Deficiency of Either Cyclooxygenase (COX)-1 or COX-2 Alters Epidermal Differentiation and Reduces Mouse Skin Tumorigenesis

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ABSTRACT

Nonsteroidal anti-inflammatory drugs are widely reported to inhibit carcinogenesis in humans and in rodents. These drugs are believed to act by inhibiting one or both of the known isoforms of cyclooxygenase (COX). However, COX-2, and not COX-1, is the isoform most frequently reported to have a key role in tumor development. Here we report that homozygous deficiency of either COX-1 or COX-2 reduces skin tumorigenesis by 75% in a multistage mouse skin model. Reduced tumorigenesis was observed even though the levels of stable 7,12-dimethylbenz(*a*)anthracene-DNA adducts were increased about 2-fold in the COX-deficient mice compared with wild-type mice. The premature onset of keratinocyte terminal differentiation appeared to be the cellular event leading to the reduced tumorigenesis because keratin 1 and keratin 10, two keratins that indicate the commitment of keratinocytes to differentiate, were expressed 8–13-fold and 10–20-fold more frequently in epidermal basal cells of the COX-1-deficient and COX-2-deficient mice, respectively, than in wild-type mice. Papillomas on the COX-deficient mice also displayed the premature onset of keratinocyte terminal differentiation. However, loricrin, a late marker of epidermal differentiation, was not significantly altered, suggesting that it was the early stages of keratinocyte differentiation that were primarily affected by COX deficiency. Because keratin 5, a keratin associated with basal cells, was detected differently in papillomas of COX-1-deficient as compared with COX-2-deficient mice, it appears that the isoforms do not have identical roles in papilloma development. Interestingly, apoptosis, a cellular process associated with nonsteroidal anti-inflammatory drug-induced inhibition of tumorigenesis, was not significantly altered in the epidermis or in papillomas of the COX-deficient mice. Thus, both COX-1 and COX-2 have roles in keratinocyte differentiation, and we propose that the absence of either isoform causes premature terminal differentiation of initiated keratinocytes and reduced tumor formation.

INTRODUCTION

The two known isoforms of COX,[2] COX-1 and COX-2, catalyze the first committed step in the synthesis of PGs (1, 2), and both isoforms are biological targets of a class of medications known as NSAIDs. The observed inhibition of tumor formation by NSAIDs provided an early indication that the COXs have roles in carcinogenesis, and NSAIDs are especially well documented for preventing colon cancer development in both humans (3, 4) and rodents (5–9). In early studies, the NSAIDs used were capable of inhibiting both COX-1 and COX-2 (5, 6); however, because COX-2 is up-regulated in a high percentage of rodent and human tumors (10), many recent studies have focused on the involvement of this isoform in tumori-

genesis. Indeed, the recently developed COX-2-selective inhibitors have proven to be effective antitumorigenic agents for several rodent tumor types (7–9, 11–15). Moreover, genetic disruption of *Ptgs-2*, the gene encoding COX-2, resulted in substantially reduced spontaneous intestinal tumorigenesis in mice carrying a mutated *Apc* (adenomatous polyposis coli) gene (16, 17).

However, observations demonstrating a role for COX-2 in tumorigenesis do not preclude a role for COX-1. We have recently reported that the genetic disruption of *Ptgs-1*, the gene encoding COX-1, leads to as great a reduction in intestinal tumorigenesis in the *Min* (multiple intestinal neoplasia) mouse as the genetic disruption of *Ptgs-2* (17). To extend our findings on the effects of COX-1 and COX-2 deficiency on tumorigenesis and to gain mechanistic insight, we examined the effects of genetic COX-1 and COX-2 deficiency (18, 19) in the mouse skin initiation/promotion model.

In skin, epidermal cell proliferation, differentiation, and death are tightly regulated, and the multistage mouse skin tumorigenesis model has been used extensively to study these processes as they relate to carcinogenesis (20). Furthermore, NSAIDs are known to decrease skin tumorigenesis in mouse skin models (13–15, 21), suggesting that COX-1 and/or COX-2 have roles in skin tumor formation. The genetically modified, COX-deficient mice provide an alternative approach to NSAID treatment for understanding the roles of COX-1 and COX-2 in tumorigenesis and the chemopreventive effects of COX-1 or COX-2 inhibition (1, 2, 18, 19, 22, 23). Here we report that targeted disruptions of the genes encoding either COX-1 or COX-2 (18, 19) lead to altered epidermal differentiation and reduced skin tumorigenesis.

MATERIALS AND METHODS

Breeding of Wild-type and COX-deficient Mice. Mice carrying the *Ptgs-1* and *Ptgs-2* mutations (18, 19) have been bred for over 20 generations. These mice have been individually maintained by the continuous breeding of heterozygous offspring from the original F_1 agouti mice (50% 129 Ola/50% C57BL/6). The wild-type and COX-null mice used in this study were also generated by heterozygous × heterozygous breedings and would also be 50% 129/50% C57BL/6. Mice were bred and housed in the animal husbandry facilities of the National Institute of Environmental Health Sciences according to the Association for the Assessment and Accreditation of Laboratory Animal Care guidelines. All studies were approved by the Animal Care and Use Committee at the National Institute of Environmental Health Sciences. Food and water were provided *ad libitum*.

Dosing and Papilloma Count. Before dosing, the dorsal surfaces of 6-week-old female COX homozygous deficient ($^{-/-}$), heterozygous ($^{+/-}$), or wild-type ($^{+/+}$) mice were shaved, and then 50 μg of DMBA (Sigma-Aldrich, Saint Louis, MO) in 200 μl of acetone were applied to an approximately 6-cm^2 area. After 1 week, thrice weekly dosing with 4 μg of TPA (Alexis Biochemicals, San Diego, CA) in 200 μl of acetone was begun and continued for 20 weeks. Papillomas were mapped and counted weekly. The conversion of papillomas to carcinomas was too infrequent in any genotype to allow meaningful comparisons.

Statistical Analysis. Because the COX-1-deficient and COX-2-deficient strains were established independently (18, 19), papilloma induction in wild-

Table 1 *Effect of COX genotype on epidermal thickness, BrdUrd labeling, and apoptosis in control and TPA-treated mice*

Shown are means $\pm$ SE for epidermal thickness, BrdUrd labeling, and apoptosis in control or TPA-treated epidermis from wild-type, COX-1$^{-/-}$, and COX-2$^{-/-}$ mice.

	Wild-type	COX-1$^{-/-}$	COX-2$^{-/-}$
Epidermal thickness (nucleated cell layers)			
Control	1.9 $\pm$ 0.2	1.9 $\pm$ 0.2	1.8 $\pm$ 0.2
TPA-20 wk	5.3 $\pm$ 0.2	4.5 $\pm$ 0.1[a]	2.8 $\pm$ 0.1[b]
BrdUrd labeling in epidermis (%)			
Control	3.1 $\pm$ 0.4	2.6 $\pm$ 0.3	1.9 $\pm$ 0.4[c]
TPA-20 wk	39 $\pm$ 1	33 $\pm$ 2[c]	27 $\pm$ 1[b]
BrdUrd labeling in papillomas (%)			
TPA-20 wk	50 $\pm$ 2	38 $\pm$ 2[a]	37 $\pm$ 2[b]
Apoptosis (apoptotic cells/10,000 cells)			
Epidermis	0.9 $\pm$ 0.4	1.8 $\pm$ 0.6	1.7 $\pm$ 0.5
Papilloma	1.0 $\pm$ 0.4	0.9 $\pm$ 0.5	0.9 $\pm$ 0.5

[a] $P < 0.01$ compared to wild-type animals with the same treatment.
[b] $P < 0.001$ compared to wild-type animals with the same treatment.
[c] $P < 0.05$ compared to wild-type animals with the same treatment.

type mice from the COX-1-deficient and COX-2-deficient breeding lines were compared. Using the Cox proportional hazards Wald test (24) for time to first papilloma and a corrected Mann-Whitney *t* test (25) for the maximum number of papillomas, the differences in papilloma induction on wild-type mice from the two lines were not significantly different. Therefore, data from wild-type mice of the two strains were combined for subsequent analysis. Statistical analysis of experiments used the Mann-Whitney *t* test (Fig. 1) or Student's *t* test (Fig. 2; Fig. 4, *s* and *t*; and Table 1).

Sample Collection. Skin samples for analysis were taken at the times described in the individual experiments. Because the hair growth cycle is known to influence epidermal physiology, skin was collected in the telogen phase of the hair cycle to standardize the samples.

DNA Adduct Formation. Female 11–12-week-old wild-type COX-1$^{-/-}$ or COX-2$^{-/-}$ mice were treated as follows. An area of dorsal skin was shaved, and the next day, the shaved area was topically treated with 50 μg of DMBA in 100 μl of acetone. After 4 or 24 h, the mice were sacrificed, and the treated area of skin was excised and snap-frozen. Epidermis from each group was ground in liquid nitrogen and divided into two equal samples for purification of DNA and duplicate analyses of the stable DNA adducts by the ^{32}P postlabeling method (26). Four mice of each genotype were examined at each time point.

PG Determination. Control, DMBA/TPA-treated skin, or papillomas were snap-frozen, and 0.10 g was homogenized in 4°C PBS [2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 137 mM NaCl, and 8 mM Na$_2$HPO$_4$-7H$_2$O (pH 7.4)] containing 60 μM indomethacin. Homogenates were centrifuged at 35,000 $\times$ *g,* and PGE$_2$ levels in the supernatant were determined using a radioimmunoassay kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions.

Immunohistochemical Detection of the COXs and Cytokeratins. Strips of mouse skin or papillomas and adjacent skin were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5-μm thickness, and mounted on SuperFrost Plus slides (A. Daigger, Lincolnshire, IL). Because expression of K1 and K10 is reduced and becomes variable in larger papillomas (27), papillomas of 3–5 mm in diameter were used in these studies. Antigen retrieval was performed in de-gassed 0.01 M sodium citrate by heating in a microwave oven. Antibodies to the keratins and loricrin were obtained from Covance (Richmond, CA). The primary antibody for COX-1 was kindly provided by Dr. David DeWitt (Michigan State University) or purchased from Cayman Chemical (Ann Arbor, MI). The COX-2 antibody was obtained from Cayman Chemical. Incubations with the primary antibodies were carried out overnight at room temperature in a humidity chamber. Antibody binding was detected using a VectaStain Elite kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The absence of staining in the corresponding COX$^{-/-}$ mice served as the negative control.

BrdUrd Labeling and Keratinocyte Transit. DMBA/TPA-treated mice were injected i.p. with 100 mg/kg BrdUrd (Sigma-Aldrich) in 200 μl of PBS 17 h after the last TPA dose (28). Tissue was collected 1 h after BrdUrd injection for measurement of cell replication. Tissue for keratinocyte transit was collected after BrdUrd injection at the times specified. Incorporated BrdUrd was detected with an antibody from Harlan-Sera-Lab (Laughborough, United Kingdom). Twenty microscope fields were counted for each mouse (4 mice/genotype/time point).

Apoptosis. Apoptotic cells were detected in the paraffin-embedded epidermis from DMBA/TPA-treated mice by terminal deoxynucleotidyl transferase-mediated nick end labeling kit using a TACS Assay kit (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. We analyzed the epidermis or papillomas from 4 mice/genotype. Twenty microscope fields were counted for each mouse.

Nucleated Cell Layers in the Epidermis. Counts (20 fields/mouse, 4 mice/genotype) were made on an Olympus BX-50 microscope with 40$\times$ UPlanApo lens and WH10X/22 eyepiece using an eyepiece micrometer reticle.

RESULTS

Deficiency of COX-1 or COX-2 Reduces Mouse Skin Tumorigenesis. The tumorigenic responses of wild-type ($^{+/+}$), heterozygous ($^{+/-}$), or homozygous deficient ($^{-/-}$) COX-1 or COX-2 mice are shown (Fig. 1, *a* and *b*). The tumor numbers in both homozygous-deficient strains were reduced by about 75% compared with wild-type mice (Fig. 1*a*). The tumor incidences were also reduced in the COX-1$^{-/-}$ and COX-2$^{-/-}$ mice (to 60% and 30%, respectively) compared with an 84% incidence in wild-type mice (Fig. 1*b*). The number of papillomas on mice heterozygous for COX-1 or COX-2 was decreased to 35% and 60%, respectively, of the wild-type number (Fig. 1*a*). About 75% of heterozygous COX-1 and COX-2 mice developed tumors (data not shown), an incidence only slightly lower than that of the wild-type mice (Fig. 1*b*). Overall, the data show that gene dosage-
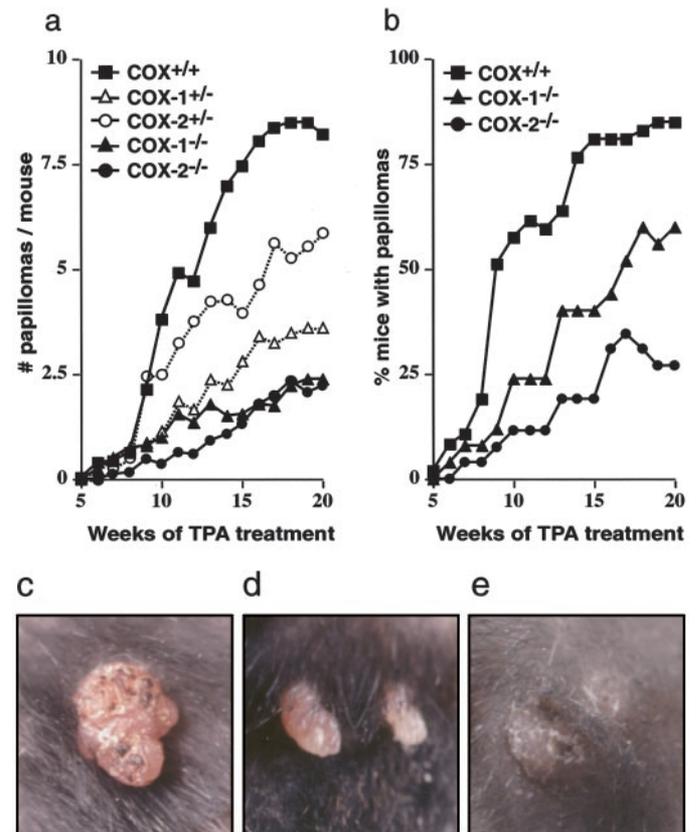

Fig. 1. Genetic deficiency of COX-1 or COX-2 reduces tumorigenesis. Fifty μg of DMBA were applied to the shaved dorsum of the mice, followed by thrice weekly dosing with 4 μg of TPA for up to 20 weeks. Tumors were scored weekly. *a,* tumor numbers in both of the COX-deficient strains are reduced in a gene dosage-dependent manner compared with similarly treated wild-type mice (Mann-Whitney one-sided test, $P < 0.001$ for both the COX-1$^{-/-}$ and COX-2$^{-/-}$ groups compared with the wild-type group). *b,* tumor incidence is reduced in the COX-deficient mice. Wild-type (■; *n* = 47), COX-1$^{-/-}$ (▲; *n* = 25), COX-2$^{-/-}$ (●; *n* = 26), COX-1$^{+/-}$ (△; *n* = 21), and COX-2$^{+/-}$ (○; *n* = 20) values are combined from replicate experiments. *c*–*e,* papilloma morphologies from wild-type (*c*), COX-1$^{-/-}$ (*d*), and COX-2$^{-/-}$ (*e*) mice after 16 weeks of TPA treatment.

dependent decreases of papilloma formation resulted from the targeted disruption of the genes encoding COX-1 or COX-2.

Alterations in papilloma morphology also resulted from COX-1 or COX-2 deficiency. Papillomas on the wild-type mice were typically stalked with apical enlargement (Fig. 1c), whereas those on the COX-1$^{-/-}$ mice were generally smaller and had less apical enlargement (Fig. 1d). Papillomas on COX-2$^{-/-}$ mice were small and sessile rather than stalked (Fig. 1e). Papillomas on mice heterozygous for either isoform did not appear to be significantly different morphologically from those on wild-type mice. Thus, the absence of either COX isoform resulted in reduced size and altered gross morphology of papillomas, although the absence of COX-2 appeared to have the greater effect.

Stable DNA Adducts Are Increased in COX-1$^{-/-}$ or COX-2$^{-/-}$ Mice. The COXs can metabolize some chemical carcinogens to DNA-reactive intermediates (4, 29), and the initiating activity of DMBA correlates with the level of stable DMBA-DNA adducts (30). Four h after DMBA treatment, the level of stable DMBA-DNA adducts in the epidermis of wild-type mice was $1.4 \pm 0.2 \times 10^{-8}$ mol/mol DNA phosphate, whereas stable adducts were elevated about 2-fold in both COX-1$^{-/-}$ and COX-2$^{-/-}$ mice to $2.8 \pm 0.2 \times 10^{-8}$ and $2.7 \pm 0.4 \times 10^{-8}$ mol/mol DNA phosphate, respectively. At 24 h after DMBA application, stable DNA adducts in the COX-deficient mice also were increased about 2-fold compared with wild-type mice (data not shown). The pattern of DMBA adducts did not show genotype-dependent differences and resembled those reported previously (26). These data indicate that the reduced papilloma formation in COX-deficient mice did not result from decreased initiation.

PGE$_2$ Levels in Skin and Papillomas. Because we had previously shown that COX-1 or COX-2 deficiency altered PG levels (18, 19), we wanted to determine the relative contributions of COX-1 and COX-2 to PG levels in skin and in papillomas. PGE$_2$ was measured as an indicator of PG production (17). PGE$_2$ levels in untreated skin of wild-type and COX-2$^{-/-}$ mice were not significantly different, but PGE$_2$ levels were reduced by about 99% in COX-1$^{-/-}$ mice (Fig. 2). After 20 weeks of TPA treatment, PGE$_2$ levels in the skin increased in both wild-type and COX-deficient mice. PGE$_2$ levels in the skins of the COX-deficient groups indicated that both COX-1 and COX-2 contribute to this increase (Fig. 2). PGE$_2$ levels were further elevated in papillomas, and the data from the COX-2$^{-/-}$ and COX-1$^{-/-}$ mice indicate that both COX-1 and COX-2 contributed to PGE$_2$ production in papillomas (Fig. 2). However, COX-1 was the major source of PGE$_2$ in untreated skin, TPA-treated skin, and papillomas.
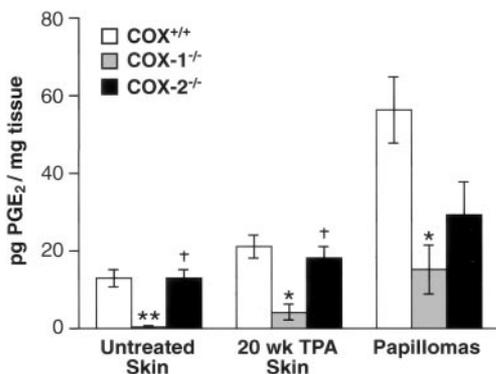

Fig. 2. COX-1 is the major source of PGE$_2$ in skin and papillomas. Tissue PGE$_2$ concentrations were determined by RIA from snap-frozen samples homogenized in PBS containing 60 μM indomethacin. Columns (wild-type, □; COX-1$^{-/-}$, ▨; COX-2$^{-/-}$, ■) show the PGE$_2$ levels (mean $\pm$ SE; $n = 6$) from the skin of untreated control mice or from papillomas or surrounding tissue of TPA-treated mice. ∗, $P < 0.05$ compared with wild-type mice with the same treatment; ∗∗, $P < 0.001$ compared with wild-type mice with the same treatment; †, $P < 0.005$ compared with COX-1$^{-/-}$ mice with the same treatment.
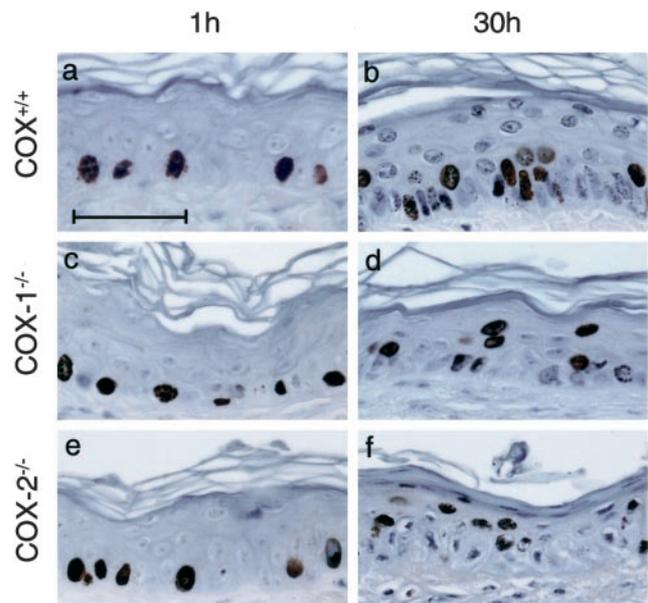

Fig. 3. Transit of keratinocytes is altered in COX-deficient mice. Incorporation of BrdUrd (100 mg/kg, i.p., 17 h after last TPA dose) into replicating keratinocytes of wild-type (a and b), COX-1$^{-/-}$ (c and d), and COX-2$^{-/-}$ (e and f) mice is initially confined to the basal cells at 1 h after injection (a, c, and e). At 30 h after injection, progression of BrdUrd-labeled cells toward the surface is less rapid in the wild-type epidermis (b) than in the COX-1$^{-/-}$ (d) and COX-2$^{-/-}$ (f) epidermis. *Scale bar*, 50 μm.

Epidermal Hyperplasia, Keratinocyte Proliferation, Death, and Transit in Wild-type and COX-deficient Mice. The number of nucleated cell layers in the untreated epidermis of wild-type and COX-deficient mice was not significantly different (Table 1). However, after TPA treatment, epidermal hyperplasia was induced in all genotypes, although the number of nucleated cell layers was smaller in the COX-deficient mice than in the wild-type mice (Table 1).

Because the hyperplastic responses of the epidermis of COX-1$^{-/-}$ and COX-2$^{-/-}$ mice were reduced, we investigated whether decreased levels of keratinocyte proliferation and/or altered cell transit and death could account for the reduced hyperplasia. PGs are known to influence keratinocyte proliferation (31), and Table 1 shows that untreated COX-deficient mice had fewer BrdUrd-labeled epidermal cells than did wild-type mice. After 20 weeks of TPA treatment, BrdUrd labeling was increased in all genotypes; however, the COX-deficient mice still exhibited less BrdUrd labeling than did the wild-type mice (Table 1). In all genotypes, epidermal basal cells were the predominant cells incorporating BrdUrd at 1 h after administration (Fig. 3, a, c, and e). After 30 h, epidermal basal and spinous cells of the wild-type mice retained most of the BrdUrd-labeled nuclei (Fig. 3b), whereas in the COX-1$^{-/-}$ and COX-2$^{-/-}$ mice, labeled cells had reached the more differentiated granular and cornified layers, respectively (Fig. 3, d and f). Thus, keratinocyte transit was more rapid in both the COX-1$^{-/-}$ and COX-2$^{-/-}$ mice.

Increased apoptosis and/or premature onset of terminal differentiation of basal or transit amplifying cells could account for both the reduced basal cell replication and the increased keratinocyte transit in the COX-1$^{-/-}$ and COX-2$^{-/-}$ mice. Because previous studies indicated that COX-2 overexpression reduced apoptosis (32), we hypothesized that the COX-deficient mice could have increased epidermal apoptosis. However, the levels of apoptosis in the epidermis or papillomas of COX-1$^{-/-}$ or COX-2$^{-/-}$ mice were slightly increased compared with the levels in wild-type mice, but the differences were not statistically different (Table 1). Therefore, increased apoptosis did not appear to be a significant factor in reducing tumor numbers or tumor growth in the COX-deficient mice.

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Immunohistochemical Analysis of Epidermal Differentiation.
To determine whether epidermal differentiation was altered, the expression of differentiation-specific proteins from the epidermis of wild-type and COX-deficient mice was analyzed by immunohistochemistry. K5 is normally expressed in basal cells and retained in suprabasal cells (33). K5 expression in the epidermis was similar in all three genotypes (Fig. 4, *a*, *e*, and *i*). K1 is expressed as keratinocytes commit to differentiation (33). K1 was detected in only 2% of the basal cells in wild-type mice (Fig. 4, *b* and *s*), but it was detected in 17% and 26% of the basal cells of COX-1$^{-/-}$ and COX-2$^{-/-}$ mice, respectively (Fig. 4, *f*, *j*, and *s*). K10, another early marker for keratinocyte differentiation (33), was also prematurely expressed in the COX-deficient mice (Fig. 4, *c*, *g*, *k*, and *t*). The increased expression of K1 and K10 in basal cells of the COX-deficient mice indicated that this normally replicative cell population had prematurely initiated terminal differentiation. The expression of the late differentiation protein, loricrin (34), did not show significant differences between the genotypes (Fig. 4, *d*, *h*, and *l*). Thus, COX-1 and COX-2 appear to influence the early stages of keratinocyte differentiation rather than the later stages.

Because the deficiency of COX-1 or COX-2 could alter the expression of the remaining isoform, we compared the levels of COX-1 and COX-2 in the epidermis of the COX-deficient and wild-type mice by immunohistochemistry. Wild-type mice expressed COX-1 primarily
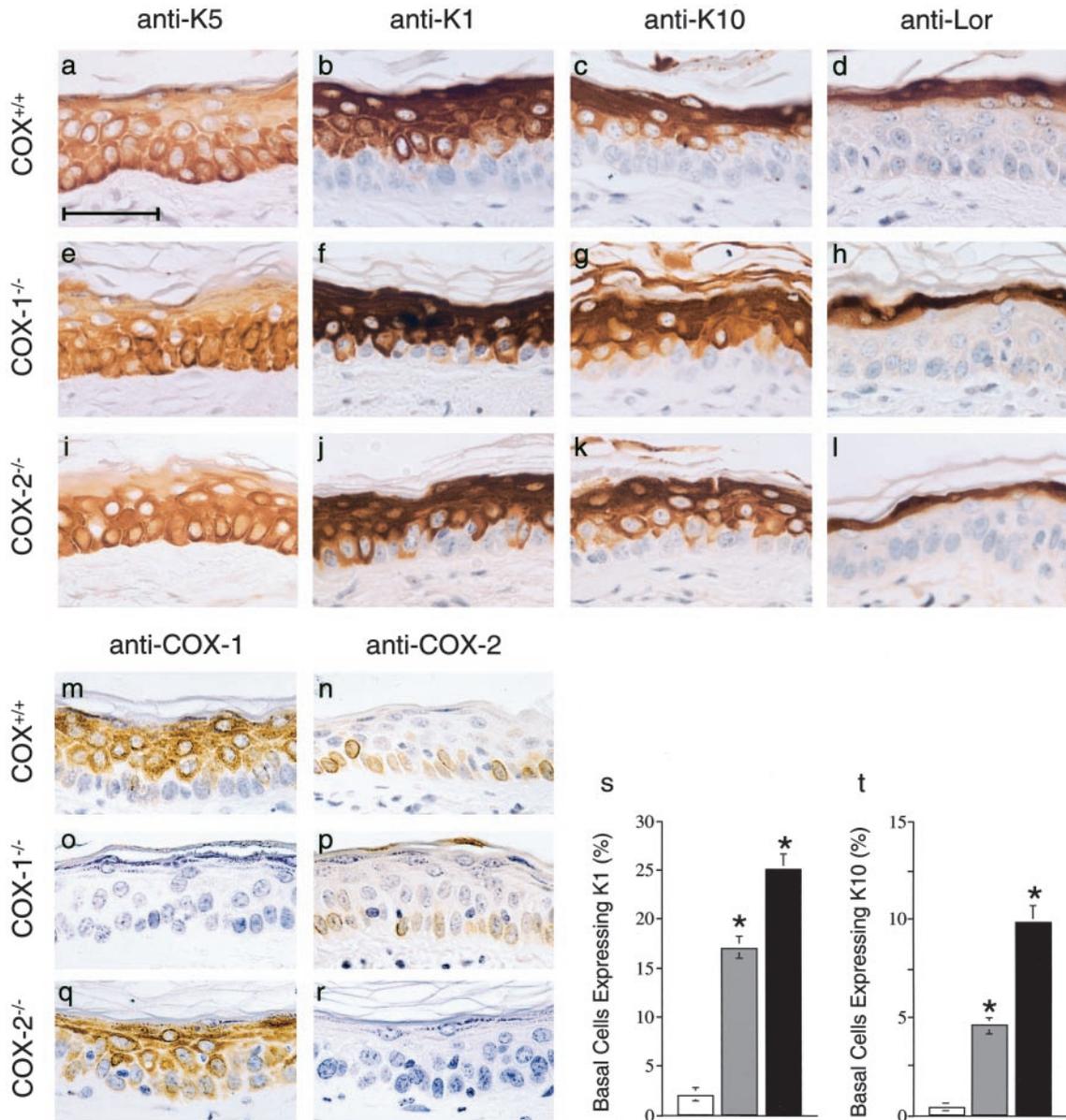

Fig. 4. Immunohistochemical analyses show altered differentiation in DMBA/TPA-treated COX-1$^{-/-}$ and COX-2$^{-/-}$ mouse hyperplastic epidermis. Tissue samples were taken 18 h after the last TPA treatment and fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5-μm thickness. Slides were stained for COX-1, COX-2, K5, K1, K10, or loricrin after antigen retrieval. *a–d,* in the wild-type mice, K5 expression begins in the basal cells (*a*), whereas K1 expression begins suprabasally (*b*), as does K10 (*c*). Loricrin expression occurs only in the uppermost layers (*d*). *e–h,* in COX-1$^{-/-}$ mice, K5 expression is similar to that seen in the wild-type mice (*e*). K1 expression begins in some basal cells (*f*), as does K10 (*g*). Loricrin expression (*h*) is similar to that seen in the wild-type mice. *i–l,* in the COX-2$^{-/-}$ mice, K5 expression is similar to that seen in the wild-type mice (*i*). Expression of both K1 (*j*) and K10 (*k*) begins prematurely in the basal cells. Loricrin expression (*l*) is similar to that seen in the wild-type mice. *m* and *n,* in the wild-type mice, epidermal expression of COX-1 protein begins primarily in the suprabasal cells (*m*), whereas COX-2 is expressed primarily in the basal keratinocytes (*n*). *o* and *p,* in the COX-1$^{-/-}$ epidermis, no immunoreactivity to the COX-1 antibody is seen (*o*), whereas expression of COX-2 remains primarily in the basal cells (*p*). *q* and *r,* in COX-2$^{-/-}$ epidermis, COX-1 expression begins in the basal keratinocytes and increases suprabasally (*q*), and COX-2 immunoreactivity is absent (*r*). *s* and *t,* graphs show the percentages of epidermal basal cells expressing K1 (*s*) and K10 (*t*) in each genotype (wild-type, □; COX-1$^{-/-}$, ▦; COX-2$^{-/-}$, ■). *, $P < 0.005$ compared with wild-type mice. *Scale bar,* 50 μm.

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in differentiated suprabasal cells (Fig. 4m). However, COX-1 was detectable in basal and suprabasal cells of the COX-2$^{-/-}$ mice (Fig. 4q). The expression of COX-1 in basal cells of the COX-2$^{-/-}$ mice, like the expression of K1 and K10, supports the conclusion of premature terminal differentiation when COX-2 is absent. However, as compared with wild-type mice, neither the location nor the intensity of COX-2 staining was altered in the COX-1$^{-/-}$ mice (Fig. 4, n and p). The specificity of the COX-1 and COX-2 immunostaining was verified with the appropriate COX-1$^{-/-}$ or COX-2$^{-/-}$ epidermis (Fig. 4, o and r). Therefore, whereas the deficiency of COX-1 expression does not alter the expression of COX-2, the deficiency of COX-2 does result in the expression of COX-1 in basal cells.

It was also observed that the arrangement of keratinocytes in the epidermis appeared less organized in the COX-deficient mice than in the wild-type mice (for example, see Fig. 4, m, o, and q). The basal cells in the COX-deficient mice often lack the columnar shape found in the wild-type mice, and the transition to the differentiated morphologies of the spinous and granular layers was less uniform, suggesting that both COX-1 and COX-2 are important in the transition of keratinocytes to a differentiated phenotype.

Cell Proliferation, Apoptosis, and Keratinocyte Differentiation in Papillomas. In papillomas collected 4 weeks after the last TPA treatment, those from the COX-deficient mice had reduced BrdUrd labeling compared with papillomas from wild-type mice (Table 1). However, at 1 h after BrdUrd treatment, label was detected in basal and suprabasal cells of papillomas from both wild-type mice and COX-deficient mice (data not shown). Thus, whereas fewer BrdUrd-labeled cells were detected in the COX-deficient mice than in the wild-type mice, labeling occurred in similar locations in all of the genotypes. The levels of apoptosis in papillomas from the three genotypes did not appear to differ (Table 1); therefore, apoptosis would not account for the smaller size of papillomas of the COX-deficient mice (Fig. 1, c–e).

We also determined whether COX-1 or COX-2 expression was altered in papillomas of the respective COX-deficient mice. In papillomas from wild-type mice, COX-1 was detected primarily supra-

basally, whereas in COX-2$^{-/-}$ mice, COX-1 was frequently detected in basal cells as well as in the suprabasal cells (Fig. 5, a and i). COX-2 expression was more variable in papillomas than in the epidermis. In the wild-type mice, COX-2 was expressed not only in the basal cells of the papillomas but also in some suprabasal keratinocytes (Fig. 5b). The absence of COX-1 did not appear to significantly alter the expression pattern of COX-2 in papillomas (Fig. 5f). The specificities of the COX antibodies are shown by the absence of binding in the respective COX$^{-/-}$ mice (Fig. 5, e and j). Thus, in papillomas, as was observed in the epidermis, the absence of COX-1 expression did not alter expression of COX-2, but the absence of COX-2 did result in premature expression of COX-1.

In papillomas of wild-type mice, K1 expression generally began in the third or fourth epidermal cell layer (Fig. 5c), whereas in papillomas of COX-1$^{-/-}$ and COX-2$^{-/-}$ mice, K1 expression began in the second epidermal cell layer (Fig. 5, g and k). K10 expression (data not shown) was similar to that of K1. Thus, in papillomas, as in the epidermis, keratinocytes from the COX-deficient mice began terminal differentiation earlier. The expression of the late differentiation marker, loricrin, was similar in papillomas of COX-deficient and wild-type mice (data not shown) and indicates that in papillomas, as in the epidermis, it is the early stages of differentiation that are affected by the deficiency of COX-1 or COX-2.

Whereas the distributions of K1 and K10 were similar in papillomas of the COX-1$^{-/-}$ and COX-2$^{-/-}$ mice, the distribution of the basal cell marker, K5, differed between the two COX-deficient genotypes. In wild-type mice, the onset of K5 expression began in the basal keratinocytes, and the protein remained throughout the suprabasal layer of the papillomas, although detectability decreased in the suprabasal layers (Fig. 5d). In the COX-deficient mice, the onset of K5 expression also occurred in the basal cells of the papillomas; however, the ability to detect K5 in the suprabasal cells of the COX-1$^{-/-}$ and COX-2$^{-/-}$ mice differed (Fig. 5, h and l). A significant decrease in staining for K5 was evident in the suprabasal layers of COX-1$^{-/-}$ papillomas compared with the suprabasal cells in papillomas on wild-type mice (Fig. 5, d and h), whereas in papillomas on

Fig. 5. Immunohistochemical analysis shows altered protein expression patterns in papillomas. Tissues from the DMBA/TPA-treated mice were prepared as described in Fig. 4, except that the papillomas were removed for study 4 weeks after the last TPA treatment. K5 is shown without antigen retrieval. a–d, in papillomas from wild-type mice, COX-1 expression is primarily suprabasal (a), whereas COX-2 appears in some suprabasal cells in addition to the basal cells (b). K1 appears in the third or fourth epidermal cell layer (c). K5 appears in the basal layer, with some immunoreactivity retained in the suprabasal keratinocytes (d). e–h, in papillomas from COX-1$^{-/-}$ mice, there is no immunoreactivity with antibody to COX-1 (e), and COX-2 expression appears similar to that seen in the wild-type mice (f). K1 appears in the first suprabasal layer of cells (g). K5 appears in the basal layer, with very little immunoreactivity retained in the suprabasal keratinocytes (h). i–l, in papillomas from COX-2$^{-/-}$ mice, COX-1 expression begins in the basal cells (i), and no COX-2 immunoreactivity is seen (j). K1 is expressed in the first suprabasal layer (k), as seen in the COX-1$^{-/-}$ mice. K5 appears in the basal layer, with immunoreactivity retained in the suprabasal keratinocytes (l). *Scale bar,* 50 μm.

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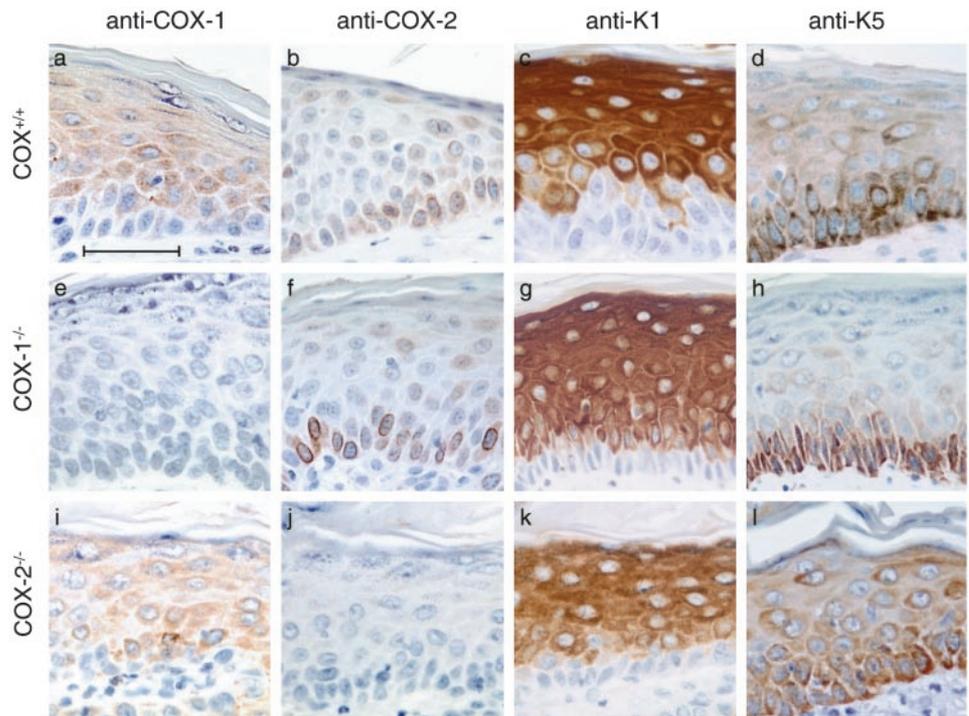
the COX-2$^{-/-}$ mice, K5 staining remained elevated throughout the papilloma (Fig. 5*l*). Thus, the appearance of K5 in the suprabasal cells of the papillomas varied among the three genotypes and shows that the deficiency of COX-1 and COX-2 had different effects on K5 staining in papillomas.

DISCUSSION

In the present study, we show that mice deficient in either COX-1 or COX-2 develop about 75% fewer skin papillomas than do the wild-type mice (Fig. 1). Furthermore, we have demonstrated that the deficiency of either COX isoform causes the premature onset of keratinocyte terminal differentiation, and we suggest that it is premature differentiation of initiated cells that is primarily responsible for the reduced papilloma formation.

The present data indicate that both COX-1 and COX-2 have significant roles in regulating epidermal differentiation. The effects of COX-1 and COX-2 deficiency on differentiation were primarily evident at the basal cell to spinous cell transition (Fig. 4). Premature differentiation of basal cells into spinous cells occurred in the absence of either COX-1 or COX-2, suggesting that both isoforms are required for normal differentiation to occur. Immunohistochemical analysis of COX-1 and COX-2 and of K1 and K10, two keratins associated with these early stages of keratinocyte differentiation, indicated that COX-1 expression must be up-regulated and that COX-2 expression must be concomitantly down-regulated for the coordinated differentiation of basal cells into spinous cells. Loricrin, a protein expressed in the later stages of keratinocyte differentiation, was not significantly altered by COX deficiency, suggesting that COX-1 and COX-2 are primarily involved in the early stages of differentiation (Fig. 4, *d*, *h*, and *l*). Papillomas from the COX-1- and COX-2-deficient mice also showed earlier expression of K1 (Fig. 5) and K10 compared with that seen in papillomas from wild-type mice. Whereas the studies reported here were conducted to determine the roles of COX-1 and COX-2 in skin tumorigenesis using an initiation/promotion model, we have observed similar effects of COX-1 or COX-2 deficiency on epidermal differentiation in untreated skin.[3] From the present studies, it is not possible to identify the specific mechanism(s) by which COX-1 and COX-2 influence epidermal differentiation, and studies are under way to elucidate the specific PGs and the PG receptors involved.

There is little precedent in the literature for a direct influence of the COXs on differentiation. It was reported previously that expression of COX-1, but not COX-2, was up-regulated when a promonocytic cell line was induced to differentiate (35). This observation is in agreement with our observation that COX-1 is induced when basal cells differentiate into spinous cells. It has been reported recently that overexpression of COX-2 in the epidermis of a transgenic mouse caused delayed expression of the differentiation markers K1, K10, and loricrin (36). These findings (36) are in agreement with our results showing that COX-2 deficiency caused premature expression of K1 and K10 in the epidermis. However, our data additionally show that COX-1 deficiency, like COX-2 deficiency, also alters epidermal differentiation.

Interestingly, it was observed that COX-1 and COX-2 deficiency caused an increase in the formation of stable DMBA-DNA adducts in the epidermis. This was unanticipated because the COXs have been demonstrated to metabolically activate some classes of chemical carcinogens (4, 29), and it was expected that their deficiency would result in a reduction of stable DMBA-DNA adducts. This increase in DMBA adduction in the epidermis of COX-deficient mice suggests that the COXs are not a major pathway for the metabolic activation of DMBA

in this tissue. Furthermore, the increase in DMBA adduction observed in the skins of the COX-deficient would be in accord with our observation that terminal differentiation begins prematurely in COX-deficient epidermis. Thus, premature epidermal differentiation may induce early expression of enzymes characteristic of differentiated cells, such as the cytochromes P450, leading to greater DMBA metabolism and ultimately leading to greater DNA adduction in the COX-deficient mice.

Whereas the present data for K1 and K10 expression suggest that the absence of COX-1 or COX-2 has similar effects on keratinocyte differentiation (Fig. 4), the observation that immunohistochemical detection of K5 in papillomas of the COX-1$^{-/-}$ and COX-2$^{-/-}$ mice differs (Fig. 5, *d*, *h*, and *i*) suggests that the isoforms do not have identical effects on papilloma growth. Whereas the basis for this difference in K5 detection is not known at present, it could be due to differences in K5 gene expression or to differences in K5 epitope masking in papillomas of the COX-1- and COX-2-deficient mice. Alterations in epidermal gene expression in these mice during skin tumor development are currently being investigated. Nevertheless, the data (Figs. 4 and 5) suggest that COX-1 and COX-2 contribute to skin tumor growth by overlapping but nonidentical mechanisms.

Genetic models or naturally occurring diseases that lead to premature differentiation in the epidermis are rare. Recently, Yuspa and co-workers (37, 38) reported that transformed keratinocytes deficient in the EGFR showed reduced ability to grow into tumors, similar to our observation of reduced papilloma growth in COX-1- and COX-2-deficient mice. However, in papillomas consisting of EGFR$^{-/-}$ keratinocytes, cycling basal tumor cells migrated prematurely into the suprabasal layer and began terminal differentiation, whereas in the COX-deficient mice, basal cell replication was reduced (Table 1), and terminal differentiation began prematurely, while the cells were still in the basal layer. Because the EGFR can induce COX-2 expression, it will be interesting to determine whether alterations in COX-2 expression have some role in the aberrant differentiation of EGFR$^{-/-}$ keratinocytes.

The failure of initiated cells to either terminally differentiate or undergo apoptosis can contribute to tumor growth (38–41). It has been reported that COX-2 overexpression in cultured cells reduces apoptosis and that NSAIDs increase apoptosis in COX-2-overexpressing cells (32) and other cell types (42). However, it is not entirely clear whether inhibition of PG production is the basis of NSAID-induced apoptosis, and COX-independent mechanisms of NSAID action may be responsible in part for their proapoptotic effects (42, 43). In the present study, we observed that epidermal apoptosis was not significantly increased in the COX-1$^{-/-}$ and COX-2$^{-/-}$ mice compared with wild-type mice, and no increase in apoptosis was observed in papillomas (Table 1). However, the levels of epidermal basal cells undergoing premature terminal differentiation in the COX-1$^{-/-}$ and COX-2$^{-/-}$ mice were elevated about 10- and 15-fold, respectively, over the levels seen in wild-type mice (Fig. 4*s*). The data in Table 1 also indicate that cell replication as determined by BrdUrd incorporation was reduced in the epidermis and papillomas of COX-deficient mice. This reduced cell replication could be accounted for by the premature onset of terminal differentiation of potentially replicative cells. Thus, premature terminal differentiation, rather then increased apoptosis, could be a mechanism by which initiated cells are removed from the pools of replicating epidermal cells in the COX-deficient mice, thus leading to the reduction in both tumor number and tumor size (Fig. 1).

Our data demonstrate that the genetic deficiency of COX-1 is as effective as the deficiency of COX-2 in reducing skin tumorigenesis and are in agreement with our previous findings that the deficiencies of either COX-1 or COX-2 were equally effective in reducing intes-

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[3] Unpublished data.

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tinal tumorigenesis in the *Min* mouse (17). The possible involvement of COX-1 as well as COX-2 in mouse skin tumorigenesis was also suggested by the studies of Fischer *et al.* (14). Furthermore, the involvement of COX-1 in tumorigenesis is also supported by our observation that COX-1, rather than COX-2, is a major source for PGE_2 production in TPA-treated skin and in papillomas (Fig. 2).

In summary, the present studies show that both COX-1 and COX-2 contribute to mouse skin tumorigenesis and that papilloma development is significantly reduced in the absence of either isoform. The premature onset of terminal differentiation, rather than increased apoptosis, appears to be the primary mechanism by which COX deficiency decreases papilloma formation in the skin. Therefore, the results demonstrate key roles for both COX-1 and COX-2 in epidermal differentiation and suggest that selective inhibition of COX-1 as well as selective inhibition of COX-2 may effectively suppress skin tumorigenesis.

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Deficiency of Either Cyclooxygenase (COX)-1 or COX-2 Alters Epidermal Differentiation and Reduces Mouse Skin Tumorigenesis

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