C-C Chemokine Ligand 2 Gene Expression in Nasal Polyp Fibroblast: Possible Implication in the Pathogenesis of Nasal Polyposis

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Key Words: CCL2, TNF-α, COX-2, Nasal Polyp, Fibroblast

RUNNING TITLE: TNF-α induces CCL2 in Nasal Polyp

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ABSTRACT

Recruitment of macrophages is essential to the pathogenesis of nasal polyp (NP) since this disease is inflammation-related. In this study, the effect of TNF-α on the expression of C-C chemokine ligand 2 (CCL2) in fibroblasts from nasal polyp (NPFs) was investigated. The roles of COX-2/PGs in the mediation of TNF-α-stimulated CCL2 expression were also detected. Northern blot showed that TNF-α stimulated the expression of CCL2 and COX-2 genes and the synthesis of CCL2 mRNA was COX-2-dependent. A transient elevation of c-Fos and c-Jun mRNAs was induced by TNF-α, whereas NS-398 and meloxicam (COX-2 inhibitors) abolished the up-regulation of c-Fos. Electrophoretic mobility shift assay revealed that TNF-α triggered AP-1/DNA binding and again, NS-398 and meloxicam inhibited this reaction via reducing c-Fos synthesis. Curcumin (AP-1 inhibitor) markedly suppressed the TNF-α-induced CCL2 expression. Immunohistochemical staining of NP surgical specimens also revealed an intimate alignment between CCL2+ fibroblasts and CD-68+ macrophage. These data suggest that NPFs may contribute to NP development by synthesizing CCL2 to promote macrophage recruitment. In addition, COX-2/PGs facilitate CCL2 transcription in NPFs via c-Fos/AP-1 signaling pathway.
INTRODUCTION

Nasal polyp (NP) is the most common mass lesion in nose. (1) Although the pathogenesis remains unclear, many authors regarded NPs to be an inflammatory disorder. (2, 3) Accordingly, the connection between nasal polyposis and infiltration of macrophage, an important element in inflammation has been established (4). Our recent investigation on surgical specimens of NPs also demonstrated the presence of essential pro-inflammatory mediators such as IL-6 and COX-2 in macrophages (5).

Chemokines can regulate the chemotaxis and metabolic activity of specific leukocyte subsets. C-C chemokine ligand 2 (CCL2), previously known as monocyte chemoattractant protein-1 (MCP-1) is a prototype of the CC chemokine subfamily that modulates monocyte chemotaxis both in vitro (6) and in vivo. (7, 8) Expression of CCL2 has been detected in a number of pathological conditions associated with monocyte aggregation, including atherosclerosis, arthritis and glomerulonephritis. (8)

Tumor necrosis factor-α (TNF-α) may play an essential role to the pathogenesis of NPs. TNF-α is related with the elevated synthesis of immunoglobulins in NPs (9). The contribution of resident stromal cells in establishing an inflammatory cascade is well accepted. We have previously demonstrated active syntheses of MMP-1, COX-2 and IL-6 inflammatory mediators in fibroblasts derived from nasal polyp (NPFs) stimulated by TNF-α and proposed the connection between inflammation and NP
development (10). However, the effect of TNF-α on CCL2 production in NPFs remains unclear.

Cyclooxygenase (COX) is the rate-limiting enzyme responsible for the conversion of arachidonic acid to prostaglandins (PGs). Two isoforms of COX have been identified: COX-1 maintains tissue homeostasis whereas inducible cyclooxygenase (COX-2) is responsible for the excessive PGs synthesis leading to pathological sequela. In addition to increasing vascular permeability to facilitate cell migration to inflammatory area, PGs also complicate the inflammatory reaction by modulating the biological behavior of proinflammatory cytokines. For example, PGE₂ can enhance the stimulatory effect of interleukin-1 (IL-1) on IL-6 synthesis in macrophages. However, the effects of the COX/PGs on chemokine synthesis remain controversial.

In this study, the effect of TNF-α on CCL2 expression in human NPFs was analyzed. The roles of COX-2/PGs in the mediation of TNF-α-stimulated CCL2 expression as well as the possible signaling pathway involved were also dissected under the rationale that this may lead to the development of new therapeutic strategies.
MATERIALS AND METHODS

Tissue samples: NPs were dissected by functional endoscopic sinus surgery (FESS) for treatment of chronic sinusitis with polyposis. All patients had no history of nasal allergy, asthma or aspirin hypersensitivity and had not taken regular topical or oral medication within 3 weeks. Informed consent was obtained before the surgery.

Primary cell cultures: Six primary cultures of fibroblast, 3 from nasal polyp (NPFs) and 3 from nasal turbinate (NFs) were established as previously described \(^4,8\) In brief, after removing the epithelial layer, the specimens were immersed in DMEM containing 10% fetal calf serum, 200 U/ml penicillin, 200 µg/ml streptomycin and 1 mg/ml amphotericin B overnight. The samples were placed in 10-cm dishes, minced into 1 mm\(^3\) fragments and covered with sterilized glass coverslips. After the fibroblasts had migrated from tissue explants and became confluent, the cells were trypsinized and subcultured. Cells between passages 3 to 6 were plated at a density of 5x10\(^5\) /ml on the 10-cm dish and subjected to different stimulations. Before different treatments, the cells were made quiescent in serum-free media for 24 h. Each cell strain was subjected to 2 independent experiments. The data presented in the article are the averages of 6 experiments.

Northern blot analysis: Total RNA was isolated by the acid guanidium
thiocyanate-phenol-chloroform method (Zol-B, Biotecx, Houston, TX, USA),
electrophoresed and transferred to a nylon membrane. RNA was immobilized by
ultraviolet cross-linking. After hybridization with radiolabeled cDNA probes for
human CCL-2, c-Fos, c-Jun and glyceraldehyde-3-phosphate dehydrogenase
(GAPDH, as internal standard), the membrane was washed under high-stringency
conditions followed by autoradiography at -80°C. The intensity of each band, after
normalization with GAPDH mRNA, was quantified by a scanning video densitometer
and software (Biomed Instrument, Fullerton, CA, USA).

Preparation of nuclear extracts: After treatment, cells were rinsed with ice-cold
PBS. Cells were scraped into 10 ml extraction buffer (10 mM HEPES [pH7.9], 10
mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF]
and 0.5% Nonidet P-40), allowed to swell on ice for 10 min and vortexed for 20s. The
nuclear pellets obtained after centrifugation (2,000 rpm, 5min) were resuspended in
100 µl of lysis buffer (25% glycerol, 20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5
mM MgCl₂, 0.2 mM EDTA, 1 mM PMSF, 0.5 mM DTT, 5nM leupetin and 5µg/ml
aprotinin) and shaken slowly in 4°C for 30 min. The supernatant obtained after
centrifugation (14,000 rpm, 4°C, 30 min) was used as nuclear extract. Protein
centration was measured by the Bradford method. The nuclear extract obtained
was further analyzed with gel electrophoretic mobility shift assay.
Electrophoretic mobility shift assay (EMSA): Five to nine micrograms of nuclear proteins were mixed with $^{32}$P-end-labeled double-stranded AP-1-binding oligonucleotide containing consensus sequence (underlined):

$5'$-CGCTTGATGAGTCA GCCGGAA-3', 2 \mu g$ dIdC and 10 X binding buffer (40% glycerol, 100 mM Tris-HCl [pH 7.5], 500 mM NaCl, 10 mM EDTA and 10 mM 2-ME) at room temperature for 1h. The DNA-protein complex formed was separated from free oligonucleotides on a non-denaturing 5% polyacrylamide gel in 1X circulating TAE at 180 V, 4°C for 3.5 h. Two methods were used to test the specificity of binding. The first was by competition with excessive unlabeled (unlabeled : labeled = 50:1) probe. The second method involved supershift analysis, in which c-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the incubation mixture for 30 min on ice and then 30 min at room temperature before separation on polyacrylamide gel.

Immunohistochemistry: Immunohistochemical staining was performed using a streptavidin-biotin method. Monoclonal antibodies of mouse anti human CCL2 (R&D, Minneapolis, USA), murine anti-rat iNOS (Transduction Laboratories, Lexington, USA) and murine anti rat monocyte/macrophage lysosomal membrane (ED-1, Serotec, Oxford, UK, for identifying macrophages) were used to detect the target molecules or cells within NPs. Synthesis of CCL2 within NPs was detected by
monoclonal mouse anti-human CCL2 (R&D, Minneapolis, USA) antibody.

*Statistical analysis:* Differences between the relative mRNA levels in each experimental group was subjected to ANOVA analysis for multiple comparisons then by Fisher’s protected least significant difference test. $P<0.05$ was considered statistically significant.
RESULTS

All the NPFs and NFs exhibited similar and reproducible responses in the expressions of CCL2 gene following stimulation (data not shown). The problem of heterogeneity derived from using primary fibroblast cultures does not exist in this experiment.

*TNF-α stimulated CCL2 expression in NPFs and NFs:* The amounts of CCL2 mRNA in NPFs and NFs stimulated by TNF-α were assessed by Northern hybridization. The results showed that TNF-α induced CCL2 expression in NPFs in a dose (Fig 1A) and time (Fig 1C) dependent manner. The maximal effect occurred at treating with 5ng/ml of TNF-α for 4h. In contrast, the expressions of CCL2 gene in NFs were much less significant than those in NPFs (Fig2). Substantial differences between NPFs and NFs in their baseline and stimulated CCL2 mRNA-producing activities were detected (Fig 2).

*TNF-α stimulated COX-2 expressions:* Elevated expression of COX-2 mRNA was also induced by TNF-α and also peaked at 4h (Fig 3A, B). COX-2 inhibitors (NS-398, meloxicam) \(^{14}\) suppressed the stimulatory effect of TNF-α on CCL2 transcription profoundly (Fig3C,D).

*Expressions of c-Fos and c-Jun mRNAs in NPFs after TNF-α stimulation:* Synthesis of c-Fos and c-Jun mRNAs increased progressively in NPFs after
incubation with TNF-α for 5 to 30 min. The stimulatory effect was transient since the amount of both mRNAs declined to baseline level after 60 min. (Fig 4A and B). To verify whether TNF-α-stimulated c-Fos and c-Jun expression involved COX-2/PGs, NS-398 and meloxicam respectively, were applied. The results demonstrated obvious attenuation by COX-2 inhibitors on c-Fos (Fig 4C) but not c-Jun expression (Fig 4D).

**TNF-α induced AP-1/DNA binding in NPFs:** The results of EMSA revealed that binding of AP-1 and DNA occurred within 5 min of treatment, peaked at 30 min and declined subsequently (Fig 5A). Competition with 50-fold of cold probe abolished the signal and confirmed the probe specificity (Fig 5A). NS-398 and meloxicam diminished AP-1/DNA interaction (Fig 5B). Supershift was clearly visible when anti-c-Fos antibodies were introduced (Fig 5B).

**Effect of AP-1 inhibitor on CCL2 expression:** To confirm the contribution of AP-1 to TNF-α-stimulated CCL2 expression, curcumin (AP-1 inhibitor, (13, 14). ) were introduced. The results showed obvious reduction of curcumin on CCL2 expressions (Fig 5C)

**Immunolocalization of CCL2, COX-2 and CD-68 in NPs:** Microscopically, NPs were lined by a layer of ciliated respiratory epithelium. Abundant CCL2 and COX-2 were identified in the subepithelial resident fibroblasts as well as the lining epithelium. An intimate alignment between the CCL2-producing fibroblasts and the
round, mononuclear CD-68\(^+\) macrophages was frequently found.
DISCUSSION

Chemotaxis of macrophages is thought to be crucial to the development of NP. In NPFs, elevated level of CCL2 mRNA was induced by TNF-α in a time- and dose-dependent manner. The stimulatory effect of the proinflammatory cytokines TNF-α on CCL2 expression in NPFs further supports the pathogenic role of inflammation in nasal polyposis.

In the present study, substantial difference between NPFs and NFs in CCL2-producing activities, either constitutively or following the stimulation of TNF-α was found. In contrast, Mullol et al compared the amounts of 5 different cytokines produced by cultured epithelial cells from NP and nasal turbinate. Statistical difference between these two cell types was found in only 2 out of the 5 cytokines examined (17). Hicks et al investigated the expressions of cytokeratins in the epithelial cells of NP and nasal turbinate. They also found an identical synthetic profile of cytokeratin (18). These reports combined with our findings of obviously altered biological behavior between NPFs and NFs propose that stroma disorder may play a more significant role than lining epithelium in mediating NP development (17-19).

Conflicting data have been reported about the modulation of COX-2/PGs on CCL2 expression depending on different cell types and agonists used. 12, 13 For
instance, PG has been reported to promote or diminish MCP-1 synthesis in vascular smooth muscle cells or astrocytes, respectively. In NPFs, 54% to 72% of the TNF-α-stimulated CCL2 expression was suppressed by specific COX-2 inhibitors. These results suggest that COX-2/PGs may contribute to NP propagation by up-regulating CCL2 expression in NPFs to modulate the infiltration of macrophages. Patriarca et al found that intranasal treatment with lysine acetylsalicylate (non-specific COX inhibitor) effectively prevented or reduced the relapse of nasal polyps. Data from the present study may provide a possible mechanism for their observations.

More detailed regulation of COX-2/PGs on TNF-α-induced CCL2 expression was investigated. COX-2/PGs function primarily by up-regulation of cyclicAMP/protein kinase A, leading to activation of AP-1 transcriptional factor that consists of homo- or hetero-dimers of Jun-Jun or Jun-Fos. (12) In NPFs, TNF-α stimulated significant synthesis of c-Fos and c-Jun mRNAs. The diminishing effect of NS398 and meloxicam on c-Fos but not on c-Jun implies the involvement of COX-2 in c-Fos expression. By using AP-1 probe containing TGAGTCA, the consensus binding sequence for Jun-Jun or Jun-Fos 10, EMSA revealed that TNF-α stimulated a time-dependent increase in AP-1/ DNA binding, peaking at 30 min. This time point coincided with the maximal expression of c-Fos. Similar to their effects on c-Fos,
NS-398 and meloxicam also diminished the interaction between AP-1 and promoter DNA. Super-shift assay revealed that c-Fos is required in the formation of TNF-α-induced AP-1/DNA complex and furthermore, COX-2/PGs regulate AP-1/DNA interaction by modulating the synthesis of c-Fos. Finally, inhibition studies using COX-2 inhibitors and curcumin confirmed that COX-2 and AP-1 were needed for TNF-α-stimulated CCL2 expression. Taken together, our results clearly demonstrated the effects of COX-2/PGs on the TNF-α-induced c-Fos/AP-1 signaling pathway leading to CCL2 expression in NPFs.

In NP, pronounced CCL2 and COX-2 were located primarily in fibroblasts, in contrast to the trivial signal in nasal mucosa from inferior turbinate. These findings highlight the significance of CCL2 and COX-2 in NP propagation. The identification of CCL2 and COX-2 in NPFs confirms our aforementioned regulatory role of endogenous COX-2 on CCL2 synthesis. Finally, an intimate spatial orientation between the CCL2-producing fibroblasts and the round, mononuclear, CD-68+ monocytes/macrophages was frequently found. This suggests the modulation of macrophages recruitment by NPFs via synthesizing CCL2.

We have previously demonstrated that the COX-2 produced by NPFs may contribute to NP development by promoting vascular dilatation/proliferation and mediating IL-6 expression. Here, our results show that COX-2/PGs may contribute
to NP propagation by up-regulating CCL2 expression in NPFs to modulate the infiltration of macrophages. Furthermore, COX-2/PGs regulate the TNF-α-stimulated CCL2 expression by promoting the induction of c-Fos transcriptional factor and the subsequent AP-1/DNA interaction. Accordingly, specific inhibitors of this signaling cascade deserve further investigation since they may to a certain extent, provide a novel therapeutic rationale for NPs.
ACKNOWLEDGEMENT

This work was supported by grants NSC92-2314-B-002-270 (Lin), 91-2314-B-002-142 (Hong) and 91-2314-B-002-306, 92-2314-B-002-347 (Liu) from the National Science Council, Taiwan.
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FIGURE LEGENDS

**Fig 1.** Expression of CCL2 mRNAs in NPFs after TNF-α stimulation. CCL2 mRNAs were analyzed by Northern blot after A) treated with different amounts of TNF-α for 24h or C) with TNF-α (5ng/ml) for different incubation periods. B, D) Results from A, C) were quantified by densitometric analysis and expressed as fold change relative to mRNA level from untreated control. *: p< 0.05 vs control, each bar represents mean ±SD of 6 experiments. Note that TNF-α stimulated CCL2 transcription A, B) dose and C, D) time dependently.

**Fig 2.** Comparison between NFs and NPFs in constitutive and stimulated CCL2 transcripts. A) CCL2 mRNAs synthesized (endogenous and induced) in NFs and NPFs were subjected to densitometric analysis, normalized by GAPDH mRNA and B) expressed as optical density relative to that of NF control group. *: p< 0.05 vs control, **: p< 0.05 vs basal level in NFs, Each bar represents mean ±SD of 6 experiments. Each bar represents mean ±SD of 6 experiments. Note the far more pronounced constitutive and stimulated synthesis of CCL2 mRNAs in NPFs compared with NFs.

**Fig 3.** Expression of COX-2 mRNAs in NPFs after TNF-α stimulation. A) COX-2 mRNA was analyzed by Northern blot after treatment with TNF-α for different
incubation periods. C) NPFs were incubated with TNF-α (5ng/ml), alone or in combination with NS-398 or meloxicam (Melox) (10^{-5} M, 3h before the addition of TNF-α) for 4h and the CCL2 mRNA levels were determined. B, D) Results from A, C) were quantified by densitometric analysis and expressed as fold change relative to mRNA level from untreated control. *: p< 0.05 vs control, **: p< 0.05 vs TNF-α 5ng/ml, each bar represents mean ±SD of 6 experiments. Note that A, B) TNF-α induced COX-2 gene expression, whereas C, D) COX-2 inhibitors reduced the TNF-α-stimulated transcription of CCL2 mRNA.

Fig 4. Expressions of c-Fos and c-Jun mRNAs in NPFs. after TNF-α stimulation. A) c-Fos and B) c-Jun mRNAs in NPFs were detected by Northern blot after treatment with TNF-α (5ng/ml) for different incubation periods. NPFs were incubated with TNF-α (5ng/ml), alone or in combination with NS-398 or meloxicam (Melox) (10^{-5}M, 3h before the addition of TNF-α) for 30 min and the levels of C) c-Fos, and D) c-Jun mRNAs were determined. Note that NS-398 and meloxicam reduced c-Fos but not c-Jun expression.

Fig 5. TNF-α induced AP-1/DNA interactions in NPFs. A) NPFs were treated with TNF-α (5ng/ml) for different incubation periods. Nuclear extracts were subjected to EMSA using $^{32}$P-labeled AP-1 probe. Fifty-fold excess of unlabeled probe were used for competitive assay (competitor). B) NPFs incubated with TNF-α (5ng/ml), alone or
in combination with NS-398 or meloxicam (Melox) (10^{-5}M, 3h before the addition of TNF-\(\alpha\)) for 30 min, nuclear extracts were analyzed with AP-1 probe. Anti-c-Fos antibody was used for supershift experiment. Note that TNF-\(\alpha\) induced AP-1/DNA binding. NS-398 and meloxicam diminished this reaction through inhibiting c-Fos synthesis. C) NPFs were incubated with TNF-\(\alpha\) (5ng/ml) alone or in combination with curcumin (10\(\mu\)M) (3h before the addition of TNF-\(\alpha\)) for 4h and the CCL2 mRNA levels were determined. Note the reductive effect of curcumin.

**Fig 6.** Immunolocalization of CCL2, COX-2 and CD-68 in NPs. A) CCL2 and B) COX-2 were identified in epithelium (arrowheads) and fibroblasts (arrows). C) CD-68 was clearly visible in the round, mononuclear cells. Note the intimate alignment between CCL2\(^{+}\), fibroblasts (arrows) and the round, mononuclear macrophages (arrowheads). A-C) Immunohistochemistry, original x150.