Expression of the Transforming Growth Factor β Isoforms in Inflammatory Cells of Nasal Polyps

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Methods

**Objective:** To determine the expression and the potential role of transforming growth factor β (TGF-β) in nasal polypsis.

**Design:** Comparison of TGF-β expression between normal and inflammatory nasal mucosa and polyps: in inflammatory nasal polyps, characterization of the TGF-β isoforms expression and their potential location in macrophages and eosinophils.

**Setting:** Patients and samples were selected at the Hôpital Intercommunal, Créteil, France, and immunohistochemistry and immunoblots were performed at the Institut National de la Santé et de la Recherche Médicale U296 (Université Paris XII, France).

**Subjects:** Nasal polyps and nasal mucosa were sampled in 21 patients during ethmoidectomy, and mucosa was sampled in 6 healthy patients during rhinoplasty.

**Methods:** Immunohistochemistry and Western blot analysis were performed using specific antibodies to TGF-β₁, TGF-β₁, TGF-β₂, and TGF-β₃ isoforms. Double labeling was also performed using anti-TGF-β₁ antibody together with macrophages or eosinophil-specific antibodies.

**Results:** The expression of TGF-β₁, was significantly higher in inflammatory nasal polyps than in inflammatory nasal mucosa and higher in inflammatory nasal mucosa than in nasal mucosa from healthy patients. Transforming growth factor β₁, was the main isoform detected in inflammatory nasal polyps, and it was present in numerous macrophages and in some eosinophils.

**Conclusions:** Transforming growth factor β, mainly TGF-β₁, is strongly expressed in inflammatory nasal mucosa, where it could be produced by macrophages and eosinophils. Transforming growth factor β could induce epithelium and connective tissue modifications and therefore be involved in the pathogenesis of nasal polypsis.

SUBJECTS AND METHODS

SUBJECTS

Twenty-one adult patients with nasal polyposis and 6 healthy controls were included in the study. Patients with cystic fibrosis or primary ciliary dyskinesia were excluded because of the special features of their NPs. The diagnosis of nasal polyposis was established on the basis of medical history and symptoms, endoscopic examination of the nose, and a computed tomographic scan of nasal fossa and paranasal sinuses. Because of the failure of medical treatment (ie, systemic and local corticosteroid therapy for at least 1 year), surgery was found to be necessary to improve the patients’ symptoms. Nasal mucosa from NPs was sampled in all patients, and nasal mucosa from the inferior turbinate, free of NPs, was sampled at the same time in 11 patients at the beginning of the surgical procedure (endoscopic endonasal ethmoidectomy). Six adult patients who underwent rhinoplasty were included as controls: all were free of symptoms of nasal inflammation with a normal appearance of the nasal mucosa at endoscopic examination. Nasal mucosa from the inferior turbinate was sampled in controls during the surgical procedure. All patients were asked to stop general and/or local nasal treatment 1 month prior to surgery. This protocol was approved by the review and ethics committee of our institution.

SAMPLES

Samples from 17 patients and 6 controls were immediately fixed in formaldehyde, embedded in paraffin, and divided into 5-µm sections. For each sample, a section was systematically stained (hemalum-eosin-safran) for standard histomorphologic analysis. In 4 patients, NPs were sampled, immediately frozen in liquid nitrogen, and then stored at −80°C until examination using Western blot analysis.

PRIMARY ANTIBODIES

Monoclonal mouse immunoglobulin antihuman TGF-β1,2,3 (1835-01, Genzyme, Cambridge, Mass), polyclonal chicken immunoglobulin antihuman TGF-β1, (BDA-19, R&D Systems, Minneapolis, Minn), polyclonal goat immunoglobulin antiporcine TGF-β2, (BDA-53, R&D Systems), and polyclonal goat immunoglobulin antichicken TGF-β3, (BDA-48, R&D Systems) (both goat immunoglobulins cross-reacting with human TGF-β) were used as primary antibodies for immunohistochemistry and Western blot analysis. In addition, monoclonal mouse immunoglobulin anti-cD68 (KP-1, M0814, Dako, Glostrup, Denmark) and monoclonal mouse immunoglobulin antieosinophil cationic protein (ECP) (EG2, 10-9116-01, Pharmacia, Uppsala, Sweden) were used as primary antibodies for immunochromic detection of activated macrophages and eosinophils, respectively.

IMMUNOHISTOCHEMISTRY

After deparaffinization, nonspecific antigenic sites were saturated with human AB serum, and the slides were incubated for 30 minutes with primary antibodies diluted at 1:50 except for anti–TGF-β1,2,3 (1:10) and anti–TGF-β3, (1:25). All incubations were performed at room temperature in a moist chamber, and the slides were washed in Tris-buffered saline (0.015 mol/L; pH, 7.6) between each incubation. The detection of TGF-β1,2,3 was performed in 17 NP samples, 11 nasal mucosa samples from patients, and 6 nasal mucosa samples from controls, and the detection of TGF-β1, TGF-β3, and TGF-β2 was performed in 10 NP samples with high TGF-β1, expression using biotinylated secondary antibodies and amplification by streptavidin-peroxidase complex (Labeled Streptavidin Biotim kit K680, Dako, Glostrup, Denmark). Monoclonal mouse immunoglobulin antihuman TGF-β1,2,3 protein were pre-

RESULTS

MORPHOLOGIC FEATURES

OF NASAL TISSUES

Modifications of epithelial morphologic features were frequent in NP samples (predominance of pseudostratified ciliated epithelium in 11 cases, squamous metaplasia in 3 cases, and secretory hyperplasia in 4 cases), but exceptional in nasal mucosa from patients (1 case with predominance of squamous metaplasia) and nasal mucosa from controls. As expected, inflammatory cells were more numerous in NP samples than in nasal mucosa from patients, but were rare in nasal mucosa from controls (data not shown).

TGF-β1,3 EXPRESSION

Using immunohistochemistry, TGF-β1,3 cell labeling was always detected in NP samples (17/17), sometimes in nasal mucosa samples from patients (5/11), and seldom in nasal mucosa from controls (1/6). In lamina propria and epithelium, the labeling was restricted to scattered cells confined to either the plasma membrane or the cytoplasm (Figure 1) without immunoreactivity in the extracellular matrix. In the lamina propria, the TGF-β1,3 indices were significantly higher in NP samples (mean ± SD, 55 ± 17.4 arbitrary units) than in nasal mucosa from controls (mean ± SD, 0.2 ± 0.4 arbitrary units; P < .001) and nasal mucosa from patients (mean ± SD, 10.5 ± 20.6 arbitrary units; P < .05) (Figure 2). The difference between TGF-β1,3 indices in nasal mucosa from patients and nasal mucosa from controls was not sta-
Transforming growth factor β (TGF-β) was extracted from frozen NP samples using a previously published method. Nasal polyp extracts (80 µg of proteins on each lane) were run under nonreducing conditions on a 15% sodium dodecyl sulfate polyacrylamide gel and transferred overnight in a Tris glycine buffer (pH, 8.3) with 20% methanol onto a nitrocellulose membrane (0.45-µm pore size, Schleicher & Schuell, Keene, NH). Five nanograms of purified TGF-β1 (101-B1, R&D Systems), TGF-β2, (101-B2, R&D Systems), or recombinant TGF-β3 (243-B3, R&D Systems) was run on 1 lane of each blot to act as a positive control. Immediately following transfer, the proteins were cross-linked onto nitrocellulose membranes by dipping them in 0.5% glutaraldehyde phosphate–buffered solution. After blocking with 1% nonfat dry milk in Tris buffer (pH, 7.5), the membranes were then incubated overnight at 4°C, with anti–TGF-β1 and anti–TGF-β3, diluted to 1:1000 and the various anti–TGF-β isoform antibodies diluted to 1:400 in 1% milk Tris polysorbate buffer. After rinsing, membranes were incubated with secondary biotinylated antibodies diluted to 1: 4000 for 1 hour. The membranes were then incubated with streptavidin–peroxidase complex (RPN 1051, Amersham, Uppsala) for 45 minutes and revealed using the electrochemiluminescence detection method (RPN 2109, Amersham).

STATISTICAL ANALYSIS

The nonparametric Wilcoxon test was used to compare TGF-β1 and TGF-β3 indices between NP and nasal mucosa from patients. The nonparametric Mann-Whitney U test was used to compare TGF-β1, indices between NP or nasal mucosa from patients and nasal mucosa from controls. All analyses were performed using a personal computer (Apple, Cupertino, Calif) with Instat 2.00 software (Instat, Graphpad, San Diego, Calif).
changes, basement membrane thickening, and inflammatory cell infiltration with eosinophils). Our results obtained from nasal tissues using either immunohistochemistry or Western blot analysis were strictly concordant, showing that TGF-β was strongly expressed in NP samples almost exclusively in the β1 isoform.

As previously reported, we detected TGF-β only in NPs and inflammatory mucosa but not in nasal mucosa from controls. Transforming growth factor ββ was mainly detected in the lamina propria, where it was localized in numerous isolated cells exhibiting inflammatory cell morphologic features. This finding suggests that in the upper airways, as in other tissues, the main source of TGF-β could be the inflammatory cells. In this study, we evaluated the cell expression of TGF-β1 in eosinophils in NPs, which have been previously suspected to express the TGF-β1 gene in NPs and macrophages that are known to be able to produce TGF-β. Transforming growth factor β1 was present in many cells of each type, but not in all. Although eosinophils seemed to be more numerous than macrophages in the NPs, TGF-β1 was detected more frequently in macrophages than in eosinophils, contrasting with findings of a previous study. This discrepancy could result from technical differences concerning the method of detection of eosinophils and macrophages, but also from the level at which TGF-β was detected (protein vs mRNA) in these cells. Apart from the lamina propria, TGF-β was also detected in some epithelial areas, suggesting local production, since airway epithelial cells are able to synthesize TGF-β in vitro and are an important site of TGF-β expression in advanced pulmonary fibrosis. In any case, TGF-β expression in epithelial cells was always low compared with its expression in inflammatory cells. A weak immunoreactivity of the extracellular matrix was detected with the TGF-β isoform–specific antibodies but not with the TGF-β1–specific antibody (Figure 1). This discrepancy could be interpreted as a technical difference between the polyclonal and monoclonal nature of the antibodies. Another interpretation could be that the anti–TGF-β1 monoclonal antibody detects a secreted form of TGF-β, while TGF-β isoform–specific antibodies detect both secreted and matrix-associated TGF-β forms.
Charactetermination of TGF-β expression by immuno-

histochemistry and Western blot analysis showed that TGF-β1 was the main isoform of the TGF-β family expressed in NPs. Conversely, TGF-β2 and TGF-β3 were expressed at a low level in NPs. These findings contrast with TGF-β expression in distal lower airways where the 3 TGF-β isoforms were detected.

The major expression of TGF-β in NPs suggests that TGF-β, particularly TGF-β1, could be involved in the pathogenesis of nasal polyposis. The TGF-β present in NPs could participate in the complex regulation of local inflammation. Transforming growth factor β1 is a potent chemoattractant for neutrophils and mononuclear cells, but also has immunosuppressive effects on T lymphocytes, neutrophils, and macrophages. Further studies could help define how TGF-β can modulate local inflammation in nasal polyposis. Transforming growth factor ββ is a chemoattractant for fibroblasts, greatly enhancing fibroblast synthesis of extracellular matrix proteins on one hand and down-regulating the expression of matrix degradation enzymes on the other. Transforming growth factor ββ could therefore be involved in the pathogenesis of nasal polyposis by enhancing extracellular matrix accumulation, which seems an important mechanism of NP growth as suggested in experimental models. Another important effect of TGF-β is its ability to influence airway epithelial cell growth and differentiation. We previously showed that epithelial cell proliferation was increased in NPs and that this epithelial cell proliferation could be induced by mitogenic growth factors that are locally produced by inflammatory cells. Transforming growth factor β1, which has been shown in vitro to down-regulate epithelial cell proliferation, could limit the proliferative effects of other growth factors to control the epithelial cell proliferation in NPs. Transforming growth factor β1 and TGF-β1 have been reported to induce in vitro squamous metaplasia in airway epithelial cells, but we never detected TGF-β-positive cells near areas of epithelial squamous metaplasia. This result is consistent with a recent in vitro study in which TGF-β failed to induce squamous differentiation in human bronchial epithelial cells. In contrast, we observed that secretory hyperplasia was frequently associated with high local TGF-β expression. This finding suggests that TGF-β could induce mucus cell differentiation in upper airways, as previously proposed.

In the present study, we showed that TGF-β was present in NPs and to a lesser extent in chronic inflammatory mucosa, but not in nasal mucosa from upper airways in controls, suggesting that TGF-β could be involved in the pathophysiological development of nasal polyposis. Of the various TGF-β isoforms, only TGF-β1 was significantly detected. Transforming growth factor β1-labeled cells were mainly macrophages and eosinophils, implying that these inflammatory cells could be a major local source of TGF-β. A broad and complex spectrum of effects can result from TGF-β release in the upper airways, such as epithelium changes and extracellular matrix accumulation. As therapeutic strategies with anti-TGF-β molecules are currently being developed for other inflammatory diseases, a better understanding of TGF-β activity in nasal polyposis could have direct clinical implications in the future.

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