Abstract. Human laryngeal papilloma (LP) is a benign neoplasm mainly caused by human papillomavirus (HPV) types 6 and 11 (1). Human LP typically starts in the commissure and anterior third of the vocal folds, and subsequently affects the whole larynx, including the trachea, bronchi and lung parenchyma, thereby causing a number of morbidity and mortality cases (2). Incidence of LP is reportedly 4.3 per 100,000 among infants and 1.8 per 100,000 among adults (3). LP is characterized by recurrent lesions are often observed because surgical removal of all superficial papillomatous lesions is difficult when the disease is widespread (2). Thus, developing new methods to treat LP is critical.

Epidermal growth factor receptor (EGFR) is upregulated in LP tissues (6). Multiple signaling molecules associated with EGFR are altered, including mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (7). Previous studies revealed that cyclooxygenase-2 (COX2) expression is elevated in LP tissues and cells, accompanied by MAPK activation (8,9). Phosphorylated MAPKs, including p38 MAPK, extracellular signal-regulated kinase (ERK) 1/2 and c-Jun N-terminal kinase (JNK) are implicated in the induction of COX2 expression (10). Previous studies have shown that COX2 is critical in the progression of LP, and ISO is a potential agent for LP therapy by impeding p38 MAPK/COX2 signaling.

Introduction

Human laryngeal papilloma (LP) is a benign neoplasm mainly caused by human papillomavirus (HPV) types 6 and 11 (1). Human LP typically starts in the commissure and anterior third of the vocal folds, and subsequently affects the whole larynx, including the trachea, bronchi and lung parenchyma, thereby causing a number of morbidity and mortality cases (2). Incidence of LP is reportedly 4.3 per 100,000 among infants and 1.8 per 100,000 among adults (3). LP is characterized by recurrence of premalignant hyperplastic epithelial papillomas (4) and apoptosis resistance (5). Recurrent lesions are often observed because surgical removal of all superficial papillomatous lesions is difficult when the disease is widespread (2). Thus, developing new methods to treat LP is critical.

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ISO inhibits LP malignancy by reducing p38 MAPK/COX2 signaling remains unclear.

The results of the present study show that COX2/PGE₂ biosynthesis was significantly upregulated in LP tissues and cells. The enhancement in COX2 and PGE₂ levels was markedly attenuated by ISO treatment in LP cells. Molecular mechanism analysis revealed that the inhibitory effects of ISO treatment on COX2 expression and activation were mediated by reducing p38 MAPK activation in LP cells. Moreover, ISO administration significantly hindered proliferation and prompted apoptosis of the LP cells via the reduction of COX2 activity. These results suggest that COX2 is a potential therapeutic target of LP, and ISO may be largely beneficial for LP treatment by inhibiting p38 MAPK/COX2 signaling.

Materials and methods

Reagents. Mouse anti-human COX2 (cat. no. 4842S), p38 MAPK (cat. no. 9212), ERK1/2 (cat. no. 4696), JNK (cat. no. 3708S), phosphorylated (p)-p38 MAPK (Thr180/Tyr182; cat no. 9215S), JNK (Thr183/Tyr185; cat no. 9251S) and β-actin (cat. no. 3700) polyclonal antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse anti-human p-ERK1/2 (Thr185/Tyr187; cat. no. ab76299) polyclonal antibody was purchased from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated anti-mouse IgG (cat. no. AP124P) was obtained from Merck Millipore (Merck KGaA, Darmstadt, Germany). SB202190, a specific inhibitor of p38 MAPK, was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). Celecoxib, a selective inhibitor of COX2, was obtained from Pfizer, Inc. (New York, NY, USA). ISO was purchased from Baxter International, Inc. (Deerfield, IL, USA). All other reagents were commercially obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) unless otherwise stated.

Tissue specimens and cell culture. LP and adjacent normal laryngeal tissues were harvested from 5 patients who were undergoing curative resection in the Children's Hospital of Zhengzhou (Zhengzhou, China). None of the patients had received chemotherapy or radiotherapy prior to surgery. Demographic information of the patients is as follows: Case 1, 4-year-old male; case 2, 4-year-old male; case 3, 6-year-old male; case 4, 8-year-old female; case 5, 1-year-old female. Biopsies were used to establish primary cell cultures or frozen in liquid nitrogen until use. Epithelial explant cultures of normal laryngeal and LP cells were established in Ham's F12 with 10 µg/ml hydrocortisone and 10 ml /100 ml fetal clone II (Hyclone; GE Healthcare, Little Chalfont, UK) as previously described (17). These cultures are >99% epithelial, based on morphology, keratin expression, and episomal HPV DNA (17). Normal laryngeal cells were expanded for ≤2-3 passages, whereas LP cells were used at first passage. Cells were trypsinized and plated at 2x10⁵ cells/cm² in serum-free keratinocyte growth medium (KGM; Clonetics Corp., San Diego, CA, USA), and used for experiments while subconfluent and proliferating. Experiments were performed at least thrice with cells derived from different patients unless otherwise noted. The use of human biopsies was approved by the Institutional Review Board of Women and Infants Hospital of Zhengzhou (Zhengzhou, China). Informed consent was signed by each subject's guardian.

Experimental protocols. LP and normal laryngeal cells were cultured in KGM for 24 h, and the cells were subsequently treated without (control) or with 1.4% ISO for 0.5 h or 2 l/min in a metabolic chamber (Columbus Instruments International Corporation, Columbus, OH, USA). During ISO exposure, the ISO concentration (1.4%) was continuously verified by sampling the exhaust gas with a Datex Capnomac (Soma Technology, Inc., Bloomfield, CT, USA) (18). To investigate the inhibitory effects of SB202190 or celecoxib, cells were treated with SB202190 (10 µM) or celecoxib (5 µM) for 1 h and continuously cultured for the indicated periods.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the frozen LP tissues and cultured cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Carsbad, CA, USA) following the manufacturer's protocol. The RNA (50 µg) was treated with 2 µl RQ1 RNase-free DNase (Promega Corporation, Madison, WI, USA) in 50 µl 10X DNase buffer with 0.5 µl RNase inhibitor and diethylpyrocarbonate-ddH₂O (to a final volume of 50 µl) at 37°C for 20 min. The concentration and quality of the RNA were measured by UV absorbance at 260 and 280 nm (260/280 nm) using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Reverse transcription was performed using 3 µg RNA with SuperScript²™II Reverse Transcriptase (Invitrogen), and cDNA was generated and detected by qPCR (5 µg per reaction) using SYBR Premix Ex Taq™(Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocols. GAPDH was used as endogenous control. The relative mRNA level of COX2 was calculated using the 2⁻ΔΔCt method (19). The primers used for PCR amplification were as follows: COX2, forward, 5'-TCTCTCTGGTGTTAAGGCAATT-3', and reverse, 5'-CTGAGGCGGTCTGAGCTT-3'; and PGE₂, forward, 5'-GGAAATGCGTTGACATT-3', and reverse, 5'-CAGGCGTCTGAGCTT-3'. The amplification was performed for 35 cycles using a denaturing temperature of 94°C (1 min), annealing temperature of 58°C (1.5 min), and extension temperature of 72°C (1.5 min). Results were analyzed using ABI 7500 Real-Time PCR System software v2.0.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Measurement of PGE₂ production. At 24 h after the LP or normal laryngeal cells were treated with ISO or celecoxib or SB202190, PGE₂ production was quantified in the culture medium from 1x10⁵ LP or normal laryngeal cells. PGE₂ was measured using a radioimmunoassay (RIA) kit (Amersham Biosciences Europe GmbH; GE Healthcare, Freiburg, Germany) as previously described (20). The experiments were performed in triplicate.

Western blot analysis. The frozen tissues and cultured cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). For tissues, ~1 ml RIPA lysis buffer was added to 100 mg tissues and homogenized on ice. For cells, cells in the 6-well plates were washed with 2 ml phosphate-buffered saline (PBS; Beyotime Institute of Biotechnology) twice and then treated with 100 µl well RIPA lysis buffer on ice for 30 min. The
homogenates or cell lysates were sonicated on ice (five times for 5 sec each at 40 W, with 20-sec intervals between each sonication) with a Braun Labscono 2000 microtip sonifier (Braun, Melsungen, Germany) and centrifuged at 12,000 x g for 10 min at 4°C. The protein concentration of the extracts was measured by the bicinchoninic acid method (Pierce Biotechnology; Thermo Fisher Scientific, Inc., Rockford, IL, USA). Samples containing equal amounts of proteins (25 µg) were boiled in sodium dodecyl sulfate (SDS) sample buffer and then analyzed by 10% SDS-polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology). The proteins were electrotransferred to nitrocellulose membranes (Merck Millipore), and blocked with 5% non-fat milk in Tris-buffered saline (50 mM Tris-HCl pH 7.4, 150 mM NaCl) and 0.1% Tween 20 (TBST) with shaking at room temperature for 1 h. Membranes were incubated with primary antibodies targeting COX2 (1:2,000, p38 MAPK (1:1,500), ERK1/2 (1:1,000), JNK (1:1,000), p-p38 MAPK (Thr180/Tyr182; 1:1,000), p-ERK1/2 (Thr202/Tyr204; 1:1,000), p-JNK (Thr183/Tyr185; 1:1,000) and β-actin (1:2,000) overnight at 4°C. After washing with TBST thrice, 5 min per time, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000) in TBST at room temperature for 1 h. Equal sample loading was confirmed using β-actin. Protein expression was detected by chemiluminescence film (Amersham Biosciences) using an enhanced chemiluminescence assay kit (Pierce Biotechnology). The protein bands were quantified using Quantity One software v4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell viability assay. At 24 h after LP cells were treated with ISO or celecoxib, the number of viable cells was determined using a Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer’s instructions. In brief, cells were seeded in 96-well plates at a concentration of 1x10^3 per well and cultured for 1, 2, 3 and 4 days. CCK-8 solution (10 µl) was added into each well at the indicated time points, then the plates were stored for 2 h at 37°C. A scanning multi-well spectrometer (Bio-Tek Instruments, Inc., Winooski, VT, USA) was used to measure the absorbance at 450 nm.

5-Ethynyl-2-deoxyuridine (EdU) incorporation assay. At 24 h after LP cells were treated with ISO or celecoxib, 50 µM EdU was added and the cells were incubated for 2 h, then fixed with 4% paraformaldehyde for 20 min at room temperature. Subsequently, the cells were incubated with reaction buffer (0.5% Triton X-100; Guangzhou Ribobio Co., Ltd., Guangzhou, China) for 30 min. After washing twice with cold PBS, cells were counterstained with 4,6-diamidino-2-phenylindole (Beyotime Institute of Biotechnology). Six random fields were selected for observation and then photographed under an inverted fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany).

Cell cycle analysis. At 24 h after the LP cells were treated with ISO or celecoxib, the cells were washed with PBS, fixed with ice-cold 70% ethanol and treated with 1 mg/ml RNAse for 30 min at 37°C. DNA content staining was performed with 50 µg/ml propidium iodide (PI) at 4°C in the dark for 30 min. A FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was utilized for data measurement, and the data were analyzed using CellQuest Pro software (BD Biosciences).

Apoptosis assay by flow cytometry. For apoptosis assessment, Annexin V-fluorescein isothiocyanate (FITC) and PI staining was performed as previously described (21). In brief, at 24 h after LP cells were treated with ISO or celecoxib, the cells were harvested, centrifuged at 10,000 x g and 4°C for 5 min and resuspended in binding buffer (BD Biosciences). Annexin V-FITC (10 µl; BD Biosciences) was added, incubated at room temperature for 15 min, and counterstained with 5 µl PI for 30 min. Annexin V-FITC and PI fluorescence was analyzed using a FACSCalibur flow cytometer and the results were analyzed using CellQuest software.

Nucleosomal fragmentation assay. At 24 h after LP cells were treated with ISO or celecoxib, cell apoptosis was measured using a nucleosomal fragmentation kit (Cell Death Detection ELISA PLUS; Roche Applied, Science, Penzberg, Germany) as previously described (16). The absorbance values were normalized against those from control-treated cells to derive a nucleosomal enrichment factor.

Quantitative caspase-3 activity assay. Caspase-3 activity was detected using a Caspase-3/CPP32 Colorimetric Assay kit (BioVision, Inc., Palo Alto, CA, USA) as previously described (16). Briefly, at 24 h after LP cells were treated with ISO or celecoxib, 1x10^4 cells were incubated with 50 µl chilled lysis buffer on ice for 10 min. The supernatant was then collected after 10,000 x g centrifugation at 4°C for 10 min. Protein (150 µg) was added to 50 µl 2X reaction buffer containing 5 µl N-acetyl-Asp-Glu-Val-Asp-pNA substrate (final concentration, 200 µM). N-acetyl-Asp-Glu-Val-Asp-pNA cleavage was monitored by detecting enzyme-catalyzed release of pNA at 405 nm after incubation at 37°C for 2 h using a microplate reader (Bio-Tek instruments, Inc.).

Statistical analysis. All values are expressed as the mean ± standard derivation. Intergroup differences were determined by Student's two-tailed unpaired t-test or one-way analysis of variance, followed by Dunnett's post hoc test as appropriate. GraphPad v5.0 statistical software (GraphPad Software, Inc., San Diego, CA, USA) was used to perform data analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

COX2 expression and PGE2 production are increased in LP tissues. The mRNA and protein levels of COX2 in LP tissues and normal laryngeal biopsies were evaluated using RT-qPCR and western blot analyses, respectively. As shown in Fig. 1A, the COX2 mRNA expression level was elevated by approximately fivefold (P<0.05) in LP tissues compared with normal laryngeal tissues. Furthermore, COX2 protein expression was significantly higher in LP tissues than that in normal laryngeal biopsies (P<0.01; Fig. 1B and C). Consistently, the mRNA and protein levels of COX2 were significantly increased in cultured LP cells derived from LP tissues compared with the normal cells isolated from normal...
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laryngeal tissues (Fig. 1D-F). PGE2 production, an indicator of COX2 activity, was measured by RIA in LP and normal laryngeal cells. As shown in Fig. 1G, PGE2 level was eightfold (P<0.01) higher in LP cells than that in normal cells, which was in accordance with the expression tendency of COX2. These results suggest that COX2 expression and PGE2 generation are increased in LP.

ISO reduces COX2/PGE2 biosynthesis in LP cells. Wang et al (16) demonstrated that ISO reduces COX2
expression and PGE₂ release in Kupffer cells. We investigated whether ISO treatment inhibited COX2 expression and PGE₂ production in LP and normal laryngeal cells. As shown in Fig. 2A and B, the expression of COX2 was nearly threefold (P<0.05) lower in ISO-treated LP cells than in control LP cells. Alternatively, ISO treatment had no effect on COX2 expression in normal laryngeal cells. PGE₂ production was significantly decreased by ISO treatment in LP cells but did not change in normal laryngeal cells (Fig. 2C). As a positive control, celecoxib administration also decreased COX2/PGE₂ biosynthesis in LP cells (Fig. 2A-C). These results indicate that ISO treatment counteracts the increase in COX2 expression and PGE₂ production in LP cells.

ISO reduces COX2 expression and activity by inactivating p38 MAPK in LP cells. MAPK activation has been implicated to be involved in the upregulation of COX2 (10). The present study investigated whether ISO treatment hindered COX2 expression and PGE₂ production by reducing p38 MAPK activation in LP cells. Western blot results showed that ISO treatment significantly reduced the phosphorylation of p38 MAPK (Thr180/Tyr182) but not those of ERK1/2 (Thr185/Tyr187) and JNK (Thr183/Tyr185) in LP cells (Fig. 3A and B). However, ISO treatment had no effect on phosphorylation of MAPKs in normal laryngeal cells (Fig. 3A and B). In addition, it was found that treatment with 10 μM SB202190 for 1 h, which is a p38 MAPK activation inhibitor, led to a notable reduction of COX2 expression in LP cells (Fig. 3C and D). Furthermore, PGE₂ production was significantly reduced in SB202190-treated LP cells (Fig. 3E). These findings indicate that ISO treatment reduces COX2 expression and activity in LP cells by inhibiting p38 MAPK activation.

ISO inhibits LP cell proliferation by decreasing COX2 activity. COX2 activity is involved in increased proliferation of LP cells (11). To investigate whether the inhibitory effects of ISO on LP cell viability and proliferation depend on the reduction of COX2 activity, CCK-8, flow cytometry and EdU incorporation assays were performed. As shown in Fig. 4A, ISO administration significantly reduced the viability of LP cells. Cell cycle was arrested at the G1 phase, with 66% of
ISO-treated LP cells in G0/G1 compared with 48% of control cells (P<0.05; Fig. 4B). The percentage of EdU incorporation was also decreased, with 22% of ISO-treated LP cells compared with 64% of control cells (P<0.01; Fig. 4C). All these results were consistent with the inhibitory effects of celecoxib on LP cell viability and proliferation (Fig. 4A-C), suggesting that ISO treatment inhibits the viability and proliferation of LP cells, probably by reducing COX2 activity.

ISO promotes LP cell apoptosis by inhibiting COX2 activity. Increase in COX2 activity has been shown to inhibit LP cell apoptosis (12). Flow cytometry, nucleosomal fragmentation and caspase-3 activity assays were employed to investigate whether ISO inhibited LP cell apoptosis by inhibiting COX2 activity. Flow cytometry analysis showed that ISO treatment significantly increased the percentage of apoptotic LP cells compared with the control cells (Fig. 5A). The significant increases in nucleosomal fragmentation and caspase-3 activity were observed in ISO-treated LP cells compared with the control cells (Fig. 5B and C). The above results were consistent with the proapoptotic effects of celecoxib on LP cells (Fig. 5). Thus, ISO administration promotes the apoptosis of LP cells probably by inhibiting COX2 activity.

Discussion

Cultured LP cells are a model system for the study of HPV-infected LP. In contrast to immortalized cell lines, these primary cells closely reflect the biology of in vivo papilloma. In the present report, it was found that ISO treatment significantly inhibited proliferation and prompted apoptosis in LP cells by reducing p38 MAPK/COX2 signaling. The key findings are as follows: First, COX2 was highly expressed in LP tissues and cells compared to normal laryngeal tissues and cells. Second, ISO treatment significantly inhibited COX2 expression and PGE2 production in LP cells. Third, the inhibitory effects of ISO on COX2 expression and activity depend on the inactivation of p38 MAPK in LP cells. Finally, ISO markedly decreased the proliferation and apoptosis resistance of LP cells by inhibiting COX2 activity.

COX2, an inducible enzyme, serves a crucial function in the production of prostaglandins under physiological and pathophysiological conditions, which can be rapidly induced by various stimulants, such as growth factors, carcinogens and proinflammatory cytokines (22). COX2 expression is enhanced in a variety of inflammatory and neoplastic diseases (23). Notably, COX2 expression is upregulated in numerous types of HPV-infected cells, including respiratory papillomas (24), head and neck tumors (25-27), cervical cancers and penile cancers (28,29). Wu et al (11) reported that COX2 upregulation is mediated in part by Rac1-dependent activation of p38 MAPK in papilloma cells. Wang et al (16) demonstrated that ISO administration markedly decreased COX2 expression via the inhibition of p38 MAPK activation in zymosan-stimulated murine Kupffer cells. The present results showed that ISO treatment significantly suppressed COX2 expression and activity by inhibiting p38 MAPK activation.
Activation of p38 MAPK can exert either proapoptotic or antiapoptotic effects in different cell types and cell microenvironments (30). p38 MAPK activation has been shown to increase cell viability in LP cells (11). This effect may be due to the increased levels of COX2 induced by p38 MAPK activation (12). Previous studies showed that COX2 upregulation enhances proliferation and apoptosis resistance, and inactivation of COX2 induces cell apoptosis in cancer cells (31-33). Treatment with celecoxib, an inhibitor of COX2, could effectively suppress cell proliferation and induce cell apoptosis in LP cells, and these effects are mediated in part by PGE2 (12). Notably, excessive production of PGE2 has been reported to promote cell proliferation and inhibit cell apoptosis (34). ISO inhibits proliferation of several human cancer cell lines (15). The present results showed that ISO treatment markedly decreased LP cell viability and proliferation by inhibiting COX2 activity. Additionally, ISO has been demonstrated to induce apoptosis in different cell types (35,36), and prolonged ISO treatment can induce apoptosis in cancer cells (15). A previous study suggested that the effect of ISO on apoptosis depends on the mitochondrial pathway (37), which is regulated by Bcl-2 family proteins; this pathway also involves the release of cytochrome c from the mitochondria to the cytosol (38). The released cytochrome c activates caspase-9, and consequently induces caspase-3 activation, ultimately leading to cell apoptosis (39). Thus, the present findings showed that ISO treatment significantly increased LP cell apoptosis by inhibiting COX2 activity.

In summary, the present study showed that COX2 is highly expressed in HPV-infected LP tissue and cells compared with normal laryngeal tissues and cells, and PGE2 production is increased in LP cells. Notably, it was found that ISO treatment significantly reduces COX2 enhancement and PGE2 release in LP cells by inhibiting the activation of p38 MAPK. In addition, ISO significantly inhibits cell proliferation and apoptosis resistance by inhibiting COX2 activity. Collectively, ISO may be a potential agent for LP treatment by inhibiting p38 MAPK/COX2 activation.

References

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