Expression and Distributional Patterns of the Inhibitor of Apoptosis Protein Family and Caspase 3 in Nasal Polyps

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Objectives: To investigate the expression and distributional patterns of the inhibitor of apoptosis protein (IAP) family and caspase 3 in nasal polyps and normal nasal mucosa and to evaluate the possible effects of the IAP family and caspase 3 on the development of nasal polyps.

Design: Prospective study.

Setting: Tertiary academic institution.

Patients: Normal inferior turbinate mucosa was obtained from 20 patients undergoing surgery for augmentation rhinoplasty. Nasal polyps were obtained from 20 patients undergoing endoscopic sinus surgery for chronic polypoid sinusitis.

Interventions: Reverse transcriptase–polymerase chain reaction, immunohistochemical analysis, and Western blot analysis were performed.

Main Outcome Measures: The expression and distribution of cIAP1, cIAP2, XIAP, survivin, and caspase 3 were evaluated in normal turbinate mucosa and nasal polyps.

Results: cIAP1, cIAP2, and XIAP were expressed in normal human nasal mucosa, where they were detected in submucosal glands, epithelial cells, vascular endothelial cells, and inflammatory cells. However, cIAP1 was not expressed in nasal polyps, whereas cIAP2 and XIAP were expressed in submucosal glands, epithelial cells, vascular endothelial cells, and inflammatory cells. Caspase 3 was localized to a portion of the epithelial cells in normal nasal mucosa and nasal polyps. Survivin was not expressed in any samples. Furthermore, cIAP2, XIAP, and caspase 3 did not show a significant difference in their expression levels between normal nasal mucosa and nasal polyps.

Conclusion: The present results indicate that cIAP1, cIAP2, XIAP, and survivin were not expressed in nasal polyps. Caspase 3 may take part in the pathogenesis of nasal polyps.


Nasal polyps are a common chronic inflammatory disease of the nasal or paranasal sinus mucosa characterized by the protrusion of benign grapelike formations from the sinus meatus in the ethmoid region into the nasal cavities. This condition, common in both atopic patients and the general population, causes variable degrees of upper airway obstruction. Nasal polyps can cause disabling symptoms and require long-term treatment with corticosteroids or surgery. The histopathologic findings of nasal polyps typically consist of an underlying edematous or myxomatous stroma with an overlying intact respiratory epithelium, which shows various degrees of tissue remodeling in the epithelium, glands, connective tissues, and vessels. Although the pathogenesis of this nasal disease has recently been dealt with in numerous articles, the etiology and pathogenesis of nasal polyps are still controversial, and additional investigations are needed.

Much evidence has shown that the apoptotic mechanism induces the secondary changes in chronic inflammation of various organs, including epithelial hyperplasia or tissue remodeling. In this respect, molecular characterization of the mechanisms involved in the regulation and execution of apoptosis could provide crucial information for understanding the pathogenesis of nasal polyps. The inhibitor of the apoptosis protein (IAP) family is among the most important regulators of the cell suicide process. The IAPs are genes that suppress apoptosis induced by various triggers and involve tumor progression. In human organs, the family of...
IAPs (eg, cIAP1, cIAP2, and XIAP) are constitutively expressed, but survivin is expressed in fetal ages and then suppressed in adult organs. Classic apoptosis is triggered by caspase, which is activated by triggers that induce apoptosis. The IAPs suppress the activation of caspase. Of the caspase family, caspase 3 takes part in the last stage of apoptosis, resulting in the proteolysis of cytoplasmic proteins.7,8

Until now, the expression and distribution of IAPs and caspase 3 have not been observed in nasal polyps. This study aims to investigate the expression and distributional patterns of cIAP1, cIAP2, XIAP, survivin, and caspase 3 in nasal polyps and normal nasal mucosa to understand the pathophysiological mechanisms of the formation of nasal polyps.

**METHODS**

**SAMPLE PREPARATIONS**

Tissues from normal inferior turbinates were obtained from 20 patients (13 men and 7 women; age range, 20-35 years) undergoing surgery for augmentation rhinoplasty. All of these patients were free of nasal symptoms at the time of investigation, and rhinoscopy did not reveal any anatomical abnormalities or signs of mucosal injury. Nasal polyps were obtained from 20 patients (17 men and 3 women; age range, 35-50 years) undergoing endoscopic sinus surgery for chronic polyoid sinusitis. None of these patients had a history of nasal allergy, asthma, aspirin sensitivity, or ongoing drug treatment. Before the tissue specimens were obtained, the protocols and informed consent form were approved by the institutional review boards for human beings at our institution.

Tissues (15 turbinates and 15 polyps) were cut into 2 portions; the first and second portions were dissected, frozen in liquid nitrogen, and stored at −70°C for subsequent RNA and protein isolation. For immunohistochemical analysis, other samples (5 turbinates and 5 polyps) were immersed overnight in a freshly prepared fixative that contained 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. These samples were then dehydrated in a graded series of ethanol to xylene and embedded in paraffin wax.

**TOTAL RNA ISOLATION AND REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION**

The frozen tissues were homogenized in TRIzol Reagent (GIBCO BRL, Grand Island, New York), and equal amounts of total RNA (1 µg) from each sample were reverse transcribed in 20 µL of a reaction mixture that contained 2.5 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) and 50 pmol of random hexanucleotides at 42°C for 60 minutes. The RNA integrity and the success of the reverse transcription reaction were monitored by polymerase chain reaction (PCR) amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. Negative controls consisted of omission of the reverse transcriptase enzyme from the complementary DNA (cDNA) synthesis for each specimen. Primer sequences of each gene and the GAPDH gene (GenBank M33197) used in this study are listed in the **Table**. Amplified PCR products were resolved in 2% agarose gels, stained with ethidium bromide, and photographed under UV light. The identity of each PCR product was confirmed by sequencing and found to be identical with the messenger RNA (mRNA) sequence of each gene as deposited in the National Center for Biotechnology Information database.

Semiquantitative reverse transcriptase PCR was performed on the samples to assess for differential expression of each gene in normal nasal mucosa and nasal polyps. The optimum number of PCR cycles for each cDNA species was determined by plotting the PCR product yield of different cycles on a semilogarithmic graph, and the cycle number that represented the exponential amplification was chosen for the final amplification. For quantification of the PCR products of all samples, the expected bands were analyzed by densitometry, and data were expressed as the ratio between each gene and the corresponding GAPDH cDNA. The data are presented as mean (SD).

The statistical significance of differences between groups was evaluated by the Mann-Whitney test, and the level of statistical significance was set at P < .05.

**IMMUNOHISTOCHEMICAL AND WESTERN BLOT ANALYSIS**

Immunohistochemical staining was performed using a peroxidase-labeled streptavidin-biotin technique. Briefly, tissue sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide in methanol for 15 minutes to quench endogenous peroxidase activity. After washing in 10mM PBS (pH 7.4), antigen retrieval was performed by heating the slides for 25 minutes in a 0.01M Tris-EDTA buffer (pH 9.0) in a staining jar. Afterward, sections were incubated with 10% normal goat serum to block nonspecific binding. Sections were then incubated overnight at 4°C with 1:30 dilution of anti-clAP1, 1:50 of anti-clAP2, 1:50 of anti-XIAP, 1:100 of antisurvivin (Santa Cruz Biotechnology Inc, Santa Cruz, California), or 1:20 of anticaspase (BD Science Biotechnology Inc, San Jose, California) primary polyclonal rabbit antibodies. After being washed in PBS, sections were treated with biotinylated goat anti–rabbit IgG and subsequently with a streptavidin-peroxidase conjugate (Vector Laboratories, Burlingame, California). The 0.02% diaminobenzidine hydrochloride that contained 0.03% hydrogen peroxide was used as a chromogen to visualize the peroxidase activity. The negative immunohistochemical control procedure included omission of the primary antibodies and replacement of the primary antibodies by normal rabbit IgG in appropriate concentrations.

**Table. Primer Sequences for IAPs and Caspase 3**

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>cIAP1</td>
<td>S: GTCACATGTCGAAGTCGAGAG&lt;br&gt;A: GTCCTTCTCTGTTGACATTC</td>
</tr>
<tr>
<td>cIAP2</td>
<td>S: AACAGCAGAAAACAAAATGAA&lt;br&gt;A: CTTCCTGACACAGAGCGAAA</td>
</tr>
<tr>
<td>XIAP</td>
<td>S: GGGGTCATTTCCAAGGACA&lt;br&gt;A: CGCTTCATGTCCTGATCATG</td>
</tr>
<tr>
<td>Survivin</td>
<td>S: GGTGGCAGGAGTGTTTTGTT&lt;br&gt;A: AAAATTAGCCAGGAGCATGTT</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>S: TGTGTTGTGCTTGTCCAGG&lt;br&gt;A: TCAAAGTTGTCGGCACACTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>S: ATCTCCAGAGAGGAGACAC&lt;br&gt;A: ACCACTGACACGTTGGCAGT</td>
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Abbreviations: AS, antisense; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IAP, inhibitor of apoptosis protein; S, sense. aThe annealing temperature was 57°C for all primers.
polyps. (0.06) and P=0.32 (0.07) for XIAP, and T=0.32(0.04) and P=0.31(0.06) for cIAP1, T=0.76(0.10) and P=0.69(0.14) for cIAP2, T=0.33
the internal control. The mean (SD) values were as follows: T=0.79(0.03)
sal polyps (P). GAPDH indicates glyceraldehyde-3-
were expressed in nasal polyps (P). GAPDH indicates glyceraldehyde-3-
were expressed in normal inferior turbinate (T) mucosa, whereas cIAP2, XIAP, and caspase 3 messenger RNA were detected in normal nasal mucosa and nasal polyps.
scanning, and the relative amount of each gene was calculated by dividing by the internal control. The mean (SD) values were as follows: T=0.79 (0.03) and P=0 for cIAP1, T=0.76 (0.10) and P=0.69 (0.14) for cIAP2, T=0.33 (0.06) and P=0.32 (0.07) for XIAP, and T=0.32 (0.04) and P=0.31 (0.06) for caspase 3. Error bars indicate SD; open bars, turbinate; and closed bars, polyps.

For Western blot analysis, samples frozen in liquid nitrogen were crushed into pieces and vigorously vortexed in a buffer solution that contained 50mM Tris hydrochloride (pH 8.0), 150mM sodium chloride, 1% Triton X-100, and 1mM phenylmethylsulfonyl fluoride. A total of 50 µg of extracted protein was resuspended in sodium dodecyl sulfate sample buffer and boiled for 5 minutes. Equal amounts of total protein were separated on 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to Immobilon (Millipore, Bedford, Massachusetts). The blots underwent reaction with each antibody in Tris-buffered saline (20mM Tris hydrochloride, pH 8.0; 150mM sodium chloride; 0.05% Triton X-100; and 5% skim milk) for 2 hours at 4°C and then reacted with a biotin-conjugated anti-rabbit IgG antibody (Vector Laboratories) in PBS for 1 hour. After several washings with trypsic soy broth, the membranes were incubated with the avidin-biotin-peroxidase complex. Antibody reactions were detected using the ECL detection kit (Amersham Bioscience, Piscataway, New Jersey) followed by detection of chemiluminescence on x-ray film. As an internal control, β-actin expression was analyzed in parallel blots using the β-actin antibody (Sigma, St Louis, Missouri). The intensity of the detected bands was quantified using the scion image Beta 4.0.2 (Scion Corporation, Frederick, Maryland). Relative intensities of each protein signal were obtained by dividing the intensities of each protein signal by those of β-actin signals. Statistical significance of differences was evaluated by the Mann-Whitney test, and the level of statistical significance was set at P < .05.

RESULTS

Reverse transcriptase PCR assays were performed to detect the presence of cIAP1, cIAP2, XIAP, survivin, and caspase 3 mRNA in human inferior turbinate mucosa and nasal polyps. As shown in Figure 1A, cIAP1, cIAP2, XIAP, and caspase 3 mRNA were expressed in normal human nasal mucosa, whereas IAP2, XIAP, and caspase 3 mRNA were expressed in nasal polyps. However, survivin was not expressed in any samples (data not shown). The PCR products of the expected size corresponding to 780 base pairs (bp) for cIAP1, 581 bp for cIAP2, 183 bp for XIAP, and 245 bp for caspase 3 were detected in human inferior turbinate mucosa and nasal polyps and showed 100% sequence homology with the published sequence. The expression levels of cIAP2, XIAP, and caspase 3 mRNA were not significantly different between normal nasal mucosa and nasal polyps (Figure 1B).

Immunohistochemical staining showed a homogeneous pattern in all samples of nasal mucosa from normal inferior turbinate mucosa and nasal polyps. In normal mucosa, cIAP1, cIAP2, and XIAP were detected in submucosal glands, epithelial cells, vascular endothelial cells, and inflammatory cells (Figure 2A-C). In nasal polyps, cIAP2 and XIAP were expressed in glandular structures, epithelial cells, infiltrating inflammatory cells, and vascular endothelial cells (Figure 3A-D). In contrast to the IAPs, caspase 3 immunostaining appeared to localize to a portion of the epithelial cells in both normal nasal mucosa and nasal polyps (Figure 2D and Figure 3E). Western blot analysis also showed the presence of cIAP1, cIAP2, XIAP, and caspase 3 in normal nasal mucosa and nasal polyps (Figure 4A). In addition, cIAP2, XIAP, and caspase 3 did not show a significant difference in their expression levels between normal nasal mucosa and nasal polyps (Figure 4B). Survivin was also not detected in any samples (data not shown). Therefore, the results of Western blot confirm the expression of each gene in normal nasal mucosa and nasal polyps.

Apoptosis, a genetically controlled active cellular process, is a physiologic cell suicide program that is critical for the development and maintenance of healthy tissues. The regulation of apoptosis may play a central role in the development of neoplastic cellular proliferative processes and may exert a strong influence on tumor progression.9,10 In this study, we have evaluated the expression and localizations of cIAP1, cIAP2, XIAP, and caspase 3 in normal nasal mucosa and nasal polyps. The results of our study demonstrate for the first time, to our knowledge, that cIAP1, cIAP2, XIAP, and caspase 3 are expressed in normal nasal mucosa, whereas cIAP2, XIAP, and caspase 3 are expressed in nasal polyps. Therefore, the present results suggest that these IAPs may contribute to tissue homeostasis and differentiation in normal nasal mucosa and nasal polyps.

Survivin is an inhibitor of apoptosis protein involved in the regulation of cell division and apoptosis.11 In contrast to other members of the IAP family, which are widely expressed in normal tissues, survivin is selectively overexpressed in common human malignancies but barely detectable in normal differentiated tissues.12,13 The XIAP mRNA was observed in all adult and fetal tissue examined except peripheral blood leukocytes, indicating that it is a ubiquitously expressed member of the family.14 Ex-
pression of cIAP1 and cIAP2 is highest in the kidney, small intestine, liver, and lung and lowest in the central nervous system. These findings are consistent with our findings that indicate that survivin was not expressed in any samples of normal nasal mucosa or nasal polyps. In normal turbinate mucosa, cIAP1, cIAP2, and XIAP were detected in submucosal glands, epithelial cells, vascular endothelial cells, and inflammatory cells. These findings are consistent with results of a recent study in which the expression of cIAP1, cIAP2, and XIAP was analyzed in a large panel of normal human tissues. The biological significance of the constitutively expressed IAPs in normal nasal mucosa is currently unknown, but a recent report showed a crucial role of constitutive IAPs in the survival of thymocytes. Therefore, the basal expression of IAPs may be important for the maintenance of normal nasal mucosa survival in a physiologic situation. However, cIAP2 and XIAP in nasal polyps were expressed in glandular structures, epithelial cells, vascular endothelial cells, and inflammatory cells. That is, cIAP1 was expressed in normal inferior turbinate but not in the nasal polyps. Taken together, the present study suggests that different IAPs may play an antiapoptotic role in normal nasal mucosa and nasal polyps. Our data also raise the question of whether down-regulation of cIAP1 may play a role in the pathogenesis of nasal polyp formation. Down-regulation of cIAP1 has been demonstrated in cervical carcinoma and colon cancer. All these observations indicate that IAP expression levels are closely related to the tumor type and also suggest that the multifaceted functions of various IAPs may depend on cell type–specific factors. Similar results were found in normal pan-

Figure 2. Immunohistochemical localization in the inferior turbinate mucosa. A, cIAP1 (original magnification ×100); B, cIAP2 (original magnification ×100); C, XIAP (original magnification ×200); and D, caspase 3 (original magnification ×200). cIAP1, cIAP2, and XIAP are localized in the submucosal gland, vascular endothelium (arrows, A, B, and C), inflammatory cells (arrowheads, A, B, and C), and epithelial layer in the inferior turbinate mucosa. Caspase 3 was localized in the epithelial layer.

Figure 3. Immunohistochemical localization in the nasal polyps. A and B, cIAP2 (original magnification ×200); C and D, XIAP (original magnification ×200); and E, caspase 3 (original magnification ×200). cIAP2 and XIAP are localized in the epithelial layer (arrows, A and C), submucosal gland (G, A and D), vascular endothelium (VE, B), and inflammatory cells (arrowheads, B, C, and D). Caspase 3 was localized in the epithelial layer.
creatic tissues and cancer. However, only a few studies have evaluated expression of these IAPs in benign lesions such as nasal polyps, in which etiopathogenesis is still poorly investigated. Additional studies are required to draw conclusions regarding the functional implications of cIAP expression in the pathogenesis of nasal polyp formation.

In contrast to the IAPs, caspase 3 immunostaining appeared to localize to a portion of the epithelial cells in both normal nasal mucosa and nasal polyps. Because caspase 3 is the most downstream caspase and correlated with apoptosis, many reports have described the relationship between caspase 3 and prognosis in nonsmall cell lung cancer, neuroblastoma, and gastric cancer. However, caspase 3 activities and localization in chronic inflammatory diseases of the upper airway are not well known. In this study, caspase 3 was not expressed in any subepithelial constituent, but it was expressed in only the epithelium of the inferior turbinate and nasal polyps. Therefore, it is possible that the caspase pathway may not be an important proapoptotic mechanism in the subepithelial area, but it may be a key molecule in epithelial renewal and proliferation. Increased epithelial cell proliferation could be an important factor in the growth of nasal polyps, and many reports have shown that increased expression of proliferation cell nuclear antigen was observed in the epithelium of the nasal polyps compared with the nasal mucosa. In this study, caspase 3 was also expressed in both the inferior turbinate and nasal polyps. Therefore, caspase 3 could be involved in epithelial cell shedding and renewal in normal nasal mucosa and nasal polyps.

Nasal polyps characteristically contain leukocyte infiltrate in which eosinophils are often remarkably prominent. The abundance of eosinophils in nasal polyps has been partly explained by a prolonged survival of these cells, depressed apoptosis. Interleukin 3, interleukin 5, and granulocyte colony-stimulating factor appear to play significant roles in the promotion of survival and activation of mature eosinophils. On the other hand, increasing evidence indicates that members of the IAP family appear to be responsible for delayed apoptosis of many inflammatory cells. However, on the basis of observations from the current literature, the expression patterns of various IAPs in inflammatory cells under physiologic and pathologic conditions tend to show some inconsistency. For example, a recent experiment using peripheral blood eosinophils suggested that XIAP is present in eosinophils under physiologic conditions, whereas cIAP2 and survivin are expressed in eosinophils cultured with granulocyte-macrophage colony-stimulating factor. In contrast, another study reported that XIAP, cIAP1, and cIAP2 were undetectable in freshly isolated eosinophils and in eosinophils cultured with 96 hours. O’Neill et al showed that neutrophils from septic patients with delayed apoptosis show an increase in XIAP, with no change in cIAP1 or cIAP2 mRNA, demonstrating that different mechanisms contribute to the delay in neutrophil apoptosis. However, another study showed the up-regulated expression of cIAP2 among these members after in vitro stimulation. These findings suggest that the expression of the IAP family members may be regulated by different mechanisms, depending on the cells and the stimuli used. In addition, the present study demonstrated that the inflammatory cells in nasal polyps showed immunoreactivity for cIAP2 and XIAP but not survivin. Therefore, additional studies with many cases are required to establish the possible pathogenetic role of dysregulation of IAP expression and its underlying mechanism in nasal polyps.

In conclusion, apoptosis mediates several important physiologic and pathologic processes, such as homeostasis, remodeling, and atrophy of normal tissues. In the present study, the IAPs were differently expressed in normal nasal mucosa and nasal polyps. That is, cIAP1, cIAP2, and XIAP in normal nasal mucosa were localized in the epithelial layer, submucosal glands, vascular endothelial cells, and inflammatory cells. In nasal polyps, cIAP2 and XIAP were expressed and localized in the epithelial layer, submucosal glands, vascular endothelial cells, and inflammatory cells. However, survivin was not expressed in any normal nasal mucosa or nasal polyp samples tested in this study. In addition, caspase 3 was localized in the epithelial layer. Therefore, the present results indicate that cIAP1, cIAP2, XIAP, and caspase 3 may regulate the homeostasis of normal nasal mucosa, whereas the absence of cIAP1 may take part in the pathogenesis of nasal polyps.

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Author Contributions: Dr Sang Hag Lee had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data.
The spinal muscular atrophy disease gene

References