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AUTHORITY
USAMRMC ltr, dtd 15 May 2003

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AD _____

Award Number: DAMD17-99-1-9113

TITLE: Matrix Dependent Mechanisms Involved in Tumor Promotion
in Initiated Human Mammary Epithelium by Reactive Stroma

PRINCIPAL INVESTIGATOR: Bryony Wiseman, Ph.D.
Zena Werb, Ph.D.

CONTRACTING ORGANIZATION: University of California
San Francisco, California 94143-0962

REPORT DATE: June 2001

TYPE OF REPORT: Annual Summary

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

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REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE June 2001	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jun 00 - 31 May 01)
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4. TITLE AND SUBTITLE Maxtrix Dependent Mechanisms Involved in Tumor Promotion in Initiated Human Mammary Epithelium by Reactive Stroma	5. FUNDING NUMBERS DAMD17-99-1-9113
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6. AUTHOR(S)
Bryony Wiseman, Ph.D.
Zena Werb, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
San Francisco, California 94143-0962

E-Mail: bryony@itsa.ucsf.edu

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

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12b. DISTRIBUTION CODE

13. ABSTRACT (*Maximum 200 Words*)
The mammary gland relies on stromal-epithelial interactions for proper development. Tumorigenesis occurs when these signals are misinterpreted. Here we show that stromal matrix metalloproteinases (MMPs) have distinct roles in mammary gland branching morphogenesis. MMP activity is required for normal mammary gland development since mice treated with a synthetic MMP inhibitor, GM6001, have retarded ductal development. Furthermore, 3D cultures of mammary organoids also require MMP activity to branch in response to growth factors, indicating a mammary specific response. We also show that specific MMPs are required for distinct aspects of mammary gland morphogenesis. Specifically, MMP-3 is needed for branching because MMP-3 null mice have significantly fewer branches than controls. However, the ductal length is normal. In contrast, MMP-2 is required for ductal elongation, since the ducts of MMP-2 null mice are retarded in their penetration into the fat pad. However, the branching of the MMP-2 null ducts is normal. MMP-14 is a known activator of MMP-2 and we show it is highly expressed in front of terminal end buds. Mammary glands deficient in MMP-14 grown under the kidney capsule of nude mice also have defective ductal morphogenesis. Thus, these stromal enzymes are important, and have distinct roles, in patterning the mammary gland.

14. SUBJECT TERMS
Matrix metalloproteinases, stromal epithelial interactions development

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT
Unclassified

18. SECURITY CLASSIFICATION OF THIS PAGE
Unclassified

19. SECURITY CLASSIFICATION OF ABSTRACT
Unclassified

20. LIMITATION OF ABSTRACT
Unlimited

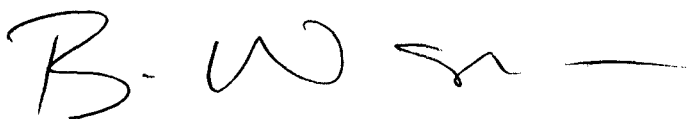
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Foreword

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

X-In conducting research using animals, the investigator 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)

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A handwritten signature in black ink, appearing to read "B. Wiseman". The signature is written in a cursive style with a long horizontal stroke at the end.

Bryony Wiseman Ph.D. May 17 2001

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Introduction

The mammary gland is a dynamic organ that undergoes cycles of proliferation, differentiation and apoptosis during an individual's life cycle, as that individual goes through puberty and pregnancies. Mechanisms exist to regulate these processes and to prevent their occurrence when not required. However disruption of these mechanisms can lead to inappropriate activation of proliferation and lead to tumorigenesis. Thus we need to elucidate the mechanisms that regulate normal breast development, in order to understand what mechanisms are appropriated by breast cancers. It is becoming clear that the stromal compartment underlying the basement membrane of the epithelium contributes both to normal epithelial development and to the promotion of carcinogenesis. In addition, matrix metalloproteinases (MMPs), which are mostly expressed by stromal cells, are important in normal development and tumorigenesis. Inappropriate activation of MMP-3 and MMP-14 activity has been shown to induce mammary tumorigenesis in mice. Here we show that MMPs are also required for normal mammary development and individual MMPs have distinct roles in the proper patterning of the mammary gland.

Body

One goal for this research proposal was to develop *in vivo* systems to study the promotion of mammary epithelial tumorigenesis by stromal factors, specifically MMPs. I reported favorable initial results in last year's annual report, however these results have not been reproducible (please refer too that report for the details). I was unable to reproducibly demonstrate the promotion of tumorigenesis by the human mammary carcinoma cell-line, MCF-7 by the co-injection of mouse embryo fibroblasts (MEFs) subcutaneously in the presence of matrigel. Seven independent experiments, using 5-6 mice (and two injection sites on each mouse) for each point, failed to identify an ability of MEFs to promote tumorigenesis by MCF-7 cells. Further, to determine if stromally produced MMPs had a role in tumor promotion, these experiments were also performed with MEFs from mice that were null for MMP-2, MMP-3 or MMP-9. No statistical difference was found between MCF-7 derived tumors formed in the presence of these cells, as opposed to wild type MEFs or in the absence of fibroblasts. The reasons for the lack of difference between the conditions described is due to the great variability in tumor sizes, which appeared to be dependent on the individual mice rather than the cells injected. To overcome this problem we have set up a collaboration with Agnes Noel who initially published this system and consistently observes an increase in MCF-7 derived tumor growth in the presence of MEFs. We have given her the MMP-null cells to see if she see a requirement for specific MMPs in this process. We are currently waiting for the initial results.

In last years annual report I reported initial results indicating a requirement for MMPs in normal mammary development. This work has now been expanded.

MMP Activity Is Required For Mammary Gland Morphogenesis

To determine whether MMPs were required for the development of the mammary gland mice were treated with a general chemical inhibitor of MMPs, GM6001 or the control vehicle between 3.5 and 10.5 weeks. The inguinal or number 4 mammary glands were removed and analyzed at various times up to 10.5 weeks. Mice treated with the MMP inhibitor had a dramatically diminished mammary gland when compared to mice treated with the vehicle alone. Inhibited glands were retarded in their penetration into

Note: This report contains unpublished data the stromal fat pad and also showed supernumerary branching or budding that was not seen in the age and estrus matched controls. At 6.5 and 10.5 weeks of age the GM6001 ducts were only 45% and 62% (P,0.00001) the length of the vehicle treated controls. To determine that GM6001 was only inhibiting MMP activity and had not irreversibly disrupted glandular development, we attempted to reverse the effects of GM6001 treatment, by halting the treatment of mice at 6.5 weeks and allowing the glands to develop for another 4 weeks in the absence of inhibitor. The glands of mice treated in this way had penetrated further into the fat pad than mice with continual GM6001 treatment, indicating that relieving inhibition of MMP activity allowed partial recovery of the ductal growth. The glands relieved of MMP inhibition did not completely recover, but this may occur over time

It was possible that the mammary gland phenotypes we had observed in response to MMP inhibition were due to systemic effects caused by inhibition of MMP activity within the whole animal, rather than local inhibition of MMPs within the mammary gland. To test that the mammary gland directly required MMPs for ductal development we used an in vitro three-dimensional culture system. Mammary organoids were cultured within collagen gels. Upon addition of either EGF or KGF they extend multi-cellular luminal branches. However, if GM6001 or recombinant human TIMP1 is added the effect of the growth factors is inhibited, and both the ability of the organoids to extend branches and the number of branches per organoid, is greatly reduced. Thus direct inhibition of MMP activity in mammary tissue retards the extension and branching of mammary ductal structures. This indicates that the effects we see in vivo are due to local inhibition of MMP activity. However this does not indicate which MMPs are required.

MMP-9 is expressed continually in the mammary gland throughout pubertal development. To address whether MMP-9 has a specific role in mammary gland development the mammary glands of MMP-9 null mice were examined at various stages of pubertal development. However, glands from MMP-9^{-/-} mice demonstrated no developmental defects with respect to ductal penetration or branching when compared to age and estrus matched controls. This indicates that expression of MMP-9 is not necessary for mammary morphogenesis.

Note: This report contains unpublished data

A role for MMP-3 in mammary gland development has already been suggested by the phenotype of mice that overexpress active MMP-3 from a mammary specific promoter. These mice have excessive arborization of their mammary glands and nulliparous mice display a phenotype suggestive of early pregnancy. The number of alveolar-like buds is greatly increased while the distance in between branches is significantly decreased in the transgenic gland compared to the non-transgenic age and estrus matched control. Thus we examined the phenotype of the MMP-3 null mammary gland. Contrary to the excess of branching observed with constitutively activated MMP-3, the absence of MMP-3 inhibited branching. Such that at 7.5 weeks of age, MMP-3^{-/-} mice had 44% less branches ($p < 0.001$) than age and estrus matched controls. However, in contrast to the phenotypes we observed when MMPs were inhibited, the lack of MMP-3 did not affect the ability of the mammary glands to penetrate into the fat pad. The ductal lengths of the glands were not significantly different, implying that another MMP must be responsible for the phenotype we observed with MMP inhibitors.

MMP-2 is upregulated both in message and activity during pubertal development. The mammary glands of these mice were examined and compared to estrus and age matched controls. In contrast to MMP-3 null mice, MMP-2^{-/-} glands were retarded in their early penetration into the stromal fat pad, such that at 4.5 weeks of age the ductal length was reduced to 69% that of controls ($p < 0.0005$). After time the difference lessened and lost significance by 7 weeks. In contrast to the MMP-3 null gland, there was no difference in the number of branch points relative to primary ductal length, indicating that MMP-2 is not required for branching. These data indicate that MMP-2 assists the extension of the epithelial ducts through the fat pad, but has no essential role in branching.

We have shown by in situ hybridization that MMP-14/MT1-MMP is specifically expressed around the TEBs in the mammary gland during pubertal development suggesting a potential role. Moreover, activation of MMP-2 is thought to be dependent on the sub-family of membrane-type MMPs (MMP14-18, 24, 25) in complex with TIMP-2. MMP-14 is expressed at a location where activation of MMP-2 is necessary to assist penetration of the epithelial ducts into the stromal fat pad. MMP-14 null mice die post-natally from defects in bone development and therefore most of the mice do not reach puberty, and thus we cannot assess the morphological development of their mammary glands. To overcome this

Note: This report contains unpublished data. In a previous problem we grafted the inguinal mammary glands of 1 day old MMP-14^{-/-} mice under the kidney capsule of immunocompromised female hosts, where they respond to the adult estrus cycle and develop in a similar manner to the pubertal gland. To control for differences between hosts, the contralateral kidney was grafted with a gland from an MMP-14^{+/+} litter-mate. The phenotypes of the MMP-14^{-/-} glands were variable, indicating the effect of the gene deletion was not fully penetrant, however, in general the ductal trees were smaller and had fewer TEBs than MMP-14^{+/+} glands. Furthermore, five out of thirteen MMP-14 null glands were severely retarded in their ductal development, while 100% of the wild type glands grew normally.

Key Research Accomplishments

- Identified requirement for MMPs in mammary gland development
- Demonstrated local versus systemic requirement for MMPs in mammary ductal development
- Identified distinct requirement for MMP-3 in branching and MMP-2 in ductal elongation
- Identified requirement for MMP-14 in morphogenesis of gland

Conclusions

MMPs are important both in development and tumorigenesis. We have demonstrated different roles for MMPs within the morphogenesis of the mammary gland. This indicates that they have specific functions. A role for MMPs within tumorigenesis has been clearly demonstrated in the late stages of tumorigenesis as general proteinases that allow cells to break through barriers. However evidence is also building to suggest a role for MMPs in earlier stages of tumorigenesis which indicates a more defined and subtle mode of action. That we have demonstrated that MMPs have different roles to play with very different developmental consequences pushes forward this point of view. It is not yet clear what role or roles the MMPs have. Possibilities include: changing cell-cell or cell-ECM adhesion; generating or releasing bioactive ECM fragments or factors; or clearing the way for developing epithelium. But disruption of their activity leads to mammary gland tumorigenesis, as do a great number of other factors involved in mammary gland development. Thus elucidating the mechanisms involved in normal development will undoubtedly assist in understanding what goes wrong in tumorigenesis.

Reportable Outcomes

Platform Presentations

Matrix Metalloproteinases Regulate Distinct Aspects of Mammary Gland Branching Morphogenesis.

Bryony S. Wiseman

Mammary Gland Biology Gordon Conference, Rhode Island, June 3-8 2001

Published Abstracts

Matrix Metalloproteinases Regulate Distinct Aspects of Mammary Gland Branching Morphogenesis.

Bryony S. Wiseman, Mark D. Sternlicht, Leif Lund, Mari E. Sciabica and Zena Werb

Mammary Gland Biology Gordon Conference, Rhode Island, June 3-8 2001

Stromally Derived Matrix Metalloproteinases Foster Mammary Tumorigenesis

B.S.Wiseman, K. Cheung and Z.Werb. 40th American Society of Cell Biology Annual Meeting (#2591), San Francisco.

Dec 9-13 2000.

Roles for Stromal Matrix Metalloproteinases in Mammary Tumorigenesis and Morphogenesis.

B.S.Wiseman, M.D.Sternlicht, L.Lund, M.Sciabica and Z.Werb

VIIIth International Meeting of the Metastasis Research Society, London, UK. Sept 24-27 2000.

Stromal Metalloproteinases in Morphogenesis, Remodelling and Cancer

M.D.Sternlicht, B.S. Wiseman, M.Simian, M.J.Bissell, Z.Werb. Proc. ENDO 2000 The Endocrine Society 82nd Annual Meeting, 17 (# 57) June 23, 2000.

Matrix Metalloproteinases and Tissue Inhibitors of Matrix Metalloproteinases in the Stroma Regulate Mammary Ductal Morphogenesis. J.L. Rinkenberger, M.D. Sternlicht, B.S.Wiseman, M.Sciabica, K. Holmbeck, J. Wiesen, Z. Werb. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Abstract # Z-10. June 8, 2000.

Training

UCSF CAR/ACF Training and Certification Program

Animal Care Facility Training Workshop on animal handling and aseptic techniques



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