Inducible Cyclooxygenase and Interleukin 6 Gene Expressions in Nasal Polyp Fibroblasts

Possible Implication in the Pathogenesis of Nasal Polyposis

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Background: Inflammation is believed to be related to the pathogenesis of nasal polyp (NP). Inducible cyclooxygenase (COX-2) and interleukin (IL) 6 are important mediators of inflammation. However, no information is available regarding the expression of these mediators in nasal polyp fibroblasts (NPFs). The inductive effects of proinflammatory cytokines (IL-1β or tumor necrosis factor α) alone or in combination with prostaglandin E2 on IL-6 and COX-2 messenger RNA (mRNA) synthesis in NPFs were investigated.

Design: The expressions of IL-6 and COX-2 mRNAs in NPFs and in 34 surgical specimens of NP were detected by Northern blot and in situ hybridization.

Results: Significant amounts of constitutive IL-6 and COX-2 mRNAs were produced in NPFs. Cytokines induced IL-6 and COX-2 mRNA synthesis in NPFs. Meloxicam (a specific COX-2 inhibitor) suppressed the induction of cytokines on IL-6 mRNA levels, and these effects could be reversed by exogenous prostaglandin E2. In situ hybridization revealed that IL-6 and COX-2 mRNAs were detected primarily in fibroblasts, macrophages, and plasma cells. Aggregation of plasma cells as well as collagen deposition in vicinity to IL-6 mRNA-producing fibroblasts was found. Rich vascularity around COX-2 mRNA+ fibroblasts was also identified.

Conclusions: The pathogenesis of nasal polyposis involves NPFs through synthesizing IL-6 to modulate the activation of immune responses (plasma cell formation) and synthesis of stroma. Inducible cyclooxygenase also contributes to NP development by promoting vasodilation and modulating the cytokine-induced IL-6 gene expression in NPFs.


NASAL POLYP (NP) is the most common mass lesion in nose. Although the pathogenesis remains unclear, NP has been regarded, at least by some authors, to be of inflammatory origin. During inflammation, a complex series of reactions including vascular proliferation or dilatation, active extracellular matrix (ECM) remodeling, and activation of immune mechanism will be elicited to promote wound healing. Elevated levels of prostaglandins (PGs) and interleukin (IL) 6 are connected with the pathogenesis of several fibrotic lesions such as systemic sclerosis and cystic fibrosis in humans and pulmonary fibrosis in mice.

Cyclooxygenase (COX) is the rate-limiting enzyme responsible for the conversion of arachidonic acid to PGs. Two isoforms of COX have been identified: COX-1 maintains tissue homeostasis whereas inducible cyclooxygenase (COX-2) is responsible for the excessive PG synthesis leading to pathological sequelae. Of the PG family, epoprostenol and prostaglandin E2 (PGE2) increase vascular permeability and thereby facilitate cell migration to inflammatory area. Prostaglandins also complicate the inflammatory reaction by modulating some important biological behavior of the proinflammatory cytokines. For example, PGE2 can enhance the stimulatory effect of IL-1 on matrix me-
PATIENTS, MATERIALS, AND METHODS

TISSUE SAMPLES
Nasal mucosal tissues were obtained from inferior turbinate of patients receiving turbinectomy to relieve nasal obstruction. Prior to surgery, the tissue appeared to be free of inflammation and had a normal appearance. Nasal polyps were dissected by functional endoscopic sinus surgery for treatment of chronic sinusitis with polyposis. The patients had no history of nasal allergy, asthma, or aspirin sensitivity and had not taken regular topical or oral medication within 3 weeks. Informed consent was obtained before surgical procedure.

PRIMARY CELL CULTURES
Six primary cultures of fibroblasts, 3 from NP (NPFs) and 3 (including 1 from the patient with NP) from nasal turbinate (NFs) were established. Cultured cells were subjected to 3 independent experiments. Consequently, each datum presented in the Northern blot hybridization was the average value obtained from the results of 9 independent experiments. The primary cell cultures were established as described in our recent study.20 In brief, after removing the epithelial layer, the specimens were immersed overnight in Dulbecco Modified Eagle Medium containing 10% fetal calf serum, 200 U/mL of penicillin, 200 µg/mL of streptomycin, and 1 mg/mL of amphotericin B. The samples were placed in the 10-cm Petri dish, minced into 1-mm fragments, and covered with a sterilized glass coverslip. After fibroblasts had migrated from tissue explants and became confluent, cells were trypanized and subcultured.

PROBE PREPARATION
For Northern blot, DNA plasmids of human IL-6 and COX-2 (Cayman Chemical, Ann Arbor, Mich) were radiolabeled with [³²P] deoxycytidine 5’-triphosphate (¹²⁵[P]dCTP) by random prime labeling method (Amershams Biosciences, Little Chalfont, England). For in situ hybridization, the probes were labeled using digoxigenin-11-deoxyuridine triphosphate (DIG-11-dUTP) by random priming (Boehringer Mannheim, Indianapolis, Ind).

NORTHERN BLOT ANALYSIS
Following stimulation, total RNAs were isolated by acid guanidinium thiocyanate-phenol-chloroform method (Zol-B; Biotec Laboratories Inc, Houston, Tex), electrophoresed, and transferred to a nylon membrane. The RNAs were immobilized by UV cross-linking. The membrane was hybridized with radiolabeled complementary DNA probes for IL-6, COX-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, as internal standard), washed under high stringency conditions, and autoradiographed at –80°C. The intensity of each band, after normalization with GAPDH mRNA abundance, was quantified by scanning video densitometer and software (Image Reader V1.8; Biomed Instruments Inc, Fullerton, Calif). As described above in “Primary Cell Cultures”, each densitometric value, expressed as mean ± SD, was obtained from 9 independent experiments.

IN SITU HYBRIDIZATION
Thirty-four surgical specimens of NP and 15 cases of nasal mucosa were collected. They were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections of 5 µm were cut and mounted on Superfrost/Plus slides (Fisher Scientific International, Pittsburgh, Pa). In situ hybridization was performed as described in our recent article.20 In brief, sections were treated with 0.1% pepsin/0.2N hydrochloric acid, postfixed with 0.4% paraformaldehyde, and acetylated with acetic anhydride. After prehybridization with hybridization buffer (4X sodium chloride and sodium citrate, 50% deionized formamide, 1X Denhardt solution, 5% dextran sulfate, 0.5 mg/mL of salmon sperm DNA, and 0.25 mg/mL of yeast transfer RNA), hybridization was carried out overnight with hybridization buffer containing 0.5 to 1.0 µg/mL of digoxigenin-labeled probes at 42°C. After a series of stringent washings, slides were incubated with alkaline phosphatase-conjugated sheep antidigoxigenin antibody overnight at 4°C. The sections were incubated in a chromogen solution (0.1M Tris hydrochloride [pH 9.5], 0.1M sodium chloride, 0.05M magnesium chloride, 0.45% 4-nitroblue tetrazolium, and 0.35% 5-bromo-4-chloro-3-indolylphosphate), counterstained with methyl green, and examined by light microscopy. Sections digested with ribonuclease A before hybridization served as negative controls.

STATISTICAL ANALYSIS
The difference between the relative mRNA level in each experimental group was analyzed by 1-way analysis of variance for multiple comparisons and then by the Fisher protected least significant difference test. P<.05 was considered statistically significant.

tallloproteinase 1 or IL-6 synthesis in macrophages or sy
novial fibroblasts, respectively.

Interleukin 6 and COX-2 serve to modulate a complex of responses in an inflammatory tissue. However, these reactions tend to become overexpressed and subsequently lead to tissue destruction instead of wound healing. Recent studies had identified increased levels of pro-inflammatory cytokines such as IL-1 and tumor necrosis factor α (TNF-α) as well as IL-6 and COX-2 in NP and proposed an association between the overexpression of these mediators and disease propagation. However, most of these experiments concentrated primarily on detecting the presence of these mediators, and the mechanisms responsible for the synthesis of these molecules remained unclear. It is important to clarify these processes in that this may lead to the development of new therapeutic strategy. Since the resident stromal cells in addition to the infiltrating inflammatory cells are also important contributors to the inflammatory cascade, we studied the stimulatory effects of IL-1α or TNF-α on IL-6

and COX-2 mRNAs production in nasal polyp fibroblasts (NPFs) to further understand the pathogenesis of nasal polyposis. The modulating effects of COX-2 and PGs in these cytokine-induced reactions were also investigated. The in vivo expressions of IL-6 and COX-2 mRNAs were examined in surgical samples of NP. The results suggest that IL-6 and COX-2 produced by NPFs may contribute to NP development. To verify this proposal, we also compared the synthesis of IL-6 and COX-2 mRNAs between NPFs and fibroblasts derived from nasal turbinate where NP never occurs.

## RESULTS

All the NPFs exhibited similar and reproducible responses concerning the expressions of IL-6 and COX-2 genes following stimulation (data not shown). The problem of heterogeneity derived from using primary fibroblast cultures does not exist in this experiment.

**IL-6 GENE EXPRESSIONS IN NPFs FOLLOWING IL-1α, TNF-α STIMULATION**

After exposure to various stimuli, total RNAs in NPFs were subjected to Northern blot hybridization. A prominent amount of constitutive IL-6 mRNA was found. Exposure to IL-1α and TNF-α resulted in further elevated levels of IL-6 mRNA (2.0-fold to 1.9-fold) compared with the control groups (Figure 1A-B). Approximately 40% of the cytokine-stimulated IL-6 mRNA production in NPFs was suppressed by the COX-2–specific inhibitor meloxicam. Exogenous PGE2 restored the inhibitory effects of COX-2.
Figure 2. Induction of inducible cyclooxygenase (COX-2) messenger RNA (mRNA) transcripts in nasal turbinate fibroblasts and nasal poly fibroblasts (NPFs) of a patient with nasal poly. After incubating in serum-free medium for 48 hours, cells were untreated (C) or treated for 24 hours with 10 ng/mL of interleukin (IL) 1α (I), 10 ng/mL of tumor necrosis factor-α (TNF-α) (T), 1 µg/mL of prostaglandin E2 (PGE2) (P), 10 ng/mL of IL-1α + 1 µg/mL of PGE2 (P+I), and 10 ng/mL of TNF-α + 1 µg/mL of PGE2 (P+T). A, Total RNAs were subjected to COX-2 mRNA analysis by Northern blot hybridization, quantified by densitometric analysis after glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA standardization and expressed as relative optical density to individual control group. B, Relative COX-2 mRNA level. Each bar represents mean ± SD of 9 individual experiments. The asterisk indicates *P<.05 vs control.

COX-2 GENE EXPRESSIONS IN NPFs

Similar to IL-6, significant amount of constitutive COX-2 mRNA was also found in NPFs. Interleukin 1α and TNF-α also induced COX-2 gene expressions (2.1-fold to 1.8-fold) compared with the control groups. Although cytokines plus PGE2 also stimulated significant COX-2 gene expressions, the cytokine-induced COX-2 mRNA production in NPFs was not affected by PGE2 (Figure 2A and B).

Figure 3. Production of interleukin (IL) 6 (A) and inducible cyclooxygenase (COX-2) (B) messenger RNA (mRNAs): comparison between nasal poly fibroblasts (NPFs) and nasal turbinate fibroblasts (NFs). After incubating in serum-free medium for 48 hours, cells were untreated (C) or treated for 24 hours with 10 ng/mL meloxicam (M). Total RNAs were subjected to IL-6 and COX-2 mRNA analysis by Northern blot hybridization. The level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also analyzed and served as an internal standard. The effects of meloxicam on the endogenous IL-6 mRNA levels in NPFs and NFs were also investigated.

IN VIVO EXPRESSIONS OF IL-6 AND COX-2 GENES IN NPS

Microscopically, NPS were lined by a layer of ciliated respiratory epithelium. Abundant IL-6 mRNAs were identified in the subepithelial resident fibroblasts as well as the infiltrating plasma cells and mononuclear round cells in contrast to the faint, weak signal in the lining epithelium. An intimate alignment between the IL-6 mRNA-producing fibroblasts and plasma cells was frequently found (Figure 4A). The ECM around some IL-6 mRNA+ fibroblasts showed a certain degree of collagen deposition in contrast to the commonly seen loose, edematous stroma (Figure 4C). Interestingly, these IL-6 mRNA+ fibroblasts as well as their associated collagen were located in close vicinity to the lining epithelium (Figure 4B). Pronounced COX-2 mRNAs were detected primarily in plasma cells, fibroblasts, and mononuclear round cells (Figure 4E), whereas only a weak signal appeared in some of the lining epithelium. Furthermore, examination of the adjacent sections revealed that the ECM around some COX-2 mRNA+ NPFs also contained rich vascular supply (Figure 4D). However, expressions of COX-2 and IL-6 mRNAs were insignificant in nasal mucosa from inferior turbinate (data not shown). Little or no signal was found in the negative control groups.

Elevated levels of IL-6 have been demonstrated in NP compared with the normal controls sampled from inferior turbinate.16 Immunohistochemical staining and in situ hybridization also indicated that macrophages, eosinophils, and lining epithelium were the main cellular sources of IL-6.16 In the present study, the significant IL-6 mRNA levels (constitutive or stimulated) synthesized in NPFs suggest that sources other than inflammatory cells or lining epithelium also contribute to the pronounced levels of IL-6.
of IL-6 detected in NP. To further confirm the data from our in vitro assay, in situ hybridization in surgical specimens was performed, and the results revealed the presence of abundant IL-6 mRNAs in NPFs. In addition, an intimate spatial orientation between the IL-6 mRNA-producing fibroblasts and plasma cells was found. These observations suggest that NPFs may modulate the immune responses in NP through synthesizing IL-6 to induce plasma cell formation.

Studies on the biological behavior of respiratory cells have clearly demonstrated the influence of stroma or ECM on the attachment and proliferation and differentiation of lung epithelium. In these studies, collagen gel is necessary for the cultured tracheal epithelium to main-

Figure 4. Detection of inducible cyclooxygenase (COX-2) and interleukin (IL) 6 messenger RNA (mRNAs) in nasal polyps by in situ hybridization. A, Interleukin 6 was identified in the cytoplasm of plasma cells (arrows) and macrophages and fibroblasts (arrowheads). B, A close spatial orientation between these collagen bundle and lining epithelium, even in direct contact. C, Collagen deposition in vicinity to some IL-6 mRNA+ fibroblasts (arrowheads) was found. D, The extracellular matrix around these COX-2 mRNA+ cells was well vascularized. E, Expression of COX-2 mRNA in plasma cells (arrows), fibroblasts (arrowheads), and mononuclear round cells was rather pronounced, whereas only weak signals were found in some lining epithelium (A, C, and E, in situ hybridization; B and D, hematoxylin-eosin; original magnification: A, ×300; B-E, ×150).
tain their mucociliary phenotype. In addition, the number of ciliated cell is obviously reduced when collagen gel is depleted from the culture dishes.\(^2\) Larsen et al\(^3,4\) also propose that a rapid ECM remodeling is necessary to support the growth and elongation of mucoserous glands and subsequently, polyp proliferation. Although the significance of ECM on the biological behavior of NP has been widely accepted, the regulatory mechanism(s) accounting for ECM remodeling in NP is still not clearly understood. In the present study, pronounced endogenous as well as cytokine-stimulated IL-6 mRNAs were produced in NPFs. Surgical specimens also revealed significant collagen deposition around the IL-6 mRNA \(^*\) fibroblasts. Since IL-6 has also been proposed to be fibrogenic\(^5,6\) in addition to its modulating role in inflammatory reaction, these results suggest that the IL-6 released from NPFs may promote collagen deposition in NP. Furthermore, IL-6-mRNA \(^*\) fibroblasts and their associated collagen were also located in close vicinity to the lining epithelium, or even in direct contact. This observation further implies that IL-6 may mediate the interactions between NPFs and the lining epithelium. Our recent experiment also reveals substantially high amounts of matrix metalloproteinase 1 mRNAs (constitutive or cytokine stimulated) in NPFs.\(^7\) Since matrix metalloproteinase 1 is an essential enzyme for degrading collagen fiber, data from our present and previous studies highlight the significance of NPFs in NP progression. Namely, this cell may promote active ECM remodeling through producing IL-6 and matrix metalloproteinase 1 to modulate collagen deposition and breakdown, respectively.

In the present study, obvious COX-2 mRNAs were located primarily in fibroblasts, plasma cells, monocellular round cells, and to a less extent, lining epithelium in NP in contrast to the trivial signal in nasal mucosa from inferior turbinate. Since PGE\(_2\) and epoprostenol are potent vasodilators and COX-2 is the rate-limiting enzyme for PG synthesis during inflammation,\(^8,9\) our findings of the well-vascularized and edematous stroma around the COX-2 mRNA-producing NPFs may be due to an excessive release of PGS.

Northern blot hybridization revealed obvious synthesis of COX-2 mRNAs in NPFs either constitutively or following the stimulation of IL-1\(\alpha\) or TNF-\(\alpha\). In addition to the originally reported 4.1-kilobase (kb) COX-2 complementary DNA, a smaller band (approximately 2.5 kb) of mRNA was also presented. This smaller band probably reflects a different site of polyadenylation as reported previously for murine COX-2.\(^5,6,10\) In NPFs, 34% of the constitutive and 40% of the cytokine-induced IL-6 mRNA levels were suppressed by the COX-2 specific inhibitor meloxicam.\(^11\) These abolishing effects could be reversed by exogenous PGE\(_2\). This implies the regulatory role of endogenous COX-2 and PGE\(_2\) on IL-6 synthesis in NPFs. Taken together, these data suggest that the COX-2 produced by NPFs may contribute to NP propagation in the following 2 ways: (1) to promote vascular dilatation and proliferation through synthesizing PGs and (2) to promote collagen synthesis by modulating IL-6 synthesis. In their clinical investigations, Patriarca et al\(^12-14\) found that intranasal treatment of lysine acetylsalicylate (nonspecific COX inhibitor) effectively prevents or reduces the relapse of nasal polyps, which supports our proposal indirectly.

To further elucidate the experimental proposal that the IL-6 and COX-2 produced by NPFs contribute to NP development, the levels of these molecules in fibroblasts (NFs) from nasal turbinates where NP never occurs was also analyzed. The results showed that substantially high amounts of constitutive IL-6 and COX-2 mRNAs were produced in NPFs compared with NFs. In addition, meloxicam diminished significantly the endogenous IL-6 mRNA level in NPFs but did not exert any influence in NFs. Mullol et al\(^15\) compared the amounts of 5 different cytokines produced by cultured epithelial cells from NP and nasal turbinates. A statistically significant difference between these 2 cell types was found in only 2 of the 5 cytokines examined.\(^16\) Hicks et al\(^17\) examined the expressions of cytokeratins in the epithelial cells of NP and nasal turbinates. The results also showed an identical profile of cytokeratin production in these 2 cell types.\(^18\) Accordingly, these authors, together with others, suggest that local stroma disorder may play a more notable role than lining epithelium in NP development.\(^19,20\) In our experiment, the data demonstrating an obviously altered biological behavior between NPFs and NFs seem to provide further support for this hypothesis.

The experimental data suggest the involvement of NPFs in the pathogenesis of nasal polyposis through synthesizing IL-6 to modulate the immune responses (formation of plasma cells) and ECM synthesis. Inducible cyclooxygenase also contributes to the development of NP by promoting vascular dilatation/proliferation and modulating the cytokine-triggered IL-6 mRNA synthesis in NPFs. Furthermore, the stimulatory effects of proinflammatory cytokines (IL-1\(\alpha\) or TNF-\(\alpha\)) on IL-6 and COX-2 gene expressions in NPFs support the pathogenic mechanism of inflammation on nasal polyposis.

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