Epidermal Growth Factor–Induced Cyclooxygenase-2 Expression Is Mediated through Phosphatidylinositol-3 Kinase, Not Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase, in Recurrent Respiratory Papillomas

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Abstract

Purpose: Recurrent respiratory papillomas, caused by human papillomaviruses, are premalignant tumors that overexpress the epidermal growth factor receptor (EGFR). The goals of this study were as follows: (a) to evaluate the expression of cyclooxygenase-2 (COX-2) in papillomas, (b) to investigate the role of EGFR signaling in COX-2 expression, and (c) to determine whether COX-2 activity is important for the growth of papilloma cells.

Experimental Design: Immunohistochemistry, Western blotting, and real-time PCR were used to determine levels of COX-2 in papilloma and normal laryngeal tissue. Explant cultures of both normal laryngeal and papilloma cells were used to define the signaling pathways that regulate COX-2 expression and investigate the potential of targeting COX-2 as a strategy to suppress papilloma growth.

Results: COX-2 levels were markedly increased in papillomas. In vitro studies suggested that overexpression in papillomas reflected activation of EGFR—phosphatidylinositol 3-kinase signaling. Treatment with prostaglandin E2 (PGE2) induced COX-2, whereas celecoxib, a selective COX-2 inhibitor, suppressed levels of COX-2, suggesting a positive feedback loop. Moreover, treatment with PGE2 stimulated papilloma cell growth, whereas celecoxib suppressed proliferation and induced apoptosis.

Conclusions: Overexpression of COX-2 in papillomas seems to be a consequence of enhanced EGFR—phosphatidylinositol 3-kinase signaling. We propose a positive feedback loop for COX-2 expression, with induction of COX-2 resulting in enhanced PGE2 synthesis and further expression of COX-2 that contributes to the growth of papillomas in vivo. These data strengthen the rationale for evaluating whether nonsteroidal anti-inflammatory drugs, prototypic COX inhibitors, will be useful in the management of respiratory papillomas.

Recurrent respiratory papillomatosis is a disease primarily caused by human papillomaviruses type 6 or 11 (1), which usually involves the larynx but can also involve the trachea, bronchi, and lungs. It is characterized by repeated recurrences of premalignant hyperplastic epithelial papillomas with defects in terminal differentiation (2). We previously reported that papillomas overexpress the epidermal growth factor receptor (EGFR; refs. 3, 4) and that multiple signal transduction pathways linked to the EGFR are altered. These alterations include constitutive activation of the p42/p44 extracellular signal-regulated kinases (ERK; ref. 3), elevated phosphatidylinositol 3-kinase (PI3K) activity but reduced activation of Akt due to overexpression of PTEN (5), increased activation of nuclear factor-κB (6), and reduced activation of STAT-3 (7), which contributes to the abnormal differentiation (8). Cyclooxygenase (COX)-1 and COX-2 are enzymes that metabolize arachidonic acid to prostaglandin H2 (PGH2). Specific isomerases then convert PGH2 to several prostaglandins, including PGE2. COX-1 is generally constitutively expressed and involved in normal physiologic functions. In contrast, COX-2 is not detected in most normal tissues. However, it is rapidly induced by growth factors, oncogenes, carcinogens, tumor promoters, and proinflammatory cytokines. Overexpression of COX-2 has been found in a variety of...
inflammatory and neoplastic conditions (9). Notably, COX-2 is overexpressed in human cervical and penile cancers, which are caused by human papillomaviruses (10, 11), and is elevated in respiratory papillomas (12) and other premalignant and malignant tumors of the head and neck (13–15).

A variety of signaling molecules can regulate COX-2 expression (16). In some cells, stimulation of the EGFR activates both the PI3K-Akt and Ras-ERK pathways, resulting in induction of COX-2. Moreover, several studies have shown that COX-2–derived PGE2 can stimulate EGFR signaling (17–19). However, nothing is known about the regulation of COX-2 expression in respiratory papillomas. In this study, we compared amounts of COX-2 in papillomas versus normal epithelium, and investigated whether its expression was linked to EGFR and PI3K activation in cultured papilloma cells. Inhibition of either EGFR tyrosine kinase activity or PI3K, but not ERK, suppressed COX-2 expression in papilloma cells. Importantly, COX-2 seems to regulate its own expression, suggesting a positive feedback loop that could have an impact on papilloma growth.

Materials and Methods

Tissues and cultured cells. Laryngeal papillomas and normal laryngeal tissues were obtained from surgical biopsies. The use of human tissues and cultured cells was approved by the Institutional Review Board of the Institute for Medical Research of the North Shore-LIJ Health System, in accordance with an assurance filed with and approved by the Department of Health and Human Services. Informed consent for use of tissues for research was obtained from each subject or the subject's guardian. Biopsies were used to establish primary cell cultures or were snap frozen in liquid nitrogen until used. Epithelial explant cultures of both normal laryngeal cells and papilloma cells were established in Ham's F12 with 10% fetal bovine serum and papilloma cells were used at first passage. These cultures are >99% epithelial, based on histologic and immunocytochemical studies.

Epithelial explant cultures of both normal laryngeal cells and papilloma cells were established in Ham's F12 with 10 μg/mL hydrocortisone and 10 mL/100 mL fetal clone II (Hyclone, Logan, UT) as described (20). These cultures are >99% epithelial, based on morphology and keratin expression, and the papilloma cells contain episomal human papillomavirus DNA (20). Normal laryngeal cells were expanded for not more than two to three passages on mitomycin C–treated I23T3 feeder cells, whereas papilloma cells were used at first passage. Cells were trypsinized and plated at 2 × 105 cells/cm2 in serum-free keratinocyte growth medium (KGM), which was made of keratinocyte basal medium (Clonetics, San Diego, CA) supplemented to a final concentration of 1 ng/mL EGF, 5 μg/mL insulin, 2 μg/mL transferrin, 0.5 μg/mL hydrocortisone, 10−5 mol/L retinoic acid, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were used for experiments while just subconfluent and proliferating. Experiments were done at least thrice with cells derived from different patients unless otherwise noted.

Immunohistochemistry. Normal laryngeal and papilloma specimens were fixed in 10% buffered formalin, paraffin-embedded, and processed as described (20). These cultures are >99% epithelial, based on histologic and immunocytochemical studies. Normal laryngeal cells and papilloma cells were established in Ham's F12 with 10% fetal bovine serum and papilloma cells were used at first passage. These cultures are >99% epithelial, based on histologic and immunocytochemical studies. Normal laryngeal cells were expanded for not more than two to three passages on mitomycin C–treated I23T3 feeder cells, whereas papilloma cells were used at first passage. Cells were trypsinized and plated at 2 × 105 cells/cm2 in serum-free keratinocyte growth medium (KGM), which was made of keratinocyte basal medium (Clonetics, San Diego, CA) supplemented to a final concentration of 1 ng/mL EGF, 5 μg/mL insulin, 2 μg/mL transferrin, 0.5 μg/mL hydrocortisone, 10−5 mol/L retinoic acid, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were used for experiments while just subconfluent and proliferating. Experiments were done at least thrice with cells derived from different patients unless otherwise noted.

Immunohistochemistry. Normal laryngeal and papilloma specimens were fixed in 10% buffered formalin, paraffin-embedded, and processed for immunohistochemistry by conventional methods. Sections were immunostained, incubated with goat polyclonal anti–COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50 dilution, detected by goat polyclonal anti–anti-goat IgG and antirabbit IgG (Pierce) at 1:3,000 dilution. The immunoreactive species were detected with Super Signal West Pico chemiluminescent substrates (Pierce). Signal intensity was quantified by UN-SCAN-IT Program (Silk Scientific, Inc., Orem, UT), adjusted for total protein in the lane and normalized to the control within each experiment.

Western blot analysis. Pulverized frozen tissues and cultured cells were extracted as previously described (6). Briefly, powered tissue or cells were suspended in ice-cold hypotonic buffer [100 mmol/L HEPES (pH 7.6), 10 mmol/L KCl, 3 mmol/L NaCl, 3 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L DTT, and 10% (v/v) glycerol] plus the complete protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitors (20 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, and 30 mmol/L sodium fluoride). Protein concentrations were determined by micro-BCA (Pierce, Rockford, IL). Cytoplasmic proteins (40 μg/lane) were separated on 10% SDS-PAGE and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Standard molecular weight markers (broad range; Bio-Rad, Hercules, CA) were used for molecular weight estimation. Membranes were stained with fast green (21) to visualize total protein in the lane, blocked with dried milk, incubated with primary antibody overnight at 4°C, washed, and incubated with secondary antibody. After detection, blots were stripped and reprobed with antibodies to β-actin to confirm equivalence in loading and transfer. Primary antibodies were as follows: goat polyclonal anti–COX-1 and anti–COX-2 were used at a dilution of 1:500, goat anti–β-actin at 1:1,000 (Santa Cruz Biotechnology), rabbit polyclonal anti–phospho-ERK, ERK, phospho-Akt, and Akt were used at a dilution of 1:500 (Cell Signaling Technology, Inc., Waltham, MA). Secondary antibodies were horse-radish peroxidase–conjugated goat IgG and antirabbit IgG (Pierce) used at 1:3,000 dilution. The immunoreactive species were detected with Super Signal West Pico chemiluminescent substrates (Pierce). Signal intensity was quantified by UN-SCAN-IT Program (Silk Scientific, Inc., Orem, UT), adjusted for total protein in the lane and normalized to the control within each experiment.

Quantitative analysis of cyclooxygenase-2 messenger RNA. Total RNA was extracted from powdered frozen tissue using the RNeasy Mini kit (Qiagen, Inc., Chatsworth, CA) and digested with DNase (Qiagen). The concentration was determined by UV absorbance. One microgram of RNA from each sample was used to synthesize cDNA (Promega Corp., Madison, WI). COX-2 cDNA was amplified for 30 cycles using TaqMan primers and probes designed using Primer Express software version 1.5 (Applied Biosystems, Inc., Foster City, CA). Real-time PCR was done as previously described (23). Data was analyzed using Sequence Detection System software version 1.6.3. Relative expression of COX-2 mRNA in papilloma samples compared with normal tissues was calculated by the ΔΔCt method (User Bulletin 2, Applied Biosystems). The COX-2 primers were 5′-TCCTCTGGTACGACCAATT-3′, which anneals between residues 7,124 to 7,145 with a Tm of 59°C, and 5′-CTGGGCGCTGTTCTGTT-3′, which anneals between residues 7,231 to 7,214 with a Tm of 59°C. The COX-2 TaqMan probe was 5′-FAM-CAGTGACGTCATCCGA-TAMRA-3′, which anneals between residues 7,159 and 7,182 with a Tm of 70°C. 36B4 TaqMan probe, 5′-FAM-CCCTCCTCTTTGGGCTGGT-TAMRA-3′, was used as an internal control. Absorbance was measured at 450 nm with an ELx 800 reader (Bio-Tek Instruments, Inc., Winooski, VT).

Effects of exogenous growth factors and inhibitors of signal transduction. Cells were cultured in KGM until subconfluent, then used as described below. For growth factor stimulation studies, cells were cultured in KGM without EGF or insulin for 24 hours and then fed with complete KGM containing either 10 ng/mL EGF or 1 ng/mL EGF plus 5 μg/mL insulin for the indicated times before extraction. For inhibition studies, cells were preincubated with inhibitor for 1 hour, maintained with inhibitor plus indicated growth factors, and then analyzed by immunoblot for COX-2 and signal transduction intermediates. Inhibitors were 1 μmol/L PD153035 (Calbiochem, San Diego, CA), a specific inhibitor of the EGFR tyrosine kinase, 50 μmol/L PD98059 (Calbiochem), a selective inhibitor of
mitogen-activated protein/ERK kinase, and 25 μmol/L LY294002 (Calbiochem), a specific PI3K inhibitor. Control cells were incubated with KGM containing an equal concentration of DMSO, the solvent for the inhibitors.

Cell proliferation and apoptosis. To measure the effects of PGE2 on cell number, papilloma cells were cultured in KGM containing 250 or 500 nmol/L PGE2 (Calbiochem) or an equal volume of DMSO for 24 hours, and the relative measure of viable cells determined by bioreduction of a tetrazolium compound (CellTiter 96 Nonradioactive Cell Proliferation Assay; Promega) according to the instructions of the manufacturer. To measure suppression of proliferation or induction of apoptosis with celecoxib, papilloma cells were incubated in KGM containing 2.5, 5, or 7.5 μmol/L celecoxib (Pfizer Pharmaceuticals, Inc., Groton, CT) or vehicle for 24 hours. Proliferation was measured by addition of 5 μg/ml bromodeoxyuridine (BrdUrd) for the last 18 hours, cells were fixed, and BrdUrd incorporation into the nucleus was detected by immunohistochemistry with mouse monoclonal anti-BrdUrd (Oncogene Research, La Jolla, CA) as per the directions of the manufacturer. Ten random fields (500 cells/field) were counted to determine labeling index with the value for control cells ~20%. Apoptosis was measured by cytoplasmic release of nucleosomal fragments using a sandwich ELISA (Cell Death Detection ELISAPLUS; Roche) detected photometrically with 2,2'-azino-di-3-ethylbenzthiazoline sulfonate as substrate.

Statistical analysis. Student’s t test or Tukey-Kramer multiple comparison test were used to determine statistical significance. Values were expressed as mean ± SD of multiple experiments using tissues or cells from different patients. A difference between groups of P < 0.05 was considered significant.

Results

Quantitation of cyclooxygenase-2 expression in papillomas. We first confirmed that COX-2 was overexpressed in biopsies of human laryngeal papillomas compared with normal laryngeal epithelium when evaluated by immunohistochemical staining (12). COX-2 staining was especially pronounced in the lower and middle spinous layers of the papilloma tissue (Fig. 1A). In contrast, normal tissues showed no detectable COX-2. We then used Western blots to estimate relative COX-2 protein levels and real-time reverse transcription-PCR to measure COX-2 mRNA levels (Fig. 1B and C). COX-2 protein was elevated ~5-fold in papilloma tissues compared with normal laryngeal epithelium (*P < 0.01) at levels approaching those in a squamous cell carcinoma of the head and neck used as a positive control. COX-1 levels were extremely low and

Fig. 1. COX-2 is overexpressed in respiratory papillomas. A, immunohistochemical staining of formalin-fixed, paraffin-embedded normal laryngeal and papilloma tissues. Tissue sections were stained with goat polyclonal anti–COX-2 or with PBS (control), detected using 3,3'-diaminobenzidine and lightly counterstained with hematoxylin. Note abundant COX-2 in the cytoplasm of papilloma tissues. Arrows, location of the basement membrane. Bar, 24 μm. B, representative Western blot showing expression of COX-2 levels in three papillomas and two samples of normal laryngeal tissue. A squamous carcinoma of the head and neck served as a positive control. Equal amounts of protein (40 μg/lane) were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, incubated with goat polyclonal anti–COX-2 antibody followed by goat IgG-horseradish peroxidase, and detected by chemiluminescence. Membranes were then stripped and incubated with goat polyclonal anti–β-actin. Columns, mean for COX-2 expression, analyzed by densitometry, normalized to actin, and expressed as relative level of COX-2 in six papillomas compared with seven normal tissues; bars, SD (*P < 0.01). C, analysis of COX-2 mRNA levels in papilloma and normal laryngeal tissues. COX-2 mRNA was quantified by real-time reverse transcription-PCR assays with specific COX-2 primers and expressed relative to mRNA for ribosomal phosphoprotein PO (36B4). Columns, mean of three normal tissues and three papilloma tissues from different patients; bars, SD. COX-2 mRNA was elevated nearly 7-fold in papillomas compared with normal laryngeal tissue (*P < 0.01). D, amounts of PGE2 produced by papilloma and normal cells. Cell culture medium from 10⁶ normal laryngeal cells or papilloma cells was analyzed for PGE2 levels by enzyme immunoassay. Papilloma cells produced more PGE2 than normal laryngeal cells, consistent with differences in amounts of COX-2. Columns, mean from three separate experiments; bars, SD (*P < 0.01).
comparable in both types of tissues (data not shown). COX-2 mRNA, normalized to transcripts for a ribosomal protein (36B4), was elevated ~7-fold in papilloma tissues compared with normal laryngeal tissues (*P < 0.01). Overexpression of COX-2 does not necessarily imply that its enzymatic products are elevated because the COX-2 substrate, arachidonic acid, is limited in some cells. We, therefore, measured PGE2 production in cultured cells derived from both papilloma and normal tissues (Fig. 1D). PGE2 production was 4-fold higher in papilloma cells than in normal cells (*P < 0.01). The observed increase in PGE2 biosynthesis was consistent with the increase in amounts of COX-2 in papilloma cells.

**Growth factors induce cyclooxygenase-2 in papilloma cells.** We next investigated whether the expression of COX-2 in cultured normal cells and papilloma cells was regulated by growth factor stimulation (Fig. 2). In the absence of growth factors, the basal level of COX-2 protein was nearly 2-fold higher in papilloma cells than in normal cells (*P < 0.05). Treatment with either 10 ng/mL EGF (Fig. 2A) or 1 ng/mL EGF plus 5 μg/mL insulin (Fig. 2B) caused significant induction of COX-2 in both types of cells (*P < 0.05), with the level in EGF-treated normal cells comparable with the basal level in papilloma cells. Therefore, EGF alone is sufficient to induce COX-2. There was no change in amounts of COX-2 in either type of cell after addition of insulin alone (data not shown). COX-1 levels remained very low with growth factor stimulation and were not elevated in papilloma cells compared with normal cells (data not shown).

**The role of phosphatidylinositol 3-kinase and extracellular signal-regulated kinase in regulating the expression of cyclooxygenase-2.** Activation of EGFR signaling can stimulate both PI3K and ERK. Previously, we reported that EGF stimulates ERK activity in both papilloma and normal laryngeal cells (3). We have now shown that EGF also stimulated the PI3K-Akt pathway in these cells (Fig. 3A). Inhibition of Akt phosphorylation with the PI3K inhibitor LY294002 confirmed that EGF-induced Akt phosphorylation was dependent on PI3K.

To assess the relative importance of ERK and PI3K as determinants of COX-2 overexpression in papillomas, we measured the effects of PD153035, a specific inhibitor of the EGFR tyrosine kinase, PD98059, a mitogen-activated protein/ERK kinase inhibitor that prevents ERK activation, and LY2940042 on COX-2 levels in papilloma and normal cells stimulated with either EGF alone or EGF plus insulin. When stimulated by EGF (Fig. 3B), increased levels of COX-2 could be detected as early as 5 hours after stimulation. Inhibition of EGFR signaling with PD153035 led to a significant reduction in levels of phospho-ERK, phospho-AKT, and COX-2 in papilloma cells. Surprisingly, treatment with PD98059 caused a marked decrease in amounts of phospho-ERK without a corresponding decrease in amounts of COX-2. Inhibiting PI3K with LY294002 caused a significant reduction in COX-2 levels in both normal and papilloma cells. Taken together, these results suggest that EGF induces COX-2 by activating the EGFR—PI3K pathway. Similarly, inhibiting EGFR tyrosine kinase or PI3K but not mitogen-activated protein/ERK kinase suppressed levels of COX-2 in papilloma cells stimulated with both EGF and insulin (Fig. 3C and D). Consistent with the observed effects on COX-2 expression, treatment with inhibitors of EGFR or PI3K led to a significant reduction in PGE2 production by papilloma cells (Fig. 3E).

**Prostaglandin E2 increases and celecoxib decreases levels of cyclooxygenase-2 in papilloma cells.** Other investigators have reported that PGE2 can indirectly stimulate the EGFR (17–19). Because activation of the EGFR stimulated COX-2 expression in papilloma cells, we reasoned that PGE2 might further increase COX-2 levels, promoting a positive feedback loop. To test this possibility, papilloma cells were incubated for 48 hours with 500 nmol/L PGE2 or with celecoxib, a selective COX-2 inhibitor. The cells showed a significant increase in amounts of COX-2 after treatment with PGE2 and a reduction in levels of COX-2 after treatment with celecoxib (Fig. 4). Secreted PGE2 levels were reduced from 400 to 57 pg/mL (P < 0.01) in cells treated with celecoxib, confirming that 3 μmol/L celecoxib was sufficient to significantly inhibit COX-2 activity (data not shown). Importantly, addition of exogenous PGE2 reversed the
suppressive effect of celecoxib on levels of COX-2. Inhibiting EGFR tyrosine kinase activity with PD153035 also suppressed COX-2 levels. Notably, PGE2-mediated induction of COX-2 was suppressed by cotreatment with the EGFR inhibitor. These results support the possibility that COX-2–derived prostaglandins stimulate a positive feedback loop that is, at least in part, mediated through the EGFR and that contributes to the marked expression of COX-2 in papilloma tissues.

**Prostaglandin E2 enhances papilloma cell proliferation, whereas celecoxib enhances apoptosis.** COX-2 expression has been linked to enhanced proliferation and resistance to apoptosis in tumor cells (24). These effects are believed to be mediated, in part, by PGE2. Therefore, we determined whether PGE2 or celecoxib altered the proliferation or apoptosis of papilloma cells. Incubating cells with 250 or 500 nmol/L PGE2 significantly increased the number of metabolically active cells (Fig. 5A). In contrast, incubating cells with increasing doses of celecoxib reduced the fraction of viable cells that were proliferating (Fig. 5B). Papilloma cells express survivin (25) and are relatively resistant to apoptosis. Incubating these cells with celecoxib also enhanced spontaneous apoptosis in a dose-dependent manner (Fig. 5C). Therefore, we conclude that COX-2 is likely to contribute to the phenotypic behavior of human papillomavirus–infected papilloma cells.

**Discussion**

We observed increased levels of COX-2 mRNA, COX-2 protein, and PGE2 production in respiratory papilloma tissues and cells. The distribution of COX-2 extended throughout the papilloma tissues, but seemed most abundant in the suprabasal and spinous layers, where early papillomavirus transcripts are most abundant (26). Activation of multiple signal transduction pathways, including stimulation of growth factor receptors, can induce COX-2 (16). Papilloma cells express nearly 3-fold more EGFR on their surface than normal cells (5.8 fmol/10^5 cells compared with 1.75 fmol/10^5 cells for normal cells) due to recycling of receptor after ligand binding (3). Papilloma cells also require 10- to 100-fold less EGF for maximal EGFR activation and show some activation in the absence of added EGF, which could reflect stimulation by basal levels of endogenous ligands such as transforming growth factor-β (3). This activation could result in elevated basal COX-2 expression. Additional stimulation from exogenous EGF in cell culture would then induce low levels of COX-2 in normal cells and would further increase COX-2 levels in papilloma cells as we have seen.

Experiments were carried out to determine the signal transduction pathway that might explain the increased levels
of COX-2 in papillomas. Interestingly, inhibitors of EGFR tyrosine kinase or PI3K, but not mitogen-activated protein/ERK kinase, suppressed levels of COX-2 in EGF-treated cells. These findings are consistent with the notion that activation of EGFR–PI3K signaling contributes to the elevated levels of COX-2 in papillomas. The studies were done with the chemical inhibitors shown rather than a combination of chemical inhibitors and genetic studies. However, the results fit with previous evidence in other systems that PI3K plays a role in regulating COX-2 expression and PGE2 production (27, 28). Activated PI3K can interact with many other signaling proteins, mediating effects that are both Akt dependent and Akt independent (29). Future studies will be needed to define the pathway downstream of PI3K-Akt that mediates the induction of COX-2 in papilloma cells. Although ERK1/2 mitogen-activated protein kinase has been linked to the induction of COX-2 in other systems (30, 31), it did not seem to be important for regulating the expression of COX-2 in EGF-treated papilloma cells. The reason for this apparent difference between papilloma cells and other cell systems is uncertain.

There is cross-talk between the EGFR and COX-2. As discussed above, EGFR signaling leads to increased levels of COX-2 and enhanced production of PGE2 (10, 32). Importantly, evidence is growing that prostaglandins derived from COX-2 can activate EGFR signaling. The binding of PGE2 to one or more of its receptors can induce the intracellular activation of the EGFR (17, 18) or enhance the expression and release of EGFR ligands resulting in receptor activation (19, 33). We have shown that COX-2 seems to regulate its own expression in papilloma cells through a positive feedback loop. COX-2 protein expression was elevated in papilloma cells incubated with exogenous PGE2 and reduced by treatment with celecoxib. The increase mediated by PGE2 was abrogated by inhibition of EGFR tyrosine kinase activity, implicating EGFR in the feedback loop. It is not known yet whether EGFR activation in papilloma cells is mediated intracellularly or by elevated expression of endogenous ligands. Lin et al. (34) reported that COX-2 expression and/or PGE2 activated PI3K, and PI3K inhibition reduced COX-2 levels. Thus, activation of PI3K via PGE2 receptors, in addition to the EGFR, might play a role in the proposed feedback loop in our cells. COX-2–derived prostaglandins have been reported to increase cell proliferation and

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**Fig. 4.** The activities of COX-2 and EGFR are determinants of COX-2 expression. Papilloma cells were incubated for 48 hours with vehicle (control), 500 nmol/L PGE2, 3 μmol/L celecoxib, 3 μmol/L celecoxib plus 500 nmol/L PGE2, 1 μmol/L PD153035, or 500 nmol/L PGE2 plus 1 μmol/L PD153035. Representative Western blots from three separate experiments are shown. Treatment with PGE2 induced COX-2 whereas celecoxib reduced COX-2 protein levels. The suppressive effect of celecoxib was reversed by cotreatment with PGE2. The EGFR tyrosine kinase inhibitor PD153035 suppressed COX-2 expression. The induction of COX-2 by PGE2 was suppressed by cotreatment with PD153035. COX-2 expression was normalized to total protein in each lane and expressed relative to control cells. Columns, mean of three separate experiments; bars, SD (P < 0.01 compared with control).

**Fig. 5.** Effects of PGE2 and celecoxib on cell proliferation and apoptosis. A, assay of cell growth measuring metabolic reduction oftetrazolium as are surrogate measure of cell number. Treatment of papilloma cells for 48 hours with PGE2 stimulated cell growth (P < 0.01). B, celecoxib caused dose-dependent inhibition of proliferation of papilloma cells. Cells were treated with 2.5, 5.0, or 7.5 μmol/L celecoxib or vehicle (control) for 24 hours, with BrdUrd added for the last 18 hours. Reduction in the relative fraction of cells incorporating BrdUrd into nuclear DNA was indicative of inhibition of proliferation (P < 0.05). The proliferating fraction in the control cells was ~20%. C, apoptosis assay, measured by nucleosome release into the cytoplasm, showing a dose-dependent increase in apoptosis after 24 hours treatment of papilloma cells with 2.5, 5.0, or 7.5 μmol/L celecoxib compared with control cells treated with vehicle (P < 0.05). Columns, means from three separate experiments for (A) and two each for (B) and (C); bars, SD.
inhibit apoptosis (16, 35). Overexpression of COX-2 leads to decreased apoptosis in epithelial cells (35–37). Inhibition of COX-2 induces apoptosis in cancer cells. This effect is mediated, in part, through a reduction in levels of the antiapoptotic protein survivin (38, 39). We have shown that PGE2 stimulated proliferation of papilloma cells and that celecoxib suppressed proliferation and induced apoptosis of papilloma cells. These results suggest that nonsteroidal anti-inflammatory drugs, dual inhibitors of COX-1/COX-2, or celecoxib could be an effective therapy for respiratory papillomas. We are currently beginning a clinical trial to test the efficacy of inhibiting COX activity as an adjunct to surgery for this disease.

In summary, to our knowledge, this is the first study to show that overexpression of COX-2 in human papillomavirus–induced respiratory papillomas is mediated in part by the EGFR and that this occurs through the EGFR→PI3K pathway but not through ERK. We have also shown that induction of COX-2 in papilloma cells seems to regulate its own levels through a positive feedback loop involving the EGFR and that this affects the growth and viability of these cells.

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