NEW LIMITATION CHANGE

TO
Approved for public release, distribution unlimited

FROM
Distribution authorized to U.S. Gov’t. agencies only; Proprietary Info.; Jul 2000. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Fort Detrick, MD 21702-5012.

AUTHORITY
USAMRMC ltr, 28 Aug 2002
Award Number: DAMD17-98-1-8057

TITLE: Evaluation of Cyclooxygenase-2 as a Novel Target for Breast Cancer Prevention

PRINCIPAL INVESTIGATOR: Andrew J. Dannenberg, M.D.

CONTRACTING ORGANIZATION: Cornell University Medical College
New York, New York 10021

REPORT DATE: July 2000

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Jul 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

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.
NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-1-8057
Organization: Cornell University Medical College
Location of Limited Rights Data (Pages):

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

[Signature]

[Signature]
<table>
<thead>
<tr>
<th><strong>Title and Subtitle</strong></th>
<th>Evaluation of Cyclooxygenase-2 as a Novel Target for Breast Cancer Prevention</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Andrew J. Dannenberg, M.D.</td>
</tr>
</tbody>
</table>
| **Performing Organization Name(s) and Address(es)** | Cornell University Medical College  
New York, New York 10021 |
| **Sponsoring / Monitoring Agency Name(s) and Address(es)** | U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012 |

**Abstract**

Cyclooxygenase (Cox) catalyzes the synthesis of prostaglandins and the intracellular production of mutagens from procarcinogens. Cox-2, the inducible form of cyclooxygenase, is expressed in a wide variety of human cancers, but its role in breast cancer has not been established. Our research is designed to test whether Cox-2 contributes to mammary cancer, using Wnt-1 as a model mammary oncogene. The role of Cox-2 in mammary tumorigenesis is being tested by evaluating the incidence of mammary hyperplasia and carcinoma formation in Wnt-1 transgenic (TG) mice of the following Cox-2 genotypes: (+/+), (+/-), and (-/-). Initial breeding programs to generate F1 mice for use in the final cross have been completed, and these mice are currently being crossed to produce offspring of the required test genotypes. Thus far we have generated 18 Wnt-1 TG, Cox-2 (+/+), 25 Wnt-1 TG, Cox-2 (+/-), and 6 Wnt-1 TG, Cox-2 (-/-) mice. In parallel, we have been dissecting the molecular mechanism by which Wnt-1 activates Cox-2 transcription. We have observed upregulation of the PEA3 transcription factor family in Wnt-1-expressing cell lines and tumors, and demonstrated PEA3-mediated upregulation of Cox-2 promoter activity. We speculate that Wnt-1 may upregulate Cox-2 via upregulation of PEA3 transcription factors.
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.

X 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).

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

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

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

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

Andrew Dannenberg 6/4/00
PI - Signature Date
# Table of Contents

- **Cover**
- **SF 298**
- **Foreword**
- **Table of Contents**
- **Introduction**
- **Body**
- **Key Research Accomplishments**
- **Reportable Outcomes**
- **Conclusions**
- **References**
- **Appendices**
  - 1 abstract (DoD Era of Hope Meeting, June 2000)
  - Figures 1-3
Introduction

Cyclooxygenase (Cox) catalyzes both the synthesis of prostaglandins (PGs) and the intracellular production of mutagens from procarcinogens. The inducible form of cyclooxygenase, Cox-2, is expressed in a wide variety of human cancers and recent evidence suggests that it plays a critical role in tumorigenesis, particularly in colorectal cancer. Both epidemiological and experimental data indicate that nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit Cox activity and PG production, protect against colon cancer. In addition, experiments utilizing Cox-2 knockout mice have shown that loss of Cox-2 leads to a marked reduction in polyp formation in a mouse model of familial adenomatous polyposis coli. These results demonstrate the importance of Cox-2 in intestinal tumorigenesis. However, a role for Cox-2 in breast cancer has not been established. Our research is designed to test whether Cox-2 is important in the pathogenesis of mammary cancer, using Wnt-1 as a model mammary oncogene. Transgenic mice which express Wnt-1 from a mammary specific promoter are predisposed to develop mammary hyperplasia and subsequent carcinomas, and represent a well characterized model of mammary tumorigenesis. Female Wnt-1 transgenic mice with the following Cox-2 genotypes; (+/+), (+/-) and (-/-), are being generated by crossing Cox-2 (+/-) females with Wnt-1 transgenic Cox-2 (+/-) males. As the target mice are generated they are being monitored for development of mammary hyperplasias and adenocarcinomas, to determine whether reduced Cox-2 expression protects against formation of tumors or preneoplastic lesions. Concurrently, the molecular mechanism by which Wnt-1 upregulates Cox-2 is being elucidated in mammary cell culture models. If our research reveals that knocking out the Cox-2 gene protects against mammary tumorigenesis, it will suggest a potential use for selective Cox-2 inhibitors as chemopreventive agents in the treatment of breast cancer.

Body

Progress during the second year of the grant will be described with specific reference to the individual tasks specified in the Statement of Work.

Task 1. Generate breeding stocks of Wnt-1 transgenic and Cox-2 knockout mice for subsequent crosses.
This was completed in year 1.

Task 2. Cross Wnt-1 TG males x Cox-2 (+/-) females to generate 10-12 Wnt-1 TG, Cox-2 (+/-) male F1 mice.
This was completed in year 1.

Task 3. Analyze Cox-2 expression in mammary tissue from 5 Wnt-1 TG females and 5 wild-type female litter mates.
Western blotting has revealed increased levels of Cox-2 in mammary gland from Wnt-1 transgenic mice relative to that from wildtype animals. Cox-2 protein is present at higher levels in tumor tissue compared with the hyperplastic mammary gland from Wnt-1 transgenic mice.

**Task 4.** Cross Wnt-1 TG, Cox-2 (+/-) males x 18 Cox-2 (+/-) females to generate F2 Wnt-1 TG females with the following Cox-2 genotypes: (+/+), (+/-) and (-/-). These crosses are in progress. Currently we have generated 18 Wnt-1, Cox-2 (+/+), females, 25 Wnt-1, Cox-2 (+/-) females and 6 Wnt-1, Cox-2 (-/-) females. We are currently maintaining an active breeding program to generate further mice of the required genotypes. All mice of inappropriate genotypes have been sacrificed.

**Task 5.** Evaluate mammary hyperplasia in 5 animals each of the above F2 genotypes at 8 weeks of age.

Preliminary experiments have been conducted to evaluate the presence of mammary hyperplasia in animals of each of the following genotypes: Wnt-1, Cox-2 (+/+), Wnt-1, Cox-2 (+/-), and Wnt-1, Cox-2 (-/-). Analysis of two sets of each genotype was conducted as follows. The 3rd and 4th pairs of mammary glands from each mouse were harvested, stained with carmine alum, and whole mounts examined microscopically. As previously described, mammary glands from Wnt-1 transgenic mice exhibited striking epithelial hyperplasia compared with wildtype mice (Tsukamoto et al., 1988). However, we did not observe a significant difference between the mammary glands of Wnt-1 transgenic mice with differing Cox-2 genotypes (Figure 1). These data suggest that Cox-2 does not contribute significantly to epithelial hyperplasia in the Wnt-1 transgenic mouse. However we plan to examine further mice of each genotype in order to confirm our preliminary observations.

**Task 6.** Analyze mechanism of Cox-2 regulation by Wnt-1 in cell culture systems.

Our preliminary observations were reported in our first annual report submitted July 1999, but will be briefly reiterated here to give context for our most recent data. Previously we reported the observation of transcriptional activation of the Cox-2 gene in mouse mammary epithelial cell lines engineered to express Wnt-1. Cell lines stably expressing Wnt-1 were generated by retroviral infection with virus encoding Wnt-1, and assayed for Cox-2 by Northern and Western blotting. Expression of Wnt-1 resulted in elevated Cox-2 protein and RNA, due to transcriptional upregulation of the Cox-2 gene. These data were published in Cancer Research (Howe et al., 1999), and reprints of this paper were submitted with our previous annual report.

More recently we have focussed on identifying the molecular basis of Cox-2 upregulation in response to Wnt-1. The observation of Cox-2 upregulation in Wnt-1-expressing cell lines (Howe et al., 1999) and in tumor tissue resulting from APC mutation (Kargmann et al., 1995; Boolbol et al., 1996; Williams et al., 1996) led us to speculate that the Cox-2 gene promoter might be regulated by β-catenin, since both Wnt-1 expression and APC mutation cause β-catenin/TCF-dependent transcriptional activation. Therefore we examined the ability of β-catenin to activate Cox-2 promoter reporter constructs in transient transfection assays. In addition, since ets transcription factors of the PEA3 subfamily synergise with β-catenin to activate transcription from promoters
other than the Cox-2 promoter (Howard Crawford, personal communication), we were also interested to address the potential involvement of PEA3 in Cox-2 gene regulation. Northern blot analysis of control and Wnt-1-expressing cell lines revealed that PEA3 expression is markedly increased in Wnt-1-expressing C57MG cells (Figure 2), mirroring previously observed changes in Cox-2. Transient transfection of human embryonic kidney cell line 293 with a Cox-2 promoter reporter construct and expression vectors encoding β-catenin and PEA3 revealed only a small activation of Cox-2 promoter activity by β-catenin (Figure 3). Activity was increased by at most 100% in several experiments. Strikingly, however, PEA3 stimulated Cox-2 promoter activity 15-20 fold (Figure 3). These data suggest that Wnt-1 may activate Cox-2 transcription via intermediate upregulation of PEA3. We are currently analyzing the Cox-2 promoter to identify the site(s) responsible for PEA3 responsiveness.

Task 7. Continuously monitor Wnt-1 TG, Cox-2 (+/+) and Wnt-1 TG, Cox-2 (+/-) females (20 each) for appearance of mammary tumors over a 12 month period. Thus far we have generated 18 Wnt-1 TG, Cox-2 (+/+) and 25 Wnt-1 TG, Cox-2 (+/-) female mice. These are being maintained and monitored for tumor incidence. In addition, we are continuing breeding programs to fulfil our target of at least 20 mice per group. Several mice from each cohort have already developed tumors. However, insufficient data have been accrued to date to enable us to perform statistical analyses to compare the rates of tumor development in the two cohorts.

Task 8. Histological analysis of mammary tumors, evaluation of Cox-2 expression in tumors, and interpretation of results. This is pending awaiting generation of all of the mice and development of tumors.

Key Research Accomplishments (cumulative over 2 years)

- Breeding programs were established to generate numerous Wnt-1 transgenic and Cox-2 heterozygote mice for further breeding
- Wnt-1 transgenic and Cox-2 heterozygote mice were crossed to generate F1 Wnt-1 transgenic, Cox-2 heterozygote males for final cross
- Breeding pairs were established to generate F2 Wnt-1 transgenic mice of genotypes Cox-2 (+/+), (+/-) and (-/-)
- 18 Wnt-1, Cox-2 (+/+) and 25 Wnt-1, Cox-2 (+/-) mice have thus far been generated in which to observe tumor incidence
- We have demonstrated that Wnt-1 expression in mammary epithelial cell lines causes transcriptional upregulation of the Cox-2 gene
- We have generated evidence that Cox-2 activation in Wnt-1-expressing cells and tissues may be mediated via upregulation of PEA3 family transcription factors.
Reportable Outcomes

Poster presented at Department of Defense Era of Hope meeting, June 2000 (Abstract appended).

Research grant obtained from the Cancer Research Foundation of America, based on the observation of Cox-2 upregulation in Wnt-1-expressing cells and tissues.

Title: Evaluation of Cox-2 as a Pharmacological Target for Breast Cancer Prevention

P.I.: Louise R. Howe, Ph.D. (co-investigators: A.J. Dannenberg, M.D. and A.M.C. Brown, Ph.D)

Active: 1/15/00-1/14/01

Conclusions

Much of the progress made to date on this project has involved establishing mice colonies and breeding programs, which constitute necessary preliminary steps to evaluating the effect of Cox-2 gene dosage on Wnt-1-induced mammary hyperplasia and carcinoma formation. However, we have also demonstrated in a cell culture system that Wnt-1 causes transcriptional upregulation of the Cox-2 gene. Consistent with this, we have observed increased Cox-2 protein in mammary glands from Wnt-1 transgenic mice relative to those of control wildtype littermates. These findings are of considerable interest, suggesting that, in addition to its well-established role in colorectal cancer, Cox-2 may also be upregulated during, and contribute to, mammary tumorigenesis. Should our experiments show a reduction in mammary tumorigenesis correlating with reduced Cox-2 gene dosage, a future goal will be to determine whether pharmacological inhibition of Cox-2 protects against human breast cancer.

We are currently using our Wnt-1-expressing cell lines to analyze the molecular mechanism of Cox-2 upregulation by Wnt-1. Cox-2 is upregulated in tumors generated as a consequence of ectopic Wnt-1 expression or mutation of the APC tumor suppressor gene. Since both of these events result in stabilization of β-catenin in the cytosol, and consequently β-catenin/TCF-mediated transcriptional activation, it was tempting to speculate that the Cox-2 gene might be subject to regulation by β-catenin/TCF complexes. However, preliminary data suggest that the Cox-2 promoter is relatively insensitive to β-catenin but markedly activated by PEA3, an ets family transcription factor. PEA3 expression is upregulated in mouse mammary epithelial cells expressing Wnt-1, consistent with a potential role in mediating activation of the Cox-2 gene.

References


Appended Material

Abstract presented at Department of Defense Era of Hope Meeting, June 2000

Figure 1

Figure 2

Figure 3
Cyclooxygenase (Cox) catalyzes both the synthesis of prostaglandins (PGs) and the intracellular production of mutagens from procarcinogens. The inducible form of cyclooxygenase, Cox-2, is expressed in a wide variety of human cancers and recent evidence suggests that it plays a critical role in tumorigenesis, particularly in colorectal cancer. However, a role for Cox-2 in breast cancer has not been established. Our research is designed to test whether Cox-2 is important in the pathogenesis of mammary cancer, using Wnt-1 as a model mammary oncogene. Wnt-1 transgenic mice exhibit mammary hyperplasia and subsequently develop mammary carcinomas. We have investigated the effect of Wnt-1 on Cox-2 expression in two mouse mammary epithelial cell lines, RAC311 and C57MG, which are morphologically transformed in response to Wnt-1. Expression of Wnt-1 in these cell lines caused transcriptional upregulation of the Cox-2 gene, resulting in increased levels of Cox-2 mRNA and protein. Prostaglandin E2 production was increased as a consequence of the elevated Cox-2 activity, and could be decreased by treatment with a selective cyclooxygenase-2 inhibitor. These experiments demonstrated that Cox-2 is upregulated in response to Wnt-1 expression, and thus laid the foundation for our ongoing experiments designed to test the contribution of Cox-2 to mammary tumorigenesis. We are currently generating Wnt-1 transgenic mice of the following Cox-2 genotypes: (+/+), (+/-), and (-/-), and will then evaluate the incidence of mammary hyperplasia and carcinoma formation in these animals. We anticipate that reduced Cox-2 gene dosage may decrease the formation of mammary tumors.
Figure 1. Whole Mount Analysis of Mammary Glands.
Epithelial hyperplasia was compared in mammary glands from mice of various genotypes, by staining the 4th inguinal mammary glands with carmine alum and examining the stained mammary glands as whole mounts. Panel A shows a wildtype mammary gland. Shown in panel B are glands from Wnt-1 transgenic mice with varying Cox-2 genotypes. Expression of the Wnt-1 transgene causes marked hyperplasia (compare panels A and B), but altered Cox-2 gene dosage does not significantly affect Wnt-1-induced hyperplasia.
Figure 2. PEA3 and Cox-2 are upregulated in C57MG cells expressing Wnt-1. Total RNA was prepared from cells and 20µg of each RNA sample analysed by Northern blotting as previously described (Howe et al., 1999). The blot was probed sequentially with a murine PEA3 probe, a murine Cox-2 probe and a murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe.
Figure 3. Regulation of Cox-2 Promoter Activity. 
293 human embryonic kidney cells were transiently transfected using lipofectamine with expression vectors encoding β-catenin and/or PEA3, and with a Cox-2 promoter reporter construct comprising residues -1432 - +59 of the human Cox-2 promoter linked to the luciferase gene. Luciferase activity was measured using the Dual Luciferase Reagent kit (Promega) and normalized to that of cotransfected Renilla Luciferase. Each data point shown represents the mean (+ s.d.) of 6 replicates.
MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

[Signature]

PHYLIS M. RINEHART
Deputy Chief of Staff for Information Management