Cycloxygenase-2 Overexpression in the Skin of Transgenic Mice Results in Suppression of Tumor Development

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ABSTRACT

Significant evidence has accumulated suggesting that the inducible form of cycloxygenase (COX-2), a central enzyme in the prostaglandin biosynthetic pathway, plays an important role in tumor development. To better understand the role of COX-2 in tumorigenesis, we generated transgenic mice that overexpress COX-2 under control of the human keratin 14 promoter, which allows for expression in the epidermis and some other epithelia. Transgenic mice, referred to as K14.COX2 mice, were readily distinguished from their nontransgenic littermates by the appearance of significant alopecia. Administration of a specific COX-2 inhibitor restored hair growth, indicating that the alopecia was attributable to elevated COX-2 enzymatic activity. Unexpectedly, COX-2 overexpression was found to protect, rather than sensitize, K14.COX2 mice to skin tumor development induced by an initiation/promotion protocol. K14.COX2 transgenics developed tumors at a much lower frequency than did their littermate controls (3.3% versus 93%, respectively, on a FVB background and ~25% versus 100%, respectively, on an ICR background) and presented with significantly reduced tumor burdens (average, 0.03 versus 12.7 tumors/mouse, respectively, on a FVB background and 0.5 versus 7.1 tumors/mouse, respectively, on an ICR background). Mice fed a COX-2 inhibitor in utero and as weanlings up to the time of promotion also showed a significant resistance to tumor development. These results clearly raise questions regarding the role of COX-2 and elevated prostaglandin levels in skin tumor development.

INTRODUCTION

One of the central enzymes involved in the PG2 synthesis pathway is PG G/H synthase, also known as COX. Two isoforms have been identified, a constitutively expressed form, known as COX-1, and an inducible form, referred to as COX-2. Whereas COX-2 expression is undetectable in most tissues, including epidermis, under physiological conditions, it can be induced in response to stimuli, including cytokines, growth factors, serum, and phorbol esters (1–3). A substantial amount of evidence has accumulated suggesting that COX-2 plays a significant role in promoting tumor development in various organ systems. COX-2 mRNA and protein are overexpressed in human colorectal (4, 5), breast (6), lung (7), and prostate tumors (8), as well as in several genetic and carcinogen-induced tumor models in rodents, including murine skin tumors induced either by chemical carcinogens or by UV light (3, 5–11).

Several potential mechanisms have been invoked to account for the tumor-promoting properties associated with COX-2 overexpression. The observations that the expression level of Bcl-2 is elevated in rat intestinal epithelial cells overexpressing COX-2 (12) and in human colon cancer cells treated with PGE2 (13) led to the suggestion that COX-2 expression may promote tumor growth by imparting resistance to apoptosis. Such protection could prolong the survival of abnormal cells, thereby allowing time for the accumulation of multiple genetic mutations, ultimately resulting in a transformed phenotype. COX-2 overexpression in colon cancer cells is also associated with increased expression of metalloproteinases and increased production of vascular endothelial growth factors (14, 15). The resulting cells possess both increased metastatic potential, as well as the ability to stimulate endothelial cell growth and promote angiogenesis. Finally, suppression of the immune system in response to elevated PGE2 levels could also contribute to tumor promotion (16, 17).

Further evidence implicating COX-2 in tumor development comes from the observation that NSAIDs, which are inhibitors of COX-1 and COX-2, can block tumor formation in several genetic and chemical models of tumor induction, including in mouse skin (11, 18–20). This observation, however, is somewhat confounded by data that clearly indicate that NSAIDs can exert their antineoplastic effects through both COX-2-dependent and -independent mechanisms (21, 22). In particular, NSAIDs inhibit the proliferation of cancer cells that do not express either COX isoform (23), and sulindac sulfone, a metabolite of the NSAID sulindac, which lacks COX inhibitory activity, can still protect against both genetic and carcinogen-induced tumor formation (24–26). Thus, genetic rather than pharmacological approaches are likely to be more informative in understanding the role of PGs in neoplasia.

To better understand the role of COX-2 in skin tumor development, we generated a line of transgenic mice in which the human K14 promoter was used to regulate tissue-specific expression of a murine COX-2 transgene. This allowed us to examine in vivo the relationship between elevated COX-2 activity, the morphology and proliferative activity of the epidermis, and its response to chemical carcinogenesis protocols. Here we report on the phenotype of these mice as well as the impact of COX-2 expression on tumor induction, using a chemical initiation/promotion protocol for multistage skin tumor carcinogenesis.

MATERIALS AND METHODS

Transgene Construction. The pK14.197 expression vector was constructed by subcloning a 2.27-kb HindIII/SphI fragment of the human K14 promoter (27), which contains all of the regulatory elements necessary for tissue-specific gene expression, into the pBluescript KS vector (Stratagene). A BamHI/SacII cassette containing the rabbit β-globin intron and the SV40 poly(A) signal sequences (28) was then inserted 3′ to the K14 promoter to make pK14.197. The pK14.COX2 construct was created by ligating the EcoRV/Smil fragment from pPG5sDNA (29), which contains the complete COX-2 coding sequence, into the unique NsuBI site in pK14.197. This places the COX-2 cDNA downstream of the human K14 promoter, between the rabbit β-globin intron and SV40 poly(A) signal sequences. The orientation and integrity of the construct were determined by sequencing.

Production of K14.COX2 Transgenic Mice. The plasmid pK14.COX2 was digested with KpnI and the 5.7-kb fragment gel was purified with a kit according to the manufacturer’s protocol (QiaGen). The K14.COX2 transgene was microinjected into the pronucleus of one-cell fertilized B6D2F2 embryos. After overnight incubation, two cell embryos were transferred into the oviducts.
of pseudopregnant ICR female mice for full-term gestation. Progeny were genotyped by PCR analysis using the oligomers 5′-CTG GTT ATT GTG CTG TCT-3′ (complementary to rabbit β-globin intron sequences), and 5′-GCT GAG TTC CTT CGT GAG-3′ (specific for the murine COX-2 cDNA). Founders were mated onto the ICR Swiss Webster outbred background to produce hemizygous animals for further characterization. The transgene was also crossed onto a FVB background by 10 generations of backcrossing with inbred FVB mice.

**PGE₂ Analysis.** Epidermal PGE₂ levels were measured with a RIA kit (NEN Life Sciences, Boston, MA) as we have described previously (30).

**Northern and Western blot Analyses.** Total RNA was isolated using TriReagent (Molecular Research Corp., Cincinnati, OH), according to the manufacturer’s protocol. Northern blots were prepared and probed as we have described previously (11). Immunoblot analysis for COX-1 and COX-2, using authentic COX-2 protein (Caymen Chemical, Ann Arbor, MI) was included as a positive control. Chemiluminescence detection was performed as directed by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ).

**Histologies.** Skin samples were fixed in 10% formalin before being embedded in paraffin. Sections (5 μm) were stained with H&E, using standard protocols. Immunhistochemical staining was also performed, using anti-COX-2 antibody (Caymen) at a 1:10,000 dilution and a fluorescein-conjugated secondary antibody (Amersham) at a 1:2000 dilution. The presence of apoptotic cells was determined using the ApopTag kit (Intergen, Purchase, NY) according to the manufacturer’s protocol.

**Tumor Experiments.** Groups of 20–30 adult mice were initiated by topical application of DMBA. Two weeks later, all mice were treated twice weekly with TPA for up to 20 weeks. For mice on a FVB background, 100 μg of DMBA and 2.5 μg of TPA were used; for ICR mice, 50 μg of DMBA and 5 μg of TPA were used. The doses of DMBA and TPA used for ICR and FVB mice were those that have previously been shown to produce a reasonable number of tumors (31, 32). Mice were observed daily, and the number of tumors present was determined weekly. Tumor data were calculated in terms of incidence (percentage of mice bearing tumors) and yield (average number of tumors per mouse). For one experiment, pairs of FVB K14.COX2 mice were fed 500 ppm celecoxib (LKT Laboratories, St. Paul, MN) in AIN76 diet beginning 1 day after mating and continuing through weaning, at which time pups were placed on diets containing celecoxib, as shown in the schematic below. Celecoxib was removed from the diet 1 week after initiation. The nontransgenic controls consisted of littermates of the K14.COX2 mouse on celecoxib.

**RESULTS**

**COX-2 Expression in the Epidermis of Transgenic Mice.** A diagram of the K14.COX2 transgene is presented in Fig. 1A. In this construct, the COX-2 cDNA is flanked by the rabbit β-globin intron and SV40 poly(A) sequences to ensure proper post-transcriptional processing of the transgene. Of the seven founder mice identified, two demonstrated germ-line transmission of the transgene, and progeny from two founders demonstrated a gross phenotype with apparent differences in severity. Although both lines exhibited similar phenotypes, the line showing the highest level of expression was chosen for study.

Northern blot analysis of transgene expression revealed significant levels of COX-2 expression in the skin of FVB (and ICR; data not shown) K14.COX2 mice; however, no COX-2 message was detected in the skin of wild-type animals (Fig. 1B). The levels of COX-1 expression were essentially the same in wild-type and transgenic mice (Fig. 1B). Western blot analysis also showed that the COX-2 transgene was expressed at the protein level (Fig. 1C). COX-2 transgene expression was also observed in the forestomach, uterus, gall bladder, prostate, and esophagus of transgenic animals (data not shown), consistent with the K14 promoter driving transgene expression in several epithelial tissues (33). COX-2 expression in transgenic mice was further verified by immunofluorescence (Fig. 1D). Fluorescence intensity was greatest in the basal epithelial layer of the skin of K14.COX2 mice, including those cells lining the outer root sheath of the hair follicle. This staining pattern was in concordance with the localization of K14 expression. Only background fluorescence was observed in the epidermis of wild-type littermates. Assays performed on both wild-type and transgenic skin (Fig. 1E) demonstrated a 2-fold increase in the levels of PGE₂ in the skin of K14.COX2 mice. This observation strongly suggests that the COX-2 protein produced as a result of transgene expression is a functionally active enzyme. Because PGE₂ is readily degraded by PG dehydrogenase, which is expressed in murine epidermis, its level is not necessarily expected to be concordant with the level of COX-2 expression (data not shown).

**Gross and Histological Phenotypes of the K14.COX2 Transgenic Mice.** Both ICR and FVB K14.COX2 mice appeared healthy at birth and presented with no obvious abnormalities or reproductive problems, although the transgenic mice were slightly smaller than their wild-type littermates. Differences between transgenic and wild-type littermates became apparent when hair growth began, starting as early as 1 week after birth. Hair development occurred at a much reduced rate in the K14.COX2 mice compared with wild-type animals, manifesting in alopecia in adult animals (Fig. 2A, middle). At the microscopic level, slight differences in...
skin thickness were noted between transgenic animals and wild-type mice (Fig. 2B) such that the skin of ICR K14.COX2 animals appeared thinner and showed signs of atrophy; this was less evident in FVB K14.COX2 skin. Additionally, the hair shafts had an altered morphology in both ICR and FVB transgenic animals (Fig. 2C) and contained cells with structurally deformed nuclei lining the hair follicles. The presence of such pyknotic nuclei, a characteristic of cells undergoing apoptosis, was infrequently observed in skin from wild-type animals. However, we were unable to identify apoptotic cells by the terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling assay. Also notable in the transgenic mice was the sizable enlargement of the sebaceous gland, which frequently was larger than the hair follicle (Fig. 2C).

Administration of a COX-2 Inhibitor Restores Hair Growth.

To demonstrate that the alopecia observed in K14.COX2 mice was attributable to increased PGE2 levels, we administered the COX-2-selective inhibitor celecoxib in the diet at a dose previously shown to inhibit PGE2 synthesis in murine epidermis (11). After 4 weeks of administration, the hair coats of the K14.COX2 animals (12 of 12) were fully restored (Fig. 2A, right). These findings support the suggestion that the alopecia results from improper or incomplete development of the hair shafts brought on by high PG levels within the epidermis of the K14.COX2 mice. Several animals were followed up to several months after discontinuation of treatment. The coats appeared to remain stable for at least 12 weeks, in that no apparent hair loss was observed in these animals, although it is not known whether they underwent another round of the hair growth cycle during this timeframe. By 3 months, some hair loss was noted in all of the treated animals (data not shown). Administration of celecoxib to pregnant animals and subsequent weanlings resulted in normal hair development and normal-appearing hair follicles and sebaceous glands in the weanlings that was indistinguishable from wild-type mice (Fig. 2D). These animals were used in a subsequent tumor experiment.

COX-2 Overexpression Reduces DMBA/TPA-induced Skin Tumor Incidence.

To determine the effect of COX-2 overexpression on skin tumor development, transgenic and wild-type mice were subjected to a DMBA/TPA treatment protocol. This was carried out with both the ICR and FVB K14.COX2 mice to determine whether tumor responses were mouse strain specific. On the basis of previously reported evidence implicating an important role for COX-2 in promoting skin tumor formation (11), we expected to find either greater tumor incidence and multiplicity or larger tumor size in the transgenic mice. However, although 100% of the wild-type ICR mice developed tumors, papillomas formed in only 25% of the ICR K14.COX2 transgenic mice (data not shown). The ICR K14.COX2 animals that developed skin tumors also had lower tumor multiplicity than observed for littermates (0.5 versus 7.1 tumors/mouse), and those tumors that did arise were significantly smaller, having a high lymphatic infiltrate (data not shown). The FVB K14.COX2 mice were also resistant, with tumor incidences of only 3.3% compared with 97% in the wild-type FVB controls (Fig. 3B). There were also dramatic differences in tumor multiplicity, with the wild-type FVB producing an average of 12.7 papillomas/mouse, whereas the FVB K14.COX2 produced only 1 papilloma on 1 of 30 mice (0.33/mouse). There was no difference in the response of wild-type and FVB K14.COX2 epidermis to TPA with regard to degree of epidermal thickening (Fig. 3B) or labeling index (data not shown). Because both FVB and ICR transgenic mice showed a similar resistance to tumor development, it is unlikely that this phenomenon is a strain-of-mouse effect.

To determine whether the hair follicle abnormalities were the cause of tumor resistance, FVB K14.COX2 mice and their nontransgenic littermates that had been exposed to celecoxib in utero and as newborns and weanlings were used in a tumor experiment. These mice
Transgenic mice display pyknotic nuclei, indicating the possibility that continuous high levels of PG may prevent complete hair follicle maturation. That one or more of the PGs inhibit tumor promotion, an idea that is supported by several studies. For example, a study on the effect of topical application of various PGs during tumor promotion showed that the most significant observation was the resistance to skin tumor development conferred by COX-2 overexpression. We considered several possible explanations related to the defects in follicular development. It is possible that cells initiated by exposure to DMBA underwent apoptosis before promotion with TPA and thus were unable to contribute to tumor formation in these animals. Alternatively, the subpopulation of cells that can be initiated by DMBA might be altered or absent from the skin of transgenic mice. To address these issues, the COX-2 selective inhibitor celecoxib was administered to impregnated females and to the subsequent weanlings, resulting in histologically normal hair follicles and hair growth. These mice were maintained on the COX-2 inhibitor up through initiation with DMBA at 6 weeks of age; this was also done to remove the possibility that COX-2 might alter DMBA metabolism (36, 37). These mice, however, were still significantly resistant to tumor development, strongly suggesting that the tumor resistance phenotype is not attributable to hair follicle abnormalities or perturbation of carcinogen metabolism. The slight decrease in tumor multiplicity observed in the wild-type mice that had been fed celecoxib was possibly attributable to residual celecoxib, which would have inhibited COX-2 induced by TPA.

The precise role(s) that induction of COX-2 expression, and the consequently increased levels of PGs, may play in the tumor promotion process remain unclear. In studies using the DMBA/TPA protocol in NMRI mice, the nonselective COX inhibitor indomethacin was shown to significantly reduce tumor multiplicity (38). This effect could be reversed by topical application of PGE\textsubscript{2}, but not PGF\textsubscript{2}\alpha (38). The effect of indomethacin, however, is mouse-strain dependent, because indomethacin and another nonselective COX inhibitor, flurbiprofen, were found to enhance, rather than inhibit, promotion in SENCAR mice (39). This latter study could be interpreted as showing that one or more of the PGs inhibit tumor promotion, an idea that is consistent with the tumor resistance phenotype of the K14.COX2 mouse. Also in support of this concept, a study on the effect of topical application of various PGs during tumor promotion showed that PGE\textsubscript{2}, but not PGF\textsubscript{2}\alpha, inhibited tumor development (40). Recently, exogenous PG administration was also unexpectedly shown to significantly reduce the number of intestinal tumors in Min\textsuperscript{+} mice (41). Additionally, overexpression of COX-2 protein has been reported to induce cell cycle arrest independently of PGs, which led to induction of apoptosis (42). Thus, evidence exists in several systems that indicates that PGs may inhibit tumor development, at least under some circumstances.

The observation that topically applied PGE\textsubscript{2} can inhibit tumor promotion (40) suggests that through its signaling pathway(s), PGE\textsubscript{2} abrogates responsiveness to TPA. Topically applied PGE\textsubscript{2} alone induces CAM, which remains elevated for 90 min (43). Although TPA elicits PG synthesis (3), this occurs as a result of TPA signaling; PG signaling before TPA application, as would occur in the transgenic mouse, may interfere with TPA signaling. Further studies are needed to verify this speculation.

There are, however, numerous observations that support a critical or enhancing role for PGs in the development of skin tumors. First, it has been well established that tumor promoters induce COX-2 and that the resulting tumors have constitutively up-regulated COX-2, which correlates with elevated PG synthesis (3). Second, inhibitors of COX-2 have been shown to reduce skin tumors induced by either the

**DISCUSSION**

The sparse hair growth observed in the K14.COX2 animals appears to result from deficiencies in the development of the individual hair shafts produced within the hair follicles of these animals. Hair follicles develop during midgestation, beginning as condensations of mesenchymal cells at regularly spaced sites along the dermal-epidermal junction and proceeding as an interaction between epithelial and mesenchymal cells (34). The involvement of PGs in hair follicle development is unknown, although the data in this and another study suggest that high PG levels may have a deleterious effect (35). Similar abnormalities in hair follicle development were noted by Neufang et al. (35) in transgenic mice in which the keratin 5 promoter drives the expression of COX-2 in basal keratinocytes. Our observation that a COX-2 inhibitor could reverse alopecia in transgenic mice suggests that continuously high levels of PG may prevent complete hair follicle maturation.

Many of the keratinocytes lining the hair follicles of K14.COX2 transgenic mice display pyknotic nuclei, indicating the possibility that these cells may be undergoing apoptosis. We were, however, unable to detect any differences in the rates of apoptosis by standard methods. It is not clear whether the pyknotic nuclei represent apoptosis or an incompletely differentiated cell. Nonetheless, such a defect in keratinocyte development would likely have a negative impact on the ability of the hair follicles to support hair growth, consistent with the alopecia phenotype.

The most significant observation was the resistance to skin tumor development conferred by COX-2 overexpression. We considered several possible explanations related to the defects in follicular development. It is possible that cells initiated by exposure to DMBA underwent apoptosis before promotion with TPA and thus were unable to contribute to tumor formation in these animals. Alternatively, the subpopulation of cells that can be initiated by DMBA might be altered or absent from the skin of transgenic mice. To address these issues, the COX-2 selective inhibitor celecoxib was administered to impregnated females and to the subsequent weanlings, resulting in histologically normal hair follicles and hair growth. These mice were maintained on the COX-2 inhibitor up through initiation with DMBA at 6 weeks of age; this was also done to remove the possibility that COX-2 might alter DMBA metabolism (36, 37). These mice, however, were still significantly resistant to tumor development, strongly suggesting that the tumor resistance phenotype is not attributable to hair follicle abnormalities or perturbation of carcinogen metabolism. The slight decrease in tumor multiplicity observed in the wild-type mice that had been fed celecoxib was possibly attributable to residual celecoxib, which would have inhibited COX-2 induced by TPA.

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DMBA/TPA protocol or by UV irradiation (11, 44). Finally, mice that are deficient in COX-2 show a dramatically reduced tumor response to DMBA/TPA (45).

The tumor resistance phenotype in our K14.COX2 mice is also more surprising in light of the recent report that overexpression of COX-2, under the control of mouse mammary tumor virus, in murine mammary glands causes spontaneous tumor development. We did not observe mammary tumors in the K14.COX2 mice, although the level of expression of COX-2 in our mice appears to be lower than in the mouse mammary tumor virus transgenic mice (46).

This study also raises the question of identification of the cells that are the targets of the PGs synthesized by the keratinocytes. PGs are secreted and can act in either an autocrine or a paracrine manner through binding to specific receptors. The effects of PGE2, for example, are mediated primarily by the EP receptor family, of which there are four major subgroups. The expression of specific EP receptors, which are linked to different signal transduction pathways, is cell-type dependent; the specific receptors expressed on murine keratinocytes are not known at present. The more recent identification of nuclear receptors, the peroxisome proliferator-activated receptors, that can be activated by some PGs is intriguing (47). The γ isoform of peroxisome proliferator-activated receptor, which is expressed in murine keratinocytes (48), has been associated with cell cycle inhibition and apoptosis (49). The extent to which this is occurring in the K14.COX2 keratinocytes (48), has been associated with cell cycle inhibition and apoptosis (49). The extent to which this is occurring in the K14.COX2 mice is not well understood. Clearly, much more work is needed to resolve these questions raised by this and other studies.

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