Up-regulation of Rac1 by Epidermal Growth Factor Mediates COX-2 Expression in Recurrent Respiratory Papillomas

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INTRODUCTION

Recurrent respiratory papillomatosis (RRP) is caused by human papillomaviruses (HPVs) (1). The papillomas are characterized by the growth of hyperplastic epithelial tissue with defects in regulation of growth and terminal differentiation (2) and resistance to apoptosis due to expression of survivin (3). We previously reported that the epidermal growth factor receptor (EGFR) is overexpressed in papilloma cells, that cyclooxygenase-2 (COX-2) is induced, and that COX-2 expression in primary papilloma cells requires activation of the EGFR but not Erk. Rac1, a member of the Rho family of GTPases, is a key signaling element that is known to control multiple pathways downstream of the EGFR. Here we report that Rac1 is overexpressed in papilloma cells compared with normal laryngeal epithelial cells and that the increased levels of Rac1 are mediated by EGFR activation. Transfecting cells with Rac1-specific siRNA suppressed COX-2 expression. Surprisingly, Rac1 mediated phosphorylation of p38 mitogen-activated kinase in papilloma cells but not normal cells, and inhibition of p38 with the specific inhibitor SB202190 suppressed COX-2 expression in papilloma cells but had no effect on low-level COX-2 expression in normal cells. Thus, the signaling cascades that regulate COX-2 expression are different in HPV-infected papilloma cells, with a significant contribution by the EGFR → Rac1 → p38 pathway.

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Rac1 is a member of the Rho family of GTPases, which control multiple cell functions including cell cycle progression, gene expression, apoptosis, actin organization, cell motility, and the invasive potential of human tumor cells (12-15). Rho GTPases act as switches, coordinating and integrating multiple pathways. Rac1 is regulated by the extent of binding of GTP. It is activated by guanine nucleotide exchange factors (GEFs) that catalyze exchange of GDP for GTP and inactivated by GTPase-activating proteins (GAPs) that promote GTP hydrolysis. Notably, Rac1 protein levels are elevated in some tumors and tumor cell lines by an as yet undetermined mechanism, and the increase may contribute to signaling from activated Rac1 (16-18).

In this study, we found that Rac1 overexpression in papilloma cells is due to increased EGFR signaling and that Rac1 mediates induction of COX-2 in papilloma cells, in part through activation of p38. Furthermore, this is specific to papilloma cells, as the Rac1 → p38 → COX-2 pathway does not function in normal laryngeal cells.
MATERIALS AND METHODS

Tissues and Cultured Cells
Laryngeal papillomas and normal tissues were obtained from surgical biopsies. The use of human tissues and cultured cells was approved by the Institutional Review Board of the Feinstein Institute for Medical Research, 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. Epithelial explant cultures of normal laryngeal cells and papilloma cells were established as previously described (19). Cells were trypsinized and plated at 1 × 10^5 cells/cm^2 in serum-free KGM [keratinocyte basal medium (KBM) (Clonetics, San Diego, CA, USA) supplemented to a final concentration of 1 ng/mL EGF, 5 μg/mL insulin, 2 μg/mL transferrin, 0.5 μg/mL hydrocortisone, 10^-7 M retinoic acid, 100 U/mL penicillin, and 100 ng/mL streptomycin]. Cells were used for experiments while just subconfluent.

For growth factor stimulation studies, cells were cultured in KGM without EGF or insulin for 24 h, and then fed with complete KGM containing either 20 ng/mL EGF or 5 μM insulin for 48 h before extraction. For inhibition studies, cells were preincubated with inhibitor for 1 h, fed with KGM containing inhibitor plus indicated growth factors for up to 48 h, and analyzed by Western blot for COX-2 and Rac1, steady-state levels of phospho-p38 and signal transduction intermediates, and by enzyme immunoassay (EIA) for PGE2. Inhibitors were from Calbiochem (San Diego, CA, USA) and phosphatase inhibitors (20 μM, 50 mL mixed with 50 mL KBM plus 2 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and incubated for 20 min at room temperature to allow complexes to form. Early passage cells, cultured in KGM in 16 mm^2 wells until 60% to 80% confluent, were washed three times with PBS, and the oligonucleotide mixture was added to 200 μL KBM in the well. The cells were incubated at 37 °C for 6 h, 300 μL of complete KGM was added to the well (final concentration 104 nM), and the cells were incubated for an additional 72 h before assay.

Western Blot Analysis

Pulverized frozen tissues were extracted as previously described (8). Briefly, the powdered tissue was suspended in ice-cold hypotonic buffer [100 mM HEPES (pH 7.6), 10 mM KCl, 3 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, and 10% (vol/vol) glycerol plus complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA)] and phosphatase inhibitors (20 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 30 mM sodium fluoride). The extraction buffer for cultured cells contained 1% NP-40, 0.4 M NaCl, 1% glycerol, 1 μM dithiothreitol, and the protease/phosphatase inhibitors (6). Protein concentrations were determined by Micro BCA (Pierce, Rockford, IL, USA). Proteins (30 μg) were separated by 10% SDS-PAGE and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA), blocked with 5% dried milk, incubated with primary antibody overnight at 4 °C, washed, and incubated with secondary antibody. Standard molecular weight markers (broad range, Bio-Rad, Hercules, CA, USA) were used for molecular weight estimation. The immunoreactive species were detected with Super Signal West Pico chemiluminescent substrates (Pierce). After detection, blots were stripped and reprobed sequentially with additional antibodies. Signal intensity was quantified by UN-SCAN-IT (Silk Scientific Inc., Orem, UT, USA), adjusted for total protein as determined by β-actin signal, and normalized to the control cells within each experiment. If bands were very faint, longer exposures than those shown were used for quantitation.

Primary antibodies were as follows: mouse monoclonal anti-Rac1 (Upstate, Temecula, CA, USA) at a dilution of 1:2000, anti-phospho-p38 and anti-p38 (BD Transduction Laboratories, San Diego, CA, USA) at dilutions of 1:500 and 1:1000, respectively, anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:500, polyclonal goat anti-β-actin (Santa Cruz) at 1:1000, polyclonal rabbit anti-phospho-Akt and anti-Akt (Cell Signaling Technology Inc., Waltham, MA, USA) at dilutions of 1:500, and anti-Erk and anti-phospho-Erk (Cell Signaling) at dilutions of 1:500. Secondary antibodies were horseradish peroxidase-conjugated anti-goat, anti-mouse, and anti-rabbit IgGs (Pierce), all used at a dilution of 1:3000.

Measurement of PGE2 Levels

Cell culture medium from 1×10^5 normal laryngeal cells or papilloma cells was assayed by PGE2 EIA according to the manufacturer’s instructions (Amersham Biosciences, Piscataway, NJ, USA). We assayed 50-mL aliquots of each sam-
ple in triplicate. Absorbance was measured at 450 nm with an ELx 800 reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Production of PGE2 was normalized to protein concentration.

**Immunohistochemistry**

Normal laryngeal and papilloma specimens were fixed in 10% buffered formalin, paraffin embedded, and processed for immunohistochemical staining by conventional methods. Sections were incubated with mouse monoclonal anti-Rac1 (Upstate) or with anti-phospho-p38 (BD Transduction Laboratories) at 1:50 dilution, washed with PBS, incubated with appropriate secondary antibody, detected by the avidin-biotin-complex (ABC) method with diaminobenzidine as chromogen (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA), and counterstained with hematoxylin. Controls omitted the primary antibody.

**Determination of Cell Proliferation and Cell Death**

To measure the effect of p38 activation on cell number and spontaneous levels of apoptosis, papilloma cells cultured in KGM were treated for 48 h with 5, 10, or 15 μM SB202190 or an equal volume of DMSO, the solvent for the drug. The relative measure of viable cells was determined by bioreduction of a tetrazolium (MTT) compound (CellTiter 96 Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI, USA) according to manufacturer’s instructions. Apoptosis was measured by cytoplasmic release of nucleosomal fragments using a sandwich ELISA (Cell Death Detection ELISA, Roche Diagnostics, Indianapolis, IN, USA), detected photometrically with 2,2′-azino-di-3-ethylbenzthiazoline sulfonate as substrate.

**Statistical Analysis**

The Student t test was 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**

We first asked whether Rac1 played a role in signal transduction pathways leading to the elevated level of COX-2 in papilloma cells, and the low level of COX-2 seen in normal laryngeal cells in culture. Knockdown of Rac1 protein with specific siRNA significantly reduced COX-2 expression and production of its downstream enzymatic product, prostaglandin E2 (PGE2), in both normal and papilloma cells (Figure 1A). This result strongly suggested that Rac1 plays a central role in EGFR-mediated COX-2 expression in these cells.

Surprisingly, when we analyzed the cells treated with Rac1 siRNA by Western blot we saw elevated levels of Rac1 protein in papilloma cells compared with normal cells (see Figure 1B). Because Rac1 levels in vascular smooth muscle cells are regulated by receptor-mediated signaling pathways, we asked whether EGF stimulation would increase Rac1 levels. Stimulation of either normal or papilloma cells with 20 ng/mL EGF significantly increased Rac1 protein levels compared with baseline ($P < 0.05$) (Figure 2). The basal level of Rac1 protein in the absence of growth factors was 3-fold higher in papilloma cells than in normal cells ($P < 0.01$), and the stimulated levels in normal cells remained below the basal levels in papilloma cells. There was no change in expression of Rac1 in either type of cell.

![Figure 1](image-url)
after stimulation with insulin in the absence of EGF, suggesting that PI3K activity does not contribute to the regulation of Rac1 levels (data not shown). The EGFR inhibitor PD153035 reduced Rac1 levels in papilloma cells below the basal level, consistent with the heightened EGFR-induced signaling in papillomas cells by very low levels of ligands that are constitutively produced by these cells (5). In contrast, the inhibitor simply reduced Rac1 levels close to the basal level in normal cells.

Phosphorylation of p38, one of the potential downstream targets of Rac1, paralleled the levels of Rac1 expression in papilloma cells cultured with EGF (Figure 2). The basal level of phosphorylated p38 in papilloma cells starved of growth factors was elevated more than three-fold compared with normal cells, further increased by EGFR stimulation (*$P < 0.05$), and reduced below the basal level by treatment with the EGFR inhibitor PD153035. Although EGFR stimulation modestly increased p38 activation in normal cells, the increase was barely significant and the EGFR inhibitor had no effect on basal activation. These findings suggest that the EGFR does not significantly contribute to p38 activation in normal cells.

We considered the possibility that the increases in Rac1 levels and p38 activation were artifacts of tissue culture, in which cells are normally grown in the presence of nonphysiologic levels of growth factors. We therefore compared Rac1 expression and phosphorylation of p38 in normal laryngeal epithelium and laryngeal papillomas by Western blots, as well as by immunohistochemical staining to visualize Rac1 and phospho-p38 distribution. There was a significant increase in both Rac1 protein level (*$P < 0.05$) and phospho-p38 (*$P < 0.01$) in papilloma tissues in vivo (Figure 3A). Therefore, the observed increases were not due to tissue culture conditions. Rac1 was abundant in both the basal and spinous layers of the papilloma tissues, and phospho-p38 staining was especially pronounced in the basal and lower and mid-spinous layers, with nuclear staining clearly evident in the basal cells (see Figure 3B). This distribution paralleled the distribution of elevated levels of the EGFR in papilloma cells (4), supporting a role for heightened EGFR signaling in vivo in the overexpression of Rac1 and its activation of downstream targets.

Because changes in Rac1 levels paralleled the extent of phosphorylation of p38, we asked specifically whether Rac1 was upstream of p38 phosphorylation. Rac1 siRNA markedly reduced the phosphorylation of p38 (*$P < 0.05$) in papilloma cells (Figure 4). In contrast, there was no suppression of p38 phosphorylation when normal cells were transfected with Rac1 siRNA. Rather, there was a small but significant and reproducible increase in p38 phosphorylation and a small but insignificant increase in total p38. These results suggest that Rac1 may modestly suppress p38 activation in normal laryngeal keratinocytes and that the regulation of COX-2 by Rac1 in normal cells is not mediated through activation of p38. Phosphorylation of p38 in papilloma cells was dependent primarily on EGFR → Rac1 signaling. Inhibition of PI3K with LY294002, or blocking Erk activation with PD98059, had no effect on phospho-p38 levels (see Figure 4B). Based on the results of these studies, we conclude that at least one of the EGFR-
activated signal transduction pathways downstream of Rac1 that mediates COX-2 expression is different in papilloma cells than in normal cells.

Finally, we investigated whether p38 activation was required for COX-2 expression. Inhibition of p38-α and p38-β activity with SB202190, which also inhibits p38 phosphorylation (21), suppressed COX-2 levels in papilloma cells (Figure 5A), and inhibited both COX-2 expression and PGE2 synthesis in a dose-dependent manner (see Figure 5B). However, inhibiting these 2 p38 isoforms had no effect on COX-2 levels in normal cells (see Figure 5A). This result was consistent with the finding that reduction of Rac1 did not affect p38 activation. The inhibitor did suppress phosphorylation of Akt and Erk in normal cells as well as in papilloma cells. This finding could reflect either direct interaction of these pathways, because p38 can directly contribute to phosphorylation of Akt in some cells (22), or indirect effects on other transcription factors because the cells were treated with inhibitor for 48 h. In either case, p38 signaling does function in normal cells. These results clearly implicate EGFR → Rac1 → p38-α/β signaling as an important contributor to COX-2 expression in HPV 6/11–infected papilloma cells but not in normal laryngeal epithelial cells.

Activation of p38 can have either proapoptotic or antiapoptotic effects on cells, depending on the cell type and interactions with other signaling pathways. We had previously reported that treating papilloma cells with celecoxib, a selective COX-2 inhibitor, reduced proliferation and increased apoptosis (10). Because p38 activation increased COX-2 levels in papilloma cells, but also contributed to activation of both Erk and Akt, we investigated whether inhibiting p38-α/β with SB202190 would enhance or decrease papilloma cell survival. Incubating these cells with SB202190 significantly reduced the number of metabolically active cells and enhanced spontaneous apoptosis in a dose-dependent manner (Figure 5C). Therefore, we conclude that activation of p38 is primarily prosurvival and is likely to contribute to the resistance to apoptosis of HPV-infected papilloma cells.

**DISCUSSION**

The mechanism of induction of COX-2 varies among different cell types. We previously reported that overexpression of COX-2 in papillomas is a consequence of both EGFR and PI3K signaling. However, COX-2 expression in papilloma cells, unlike most tumor lines, does not require Erk signaling (10). Thus, we reasoned that at least one other signaling pathway mediated the EGFR/PI3K induction of COX-2 in these cells. Our studies have now shown for the first time that Rac1 plays an important role in mediating COX-2 expression in HPV-infected papilloma cells, acting in part through p38-α/β, and that the elevated levels of Rac1 in these cells are due to EGFR signaling.

Rac1 transduces signals from both the EGFR and PI3K in other cell types (23, 24, 25), consistent with their contribution to COX-2 expression in papilloma cells (10). Slice et al. (25) reported that signaling by small GTPases results in COX-2 expression in 3T3 cells through independent, parallel signaling pathways. Our
results suggest that Rac1 mediates COX-2 expression through multiple pathways, and that HPV infection alters the downstream pathway(s) regulating COX-2 expression. Clearly, the low level expression of COX-2 in uninfected cells uses different Rac1 effectors from papilloma cells. Knockdown of Rac1 reduced the expression of COX-2 but not p38 phosphorylation in normal cells, and inhibiting p38 had no effect on COX-2 expression. Reducing Rac1 levels in papilloma cells had a greater effect on COX-2 levels than on p38 phosphorylation. Phosphorylation of p38 in papilloma cells required both Rac1 and EGFR activity but not PI3K, suggesting that an EGFR-specific Rac1 GEF mediates this pathway. Tiam1 is one such GEF, acting downstream of Ras and independent of PI3K.

The different Rac1 pathways can also induce COX-2 through different mechanisms; p38 can elevate COX-2 levels by modifying posttranscriptional mRNA stability (27,28), whereas other signaling elements that function downstream of Rac1, such as NF-κB, directly increase COX-2 transcription (29). Therefore, the elevated level of COX-2 expression in papilloma cells may reflect the sum of the use of both mechanisms.

The differences in Rac1 signaling in the two cell types could result simply from elevated levels of Rac1 protein in papilloma cells, thereby altering interactions with downstream effectors. Studies are ongoing to determine the molecular mechanism for this overexpression. Clearly, EGFR signaling increases Rac1 levels in vitro, and EGFR signaling is enhanced in papilloma cells in vitro (5). A similar process may mediate increased Rac1 levels in papilloma tissues in vivo, because the EGFR is highly overexpressed and EGFR signaling, as indicated by Erk phosphorylation, is constitutively active (5). Alternatively, the activation of p38 by Rac1 in papilloma cells could reflect the effect of one or more HPV 6/11 proteins altering multiple intracellular protein interactions, as has been described for HPV 16 (30,31). Future studies will directly address this question, determining whether knockdown of HPV mRNAs affects both Rac1 levels and signaling pathways.

Activation of the different MAP kinases can either enhance or suppress apoptosis, depending on the cell type, the specific kinase, and the cell environment (32). Although the activation of p38 in papilloma cells is generally considered “proapoptotic,” we have shown that it leads to increased cell viability. This effect could be due to the induction of COX-2, the activation of Akt by p38, or a combination of the two mechanisms. Our previous studies showed that inhibiting COX-2 reduced papilloma cell viability (10). The overexpression of PTEN in papilloma cells suppresses Akt activation by PI3K (7). Activation of an Rac1-p38-Akt pathway could compensate for PTEN suppression and enhance viability. The result of increased viability would be enhanced growth of the papillomas. Our present studies suggest that Rac1 or p38 signaling may present a new source of drug targets for therapeutic treatment of recurrent respiratory papillomatosis and other HPV-induced diseases.

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**Figure 4.** Rac1 activates p38 in papilloma cells in response to EGFR signaling, but does not activate p38 in normal cells. (A) Cells were transfected with either Rac1 or luciferase siRNAs as in Figure 1, analyzed by Western blot followed by densitometry with intensity normalized to β-actin. Representative Western blots are shown. Results are the mean ± SD of the ratio of phosphorylated to total p38 in three separate experiments with each type of cells, normalized to actin and expressed relative to the ratio in normal cells transfected with luciferase siRNA (*P < 0.05). (B) Papilloma cells cultured in serum-free KGM containing both EGF and insulin were treated with PD153035 to inhibit the EGFR, PD98059 to inhibit MEK and thus prevent Erk activation, and LY294002, a specific inhibitor of PI3K. A representative Western blot from three separate experiments is shown.
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Figure 5. COX-2 expression is regulated by p38 in papilloma cells but not normal cells. (A) Cells cultured in serum-free KGM with EGF and insulin were treated for 48 h with 10 μM SB202190, a specific inhibitor of p38 which also inhibits p38 phosphorylation, and analyzed by Western blot for expression of COX-2 and for phosphorylation of Erk and Akt. A representative blot is shown. The bar graph shows the mean ± SD relative level of COX-2, normalized to β-actin and expressed relative to levels in control normal cells (*P < 0.05 compared with control cells of each type). (B) Dose-responsive inhibition of COX-2 expression and PGE2 synthesis by papilloma cells treated with SB202190. Cells cultured in KGM with EGF and insulin were treated with 0, 5, 10, or 15 μM SB202190 for 48 h, extracted and analyzed by Western blot for COX-2. Bar graph shows the mean ± SD of COX-2 levels in three experiments, normalized to β-actin, and expressed relative to levels in cells treated with DMSO, the solvent for the inhibitor (*P < 0.05, **P < 0.01). Culture supernatants from three experiments were analyzed for PGE2 by EIA (*P < 0.05, **P < 0.01 compared with controls). (C) Dose-responsive reduction in cell number and increased apoptosis in papilloma cells treated with SB202190. Papilloma cells cultured in KGM with EGF and insulin were incubated for 48 h with increasing concentrations of SB202190. Control cells received vehicle. Metabolic reduction of MTT was used as a surrogate measure of cell number, apoptosis measured by release of nucleosome fragments. Results are means ± SD from three separate experiments. SB202190 significantly reduced papilloma cell number at doses of 10 and 15 μM (*P < 0.01), and increased apoptosis even at the low concentration of 5 μM compared with control cells (*P < 0.01).
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