AD_____

AWARD NUMBER DAMD17-97-1-7106

TITLE: Promoter Regions Determining Over-Expression of Metalloproteinase Genes in Breast Cancer

PRINCIPAL INVESTIGATOR: James G. Lyons, Ph.D.

CONTRACTING ORGANIZATION: Royal Prince Alfred Hospital
Camperdown, Australia

REPORT DATE: June 1998

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1998	Annual (1 Jun 97 - 31 May 98)

4. TITLE AND SUBTITLE
Promoter Regions Determining Over-Expression of Metalloproteinase Genes in Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7106

6. AUTHOR(S)
James G. Lyons, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Royal Prince Alfred Hospital
Camperdown, Australia

8. PERFORMING ORGANIZATION REPORT NUMBER

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

10. 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 words)*

The purpose of this project is to identify the molecular and genetic basis for the abnormal destruction of extracellular matrix that accompanies breast cancer, by addressing the transcription of the genes of the matrix metalloproteinases (MMPs) that are made at high levels in and around carcinomas. The objectives of this project are to identify regions of the promoters of three MMP genes that mediate their high level of expression *in vivo*, to determine the histological distribution of MMP-producing cancer cells in tumors, to determine for each MMP gene whether it has undergone amplification.

The breast cancer cell line, BC1, is composed of two subpopulations, BC1-E and BC1-M. We have found that BC1-M cells upregulate the expression of three MMP genes in response to exposure to BC1-E cells and conditions for this response are being optimised. The BC1-E cells express only one of these genes and do so constitutively. Two clones and restriction maps of the rat collagenase-3 gene have been obtained. BC1-M cells have been stably transfected with reporter genes bearing full-length and truncated versions of the stromelysin-1 promoter. Immunological characterization of BC1-M cells is consistent with them being carcinoma cells that have undergone an epithelial-mesechymal transition.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Matrix Metalloproteinases, Invasion, Metastasis, Transcription	11
	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

[redacted]

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.

S.C.A. 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.

S.C.A. 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.

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

S.C.A. 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.

T. [redacted] 14 June 1988

PI - Signature Date

TABLE OF CONTENTS

Introduction

Rationale

The critical feature of breast cancer that makes it a malignant disease is its ability to spread from the breast to other parts of the body. It is these secondary breast cancer tumors whose growth and destruction of surrounding tissues can cause suffering and the death of the patient. The spread of the tumor, in the context of both invasion of tissues locally and in metastasis to distant organs, requires the destruction of the extracellular matrix (ECM). The purpose of this project is to identify the molecular and genetic basis for this abnormal ECM destruction by addressing the expression of the genes of a group of proteinases, the matrix metalloproteinases (MMPs), that are made at high levels in and around carcinomas.

Objectives

The objectives of this project are:

1. to identify regions of the promoters of three MMP genes that mediate their high level of expression *in vivo*

2. to determine the histological distribution of MMP-producing cancer cells in tumors

3. to determine for each MMP gene whether it has undergone amplification

Experimental Methods, Assumptions and Procedures

Experimental Methods

The methods employed are primarily *in vitro* molecular and cellular biological methods, as described under "*Procedures*". *In vivo* tumorigenesis and metastasis assays in laboratory rats will be used in future phases of the project. The experimental model is based on the BC1 cell line, established from a mammary carcinoma that arose spontaneously in a female of the DA inbred strain of *Rattus norvegicus* [1]. These cells grow continuously in serum-free culture and, when injected into immunocompetent, syngeneic host animals, produce tumors that metastasize and ivade bone and muscle.

Assumptions

The clinical relevance of this project is based on the overall assumption that the molecular pathological basis of the rat model system being used for the experiments is similar to that of human breast cancer. This is supported by the behaviour of the tumours *in vivo*, in that they metastasize to draining lymph nodes and lungs, are able to invade bone and produce mRNAs

for MMPs. At present, there is no practical or ethical means for addressing the first objective in human subjects, although the findings of the second and third objectives may be verifiable using biopsy or autopsy samples of breast cancer obtained from patients. The rationale for examining MMPs is based on the assumption that they are, in fact, essential for invasion and metastasis, a proposition that has not been tested scientifically in a rigorous, *in vivo* model of tumor invasion and metastasis. Since invasion and metastasis require destruction of the extracellular matrix, and the MMPs are able to do this efficiently and are the only known enzymes that are able to degrade interstitial collagens, it is reasonable to assume that at least come of them will play a role. Whether the MMPs used for invasion and metastasis are produced by the cancer cells, themselves, or by non-neoplastic host cells, is still a matter for conjecture. The ability of neoplastic epithelial cells that have acquired stromal characteristics to produce MMPs (described in "Results and Discussion") suggests that many previously published papers that concluded that non-neoplastic stromal cells are the source of MMPs in carcinomas may have done so in error, due to the misidentification of the MMP-producing cells. The current study has the potential to identify cells as being of cancer or host origin unequivocally, by virtue of the cancer cells being genetically tagged with the *E. coli LacZ* gene.

Procedures

The BC1 cell line and its clonal derivatives were grown under continuous serum-free conditions as described [2]. Transfections were performed using Lipofectamine (GIBCO-BRL) according to the manufacturer's instructions. Collagen-degrading activity was measured as described previously [3]. Absolute levels of MMP mRNAs were measured by competive PCR assays developed in this laboratory [4]. E-cadherin and keratin were detected by immunoblotting [5] using specific antibodies (Transduction Laboratories and DAKO, resp.). Southern blotting was done according to standard procedures [6]. Karyotypes were obtained as described [7].

Results and Discussion

Cellular interactions determine the expression of MMP genes in a BC1 subpopulation.

The BC1 cell line that is being used as a model for breast cancer is composed of epithelial (E) cells, that have retained an epithelial morphology, and metaplastic (M) cells that have not. These 2 phenotypes are stable within the cell line, in that they persist for over 100 flask passages and clones representative of each do not change spontaneously from one phenotype to another. Preliminary data demonstrated that BC1-E cells were the major source of gelatinase B (MMP9), whereas BC1-M cells were the major source of collagenase-3 (MMP13) and stromelysin-1 (MMP3).

We have employed clonal derivatives representative of each cell type (BC1-E2 and BC1-M3) to characterize the expression of the MMP genes further, by measuring mRNA levels using

competitive PCR assays. BC1-E2 cells grown in isolation produce high levels of gelatinase B mRNA, in keeping with their previously observed ability to secrete gelatinase B protein [8]. Co-culture with BC1-M3 cells did not significantly modulate this constitutive expression of the gelatinase B gene or induce the expression of the collagenase-3 or stromelysin-1 genes. In contrast, the BC1-M3 cells express all 3 MMP genes at a low level when grown in isolation. However, when they are co-cultured with BC1-E2 cells, the expression of all three genes is induced, and a net collagen-degrading activity apears in the culture supernatant.

Thus, although expression of all 3 MMP genes is constitutive when examining the BC1 cell line as a whole, when the cell line is dissected into its constituents one of the MMP genes (that of gelatinase B) is expressed constitutively in one subpopulation, whereas the expression of all three is induced in the other population.

The two cellular phenotypes are being characterized in more detail, to determine how they correspond to phenotypes within human neoplasms. E-cells are positive for E-cadherin and keratin by PCR and western blotting, whereas M-cells are not. The loss of these epithelial characteristics suggest that the M-cells have undergone an epithelial-mesenchymal transition.

Experiments have been performed to optimize the level of induction of the stromelysin-1 gene in BC1-M cells, by varying the plating ratio of E:M and the length of time of induction. The degree of induction of stromelysin-1 gene expression in M-cells was found to increase as the proportion of E-cells in the co-culture increases. Thus, a high E:M cell ratio will be used in future induction studies. Experiments are under way to determine whether the E-cell factor that induces MMP gene expression in M-cells is released in a soluble form or relies on cell-to-cell contact. This is being done using microporous membranes to separate the two subpopulations in culture.

Stable transfection of BC1-M3 cells with reorter genes for the stromelysin-1 promoter

BC1-M3 cells have been stably transfected with reporter gene plasmids in which transcription of the *neo* gene, whcih confers resistance to G418, is driven by a full-length (-1100 to + 8, with respect to transcription start site) stromelysin-1 promoter and transcription of the *CAT* gene is being driven by either the full-length promoter or partially truncated promoters or, in the control case, by no promoter. Selection of stably transfected cells was performed by co-culturing the transfected BC1-M3 cells with BC1-E2 cells in a concentration of G418 (200µg/ml) that was lethal to untransfected BC1-M3 cells, but not to BC1-E2 cells. The appearance of G418-resistant colonies in transfected cultures but not in untransfected control cultures indicated that the full-length proximal promoter is transcriptionally active in co-cultured BC1-M3 cells. The possibility of more distant control elements will be explored by DNase hypersensitivity site (DHS) analysis (see below). The amount of stromelysin-1 transcriptional activity that is inducible by co-culture with BC1-E2 cells and the identification of the elements that are responsible for this will be determined when the optimization of conditions for induction is completed (see above).

MMP gene structure and chromosomal location

At the suggestion of the grant application reviewers, DHS analysis will be used to identify transcriptional control regions in the MMP genes. As a first step towards this goal, Southern blots of BC1 DNA have been used to generate restriction maps of the genes, a pre-requisite for DHS analysis. In the case of collagenase-3, PCR primers were designed to amplify a region of the proximal promoter which was used to probe the membrane. Several useful restriction enzyme combinations were identified. Additionally, these primers were used to screen a commercial P1 rat genomic DNA library (Genome Research Inc.). The method originally proposed to obtain the rat collagenase-3 gene was ligation-mediated PCR. However, the commercial route was determined to be preferable, as it was an efficent use of resources in terms of both time and finances and had the potential to yield several 10s of kilobases of the gene, which ligation-mediated PCR is not able to do. Two clones were obtained and are currently being restriction-mapped to determine which part of the collagenase-3 gene they contain.

G-banded karyotypes of BC1-E2 and BC1-M3 cells, as well as normal fibroblasts, have been obtained. Preliminary analysis suggests that a marker chromosome, possibly involving a translocation of chromosome 1, exists in the BC1 cells.

Discussion

This ability of one cancer cell subpopulation to induce MMP gene expression in the other has potentially important implications. Cancer cells can be extremely heterogenous, even when isolated from a single tumor. This heterogeneity can arise from genetic instability [9] or normal differentiation processes [10]]. An epithelial-mesenchymal transition, as appears to have occurred in order to give rise to the M-cell subpopulation, can result from either of these two causes [11]. The genesis of phenotypic diversity in the cancer cell population can faciltate the progession of tumors to a more highly aggressive form by allowing the generation of cells with a phenotype that is inherently more aggressive than that of its predecessors e.g. by a faster rate of proliferation, ECM destruction or migration. These cells and their descendants will tend to dominate the tumor cell population in a simple Darwinian "survival of the fittest" fashion [9]. The results obtained above, in which BC1-E and BC1-M cells co-operate to produce MMPs, suggest a second way in which phenotypic diversity can facilitate tumor progression: by giving rise to two or more subpopulations that can interact to attain, together, a faster rate of proliferation, ECM destruction or migration. Thus, diverse tumor cell populations may behave more aggressively in co-existence than in isolation. This could be analogous, in evolutionary terms, to symbiosis. This alternative model for tumor progression is deserving of more investigation, and it is intended to do so, within the context of the present project, insofar as it is directly relevant, and in other projects.

The identity of the BC1-E cell factor that induces MMP gene expression in the BC1-M cells is not known. It may be a previously identified factor, such as tranforming growth factor-alpha or emmprin [12] or a novel factor. The identification of this factor does not fall directly within the Statement of Work of this project and so its pursuit will depend on finding additional resources.

Recommendations in relation to the Statement of Work

The state of progress is currently a few months behind that originally predicted in the Statement of Work in the original proposal. Two reasons have been identified. Firstly, the post-doctoral fellow position was not filled for five of the first twelve months, due to the decision of the first postdoctoral fellow to leave research for a career elsewhere after five months and the lag time in both instances between advertising and finding an applicant with appropriate experience. There is also inevitably a lag time between a new scientist beginning a project and reaching maximal productivity, due to the "learning curve" in some new techniques and reagents, a new field of research and a new physical workspace; this has happened twice. Secondly, the suggestion of the reviewers and the comitteee of the original grant to undertake DHS analysis of the MMP promoters, while certainly a scientifically worthwhile objective, was not included in the timeframe of the original statement of work. However, the second postdoctoral fellow has commenced the DHS analysis and is becoming increasingly productive. I expect the gap between projected and actual time courses for completion of tasks to narrow over the next 2 years.

There are also practical implications of the 3 MMPs being inducible in the BC1-M cells by the BC1-E cells. This observation should, in fact, facilitate the studies. For instance, DHS analysis can now be performed by comparing the DHS of MMPs in non-stimulated BC1-M cells (i.e. those cultured alone) with those that have been stimulated (i.e. by co-culture with BC1-E cells). In this way, it will be possible to distinguish between those DHS that are intrinsically sensitive to DNase and those whose sensitivity depends on a transcriptionally active gene and which, therefore, are more likely to be involved in controlling transcription. Similarly, the DHS or the intrinsically active gelatinase B gene in BC1-E cells can be compared with the inactive gelatinase B gene of BC1-M cells cultured in isolation.

Conclusions

1. The BC1-M cells upregulate the expression of stromelysin-1, gelatinase B and collagenase-3 genes in response to exposure to BC1-E cells and conditions for this response are being optimised.

2. The BC1-E cells express only the gelatinase B gene and do so constitutively.

3. Two P1 clones and restriction maps of the rat collagenase-3 gene have been obtained as a prelude to DHS analysis.

4. BC1-M cell clones have been stably transfected with reporter genes bearing full-length and truncated versions of the stromelysin-1 promoter.

5. Immunological characterization of the BC1-M cells that produce all 3 MMPs is consistent with them being carcinoma cells that have undergone an epithelial-mesechymal transition.

References

[1] O'Grady, R. L., Upfold, L. I. and Stephens, R. W. (1981)
"Rat mammary carcinoma cells secrete active collagenase and activate latent enzyme in the stroma via plasminogen activator."
Int. J. Cancer **28**, 509-515.

[2] Stevenson, G. A., Lyons, J. G., Cameron, D. A. and O'Grady, R. L. (1985)
"Rat carcinoma cells in long-term, serum-free culture provide a continuing supply of collagenase."
Biosci. Rep. **5**, 1071-1077.

[3] Nethery, A., Lyons, J. G. and O'Grady, R. L. (1986)
"A spectrophotometric collagenase assay."
Anal. Biochem. **159**, 390-395.

[4] Martorana, A. M., Zheng, G., Springall, F., Iland, H. J., O'Grady, R. L. and Lyons, J. G. (submitted)
"Absolute quantitation of specific mRNAs in cell and tissue samples incorporating an external standard."

[5] Harlow, E. and Lane, D. (1988)
"Antibodies. A laboratory manual."
Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

[6] Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1992)
"Short Protocols in Molecular Biology"
2nd. John Wiley and Sons, New York.

[7] Lukeis, R., Ball, D., Irving, L., Garson, O. M. and Hasthorpe, S. (1993)
"Chromosome abnormalities in non-small cell lung cancer pleural effusions: cytogenetic indicators of disease subgroups."
Genes Chrom. Cancer **8**, 262-269.

[8] Lyons, J. G., Birkedal-Hansen, B., Moore, W. G. I., O'Grady, R. L. and Birkedal-Hansen, H. (1991)
"Characteristics of a 95kDa matrix metalloproteinase produced by mammary carcinoma cells."
Biochemistry **30**, 1449-1456.

[9] Vogelstein, B. and Kinzler, K. W. (1993)
"The multistep nature of cancer."
Trends Genet. **9**, 138-141.

[10] Whitehead, R. H., Bertoncello, I., Webber, L. M. and Pederson, J. S. (1983)
"A new human breast carcinoma cell line (PMC42) with stem cell characteristics. I. Morphologic characterization."
J. Natl Cancer Inst. **70**, 649-661.

[11] Birchmeier, C., Birchmeier, W. and Brand-Saberi, B. (1996)
"Epithelial-mesenchymal transitions in cancer progression."
Acta Anat. **156**, 217-226.

[12] Kataoka, H., DeCastro, R., Zucker, S. and Biswas, C. (1993)
"Tumor cell-derived collagenase-stimulatory factor increases expression of interstitial collagenase, stromelysin, and 72-kDa gelatinase."
Cancer Res. **53**, 3154-3158.