Lipopolysaccharide Induction of Cyclooxygenase-2 in Long-term Nasal Epithelial Cultures in Cystic Fibrosis

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Objective: To evaluate the lipopolysaccharide induction of cyclooxygenase-2 (COX-2) in long-term epithelial cultures from nasal polyp tissue of patients with cystic fibrosis (CF) and severe nasal polyposis.

Design: Experimental and histologic study.

Setting: Department of Otorhinolaryngology, University of Ulm, Ulm, Germany.

Participants: Nasal polyp tissue was evaluated from 9 patients with CF and obstructing nasal polyps undergoing elective sinus surgery. Nasal mucosa from the hypertrophic inferior turbinate of 9 patients without a history of CF or aspirin intolerance undergoing nasal corrective surgery served as control specimens.

Interventions: Tissue culturing, Western blotting, and tissue staining with hematoxylin-eosin.

Main Outcome Measures: The expression and lipopolysaccharide induction of COX-2 was detected and compared with those of the control group. Tissue was analyzed for the presence of inflammatory cells such as neutrophils, eosinophils, mast cells, plasma cells, lymphocytes, and monocytes.

Results: COX-2 was detectable in tissue specimens from all of the patients with CF and control subjects. In patients with CF, however, COX-2 expression was significantly lower in lipopolysaccharide-stimulated, long-term cultured epithelial cells compared with control tissue. Polyps from patients with CF contained markedly more neutrophils, macrophages, and plasma cells than did nasal mucosa from hypertrophic inferior turbinates of controls.

Conclusions: COX-2 expression in lipopolysaccharide-stimulated, long-term cultured epithelial cells in patients with CF is decreased compared with that in hypertrophic turbinate mucosa. The estimated defect in lipopolysaccharide responsiveness and the reduced induction of COX-2 needs further investigation.


Cystic fibrosis (CF) is the most prevalent lethal autosomal recessive disease in the white population. Patients with CF generally have involvement of the paranasal sinuses and often develop recurrent nasal polyps (NPs). The incidence of nasal polyposis in CF varies from 6% to 48%. Cystic fibrosis is caused by mutations in a single gene responsible for coding of the CF transmembrane conductance regulator, an adenosine triphosphate–gated Cl− channel in the apical membrane of diverse epithelial cells. Arachidonic acid (AA) is the primary substance of the so-called AA cascade, resulting in proinflammatory and anti-inflammatory mediators. Cyclooxygenase (COX), an enzyme also called prostaglandin H synthase, has a key role in the conversion of AAs to prostaglandins, thromboxanes, and prostacyclins. Conversion occurs through 2 distinct reactions—COX activity, which transforms AAs into prostaglandin G2, and peroxidase activity, which further converts prostaglandin G2 to prostaglandin H2. Prostaglandin H2 is then transformed into prostacyclin, thromboxane A2, and prostaglandins by specific synthases.

To date, 2 isoforms of COX have been discovered. In general, COX-1 is a constitutive, or “housekeeping,” enzyme that is expressed in most tissues. COX-2 is considered an “inducible” form of the enzyme. The expression of this isoform can be induced by various substances, such as lipopolysaccharides, cytokines (eg, interleukin 1β), growth factors (eg, tumor necrosis factor α), phorbol esters (12-O-
tissues, such as lipopolysaccharides, the objective of this study was to investigate the expression and lipopolysaccharide induction of COX-2 protein in cultured human sinonasal cells. Pujols et al showed low COX-2 expression in aspirin-tolerant and aspirin-intolerant patients with NPs compared with that in healthy NM. Patients with chronic rhinosinusitis without polyps had high expression of COX-2, whereas patients with NPs showed substantially lower COX-2 expression in nasal polypous epithelial cells. Whereas Pérez-Novo et al demonstrated downregulation of COX-2, especially in aspirin-intolerant patients with chronic rhinosinusitis and NPs, Liu et al showed upregulation of COX-2 in aspirin-tolerant patients and Owens et al in aspirin-tolerant and aspirin-intolerant patients.

Roca-Ferrer et al studied the expression of COX-2 in NPs of patients with CF and observed significant upregulation of COX-2 protein levels compared with that in non-CF polyp tissue or NM. Owens et al recently also studied the expression of COX-2 in the sinonasal mucosa of patients with CF. Expression of COX-2 in columnar epithelium was noted in all of the CF specimens. COX-2 was expressed in the submucosal glands and in the cytoplasm in 86% of the CF specimens.

The invasion of the respiratory epithelium by neutrophils is a typical finding in CF airway mucosa, indicating bacterial colonization or mucosal infection. The expression of COX-2 can be induced by various substances, such as lipopolysaccharides, the objective of this study was to investigate the expression and lipopolysaccharide induction of COX-2 protein in cultured human NP explants from patients with CF. In addition, we examined the morphologic features of the nasal epithelium and the presence of inflammatory cells in CF polyp sections and in hyperplastic NM of control subjects.

METHODS

PARTICIPANTS

Patients were recruited from the Department of Otorhinolaryngology at the University of Ulm during a 3/2-year period. After an initial 1-year pilot study including 4 patients with CF, an additional 5 patients with CF undergoing functional endoscopic sinus surgery for nasal polyposis were included in the study. Cystic fibrosis was diagnosed by positive sweat chloride test results and characteristic genotype abnormalities, as reported elsewhere by the same working group. The mean age of the 9 patients with CF (3 males and 6 females) was 21 years (age range, 12-35 years; median NP score, 2). Nine patients undergoing septoplasty or septrhinoplasty because of anatomical variations and not from having any sinus disease were considered controls (NM group). None of the patients in the NM group had bronchial asthma or evidence of CF or aspirin-intolerance syndrome. The mean age of the controls (4 males and 5 females) was 22 years (age range, 18-42 years; no nasal polyposis). Patients with known immunodeficiency disorders or use of topical or systemic corticosteroids were excluded from the study.

Tissue samples of NPs protruding from the ethmoid of patients with CF and NM from the anterior/inferior part of the hypertrophic inferior turbinate of patients without CF were obtained during nasal surgery. At the time of surgery, patients with CF and controls were not taking corticosteroids or nonsteroidal anti-inflammatory drugs. All of the participants agreed to participate in the study, which was approved by the institutional ethics committee of the University of Ulm.

Tissue Handling

All nasal samples for histopathologic examination were immediately fixed in formaldehyde and embedded in paraffin. For each NP and NM sample, a section was systematically stained with hematoxylin-eosin for standard histomorphologic analysis and morphologic evaluation according to the method of Lesprit et al. Immediately after removal, NPs and NM for COX detection were cut into approximately 1-mm³ cubes, and these fragments were placed in 10-ml test tubes with 5 ml of Dulbecco modified eagle medium, on room temperature, containing 0.1% Accutase, penicillin (10⁵ U/L), streptomycin sulfate (100 mg/L), and gentamicin sulfate (50 mg/L). After 1 hour of gentle shaking, fetal bovine serum (FBS) was added to neutralize the Accutase. The solid parts of the polyp were discarded, and the remaining epithelial suspension was washed twice with Dulbecco modified eagle medium/F12 1:1 culture medium containing 10% FBS and antibiotics as described previously herein. The pellet was resuspended in 5 ml of culture medium in a small flask and was incubated on a controlled atmosphere at 37°C with 5% carbon dioxide. During the first 3 to 4 hours, the flask was gently moved to reduce attachment of cell sheets to the bottom of the flask. The medium was changed daily for the first 2 days and then 3 times weekly. From the third day, gentamicin was omitted from the medium. Cells were then seeded in a 6-well culture plate. After a minimum of 3 hours, the medium was changed to a serum-free medium (culturing medium without FBS), and cells were grown to confluence.

Western Blotting

On the basis of a preliminary study on NP immortalized cells from patients with CF, COX-2 detection in this type of cell was established. After 72 hours in culture and the cells growing to confluence (approximately 106 cells per sample), the medium was changed to a serum-free medium (culturing medium without FBS) with or without lipopolysaccharide supplementation. We used lipopolysaccharide, 10 μg/mL, for stimulation for 20 hours.

After 20 hours of incubation at 37°C with 5% carbon dioxide, the cell medium was discarded and the cells were trypsin-
ized with 500 µL of trypsin-EDTA solution for 5 minutes. Trypsinized cells were transferred into labeled Eppendorfs vials containing 500 µL of culture medium supplemented with 10% FBS to stop the trypsin effect. The cell suspension was then centrifuged with 400 g at 4°C for 5 minutes. Supernatant was carefully discarded. Cells were resuspended in lysate buffer (100 µL of 1% Triton-X-100 in phosphate-buffered saline [PBS] supplemented with a Complete protease inhibitor cocktail tablet [Roche, Basel, Switzerland]) and were left on ice for 30 minutes at 4°C. Cell lysates were sonicated twice for 20 seconds. As a next step, cells were centrifuged for 15 minutes at 4°C with 10 000 g.

Ten microliters of sample-loading buffer (4X NuPAGE LDS; Invitrogen, Carlsbad, California) was added to 10 µL of the supernatant. All the samples were heated in a 70°C water bath for 10 minutes. Rainbow molecular weight marker (15 µL) (Amersham Biosciences, San Francisco, California), positive control for COX-2 (7 µL) (BD Transduction Laboratories, BD Biosciences, Mississauga, Ontario, Canada), and samples were loaded on a 4% to 12%, 15-well NuPAGE Bis-Tris ready gel (Invitrogen). After electrophoresis, proteins were transferred to a polyvinyl difluoride membrane (Bio-Rad Laboratories, Hercules, California). Blot was run at 0.8 mA/cm² (2 blots: 27.5 cm = 60 mA for 2 gels), minimal voltage (10 V), for approximately 1 hour. We used the semidry blotting technique (Pegasus; Phase GmbH, Lubeck, Germany). After blotting, polyvinyl difluoride membranes were washed with aqua dest and were placed in blocking buffer (10% skim milk–PBS solution) overnight at 4°C to block nonspecific binding sites.

Membranes were washed in PBS and were incubated with the primary antibody solution (antibody dilution buffer; Invitrogen; anti–COX-2 antibody, 1:500, mouse IgG1; BD Transduction Laboratories, BD Biosciences). As a next step, membranes were washed 3 times with 0.05% PBS-polyborate solution, and then the secondary antibody solution was applied (1:1000, peroxidase-linked anti–mouse IgG from sheep; Amersham Biosciences).

After a new series of washes, COX-2 immunoreactive bands were visualized using a light-emitting chemiluminescent method (SuperSignal Femto Maximum; Invitrogen). Light emission was detected by exposure to blue light–sensitive autoradiography film (Hyperfilm ECL; Amersham Biosciences).

Chemiluminescent intensity was semiquantitatively assessed and was graded as none, low, medium, or high for each sample. Evaluations were performed by the same person (A.R.) for all samples to reduce rater-related variability. Statistical analysis was performed to compare the chemiluminescent intensities in samples from patients with and without CF using the Fisher exact test. Statistical significance was defined as P < .05.

RESULTS

SPONTANEOUS REGULATION OF COX-2 EXPRESSION

In all of the cell lines, spontaneous but low-level expression of COX-2 in long-term cultured epithelial cells could be seen. Comparing the 2 different groups, cultured cells from the NPs of patients with CF without lipopolysaccharide stimulation had a nonsignificantly higher level of COX-2 content than did NM cells from patients without CF (P > .05) (Figure 1).

EFFECT OF LIPOPOLYSACCHARIDE ON COX-2 EXPRESSION

COX-2 was only slightly upregulated when stimulated with lipopolysaccharide in cultured cells from CF NPs (P > .05) (Figure 2). Cultured cells from NM of inferior turbinates of controls showed stronger upregulation of COX-2 after stimulation with lipopolysaccharide (P < .05) (Figure 2).
The NPs of patients with CF showed various patterns of acute inflammation (ie, only a few or missing glands in the stroma) and chronic inflammation (ie, seromucinous glands present in the stroma) (Figure 3). Neutrophils were numerous in CF polyp tissue, infiltrating intraepithelially and subepithelially. Only low numbers of eosinophils were seen in CF NPs. In non-CF NM, relatively few macrophages, lymphocytes, and granulocytes were found.

HISTOLOGIC EXAMINATION

The etiology and pathogenesis of nasal polyposis in CF are poorly understood. Chronic neutrophilic airway inflammation is a main characteristic of CF. Previous studies8-10,19,20 on proinflammatory leukotrienes and the COX and 5-lipoxygenase pathways of the respiratory epithelium have shown an impact of the AA cascade on respiratory inflammation in CF.

The objective of this study was to investigate whether there is an abnormality in the AA cascade, especially of the COX pathway similar to that in aspirin-intolerant asthma,14 in the pathogenesis of NPs in CF. For this purpose, the presence of COX-2 in NP tissue in patients with CF and severe nasal polyposis and its induction by incubation with lipopolysaccharide was evaluated. In addition, histologic examinations of NPs and evaluation of neutrophilic infiltration of the epithelium were performed.

Nasal polyp tissue was evaluated from 9 patients with CF undergoing sinus surgery. Tissue was analyzed for the presence of neutrophils, indicating neutrophilic inflammation. Additional NP tissue was obtained for COX-2 determination. Conchal mucosa from the inferior turbinate of 9 controls undergoing corrective surgery served as control tissue. Polyps from patients with CF contained markedly more neutrophils than did NM from controls. COX-2 was detectable in all patients with and without CF. Cultured cells from the NPs of patients with CF without lipopolysaccharide stimulation had a nonsignificantly higher level of COX-2 content than did nasal mucosal cells from controls. In patients with CF, however, COX-2 expression was significantly lower in lipopolysaccharide-stimulated long-term cultured epithelial cells compared with that in control tissue.

These data indicate that in patients with CF and NPs, a feasible defect in lipopolysaccharide responsiveness seems to be responsible for significant downregulation of COX-2 after lipopolysaccharide stimulation compared with control tissue from the inferior turbinate.

Another study13 has reported spontaneous upregulation of COX-2 with the time in cultured NP explants from aspirin-tolerant patients without CF and in normal NM. According to the time curve related to the level of COX-2 content in a certain amount of cells, spontaneous upregulation of COX-2 in normal NM can be seen starting from the zero point at the time of nasal surgery, reaching its peak 24 hours after surgery. A lower and delayed peak could be seen in NPs from aspirin-tolerant patients 72 hours after putting polyp explants into culture. Under stimulation with proinflammatory cytokines, normal NM showed upregulation of COX-2 expression, whereas in NPs, delayed upregulation of COX-2 was seen. The present findings of weaker spontaneous upregulation of COX-2 in CF NPs are in contrast to these results in non-CF NPs and underline the possible different etiology of NPs in CF disease.

Neutrophilic inflammation was histologically found in polyp specimens from all the patients with CF. Expression of COX-2 is induced by bacterial products from Pseudomonas aeruginosa, a bacteria often found in patients with CF and NPs.25 This observation may explain the finding of an upregulated COX-2 protein level in NPs of CF detected by our and other work groups.19,20 However, the mechanism of induction and suppression of COX-2 in CF NPs is not yet completely understood.26

In summary, COX-2 expression in lipopolysaccharide-stimulated, long-term cultured epithelial cells in patients with CF is decreased compared with expression in hypertrophic turbinate mucosa. The estimated defect in lipopolysaccharide responsiveness and the reduced induction of COX-2 needs further investigation.

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Figure 3. Inflammatory polyoid nasal tissue from a patient with cystic fibrosis (hematoxylin-eosin, original magnification ×360). The epithelium (upper part of the figure) is broad and edematous. Note the neutrophils infiltrating the subepithelial connective tissue and lamina propria (residual neutrophils) (a) and the epithelial layer of the nasal polyp (b). The subepithelial connective tissue and lamina propria are dominated by lymphoid cells (c), plasma cells (d), and macrophages (e). Eosinophils were not regularly seen. f Indicates stromal cell.

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