HER2/neu-Induced Mammary Tumorigenesis and Angiogenesis Are Reduced in Cyclooxygenase-2 Knockout Mice

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Abstract

The inducible prostaglandin synthase cyclooxygenase-2 (Cox-2) is overexpressed in ~40% of human breast cancers and at higher frequencies in preinvasive ductal carcinoma in situ (DCIS). Cox-2 expression is particularly associated with overexpression of human epidermal growth factor receptor 2 (HER2/neu). To definitively interrogate the role of Cox-2 in mammary neoplasia, we have used a genetic approach, crossing Cox-2-deficient mice with a HER2/neu transgenic strain, MMTV/NDL. At 20 weeks of age, mammary glands from virgin MMTV/NDL females contained multiple focal tumors, or mammary intraepithelial neoplasias, which histologically resembled human DCIS. Mammary tumor multiplicity and prostaglandin E2 (PGE2) levels were significantly decreased in Cox-2 heterozygous and knockout animals relative to wild-type controls. Notably, the proportion of larger tumors was decreased in Cox-2-deficient mice. HER2/neu-induced mammary hyperplasia was also substantially reduced in Cox-2 null mice. Additionally, mammary glands from Cox-2 knockout mice exhibited a striking reduction in vascularization, and expression of proangiogenic genes was correspondingly reduced. Decreased vascularization was observed both in dysplastic and normal-appearing regions of Cox-2-null mammary glands. Our data provide the first genetic evidence that Cox-2 contributes to HER2/neu-induced mammary tumorigenesis. This finding may help to explain the reduced risk of breast cancer associated with regular use of nonsteroidal anti-inflammatory drugs.

Introduction

Cyclooxygenase-2 (Cox-2), an inducible enzyme involved in prostaglandin synthesis, is strongly implicated in cancer. The relationship between Cox-2 and cancer was first recognized in the setting of colorectal cancer, but it has now become apparent that Cox-2 is overexpressed in human breast carcinomas as well as in numerous other cancers (1–4). Immunohistochemical analyses have detected Cox-2 protein in ~40% of human breast cancers and 60% to 80% of preinvasive ductal carcinoma in situ (DCIS) lesions (5–8). Additionally, Cox-2 expression has been detected in focal regions of normal breast in association with silencing of CDKN2A (p16INK4a; ref. 9). Cox-2 expression in human breast carcinomas correlates with poor prognosis and several associated clinical variables, particularly overexpression of human epidermal growth factor receptor 2 (HER2/neu; refs. 6, 10–14). A causal relationship between HER2/neu and Cox-2 expression is suggested by cell culture experiments, and may be mediated through signaling intermediates such as activator protein-1 and Ets transcription factors as well as by transactivation mediated by nuclear-localized HER2/neu protein (13, 15–17). Numerous additional mechanisms may also contribute to Cox-2 up-regulation during tumorigenesis, including post-transcriptional message stabilization (3, 18, 19). Consistent with the human expression data, rodent mammary tumors also express Cox-2, and transgenic Cox-2 overexpression can drive tumorigenesis in mouse mammary glands (2, 3, 20).

Epidemiologic data link regular use of nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit Cox activity, with reduced risk of several tumor types including breast cancer (2, 3). A meta-analysis of six cohort studies and eight case-control studies concluded that NSAID use was associated with a 22% decrease in breast cancer risk (21). Additionally, the Women's Health Initiative Observational Study revealed 21% and 28% reductions in breast cancer incidence associated with regular NSAID use for 5 to 9 and >10 years, respectively (22). In contrast, a recent study reported increased breast cancer risk associated with daily NSAID use (23). However, in aggregate, epidemiologic analyses support a role for Cox enzymes in breast carcinogenesis.

Based on the above data sets, rodent models have been extensively employed to evaluate the relationship between Cox-2 and breast cancer. Both selective Cox-2 inhibitors and conventional NSAIDs delay or prevent experimental breast cancer (2, 3, 24). These pharmacologic studies suggest a role for Cox-2 in breast neoplasia. However, because both selective Cox-2 inhibitors and nonselective NSAIDs may have Cox-independent effects (25–27), a genetic approach is required to definitively establish and characterize the role of Cox-2 in breast cancer. Therefore, we have used a knockout strategy to interrogate the role of Cox-2 in mammary tumorigenesis.

Here we report the effect of Cox-2 deficiency on mammary morphology and tumorigenesis in the MMTV/NDL mouse strain, in which breast cancer is driven by a mutationally activated HER2/neu allele. Knocking out Cox-2 reduced both tumor formation and...
growth and also decreased HER2/neu-induced mammary hyperplasia. Strikingly, blood vessels were virtually absent in mammary tissues from Cox-2-null mice. These results provide direct genetic evidence linking Cox-2 to mammary tumorigenesis.

Materials and Methods

Mouse experimental procedure. Cox-2+/− mice (28) were obtained from The Jackson Laboratory (Bar Harbor, ME) on a hybrid C57BL/6J:129 strain background (B6:129S7-Ptgstm1Jed) and were crossed with FVB/J wild-type mice for 10 generations to introduce the targeted Cox-2 allele onto an FVB background. FVB-Cox-2 null mice were healthier than those on the original B6:129 background, with less morbidity and increased life spans. MMTV/NL mice express a mutationally activated HER2/neu allele (Neu Deletion mutant, NDL) that induces mammary hyperplasia and tumorigenesis (29). MMTV/NDL and FVB+Cox-2+/− mice were interbred to generate MMTV/NDL, Cox-2+/− progeny, which were backcrossed with FVB-Cox-2+/− mice to generate females of the required test genotypes: MMTV/NDL, Cox-2+/−; MMTV/NDL, Cox-2−/−; and MMTV/NDL, Cox-2−/−. Genotypes were determined by PCR analysis of tail-tip-derived genomic DNA as previously described (29, 30). Virgin test females were sacrificed at 20 weeks of age, and tissues were harvested as described below.

Mammary tissue harvesting and analysis. Abdominal (#4) mammary glands were either snap-frozen in liquid nitrogen and stored at −80°C or stained with carmine alum and mammary wholemounts prepared as previously described (31). Prostaglandin E2 (PGE2) in snap-frozen mammary glands was assayed as previously described (32). Tumor multiplicity was scored in carmine alum–stained mammary wholemounts in a blinded manner by two independent investigators using an eyepiece graticule on a dissecting microscope and a threshold diameter of 0.25 mm. To evaluate the effect of Cox-2 deficiency on tumor size, tumor multiplicity was rescored using two additional threshold diameters, 0.50 and 1.0 mm. Axillary (#3) mammary glands were assayed as previously described (32). Tumor multiplicity per gland (all tumors 0.25-mm diameter) for each mouse followed Poisson distributions, and that the expected numbers of tumors followed a log-normal distribution across mice within each genotype (Cox-2−/− versus Cox-2−/− versus Cox-2+/−), we tested whether the means of the expected numbers varied between genotypes using the likelihood ratio test. We also used ANOVA to test whether there was a difference in the arcsine-transformed proportions of larger (>1.0 mm) versus smaller (0.25-0.49 mm) tumors per mouse between genotypes.

Reverse transcription-PCR analysis. Quantitative reverse transcription-PCR (RT-PCR) was used to compare gene expression in mammary glands harvested from four animals each that were MMTV/NDL, Cox-2−/− and MMTV/NDL, Cox-2−/−. Total RNA was prepared from resected, snap-frozen mammary glands and cDNA was generated by reverse transcription as previously described (34). RT-PCR primers for total vascular endothelial growth factor (VEGF), angioptioin 1 (Ang1), Ang2, Flk-1, and Flt-1 were as previously described (35). Primers for the VEGF120 isoform and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer Express 2.0 software (PE Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. PEF120 primers were forward, 5′-GGAGATCCTTCGAGGAGCACTT-3′; reverse, 5′-GGCGATTTAGCAGCAGATAAAGA-3′. GAPDH primers were forward, 5′-CAACTCATGGTCTACATGTTCCAGTATG-3′; and reverse, 5′-ATGACGGCTTGGGTCCTCA-3′. Real-time PCR was done using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) and an ABI Prism 7900 HT Sequence Detection System (PE Applied Biosystems), and calculations done as previously described (34). Target gene expression levels were normalized to GAPDH expression level for each sample and expressed as a percentage of the mean value in mammary glands from MMTV/NDL, Cox-2−/− mice.

Statistical analysis. PGE2 levels in mammary glands from MMTV/NDL mice that were Cox-2−/− or Cox-2−/− were analyzed by paired comparisons with PGE2 levels in MMTV/NDL, Cox-2−/− mammary glands using ANOVA of log-transformed data. Tumor multiplicity data were analyzed as follows. A nonlinear mixed-effects model was fitted to the data using the SAS software NLMixed procedure. Assuming that the observed tumor multiplicity per gland (all tumors ≥0.25-mm diameter) for each mouse followed
A transgenic strain was selected as a relevant model of the human disease, based on previous observations of coordinate expression of HER2/neu and Cox-2 in human breast cancers (6, 10, 13, 36). In an earlier study, we used the MMTV/neu strain, which expresses a wild-type HER2/neu allele, to show that HER2/neu-induced mammary tumorigenesis is delayed by administration of the selective Cox-2 inhibitor celecoxib (37). However, the current experiment required a strain with accelerated tumor development because of the reduced life span previously reported for Cox-2 null mice (28). Therefore, we selected the MMTV/NDL strain which develops tumors more rapidly than MMTV/neu mice due to expression of a mutationally activated HER2/neu allele that resembles a splice variant detected in human breast carcinomas (29).

Our first priority was to characterize the time course of tumor development in MMTV/NDL mice to identify a suitable time point for our experiment. Virgin females that were hemizygous towards a lower proportion of larger tumors on October 23, 2017. © 2005 American Association for Cancer Research.

The Table 1. Mammary PGE2 levels correlate with Cox-2 gene dosage

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>PGE2 (ng/mg protein)*</th>
<th>P †</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMTV/NDL, Cox-2+/+</td>
<td>7</td>
<td>0.688 ± 0.114</td>
<td>—</td>
</tr>
<tr>
<td>MMTV/NDL, Cox-2+/-</td>
<td>5</td>
<td>0.532 ± 0.146</td>
<td>0.043</td>
</tr>
<tr>
<td>MMTV/NDL, Cox-2-/-</td>
<td>5</td>
<td>0.348 ± 0.068</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

*Mean ± SD.
†Relative to MMTV/NDL, Cox-2+/+.

we selected 20 weeks as a useful experimental time point at which animals would be expected to have developed multiple MIN lesions in each gland, enabling quantitation of tumor multiplicity. As previously observed in MMTV/neu mammary glands, Cox-2 was expressed in mammary tissues from MMTV/NDL mice, confirming this strain as a relevant model of human breast cancer (ref. 37; data not shown).

Our next step was to generate Cox-2-deficient MMTV/NDL mice. Because strain background is a well-established modifier of experimental tumor formation in mouse cancer models, we first introduced the targeted Cox-2 allele into an FVB background. Subsequently, MMTV/NDL and FVB-Cox-2+/+ mice were interbred to generate test MMTV/NDL females that were Cox-2+/+, Cox-2+/-, and Cox-2-/- mice. Mammary tissues were harvested from multiple virgin females of each genotype sacrificed at 20 weeks of age. Cox-2 deficiency led to a reduction in mammary PGE2 levels in MMTV/NDL mice: mammary PGE2 was reduced to ~50% of Cox-2 wild-type levels in Cox-2-null tissue (Table 1).

Tumor multiplicity was assayed in carmine alum-stained, whole-mounted mammary glands using a threshold diameter of 0.25 mm as the lower limit for inclusion as an epithelial lesion. The total number of tumors per gland was significantly reduced in mammary glands from both Cox-2 heterozygous and null animals relative to Cox-2 wild-type controls (Fig. 2A; P < 0.001). Mean tumor multiplicity was reduced by 50% in Cox-2 null animals from 26.7 to 13.3 tumors per gland. These data provide the first genetic evidence that Cox-2 contributes to HER2/neu-induced mammary tumorigenesis.

To investigate the effect of Cox-2 deficiency on tumor size, mammary tumor multiplicity was rescored in the same whole mounts using threshold diameters of 0.5 and 1.0 mm, and the percentage of tumors in each of three size categories was calculated. There was a significant decrease in the proportion of larger tumors in Cox-2 null animals relative to Cox-2 wild-type animals (Fig. 2B; P = 0.02). Together with the observed reduction in total tumor multiplicity, these data suggest that Cox-2 contributes not only to tumor formation but also to tumor growth.

A trivial explanation for the observed effects on tumor size and multiplicity could be that expression of the NDL transgene was reduced as a consequence of Cox-2 deficiency. However,
quantitative RT-PCR analysis revealed that transgene expression levels were similar in mammary glands from MMTV/NDL mice of all three Cox-2 genotypes (data not shown). An alternative confounder of our experimental approach was also considered; that is, that the effects of Cox-2 deficiency might be ascribable to reduced animal weight or viability, since dietary restriction and consequent reduction in body weight has been shown to diminish tumor formation in several animal models. However, all test animals were overtly healthy at sacrifice and animal weights were similar in all three groups. Weights at sacrifice (mean ± SD) of randomly selected animals were MMTV/NDL, Cox-2<sup>+/+</sup>, 24.9 ± 3.6 g (n = 18); MMTV/NDL, Cox-2<sup>−/−</sup>, 26.6 ± 4.1 g (n = 16); and MMTV/NDL, Cox-2<sup>/−</sup>, 23.5 ± 1.8 g (n = 9).

In addition to assaying tumor multiplicity, mammary morphology was compared in MMTV/NDL mice that were Cox-2 wild type and Cox-2 null (Fig. 3). HER2/neu-induced mammary hyperplasia was significantly attenuated in Cox-2 knockout mice and was completely absent in mammary glands from some animals such that the morphology resembled that of wild-type animals lacking the NDL transgene (compare with Fig. 1). Mammary morphology in MMTV/NDL, Cox-2<sup>−/−</sup> animals was intermediate between that of Cox-2 wild-type and null animals (data not shown).

Interestingly, we noted a marked reduction in blood vessels in the H&E-stained tissue sections from Cox-2 null animals (Fig. 3B, compare a-e with f-j). To substantiate this observation, tissue sections were stained with anti-CD31 (platelet/endothelial cell adhesion molecule 1) antibody to reveal the vascular architecture (Fig. 4). Mammary glands from MMTV/NDL, Cox-2<sup>+/+</sup> animals were highly vascularized with large, dilated vessels adjacent to normal-looking ducts (Fig. 4, a-c) and extensive vascularization of the MIN lesions (Fig. 4, d-f). Both the number and size of blood vessels were strikingly reduced in mammary glands from MMTV/NDL, Cox-2<sup>−/−</sup> animals. In the majority of fields examined, blood vessels were completely undetectable, and when present, vessels were only the size of capillaries (e.g., Fig. 4, h and i). Notably, blood vessels were virtually absent from both dysplastic regions and normal-looking areas within the mammary gland, suggesting a previously undescribed role of Cox-2 in vascularization of the mammary gland.

Consistent with the marked reduction in vasculature in Cox-2 null mammary glands, expression of angiogenesis-associated genes was decreased. Quantitative RT-PCR was used to compare the expression levels of six genes in mammary glands from MMTV/NDL virgin females that were Cox-2 wild type or Cox-2 null. Genes examined were VEGF (total VEGF and the VEGF120 isoform), Tie-2 ligands Ang1 and Ang2, and VEGF receptors Flt-1 and Flt-1. A uniform reduction in expression of all six genes examined was observed in Cox-2 null mammary glands (Fig. 5). Comparison of individual genes revealed that VEGF, Ang1, and Flt-1 were all expressed at significantly lower levels in tissues from Cox-2 nulls compared with tissues from Cox-2 wild-type mice (P = 0.016, 0.049, and 0.010, respectively). Additionally, there was a significant global reduction in expression of all six genes in mammary glands from Cox-2 nulls relative to Cox-2 wild-type animals (P = 0.025).

**Discussion**

Here we show for the first time that genetic ablation of the Cox-2 gene protects against mammary intraepithelial neoplasia, causing decreases in both number and size of mammary tumors (Fig. 2). Similar knockout approaches have previously established roles for Cox enzymes in both intestinal and skin tumorigenesis (38–41). Consistent with our genetic data, selective Cox-2 inhibitors are protective in rodent models of breast cancer (2, 3). However, interpretation of such pharmacologic studies is limited by the knowledge that these drugs may have Cox-independent effects (25–27). In this study, Cox-2 knockout mice have been used to definitively establish a role for Cox-2 in HER2/neu-induced mammary tumorigenesis. Interestingly, lobular hyperplasia in the MMTV/NDL strain is also reduced by Cox-2 deficiency (Fig. 3), suggesting that Cox-2 may contribute to early stages of preneoplastic development. Notable in this context is the recent report of focal areas of Cox-2 expression in histologically normal human breast tissue, which coincide with loss of p16<sup>INK4a</sup> expression due to hypermethylation of the CDKN2A promoter (9). Together, these data suggest that Cox-2 may be expressed in preneoplastic foci in the mammary gland as a very early aberration and may potentially contribute to the...
hyperplasia that precedes invasive breast cancer. Our data provide an important corollary to the work of Hla et al., who showed that transgenic Cox-2 overexpression caused extensive mammary hyperplasia and tumor formation in mice that had undergone multiple cycles of pregnancy and lactation (20, 33).

Interestingly, we observed equivalent reductions in tumor multiplicity and mammary PGE2 levels in the absence of Cox-2: both were reduced to ~50% of the values observed in Cox-2 wild-type mammary glands (Table 1; Fig. 2A). This correlation suggests a causal role for prostaglandins in mammary tumorigenesis, consistent with data from the MMTV/Cox-2 strain (20, 33). A further inference from these data is that Cox-1-derived prostaglandins may be contributing to mammary neoplasia in the Cox-2-deficient mice. Consistent with this notion, previous studies using knockout mice have shown that both Cox-1 and Cox-2 can contribute to intestinal and skin tumorigenesis (38–40, 42), and we have observed Cox-1 expression in HER2/neu-induced murine mammary tumors (data not shown). Thus, it is possible that the sum total of eicosanoid production from Cox-1 and Cox-2 is an important determinant of tumor formation.

A striking reduction in vascularization was observed in Cox-2 null mammary tissues (Fig. 4). We had anticipated that Cox-2 deficiency might affect mammary tumor vascularization based on extensive data linking Cox-2 with angiogenesis (43, 44). Thus, selective Cox-2 inhibitors have been shown to reduce neovascularization in vivo (33, 45–49), and, in vitro, selective Cox-2 inhibitors can decrease endothelial tubule formation (50, 51). Additionally, tumor xenografts show markedly diminished growth and vascular density when implanted into Cox-2 null versus wild-type hosts (41), and conversely, Cox-2 overexpression in mouse mammary gland drives extensive vascular development and tumorigenesis (20, 33). Consistent with a role for Cox-2 in breast tumor angiogenesis, correlations have been identified between Cox-2 expression and microvessel density/VEGF expression in human breast cancers (14, 52–54), and Cox-2 inhibitors have been shown to decrease tumor angiogenesis in rodent breast cancer models (55–57). However, previous studies in mouse colorectal cancer models have suggested that Cox-2 contributes primarily to growth and vascularization of tumors beyond 1 mm in diameter, and consistent with this, Cox-2 expression in human colorectal cancer increases coordinately with tumor size (42, 58–60). In marked contrast, we have identified defective vascularization of both tumors and apparently normal mammary gland in Cox-2 knockout mice. Blood vessels were virtually undetectable in mammary tissues from Cox-2 null mice, either in association with MIN lesions or adjacent to normal-looking mammary ductal structures (Figs. 3 and 4), suggesting a previously unanticipated role for Cox-2 in mammary vascular development. We speculate that this may relate to the unique composition of the mammary gland, specifically the

![Figure 4. Mammary glands from Cox-2 null MMTV/NDL mice exhibit strikingly diminished vasculature.](image)

![Figure 5. Expression of angiogenesis-related genes is decreased in Cox-2 null MMTV/NDL mice. Quantitative RT-PCR was used to compare expression levels of six angiogenesis-associated genes in mammary glands harvested from 20-week-old virgin MMTV/NDL females that were Cox-2 wild type (+/+) or Cox-2 null (−/−) mice. Columns, means normalized to the mean expression level of that gene in MMTV/NDL, Cox-2+/+ mammary glands (n = 4 in each group); bars, +SE. Comparison of individual genes in Cox-2 null mammary glands relative to Cox-2 wild-type mammary glands revealed that expression of VEGF, Ang1, and Flt-1 was significantly reduced (P = 0.016, 0.049, and 0.010, respectively). The average of log values across all six genes for each mouse, representing a global effect, was significantly higher in wild-type tissues than in null tissues at P = 0.025.](image)
high adipose content of the mammary fat pad, because there appears to be an intimate relationship between adipose tissue and angiogenesis. Adipocytes express high levels of VEGF, and inhibition of angiogenesis antagonizes adipocyte differentiation (61–63). Furthermore, serum VEGF levels correlate with body mass index and visceral fat area in obese human subjects (64), while in obese mice, weight reduction and loss of adipose tissue can be induced by anti-angiogenic treatments (65). Together, these data indicate a unique reciprocal relationship between angiogenesis and adipogenesis, which may relate to the role of Cox-2 in mammary gland vascularization.

Consistent with the diminished vascularization in Cox-2 null mammary glands, we also observed significant reductions in expression levels of proangiogenic genes (Fig. 5). This is likely attributable, at least in part, to the Cox-2 gene dosage–dependent reduction in prostaglandins, because PGE2 can regulate VEGF expression (66). Cox-2–/– fibroblasts have a 94% reduction in the ability to produce VEGF relative to wild-type fibroblasts (41), and Cox-2 inhibitor treatment of a Cox-2-overexpressing colorectal cancer cell line diminishes secretion of proangiogenic factors (51).

Conversely, transgenic overexpression of Cox-2 in mammary tissues causes up-regulation of multiple angiogenesis-associated genes including VEGF, Ang1, Ang2, and Flk-1 (33). Together the Cox-2 overexpression study and our current knockout study suggest that Cox-2–derived prostanoids may regulate expression not only of VEGF and VEGF receptors but also of angiopoietins (Ang1 and Ang2) in mammary tissues. In contrast, defective uterine angiogenesis in Cox-2-deficient mice during embryo implantation correlates with diminished expression of VEGF and the VEGFR Flk-1, but angiopoietin signaling is unaltered (67). Thus, Cox-2 may differentially regulate VEGF and angiopoietin pathways in individual tissues.

In combination, previous pharmacologic studies and our current genetic data demonstrate unequivocally that Cox-2 contributes to mammary neoplasia (Figs. 2 and 3; refs. 2, 3). Because Cox-2 expression has been detected at high frequencies in human DCIS, a common precursor to invasive breast cancer, inhibiting prosta-glandin synthesis may reduce the risk of breast cancer. Clinical trials have been initiated to further evaluate this question.

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References

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