REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Final (30 Sep 97 - 29 Sep 00)

4. TITLE AND SUBTITLE

Metalloprotease/Disintegrin Proteins and Mammary Carcinoma Progression

5. FUNDING NUMBERS

DAMD17-97-1-7154

6. AUTHOR(S)

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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)*

This project was designed to identify novel integrin ligands that are expressed in breast cancer tissue, by studying members of the MDC protein family. As a model system for breast cancer progression, we are using transgenic mice that express the polyomavirus middle T oncogene in the mammary gland epithelium. Using the polymerase chain reaction (PCR), we have shown that MDC9 and MDC15 are expressed in mammary tumor tissue derived from these mice. We have been unsuccessful in developing monoclonal antibodies to these MDCs that would recognize the proteins in breast cancer tissue sections. We have also raised panels of rabbit monoclonal antibodies to proteins expressed on the surface of mammary tumor cells, and have identified several of the corresponding antigens. Even though none of these are MDCs, two of them (MFG-E8, mClCa-1) are novel integrin ligands that may play a role in regulating breast cancer cell behavior. The antibodies to these molecules will be important tools aiding further studies of breast cancer cell biology.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
10

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

AD_____

GRANT NUMBER DAMD17-97-1-7154

TITLE: Metalloprotease/Disintegrin Proteins and Mammary Carcinoma Progression

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REPORT DATE: October 2000

TYPE OF REPORT: Final

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

FOREWORD

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Grant Number: DAMD17-97-1-7154
P.I.: Robert Pytela, Ph.D.

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Title: Metalloprotease/disintegrin proteins and mammary carcinoma progression

INTRODUCTION

MDCs (also called ADAMs) are members of a large family of transmembrane proteins. Within the last few years more than 30 MDCs have been identified, but the function of most of them is still unknown. Several MDCs mediate important biological processes, such as sperm-egg interaction, myoblast fusion, and release or "shedding" of membrane-anchored proteins (e.g., TNFalpha, selectin, TGFalpha, beta-amyloid precurser protein, and HB-EGF). All MDCs are characterized by having a pro-domain, an EGF-like domain, a transmembrane domain, and a cytoplasmic tail. The pro-domain is removed by a furin-type pro-protein convertase in the secretory pathway before the protein emerges on the cell surface. Some of the cytoplasmic domains contain consensus SH3-binding sequences, suggesting that MDCs can interact with cytoplasmic signaling pathways (Howard et al., 1999).

Although all MDCs contain a disintegrin domain, in most cases it is not clear whether the domain actually interacts with integrins, and which members of the integrin family may be involved. In contrast to snake venom peptides, the disintegrin domains of MDCs generally lack the widely distributed RGD integrin recognition motif. Human MDC15 (also called metargidin) is the only exception in that it contains an RGD motif in its disintegrin-like domain. It has recently been shown that the $\alpha v\beta 3$ integrin recognizes MDC3/ADAM23 in an RGD-dependent manner (Cal et al., 2000), and that the integrin $\alpha 9\beta 1$ interacts with both MDC12 and MDC 15 in an RGC-independent way (Eto et al., 2000). Thus, interactions between MDCs and integrins may have diverse functions in cell adhesion and regulation, but the mechanisms may be different from those of the typical snake-venom peptide/integrin interactions.

Based on the intriguing structural properties of the MDCs, we hypothesize that they may be important regulators of cell adhesion, migration, and invasion, and that specific members of this family may contribute to the invasive growth of breast cancer cells. The distribution and possible function of these proteins in normal and malignant breast tissue has not been studied. In the course of this project, we have used PCR cloning in order to identify known and novel MDCs that are expressed in human and mouse mammary tumors. We have also attempted to raise monoclonal antibodies in order to determine the distribution and expression levels of individual MDCs in normal mammary gland tissue and at different stages of mammary carcinoma progression. As a mouse model of mammary tumorigenesis, we have used transgenic mice overexpressing either the polyoma middle-T or the c-neu oncogene under the control of the MMTV promoter.

BODY:

Transgenic mouse lines as models of human breast cancer: As a part of this project, we characterized two transgenic mouse lines that spontaneously develop mammary tumors, and can be utilized as models of human breast cancer. The first line was generated by introducing the polyoma middle T gene under the control of the MMTV promoter (Guy et al., 1992). These mice strongly express the middle T oncogene in their mammary galnd tissue. We have established colonies of this line and have found (as previously described, Guy et al., 1992) that 100% of females develop invasive adenocarcinomas of the mammary glands by 60 days of age. Mice are sacrificed at 100 days of age, at which time 95% of them have developed extensive metastatic lesions in the lungs. Thus,

this mouse strain provides a practical experimental model because of the rapid and consistent progression of malignancy. However, it may not provide a realistic model of human breast cancer, which develops stochastically and involves multiple steps of progression through genetic instability and random mutations. As an alternative model, we have also established colonies of a mouse strain expressing the c-neu oncogene under the control of the MMTV promoter. We found that female mice of this strain develop tumors much more slowly (5-8 months of age) and stochastically. Most of the tumors are invasive and metastasize to the lung by 12 months of age. We have collected tissues from normal mammary glands, early and late stage primary tumors, and lung metastases obtained from both of these mouse strains. In addition, we have established a series of cell lines, as well as primary cultures, from both tumor models.

Identification of MDC proteins by PCR: We initially used mixed oligonucleotide primers designed to hybridize with all members of the MDC family, because they are based on highly conserved sequences in the metalloprotease and disintegrin domains, as detailed below

Primer 1: MXHEXGHN (metalloprotease domain) (forward orientation)

Primer 2: EECDCG (forward orientation)

Primer 3: ECDLXEXC (reverse orientation)

We used these primers for PCR amplification of total cDNA obtained by reverse transcription of mRNA purified from mouse mammary tumor tissues. These tissues were derived from primary tumors and metastatic lesions arising in MMTV-middle-T transgenic mice, or MMTV-c-neu transgenic mice. When degenerate primers were used, bands of the expected size (200 bp for primers 1 and 2, 400 bp for primers 1 and 3) were obtained.

Subcloning and sequencing of the PCR products revealed the presence of MDC9 and MDC 15. No novel members of the MDC family were identified. However, analysis of sequences obtained in the course of the human genome project has revealed that the extent of the MDC family is likely to be limited to about 30 members, the sequences of which are already known. Thus, the expression of all MDCs in breast cancer tissue can now easily be studied by using specific primers for each one of the 30 cDNAs, or by applying DNA array analysis. This approach goes beyond the scope of the current grant and will be adopted in the future as part of a continuation project.

We have already used a limited set of primers specific for individual members of the family (MDC9, MDC10, MDC11, MDC12, MDC15). Of these, MDC9 and MDC15 primers amplified bands of the expected size from mouse mammary tumor cells. These results suggest that the predominant MDCs expressed in this tissue are MDC9 and MDC15; however, other MDCs may be present in smaller amounts, and analysis with primers or cDNA arrays for all members of the family may identify additional MDCs expressed in breast cancer tissue. However, for the current project we decided to focus on identifying MDCs on the protein level, by attempting to produce novel antibodies.

Production of antibodies to MDC fragments:

As outlined in the original proposal, we have raised polyclonal antibodies to synthetic peptides and to recombinant MDC protein fragments expressed in bacteria. Even though these antibodies recognized the respective immunogens, they did not cross-react with native MDC proteins and did not produce specific staining patterns of breast cancer tissues. This may be due to the complex folding of the MDC proteins, which is difficult to reproduce with short synthetic fragments or with protein fragments expressed in bacteria. We have also attempted to express MDC fragments in mammalian cells (human embryonic kidney 293 cells), but the expression levels were too low to allow purification of the recombinant proteins in the amounts needed for raising antibodies (100-200

micrograms). Therefore, we have modified our strategy by attempting to raise monoclonal antibodies to all cell surface proteins, and to screen for antibodies that react with MDC proteins.

Production of rabbit monoclonal antibodies to mammary tumor cells: We have established an efficient novel method for raising monoclonal antibodies in rabbits. This method depends on the availability of a stable subclone of the rabbit plasmacytoma cell line 240E, originally described by K. Knight and colleagues (Spieker-Polet et al.,.1995). We immunized rabbits with tumor cells obtained from a transgenic mouse mammary tumor model. We used primary cultures of tumor tissue that had been dissociated with collagenase, and injected several rabbits subcutaneously in the hind legs. After 4 injections at 1-week intervals, we collected blood and found that the serum contained a high concentration of antibodies reactive with the tumor cells. We then removed the spleens as well as the inguinal and popliteal lymphnodes and recovered the lymphocytes. The lymphocytes were suspended in 10% DMSO/90% fetal bovine serum and frozen in liquid nitrogen. We had previously shown that rabbit lymphocytes can be frozen and stored in liquid nitrogen without any loss of fusion capacity. Aliquots of the lymphocytes were fused with 240E cells, and plated in 96-well plates. After two weeks, hybridoma supernatants were screened by immunohistochemistry of frozen mouse mammary tumor tissue, to detect antibodies that produced specific tissue staining patterns. A variety of different staining patterns was observed, suggesting that many of the hybridomas produced distinct antibodies. We have used this system to produce several hundred hybridoma clones that stably secrete monoclonal antibodies reactive with mouse mammary tumors. An overview of the results is shown in the table below.

Total number of wells (30 plates)	2880
Number of wells with hybridoma colonies	1152
Number of wells with positive reaction in immunohistochemistry	157
Number of antibodies preferentially reacting with tumor cells	80
Number of antibodies reacting with both normal epithelium and tumor cells	57
Number of antibodies reactive with myoepithelial cells	10
Number of antibodies reactive with stroma	11
Number of antibodies reactive with cell-cell junctions	2
Number of antibodies detecting secreted molecules	5
Number of antibodies specific for necrotic areas of tumors	2

We have further characterized several of these antibodies and have found that they recognize the respective antigens with high affinity and specificity. Many of the antibodies can be used for western blotting, immunoprecipitation, immunohistochemistry, and immunocytochemistry. Thus, these antibodies are ideal reagents for characterizing novel proteins. In order to identify the antigens recognized by these antibodies, we have developed a rapid expression cloning protocol. We have obtained a custom-made cDNA library (Edge Biosystems, Gaithersburg, Maryland) made from the mouse mammary tumor model described above. Pools of cDNA clones from this library were transfected into mammalian cells, and screened for reaction with the respective antigens. Six different clones were identified and sequenced. Comparison of these sequences with the genbank database revealed the identity of antigens recognized. In all cases, the size of the bands recognized by these antibodies in western blotting agreed with the expected properties of the proteins identified by expression cloning. The following table summarizes the proteins identified by expression cloning:

CD24; mucin-like PI-linked peripheral membrane protein

MFG-E8; RGD-containing adhesion protein present at the cell surface and in milk fat

mClCa-1; calcium-dependent epithelial chloride channel/adhesion protein

BAP-31; ER-membrane protein involved in apoptosis
Cytokeratin 19; cytoskeletal protein
Fibronectin; extracellular matrix protein

None of the antibodies identified a protein with properties expected of MDCs. Since MDCs probably constitute a minor fraction of total cell surface proteins, it may be necessary to immunize rabbits with protein fractions highly enriched in MDCs. To this end, we attempted to use metal-chelate chromatography to enrich for cell-surface proteins interacting with Zn. Since most MDCs contain zinc-binding metalloprotease domains, and most other metalloproteases are secreted proteins, this approach is expected to highly enrich for MDCs. However, using this method only small amounts of protein could be obtained from mammary tumor cells, and no specific antibodies were obtained following immunization of rabbits with this material.

Further characterization of MFG-E8: Even though none of the proteins identified above were MDCs, several of the antibodies did produce very interesting staining patterns of mouse mammary tissue. Thus, the novel antibodies that we have generated in this project may be of considerable interest to breast cancer researchers. One of these proteins, termed MFG-E8 or lactadherin, has an especially interesting domain structure suggesting that it may be a cell surface ligand for integrins. The protein contains two EGF-like domains, one of which includes an RGD sequence that is located at the tip of a predicted surface-exposed loop. It has been shown that MFG-E8 can promote integrin-dependent cell adhesion (Taylor et al., 1997). Thus, even though it is not a member of the MDC family, MFG-E8 can be considered functionally related to MDCs. MFG-E8 was originally identified as a major component of the milk fat globule, but is now recognized to be a widely distributed protein; however, its biological function is still unknown. It has been implicated in the regulation of apoptosis, cell proliferation, and intercellular communication. Our results show that the protein is strongly expressed in the normal mammary gland epithelium, but only in a subset of cells within invasive mammary tumors. By immunofluorescence of cultured cells, we find that the protein is localized in dot-like structures at the cell surface, which partly overlap with caveolin-containing membrane domains (W. Zhu and R. Pytela, unpublished results).

Expression of mCaCl-1 in lung metastases: Another one of our rabbit monoclonal antibodies specifically detects a novel chloride channel protein, termed mClCa-1. This is a member of a family of proteins that recently have been recognized to function both as chloride channels and as cell adhesion molecules (Elble et al., 1997). Results suggest that ClCa proteins can interact with integrins and may be involved in mediating adhesion of metastatic tumor cells to the lung endothelium. Our data suggest that in the middle-T transgenic mouse system, mClCa-1 is strongly expressed on metastatic tumor cells, but not on endothelial cells. Thus, this family of proteins may be involved in the adhesion of metastatic cells, both as tumor cell surface adhesion proteins and as endothelial ligands.

CONCLUSIONS:

We have identified MDC9 and MDC 15 as the major MDC proteins expressed in mammary tumors of MMTV-mT transgenic mice. Further characterization of the role of these proteins in breast cancer is dependent on raising antibodies that recognize the respective native proteins in tissue sections. We have raised antibodies to synthetic peptides and recombinant MDCs, but these antibodies did not react with the native proteins. We have also raised antibodies to mammary tumor cell surface antigens, and have identified several novel breast cancer antigens. We characterized six of the

antigens by expression cloning. None of these turned out to be MDC family members. However, two of them are functionally connected to the overall goal of our project, i.e., to identify novel integrin ligands expressed in breast cancer cells. One of them, an RGD protein termed MFG-E8/lactadherin, has been shown to interact with the αvβ3 integrin and may be involved in regulating apoptosis and cell proliferation. The other, termed mClCa-1, is a member of a novel family of adhesion proteins that also function as chloride channels, and mediate adhesion of metastatic tumor cells. Thus, the antibodies raised in the course of this project will be valuable tools in further studies of the molecular mechanisms underlying breast cancer cell function.

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APPENDICES: None

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CAR APPROVAL LETTER

Project # 96013425

COPY

September 20, 2000

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Dept.: Medicine
Phone No.: 206-4889/206-4123

Study Title: Characterization of Mouse Integrins

APPROVAL NUMBER: A6169-13425-04

Approval Date: 09/12/00
Expiration Date: 09/15/01

This number is a UCSF Committee on Animal Research (CAR) number which should be used for ordering animals for this study. This number may only be used by the principal investigator and those listed as participants included in the protocol and should be referenced in any correspondence regarding this study. The committee must be notified in writing of any changes to the approved protocol including changes in personnel.

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SPECIES NAME	TOTAL NUMBER APPROVED					
	Purchased			Bred		
	Category A	Category B	Category C	Category A	Category B	Category C
Mice	0	30	0	0	0	0
Rabbits	0	40	0	0	0	0

Condition: In section Q., please specify bilateral thoracotomy of the rabbits.

N. W. Bunnett

Nigel W. Bunnett, Ph.D., Chair
Committee on Animal Research