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13. ABSTRACT (Maximum 200 Words) 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 <i>Wnt-1</i> 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 <i>Wnt-1</i> 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 <i>Wnt-1</i> TG, Cox-2 (+/+) mice, 25 <i>Wnt-1</i> TG, Cox-2 (+/-) mice and 6 <i>Wnt-1</i> 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 <i>Wnt-1</i> -expressing cell lines and tumors, and demonstrated PEA3-mediated upregulation of Cox-2 promoter activity. We speculate that <i>Wnt-1</i> may upregulate Cox-2 via upregulation of PEA3 transcription factors.				
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FOREWORD

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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.

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Tsukamoto, A.S., Grosschedl, R., Guzman, R.C., Parslow, T. and Varmus, H.E. Expression of the *int-1* gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell*, 55: 619-625, 1988.

Appended Material

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

Figure 1

Figure 2

Figure 3

CYCLOOXYGENASE-2 AS A NOVEL TARGET FOR BREAST CANCER PREVENTION

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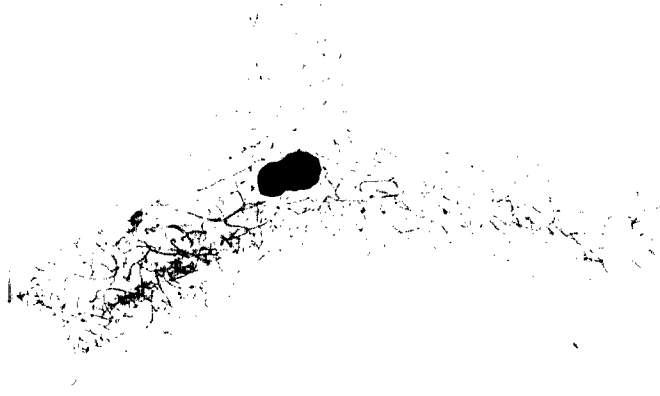
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Cyclooxygenase (Cox) catalyzes both the synthesis of prostaglandins (PGs) and the intracellular production of mutagens from procarcinogens. The inducible form of cyclooxygenase, *Cox-2*, is expressed in a wide variety of human cancers and recent evidence 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.

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8057 supported this work.

A. Wildtype

B. Wnt-1 Transgenic



Cox-2 ^{+/+}



Cox-2 ^{+/-}



Cox-2 ^{-/-}

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.

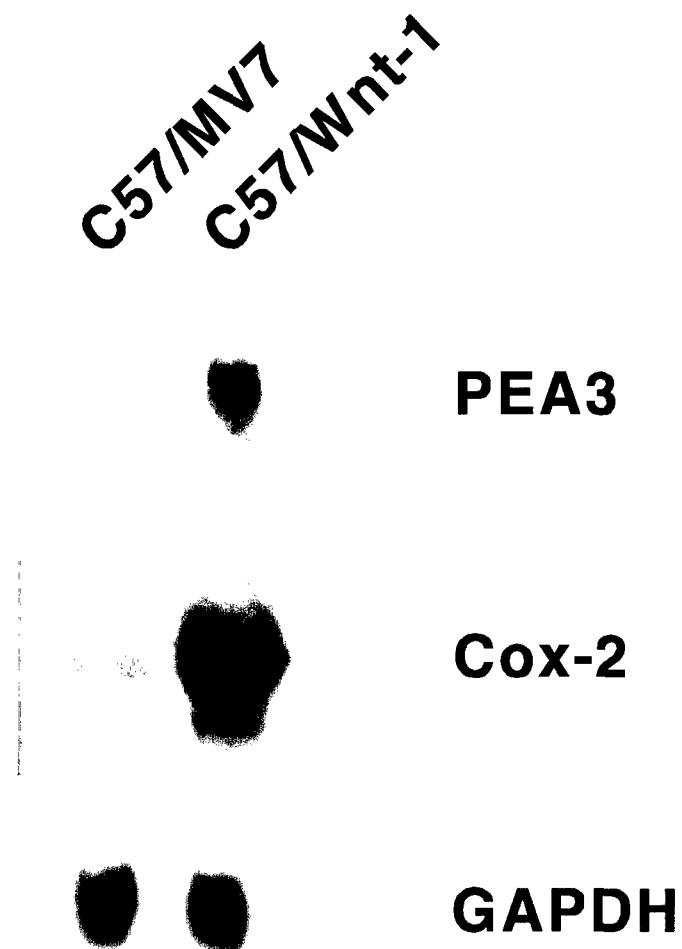


Figure 2. *PEA3* and *Cox-2* are upregulated in C57MG cells expressing *Wnt-1*. Total RNA was prepared from cells and 20 μ g of each RNA sample analysed by Northern blotting as previously described (Howe *et al.*, 1999). The blot was probed sequentially with a murine *PEA3* probe, a murine *Cox-2* probe and a murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe.

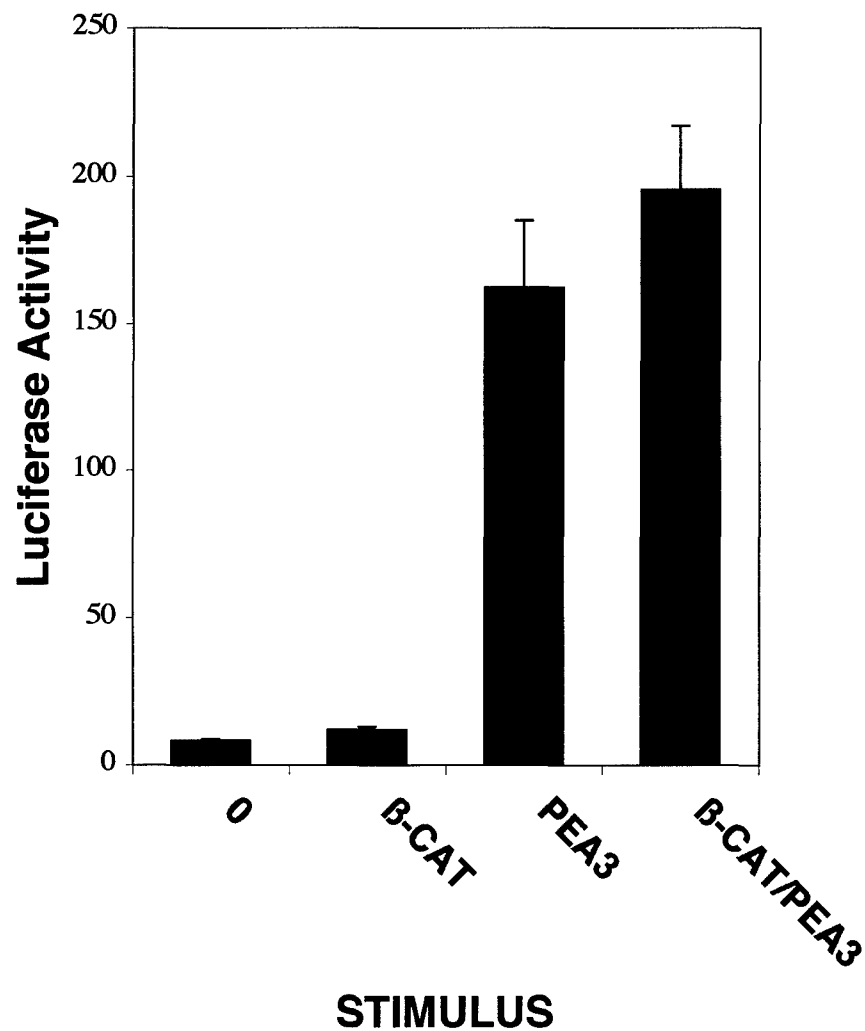


Figure 3. Regulation of *Cox-2* Promoter Activity.

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.



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