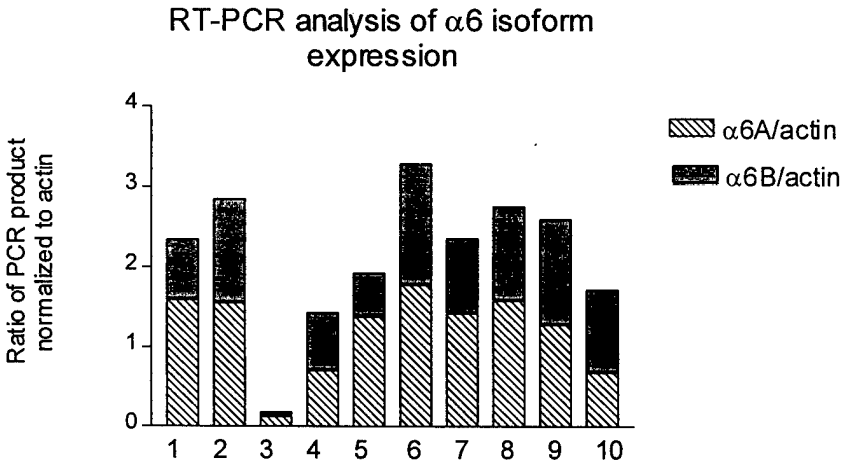


Fig. 3b