Overexpression of Cyclooxygenase-2 Is Sufficient to Induce Tumorigenesis in Transgenic Mice*

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The cyclooxygenase (COX)-2 gene encodes an inducible prostaglandin synthase enzyme that is overexpressed in adenocarcinomas and other tumors. Deletion of the murine COX-2 gene in Min mice reduced the incidence of intestinal tumors, suggesting that it is required for tumorigenesis. However, it is not known if overexpression of COX-2 is sufficient to induce tumorigenesis. We have derived transgenic mice that overexpress the human COX-2 gene in the mammary glands using the murine mammary tumor virus promoter. The human COX-2 mRNA and protein are expressed in mammary glands of female transgenic mice and were strongly induced during pregnancy and lactation. Female virgin COX-2 transgenic mice showed precocious lobuloalveolar differentiation and enhanced expression of the β-casein gene, which was inhibited by the COX inhibitor indomethacin. Mammary gland involution was delayed in COX-2 transgenic mice with a decrease in apoptotic index of mammary epithelial cells. Multiparous but not virgin females exhibited a greatly exaggerated incidence of focal mammary gland hyperplasia, dysplasia, and transformation into metastatic tumors. COX-2-induced tumor tissue expressed reduced levels of the proapoptotic proteins Bax and Bel-xL and an increase in the anti-apoptotic protein Bel-2, suggesting that decreased apoptosis of mammary epithelial cells contributes to tumorigenesis. These data indicate that enhanced COX-2 expression is sufficient to induce mammary gland tumorigenesis. Therefore, inhibition of COX-2 may represent a mechanism-based chemopreventive approach for carcinogenesis.

The cyclooxygenase (COX) enzymes, COX-1 and -2, catalyze the rate-limiting steps in the biosynthesis of prostaglandins and thromboxanes, collectively known as prostanoids. Regulation of expression of COX-1 and -2 is distinct; COX-1 is expressed ubiquitously, whereas COX-2 is induced as an immediate-early gene in most cells (1–3). Various extracellular stimuli, including growth factors, cytokines, tumor promoters, peroxisomal proliferators, and carcinogens, induce COX-2 expression (1–6). Although transcriptional regulation of COX-2 has been studied extensively, post-transcriptional mechanisms are also important for COX-2 expression (1–3).

A strong correlation has been established between the use of non-steroidal anti-inflammatory drugs (NSAIDs) and the decreased incidence of colorectal, breast, and lung cancers (4–8). In addition, NSAIDs and COX-2 inhibitors suppress carcinogen-induced tumorigenesis in animal models (9–11). Such data have prompted the examination of expression of COX-1 and -2 in human cancer tissues. COX-1 is expressed in both normal and malignant cells; however, COX-2 is up-regulated in a high percentage of tumors (4–10). This finding was established originally in gastrointestinal tumors but has been extended to carcinomas of diverse origins (4–8, 12–14). Indeed, recent studies have indicated that high levels of COX-2 are expressed in human mammary tumor tissues compared with the adjacent normal tissue (13, 14). Gene deletion studies support the concept that COX-2 plays a critical role in the development of intestinal tumors (15). This work has been confirmed not only in intestinal tumors but also in carcinogen-induced model of skin cancer (16, 17). Surprisingly however, deletion of the murine COX-1 gene also reduced the incidence of both intestinal and skin tumors (16, 17). The COX-2 gene was expressed in the mesenchymal compartment in the neoplastic tissue in the mice, which is in contrast with both mesenchymal and epithelial expression in human tumors (15). These genetic loss-of-function studies suggest that expression of COX-1 and -2 at the site of transformation or at some distal site is necessary for tumorigenesis.

In contrast, other studies show that inhibition of COX-2 by NSAIDs cannot account for their anti-tumor effects. For example, sulindac sulfone, a derivative of sulindac which lacks COX-1 or -2 enzymatic inhibitory activity, induced epithelial cell apoptosis and inhibited carcinogenesis in animal models (18, 19). Furthermore, various NSAIDs, albeit at high concentrations, inhibited growth and tumorigenicity of transformed mouse embryonic fibroblasts lacking both COX-1 and COX-2 (20). Several other targets of NSAID action have also been proposed. First, the nuclear receptor peroxisomal proliferator-activated receptor (PPAR) was induced by the APC mutation, which resulted in the transcriptional enhancement of PPARα via the β-catenin pathway. High doses of NSAIDs inhibited the action of PPARα on transcriptional regulation of downstream genes, suggesting that it might be one of the targets for NSAID inhibition of carcinogenesis (21). Second, NSAIDs at high doses inhibited the activity of the nuclear factor κB pathway and thus may promote cell death in a prostanooid independent manner (22).
Northern analysis for human Cox-2 and mouse Cox-1 was done as described. Mammary glands (200 mg) from lactating Cox-2 and normal mice animals were superfused with 12 m
non-transgenic animals with the Cox-2 antibody was negative. Developing alveolar structure indicating perinuclear staining for Cox-2. Staining of similar sections with an irrelevant mouse IgG or staining of
Panel F
eicosanoids secreted into the medium were extracted, separated by TLC, and exposed to an autoradiographic film. The bands were identified by

FIG. 1. Generation of transgenic mice that overexpress Cox-2 in mammary glands. Panel A, scheme of the construct. The MMTV promoter was cloned upstream of the human Cox-2 gene (23, 24), and the resulting construct was used to derive transgenic mice as described. Panel B, Northern analysis of mammary tissues from Cox-2 transgenic mice (Cox-2) and normal mice (NM). Mammary tissues from virgin (V, 16 weeks), pregnant (P, 18 days pregnant), lactating (L, 7 days postpartum), and weaning (W, 14 days postweaning) mice were used to purify RNA, and Northern analysis for human Cox-2 and mouse Cox-1 was done as described. Panel C, immunoblot analysis of mammary tissues. Extracts were prepared from mammary tissues of Cox-2 transgenic and normal mice, and immunoblot analysis was conducted using the Cox-2 or the β-actin antibody as described. Panel D, immunochemistry of mammary tissues from Cox-2 transgenic mice. Mammary glands from 18-days-pregnant Cox-2 transgenic mice were fixed and analyzed by immunohistochemical procedures for expression of the Cox-2 antigen. Note the specific expression in mammary epithelia of alveolar and ductal structures (asterisk, magnification ×20). The inset shows the ×40 magnification of a developing alveolar structure indicating perinuclear staining for Cox-2. Staining of similar sections with an irrelevant mouse IgG or staining of non-transgenic animals with the Cox-2 antibody was negative. Panel E, TLC analysis of radioactive eicosanoids secreted by mammary glands. Mammary glands (200 mg) from lactating Cox-2 and normal mice animals were superfused with 12 μM [14C]arachidonic acid for 30 min, and eicosanoids secreted into the medium were extracted, separated by TLC, and exposed to an autoradiographic film. The bands were identified by comigration with authentic cold standards. Panel F, mammary tissues from Cox-2 and normal mice were superfused with 12 μM arachidonic acid for 30 min, medium was extracted, and PGE2 was quantified by a radioimmunoassay as described. Results represent the mean ± S.D. from mammary glands derived from four animals.

These studies have raised questions about the causal role of Cox-2 in tumorigenesis and have identified potential non-Cox-2 targets of NSAID action.

The question of whether Cox-2 overexpression is sufficient to induce tumorigenesis has not been addressed. In this report, we describe a genetic gain-of-function approach to examine the direct role of Cox-2 in tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Generation of Transgenic Mice**—The human COX-2 gene (23) was cloned behind the murine mammary tumor virus (MMTV) promoter (24). The linearized construct was used to derive transgenic CD1 mice (Charles River Laboratories) in the UCHC transgenic core facility. Transgenic mice were identified by Southern blot analysis of tail DNA using a human Cox-2 3′-untranslated region specific probe. Three founder animals (two males and one female) were used to derive F1 and F2 hemizygotic transgenic mice. Gene expression and phenotypic analysis of the linearized construct, were bred with CD1 mice, formed as described (29) on formallin-fixed, paraffin-embedded sections

**Northern and Western Analysis of Mammary Glands**—Mammary glands were dissected and processed for whole mount analysis as described (24, 26). Some glands were fixed and analyzed by histological methods (28). Immunohistochemistry for human Cox-2 was conducted as described (12, 28) using the Cox-2 monoclonal (27) and polyclonal antisera (Cayman Chemical). Specificity of immunostaining was confirmed by competition with excess peptide antigen as described (12, 28).

**Arachidonic Acid Metabolism in Mammary Glands**—Mammary glands were dissected, cut into 1-mm³ sections, washed extensively, and incubated with 12 μM [1-14C]arachidonic acid (PerkinElmer Life Sciences) for 30 min at 37 °C. Medium was acidified, extracted, and prostanooids were separated and identified by TLC/autoradiography procedures as described previously (25). In some experiments, cold arachidonic acid was used, and medium was analyzed for PGE2 synthesis by radioimmunoassay as described (25, 27).

**Whole Mount, Histological Methods, and Immunohistochemistry**—Mammary glands were dissected and processed for whole mount analysis as described (24, 26). Some glands were fixed and analyzed by histological methods (28). Immunohistochemistry for human Cox-2 was conducted as described (12, 28) using the Cox-2 monoclonal (27) and polyclonal antisera (Cayman Chemical). Specificity of immunostaining was confirmed by competition with excess peptide antigen as described (12, 28).

**Apoptosis Assay**—Terminal nucleotidyl transferase staining was performed as described (29) on formalin-fixed, paraffin-embedded sections (4 μm) with a in situ cell death detection kit (Roche, Indianapolis) according to the manufacturer's instructions. Sections were counterstained with methyl green (Vector Laboratories, Burlingame, CA). The percentage of apoptotic cells was calculated from randomly selected fields (total of 4,000 cells/section) at a magnification of ×60. Statistical analysis was done using Student’s t test.

**RESULTS**

The MMTV promoter (24) was used to direct expression of the human COX-2 gene in transgenic mice (Fig. 1A). Three independent founder lines, obtained after pronuclear microinjection of the linearized construct, were bred with CD1 mice,
Virgin normal (NM) mice or Cox-2 mice were administered indomethacin (14 μg/ml in water) or vehicle for 12 weeks beginning at 4 weeks of age. Mammary glands were analyzed by whole mount analysis and photographed under a stereomicroscope (magnification, ×1). Panel B, histology. Mammary tissues from 16-week-old virgin normal mice or Cox-2 mice were analyzed by hematoxylin and eosin staining of histological sections (magnification, ×20). Note that alveolar differentiation is apparent in Cox-2 mice, whereas normal mice show only ductal structures. Panel C, gene expression. RNA was purified from virgin 16-week-old normal or Cox-2 transgenic mice (lanes 1 and 2) that were treated or not with indomethacin for 1 week. Expression of β-casein or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed by a Northern blot as described. Panel D, indomethacin treatment and PGE2 synthesis. Normal or Cox-2 mice were treated with indomethacin for 12 weeks as described for panel A. Synthesis of PGE2 by mammary gland explants was quantified by radioimmunoassay.

and the hemizygous F1 and F2 mice were characterized for the studies described below. By Northern analysis, human Cox-2 transgenic mRNA was detected only in mammary glands and not in liver, lung, heart, brain, spleen, or muscle tissues (data not shown). RNA samples were isolated from the mammary gland during progressive stages of maturation. Northern blot analysis was conducted to determine the expression of endogenous murine Cox-1 and Cox-2 as well as transgenic human Cox-2 mRNA. High stringency hybridization conditions (20% formamide, 65 °C) were used to discriminate between mouse and human Cox-2 mRNAs. Only endogenous murine Cox-1 mRNA was expressed in the mammary glands of virgin mice. During lactation, Cox-1 expression was induced and returned to base line after the involution of the mammary gland in weaned mothers (Fig. 1B). Murine Cox-2 mRNA was not detected by Northern analysis of total RNA preparations, suggesting that endogenous Cox-2 is expressed at extremely low levels during the development and involution of the mammary gland. Reverse transcription-polymerase chain reaction analysis for murine Cox-2 confirmed the Northern data and suggests that it is expressed at extremely low levels in normal mammary tissue (data not shown). In the human Cox-2 transgenic mouse, a low level of Cox-2 mRNA was detected in mature virgin mice, but significantly higher levels were induced during pregnancy and lactation. Concomitant with the regression of the mammary gland in weaning females, the transgenic Cox-2 expression was attenuated after removal of nursing young (Fig. 1B). The highest level of Cox-2 expression was observed during lactation, which is consistent with the activity of the MMTV promoter (24). The Cox-2 transcript was not detected by Northern analysis in non-transgenic normal mice. Expression of the human Cox-2 mRNA did not alter the expression of endogenous murine Cox-1 and -2.

Immunoblot analysis of mammary tissues indicates that the human Cox-2 polypeptide accumulates concomitantly with the transcript (Fig. 1C). Immunohistochemistry of mammary tissue sections was conducted using the human Cox-2-specific monoclonal antibody (Fig. 1D). The expression of the Cox-2 transgene was observed in the mammary epithelial cells of developing alveoli. In contrast, stromal cells in the mammary gland do not express the transgenic Cox-2. Prominent perinuclear immunostaining was observed, consistent with previous studies that demonstrated endoplasmic reticulum localization of the Cox-2 polypeptide (30).

The functionality of the transgenic Cox-2 polypeptide was demonstrated by thin layer chromatographic analysis of radioactive arachidonic acid metabolites secreted from mammary gland explants. As shown in Fig. 1E, enhanced synthesis of PGE2, 6-keto-PGF1α, PGD2, and PGF2α was observed in Cox-2 transgenic mammary glands compared with the age-matched normal counterparts. Quantitative analysis of PGE2 synthesis by mammary gland explants (Fig. 1F) was conducted next in mammary glands from normal as well as Cox-2 transgenic mice. Mammary glands from non-transgenic mice also exhibited an increase in Cox activity during lactation, which is consistent with the induction of endogenous Cox-1 mRNA expression. The human Cox-2 transgenic mice exhibited a higher level of PGE2 synthesis, which correlated with the expression of the Cox-2 mRNA and polypeptide. These data suggest that overexpression of Cox-2 was achieved in the mammary glands of transgenic mice, particularly during pregnancy and lactation.

The mammary glands of Cox-2 transgenic mice were analyzed by morphological methods. Analysis of whole mount preparations of virgin mammary glands demonstrated abnormal and hyperplastic alveolar development (Fig. 2A). Histological analysis of mammary sections confirmed precocious development of alveolar glands in Cox-2 transgenic mice (Fig. 2B). Northern blot analysis indicated that the expression of the β-casein, a gene normally expressed during late pregnancy and
Cox-2 Induction of Tumorigenesis

Mammary gland involution occurred in Cox-2 transgenic mice. Mammary glands from control mice showed expected rates of epithelial cell death and collapse of the alveolar glands at 1–2 days postweaning, resulting in the regression of the mammary gland. Although mammary glands from Cox-2 transgenic mice regressed at 7 days, the rate was delayed at early stages (days 1–2), no signs of involution were apparent (Fig. 3B). 2 weeks after weaning, the mammary glands appeared similar in both Cox-2 transgenic and normal mice. Thus enhanced Cox-2 expression at 0–2 days postweaning may have maintained the viability and function of the mammary epithelial cells.

Because cellular apoptosis contributes to the involution of the mammary gland, we measured the rates of apoptosis in mammary glands of normal and Cox-2 transgenic mice after weaning using the terminal nucleotidyl transferase method (29). As shown in Fig. 3C, significant decreases in apoptotic cells were observed in Cox-2 transgenic mice compared with non-transgenic normal mice at 2 days after weaning. These observations are consistent with the previous findings that enhanced Cox-2 expression inhibits cellular apoptosis in transfected epithelial cells (31).

The incidence of mammary tumors in Cox-2 transgenic mice and non-transgenic mice were analyzed by a combination of whole mount analysis and histological sections. As shown in Fig. 4B, >85% of Cox-2 transgenic mice that have undergone multiple cycles of pregnancy and lactation show the presence of tumors in mammary glands. In contrast, a very low incidence of tumors was seen in non-transgenic mice with similar age and multiparous. Transgenic female mice derived from all three founders showed this phenomenon, suggesting that the effects are caused by the expression of the human COX-2 gene rather than integration-dependent events. Cox-2 transgenic mice that did not undergo pregnancy and lactation did not show the increased mammary tumorigenesis. Three female Cox-2 transgenic mice (the founder female and two F1 females from a different founder) developed large mammary tumors with metastatic spread. One such animal at necropsy is shown in Fig. 4C, as an example of the presence of large metastatic tumors in and outside of the mammary glands.

Histological analyses of mammary tissue from non-transgenic mice and from Cox-2-induced tumors are shown in Fig. 5, A and B, respectively. In Fig. 5B, one can observe numerous hyperplastic glandular structures, keratinizing squamous cells (indicating metaplasia), stromal proliferation, and numerous blood vessels. In Fig. 5C, adenomatous carcinoma was observed in the peripheral lymph node. Well differentiated tumors appeared to have metastasized into the lymph nodes of the Cox-2 transgenic mice. In Fig. 5D, an example of a mammary tumor with squamous metaplasia is shown. In Fig. 5E, an example of a hyperplastic and invasive ductal structure with hemosiderin (indicating bleeding into the ducts) is shown. These data suggest that Cox-2 overexpression induced various types of invasive mammary tumors, including ductal and lobuloalveolar carcinomas.

Mammary tumors in the Cox-2 transgenic mice were stained

![Fig. 3. Delayed mammary gland involution in Cox-2 transgenic mice.](image)

Lactating Cox-2 or normal (NM) transgenic mice were weaned by removing the pups at day 0. At indicated times after weaning, mammary glands were dissected out and analyzed for expression of Cox-2 by immunoblot analysis (panel A) and histology by hematoxylin and eosin staining of sections (panel B) as described. Note that in panel B (magnification, ×40), a delay in mammary gland involution was seen in Cox-2 transgenic mice. Panel C, detection of apoptotic cells in involuting mammary glands. Mammary tissue was collected from four normal and six Cox-2 mice 2 days after weaning and analyzed by terminal nucleotidyl transferase assay on paraffin sections as described under “Experimental Procedures.” Results are shown as mean ± S.E. * indicates a p value of <0.05 as determined by Student’s t test.
with the Cox-2 antibody using immunohistochemical procedures (Fig. 5F). Numerous epithelial cells in glandular structures exhibited strong immunoreactivity in the perinuclear locale. Immunoreactivity was competed by coincubation of the antibody with the Cox-2 antigenic peptide (data not shown). These data suggest that Cox-2 is expressed in the mammary tumors of Cox-2 transgenic mice.

To determine if decreased apoptosis of mammary epithelial cells contributed to the Cox-2-induced tumorigenesis, we analyzed the expression of apoptotic regulatory proteins Bcl-2, Bax, and Bcl-xL. Expression of Bcl-2 was induced by Cox-2 overexpression in intestinal epithelial cells (31), and Bax is known to regulate NSAID-induced apoptosis (32). As shown in Fig. 6, high levels of Cox-2 polypeptide were observed in the mammary tumors and lower levels were seen in the adjacent normal tissue. In contrast, Cox-2 polypeptide was not expressed in mammary glands of multiparous, non-transgenic mice. Interestingly, expression of the proapoptotic proteins Bax and Bcl-xL was reduced in Cox-2-expressing tumors. In contrast, Bcl-2 expression was up-regulated in the tumor tissue. These data suggest that regulation of expression of apoptosis regulatory factors Bcl-2, Bax, and Bcl-XL may be a mechanism via which Cox-2 overexpression contributes to tumorigenesis.

Together, these observations support the concept that overexpression of Cox-2 alone is sufficient to transform the mammary epithelium into a tumorigenic state.

**DISCUSSION**

In this report, we describe a gain-of-function approach to evaluate the direct role for Cox-2 in tumorigenesis. Although studies utilizing various approaches, namely, epidemiologic, expression studies and genetic loss-of-function approaches, have implicated the functional role of Cox-2 in tumorigenesis, questions remain regarding the requirement and sufficiency of Cox-2 (4–8, 18, 19). Furthermore, NSAIDs were shown to inhibit various animal models of tumorigenesis; however, issues of nonspecificity have prevented the unequivocal interpretation of these studies (18–22). In this study we provide evidence that overexpression of Cox-2 alone is sufficient to induce tumorigenic transformation in a tissue-specific manner.

Overexpression of Cox-2 expression was achieved using the entire human COX-2 gene (23) under the control of the MMTV promoter (24). Previously, we attempted to derive transgenic mice using the keratin K14-driven human Cox-2 cDNA (33) and cytomegalovirus promoter-driven human Cox-2 cDNA in a tetracycline-regulated system (34). Both approaches were unsuccessful. In the former case, embryonic lethality appears to be induced by the developmental expression of the transgene. In the second case, the Cox-2 cDNA was not expressed after induction in two independent lines of transgenic mice. Therefore, we used the MMTV promoter, which is known to be induced postpartum in a mammary gland-enriched manner (24). In addition, we utilized the entire human COX-2 gene to include all of the post-transcriptional signals required for efficient expression of the gene (23, 25).

As shown in Fig. 1, MMTV-hCox-2 transgenic mice expressed high levels of Cox-2 transcript, protein, and enzymatic activity in the mammary glands. Specifically, expression of the transgenic Cox-2 polypeptide was induced during mammary gland development, achieved very high levels during pregnancy, and was maximal during lactation. Although the expression of Cox-2 is low in normal mammary glands, it can be induced by a high fat diet and carcinogen exposure (35, 36). However, significant expression of Cox-1 was observed in murine mammary tissue during all phases of mammary gland development. In addition, Cox-1 activity was induced during lactation.

Cox-2 enzymatic activity followed a kinetics of induction similar to that of the mRNA and the protein, resulting in the production of PGE₂, 6-keto-PGF₁α, PGD₂, and PGF₂α in the human Cox-2 transgenic mammary glands. After weaning, Cox-2 expression decayed rapidly, consistent with the short half-life of the mRNA and the protein (1–3). Thus the MMTV-hCox-2 transgenic mouse model provides an animal model in which persistent low level expression of Cox-2 is achieved in mammary epithelial cells, and exaggerated expression of Cox-2 can be induced during pregnancy and lactation.

Interestingly, MMTV-hCox-2 mice exhibited precocious development of the mammary glands as virgins. This is most likely the result of the secretion of prostanooids because indomethacin treatment reversed the changes. Mammary development is controlled by hormonal factors as well as by local signals (37). It is known that PGE₂ induces mammary epithelial cell proliferation (38). In addition, PGE₂ is known to induce aromatase, an enzyme involved in the local biosynthesis of estrogen, a potent inducer of mammary gland development (39). Thus, low level persistent expression of Cox-2 may induce precocious mammary gland development via the secretion of PGE₂.

MMTV-hCox-2 transgenic mice exhibited a delayed mam-
Fig. 5. Histological Analysis of Cox-2-induced mammary tumors. Panel A shows the section of a multiparous normal mouse which indicates unremarkable mammary tissue (×20). Panel B shows an example of an adenocarcinoma-type mammary tumor in multiparous Cox-2 transgenic mice (×20). Note the presence of numerous glandular structures, blood vessels, and accumulated keratin (white arrowheads), which indicates squamous metaplasia. Panel C, lymph node metastasis in Cox-2 transgenic founder mice. Abdominal lymph nodes were dissected and analyzed by histology. Note the well differentiated mammary carcinoma with abundant stroma and calcification (white arrowheads) which have invaded theymphatic tissue (white asterisk, ×20). Panel D, example of a mammary tumor in multiparous Cox-2 transgenic mice with abundant keratin, squamous metaplastic epithelial cells, and invasive tumor cells (×20). Panel E, example is shown of a mammary duct in multiparous Cox-2 transgenic mice with invasive ductal hyperplasia (asterisk) and bleeding into the duct as indicated by hemosiderin (×40). Panel F, expression of Cox-2 in mammary tumors in Cox-2 transgenic mammary glands. Sections of mammary glands from multiparous Cox-2 transgenic animals were stained with the Cox-2 antibody as described. Note the high expression of Cox-2 in glandular structures (black arrows) and weaker staining in the stroma but not in the adipocytes (black asterisks) (×64).

Fig. 6. Expression of Cox-2 and apoptotic regulatory proteins in mammary glands of Cox-2 transgenic mice. Involved mammary glands from multiparous animals were dissected, and protein extracts were analyzed for the expression of Cox-2, Bcl-2, Bax, and Bcl-xL as described. Lanes 1 and 2, normal mammary tissue from non-transgenic mice; lane 3, tumor tissue from Cox-2 transgenic mammary gland; lane 4, adjacent normal tissue from Cox-2 transgenic mammary gland.

Cox-2 Induction of Tumorigenesis

Cox-2 induction and a second mutation are required to transform the mammary epithelium completely. Indeed, proliferating epithelial cells in the pregnant mammary gland may be more susceptible to mutagenic events of critical tumor suppressor genes. This observation is similar to the phenotype of other transgenes such as MMTV-cyclin D1 and MMTV-stromelysin (41, 42). Nevertheless, these findings point out that overexpression of Cox-2 alone is sufficient to induce tumorigenic transformation.

The molecular mechanisms responsible for Cox-2 induction of tumorigenesis are unclear at present. Secreted prostanooids may interact with plasma membrane-localized G protein-coupled receptors to induce mitogenic or anti-apoptotic signals (1–3). Alternatively, nuclear receptors such as PPARγ and PPARδ, which are activated by prostanooids, may be involved (1–3, 21). The possibility that the peroxidase action of Cox-2 is involved in tumorigenesis also cannot be ruled out at present (1–3). The animal model described in this study may provide a useful system to begin to address these issues. However, Cox-2-induced tumors expressed high levels of Cox-2, the anti-apoptotic protein Bcl-2, and Bcl-xL, as described. Lanes 1 and 2, normal mammary tissue from non-transgenic mice; lane 3, tumor tissue from Cox-2 transgenic mammary gland; lane 4, adjacent normal tissue from Cox-2 transgenic mammary gland.

Many risk factors for human breast cancer induce Cox-2 expression. For example, dietary n-6 fatty acids, activation of HER/Neu signaling, Wnt signaling, as well as carcinogen exposure induce Cox-2 gene expression (3, 8, 36, 43–45). In addition, data from carcinogen-induced rodent models of mammary cancer suggest that Cox-2 inhibition reduces the incidence of mammary tumors (10, 11). Our data are consistent with the concept that overexpression of Cox-2 in the mammary epithelium is sufficient to induce mammary carcinogenesis. Coupled with the strong epidemiological evidence of NSAID usage and cancer incidence in humans (7), these findings strongly support the notion that dysregulated and exaggerated expression of Cox-2 are strong carcinogenic risk factors. Whether inhibition of Cox-2 in high risk populations would confer an anti-cancer benefit in human breast cancer warrants further study. However, efforts to define chemopreventive agents that work via Cox-2 inhibition will be greatly facilitated by this human Cox-2 transgenic model. Furthermore, mechanistic studies and identification of downstream genetic targets of Cox-2 action may be readily approached in this model.
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REFERENCES

1. Smith, W. L., DeWitt, D. L., and Garavito, R. M. (2000) Annu. Rev. Biochem. 69, 145–82.
2. Vane, J. R., Bakhle, Y. S., and Botting, R. M. (1998) Annu. Rev. Pharmacol. Toxicol. 38, 97–120.
3. Hla, T., Bishop-Bailey, D., Liu, C. H., Schaefers, H. J., and Trifan, O. C. (1999) Int. J. Biochem. Cell Biol. 31, 551–557.
4. Prescott, S. M., and Fitzpatrick, F. A. (2000) Biochim. Biophys. Acta 1470, M69–M78.
5. Taketo, M. M. (1998) J. Natl. Cancer Inst. 90, 1529–1536.
6. Taketo, M. M. (1999) J. Natl. Cancer Inst. 91, 1669–1620.
7. Thun, M. J. (1996) Annu. Rev. Pharmacol. 36, 123–127.
8. Williams, C. S., Mann, M., and DuBois, R. N. (1999) Oncogene 18, 7908–7916.
9. Reddy, B. S., Tokumo, K., Kulkarni, N., Aligia, C., and Kelloff, G. (1992) Cancer Res. 52, 652–661.
10. Nakatsugi, S., Ohta, T., Kawamori, T., Mutoh, M., Tanigawa, T., Watanabe, K., Sugie, S., Suginura, T., and Wakabayashi, K. (2000) Jpn. J. Cancer Res. 91, 886–892.
11. Harris, R. E., Alshafie, G. A., Abou-Issa, H., and Seibert, K. (2000) Cancer Res. 60, 2101–2103.
12. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. (1995) Cancer Res. 55, 3785–3789.
13. Hwang, D., Scollard, D., Byrne, J., and Levine, E. (1998) J. Natl. Cancer Inst. 90, 455–460.
14. Esler, R. A., Dannenberg, A. J., Rush, D., Woerner, B. M., Khan, K. N., Masteller, J., and Koli, A. T. (2000) Cancer 90, 2637–2645.
15. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. (1996) Cell 87, 803–809.
16. Chiu, J. C., Thompson, M. B., Mahler, J. F., Doyle, C. M., Gaul, B. W., Lee, C., Tiano, H., Mornah, S. G., Smithies, O., and Langenbach, R. (2000) Cancer Res. 60, 4705–4712.
17. Langenbach, R., Loftin, C. D., Lee, C., and Tiano, H. (1999) Ann. N. Y. Acad. Sci. 869, 92–101.
18. Piazza, G. A., Rahm, A. K., Finn, T. S., Fryer, B. H., Li, H., Stoumen, A. L., Pamukcu, R., and Ahnen, D. J. (1997) Cancer Res. 57, 2452–2459.
19. Booth, S. K., Dannenberg, A. J., Chadburn, A., Martucci, C., Guo, X. J., Ramonetti, J. T., Albrecht, M., Newmark, H. L., Lipkin, M. L., DeCosse, J. J., and Bertagnolli, M. M. (1996) Cancer Res. 56, 2556–2260.
20. Zhang, X., Morham, S. G., Langenbach, R., and Young, D. A. (1999) J. Exp. Med. 190, 451–459.
21. He, T. C., Chan, T. A., Vogelstein, B., and Kinzler, K. W. (1999) Cell 99, 335–345.
22. Yin, M. J., Yamamoto, Y., and Gaynor, R. B. (1998) Nature 396, 77–80.
23. Appleby, S. B., Ristimaki, A., Neilson, K., Narko, K., and Hla, T. (1994) Biochem. J. 302, 723–727.
24. Lane, T. F., and Leder, P. (1997) Oncogene 15, 2133–2144.
25. Hla, T., and Neilson, K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7384–7388.
26. Sympson, C. J., Tailou, R. S., Alexander, C. M., Chin, J. R., Cliff, S. M., Bissell, M. J., and Werb, Z. (1994) J. Cell Biol. 125, 681–693.
27. Ang, B. C., Sanchez, T., Schaefers, H. J., Tripan, O. C., Liu, C. H., Creminon, C., Huang, C. K., and Hla, T. (2000) J. Biol. Chem. 275, 39507–39515.
28. Narko, K., Ristimaki, A., MacPhee, M., Smith, E., Haudenschild, C. C., and Hla, T. (1997) J. Biol. Chem. 272, 21451–21460.
29. Etsuko, T., Susaki, M., and Kozuka, M. (2000) Adv. Exp. Med. Biol. 478, 369–370.
30. Morita, J., Schindler, M., Regier, M. K., Otto, J. C., Hori, T., DeWitt, D. L., and Smith, W. L. (1995) J. Biol. Chem. 270, 10902–10908.
31. Tsuchi, M., and DuBois, R. N. (1995) Cell 83, 493–501.
32. Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., and Vogelstein, B. (2000) Science 290, 989–992.
33. Byrne, C., Tainsky, M., and Fuchs, E. (1994) Development 120, 2369–2383.
34. Shockett, P. E., and Schultz, D. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5173–5176.
35. Badawi, A. F., and Archer, M. C. (1998) Prostaglandins Other Lipid Mediat. 56, 167–181.
36. Badawi, A. F., El-Sohemy, A., Stephen, L. L., Ghoshal, A. K., and Archer, M. C. (1998) Carcinogenesis 19, 905–910.
37. Cardiff, R. D. (1996) J. Mammary Gland Biol. Neoplasia 1, 61–73.
38. Imagawa, W., Bandopadhayay, G. K., Wallace, D., and Nandi, S. (1998) J. Cell. Physiol. 175, 509–515.
39. Zhao, Y., Agarwal, V. R., Mendelson, C. R., and Simpson, E. R. (1996) Endocrinology 137, 5739–5742.
40. Furr, P. A. (1989) J. Mammary Gland Biol. Neoplasia 4, 123–127.
41. Sternlicht, M. D., Bissell, M. J., and Werb, Z. (2000) Oncogene 19, 1102–1113.
42. Wang, T. C., Catiffi, R. D., Zuckukerberg, L., Lees, E., Arnold, A., and Schmidt, E. V. (1994) Nature 369, 669–671.
43. McPherson, K., Steel, C. M., and Dixon, J. M. (2000) Br. Med. J. 321, 624–628.
44. Howe, L. R., Subbaramaiah, K., Chung, W. J., Dannenberg, A. J., and Brown, A. M. (1999) Cancer Res. 59, 1572–1577.
45. Vadlamudi, R., Mandel, M., Adam, L., Steinbuch, G., Mendelsohn, J., and Kumar, R. (1999) Oncogene 18, 305–314.