

# Myofibroblasts in nasal polyposis: Regulation by topical steroids

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**GM Tremblay, M Nonaka, B Särnstrand, J Dolovich, J Gauldie, M Jordana. Myofibroblasts in nasal polyposis: Regulation by topical steroids. Can Respir J 1997; 4(4):205-210.**

**OBJECTIVES:** To compare numbers of myofibroblasts, a fibroblast phenotype associated with chronically inflamed tissue, in nasal polyp tissues from untreated and corticosteroid-treated subjects. To study whether corticosteroids can directly affect myofibroblast differentiation *in vitro*.

**PATIENTS AND METHODS:** Immunolocalization of alpha-smooth muscle actin (alpha-SMA) as a marker of myofibroblast differentiation, vimentin and transforming growth factor (TGF)-beta in nasal polyp tissues from nine patients who had taken no steroids for at least a month before polypectomy and from eight patients who had received intranasal budesonide or beclomethasone dipropionate continuously for four to 12 months before polypectomy. Cultured nasal polyp fibroblasts were exposed to budesonide for six days and alpha-SMA expression was determined by immunocytochemistry.

**RESULTS:** Nasal polyp tissues from untreated subjects were characterized by the presence of a substantial number of myofibroblasts compared with tissues from corticosteroid-treated subjects. The median percentages of alpha-SMA positive cell areas per total area for the two groups were 6.4% (0.5 to 11.5) and 1.3% (0.1 to 4.7), respectively. This difference in alpha-SMA staining between the two groups was not due to a decrease in fibroblast numbers (vimentin-positive spindle-shape cells). Numbers of TGF-beta positive cells were similar in the two groups of sub-

jects. *In vitro*, budesonide treatment decreased the number of alpha-SMA positive fibroblasts in primary lines in a dose dependent manner.

**CONCLUSIONS:** The difference in myofibroblast numbers between nasal polyp tissues from untreated and corticosteroid-treated subjects, as shown by immunolocalization of alpha-SMA, suggests a new therapeutic effect of nasal topical corticosteroids in nasal polyposis. This could be a direct effect of the drug on fibroblast differentiation and/or modulation of cytokine production.

**Key Words:** *Fibroblasts, Immunocytochemistry, Nasal polyps, Transforming growth factor-beta*

## Les myofibroblastes dans la polypose nasale : régulation à l'aide de stéroïdes topiques

**OBJECTIFS :** Comparer le nombre des myofibroblastes, un phénotype de fibroblastes associé à une inflammation chronique des tissus, dans les tissus de polypes nasals de sujets non traités et traités à l'aide de corticostéroïdes. Examiner si les corticostéroïdes peuvent directement affecter la différenciation des myofibroblastes *in vitro*.

**PATIENTS ET MÉTHODES :** Immunolocalisation de l'alpha-actine du muscle lisse (alpha-SMA) comme marqueur de la différenciation des myofibroblastes, de la vimentine et du facteur de croissance transformant (TGF)-bêta dans les tissus des polypes nasals chez neuf patients n'ayant pas reçu des stéroïdes pendant au moins un mois avant de subir une polypectomie et chez huit patients ayant reçu du budésonide par voie nasale ou du dipropionate de béclométhasone sur une base régulière pendant 4 à 12 mois avant de subir une polypectomie. Les cultures de fibroblastes des

polypes nasals ont été exposées au budésonide pendant six jours et l'expression de l'alpha-SMA a été déterminée à l'aide d'une technique immunocytochimique.

**RESULTATS :** Les tissus des polypes nasals des patients non traités étaient caractérisés par la présence d'un nombre important de myofibroblastes comparativement aux tissus des patients traités par corticostéroïdes. Les pourcentages médians des surfaces de cellules positives pour l'alpha-SMA par surface totale pour les deux groupes étaient respectivement de 6,4 % (0,5 à 11,5) et de 1,3 % (0,1 à 4,7). La différence dans la coloration de l'alpha-SMA observée entre les deux groupes n'était pas due à une diminution du nombre des fibroblastes (cellules fusiformes positives pour la vimentine). Le nombre de cellules positives pour le TGF- $\beta$  était

similaire dans les deux groupes de sujets. *In vitro*, le traitement au budésonide diminuait le nombre des fibroblastes positifs pour l'alpha-SMA dans les lignes primitives d'une manière proportionnelle à la dose administrée.

**CONCLUSIONS :** La différence dans le nombre des myofibroblastes entre les tissus des polypes nasals de patients non traités et des patients traités avec des corticostéroïdes, comme l'a montré l'immunolocalisation de l'alpha-SMA, laisse à penser que les corticostéroïdes topiques par voie nasale peuvent jouer un rôle thérapeutique nouveau dans le traitement de la polyposis nasale. Ceci pourrait résulter de l'effet direct du médicament sur la différenciation des fibroblastes et/ou la modulation de la production des cytokines.

**N**asal polyposis is a hyperplastic condition characterized by chronic inflammation and structural abnormalities including epithelial damage and thickening of the basement membrane (1). Despite the significant infiltration by numerous eosinophils (2), which are probably involved in the regulation of the inflammatory response, the bulk of nasal polyp (NP) tissue cells obtained by enzymatic dispersion are fibroblastic in nature (1). It is now believed that fibroblast effector functions and phenotypic change play an important role in the remodeling process associated with chronic airways inflammation (3).

A fibroblast phenotype associated with chronically inflamed tissue is the myofibroblast, a cell having contractile elements including alpha-smooth muscle actin (alpha-SMA) (4). Since myofibroblasts' first description in contracting experimental granulation tissue, they have been found in different tissues including the lungs of normal subjects and those with pathological conditions (5). Possible myofibroblast contribution to the disordered extracellular matrix production associated with asthma and pulmonary fibrosis (6,7) suggests that they could also be involved in nasal polyposis where a similar structural abnormality has been observed. In this regard, we have previously demonstrated the presence in NP of transforming growth factor (TGF)- $\beta$  and granulocyte macrophage colony-stimulating factor (GM-CSF) (8,9), two cytokines that can induce alpha-SMA expression in fibroblasts (10-12). Moreover, recently the presence of myofibroblasts in NP tissue has been demonstrated (1,13), whereas they are nearly absent from normal turbinate tissue (1). Myofibroblasts may be the NP fibroblastic cells recently described as showing an increased rate of proteoglycan synthesis (14).

The modulation of myofibroblast alpha-SMA expression by different cytokines has received considerable attention (15). In contrast, there is no information to our knowledge regarding the effect of corticosteroids on alpha-SMA expression in fibroblasts. Topical corticosteroids have been used for their anti-inflammatory effects in asthma and allergic rhinitis (16) as well as in nasal polyposis (17), where they have been shown to decrease the number of both activated eosinophils (18) and cells localizing GM-CSF (unpublished results). In addition, their effects on numerous fibroblast functions (19) suggest that corticosteroids could also have a direct regulatory effect on fibroblast alpha-SMA expression as shown for smooth muscle cells (20).

The purpose of this study was to investigate whether topical treatment with corticosteroids affects the number of myofibroblasts (ie, alpha-SMA positive fibroblasts) in NP tissue and whether corticosteroids can directly affect myofibroblast differentiation *in vitro*.

## PATIENTS AND METHODS

**Subjects:** Seventeen patients who underwent polypectomy for nasal obstruction were included in the present study. Nine patients (seven males and two females), including four asthmatic (three atopic), aged 37 to 65 years (mean 52 years) had taken no steroids or antibiotics for at least one month before polypectomy. Eight patients (four males and four females), including four asthmatics [two atopic], aged 34 to 67 years (mean 51 years) were treated with either intranasal budesonide or beclomethasone dipropionate (200 to 400  $\mu$ g/day) at the time of surgery. These patients had taken the steroids continuously for four to 12 months before polypectomy. The two groups were similar with regard to demographics and history of asthma and atopy. Patients were not randomized, and the decision to treat with steroids was left to their physician. None of the subjects had aspirin sensitivity or cystic fibrosis. The study was approved by the local Ethics Committee, and subjects gave informed written consent.

**Nasal polypectomy and tissue processing:** Nasal polyp tissues were removed at surgery, immediately placed in F-12 Nutrient Mixture (Ham) (GIBCO BRL, New York) and kept on ice. Tissues were fixed in periodate-lysine-paraformaldehyde (PLP) and washed in increasing concentrations of sucrose. The tissues were then frozen in Tissue-Tek OCT compound (Miles Inc, Indiana) in  $-70^{\circ}\text{C}$  isopentane and stored at  $-70^{\circ}\text{C}$ . Serial cryostat sections (6  $\mu$ m) were cut onto slides coated with 3-aminopropyl-triethoxysilane (Sigma, Missouri).

**Immunohistochemical staining of vimentin and TGF- $\beta$ :** To remove OCT compound and to obtain subsequent enhanced permeability and blocking of nonspecific reactions, slides were rinsed in Dulbecco's phosphate-buffered saline (PBS) (GIBCO BRL) supplemented with 0.01 M HEPES buffer and 0.01% saponin (Sigma) (DPBS [H+S]) for 10 mins at room temperature (RT). For TGF- $\beta$  staining, the sections were digested with 1 mg/mL of hyaluronidase in 0.1 M sodium acetate and 0.15 M sodium chloride (pH 5.5) for 30 min at RT. To block further nonspecific binding, sec-

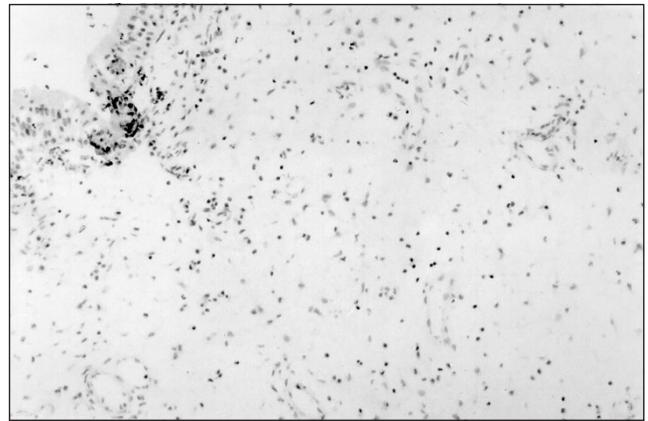
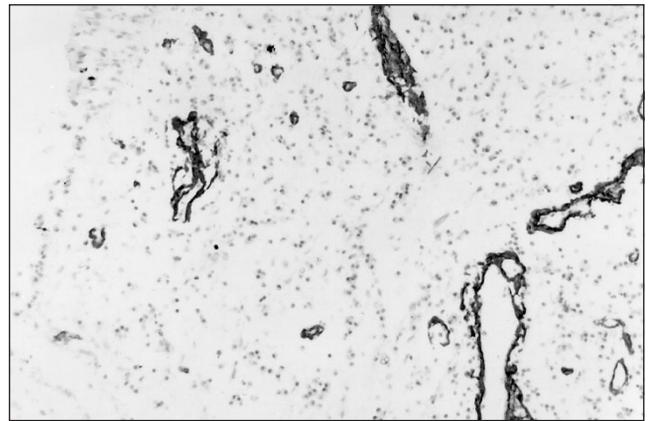
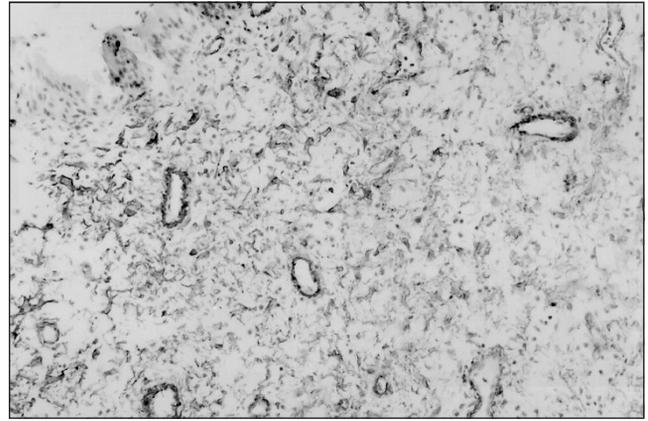
tions were preincubated in DPBS (H+S) containing 75% heat inactivated human AB sera for 1 h and then in the same buffer with 25% heat inactivated normal rabbit serum (Sigma) for 30 mins at RT. Mouse monoclonal anti-human vimentin (clone V9, Boehringer Mannheim Canada) or mouse monoclonal anti-human TGF-beta<sub>1,3</sub> (Genzyme, Massachusetts) was diluted in DPBS (H+S), containing 1% bovine serum albumin (Sigma) at a concentration of 2 µg/mL and 75 µg/mL, respectively. After brief washing with DPBS (H+S), sections were incubated with the monoclonal antibody overnight at 4°C and then washed in the same buffer. Labelling of the monoclonal antibody was detected by the alkaline phosphatase anti-alkaline phosphatase method (APAAP Kit, Dako, California) following the manufacturer's instructions. Counterstaining was performed with Mayer's hematoxylin solution (Sigma), and specific controls were included in each staining run. Mouse immunoglobulin (Ig) G<sub>1</sub> (Zymed Laboratories Inc, California) was used as negative control.

**Immunohistochemical staining of alpha-SMA:** The streptavidin-biotin system (Zymed) was used. Slides were rinsed twice in PBS (0.01 M, pH 7.4) supplemented with 0.1% tween 20 (PBS+) for 5 mins at RT. To block endogenous peroxidase, sections were preincubated for 30 mins in 0.6% hydrogen peroxide in methanol. To block further non-specific binding, sections were then preincubated for 1 h in 100% heat inactivated normal rabbit serum (Sigma) at RT.

The monoclonal antihuman alpha-SMA (Sigma) was diluted in PBS+ containing 1% bovine serum albumin at a concentration of 2 µg/ml. After brief washing with PBS+, sections were incubated with the monoclonal antibody overnight at 4°C. Slides were then washed in PBS+ and incubated with biotinylated rabbit antimouse antibody (Zymed) for 30 mins at RT. All incubations were followed by three washes of 5 mins each in PBS+. Slides were incubated with peroxidase conjugated streptavidin (Zymed) for 30 mins at RT. The chromogen solution of 3-amino-9-ethyl-carbazide (Zymed) was then applied. Counterstaining was performed with Mayer's hematoxylin, and specific controls were incubated in each staining run. Mouse IgG<sub>2a</sub> was used as negative control.

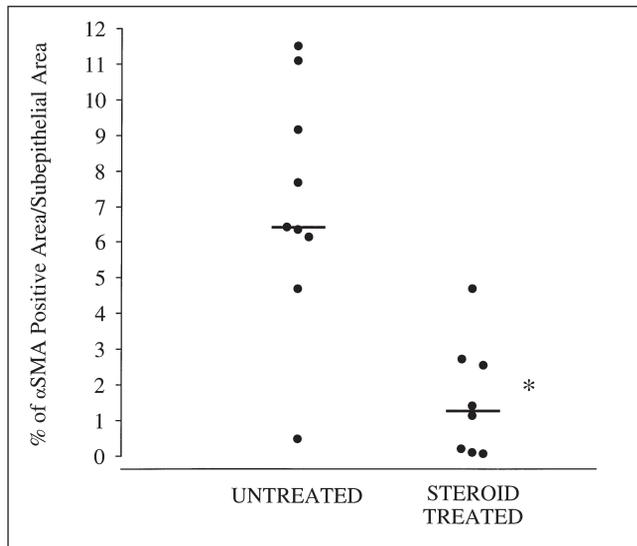
**Quantification of immunohistochemical staining:** All NP tissue samples were identified by code to prevent bias, and sections were quantified with computer attached Zeiss microscope. Quantification was done in five to six fields in an area of 1 mm<sup>2</sup>, extending 1 mm in depth from the lower border of the basement membrane. For both vimentin and TGF-beta staining, results were expressed as the number of stained cells per mm<sup>2</sup>. A computer colour imaging system was used to detect the brown peroxidase reaction product for the quantification of alpha-SMA staining only. The colour image was acquired from the Zeiss microscope via a Sony 3 CCD colour video camera (model DXC-930). The segmented colour images were entered into a Vidas Image System (Kontron, Munich, Germany). The mean percentage of stained area per total area of the tissue excluding blood vessels was calculated from the same fields as above.

**Fibroblast cell lines:** Nasal polyp fibroblasts were obtained



**Figure 1** Immunolocalization of alpha-smooth muscle actin (SMA) in nasal polyp tissues. **Top** Tissue from an untreated subject stained with anti-alpha-SMA antibody. **Middle** Tissue from a corticosteroid-treated subject stained with anti-alpha-SMA antibody. **Bottom** A nasal polyp tissue stained with control antibody (original magnification  $\times 160$ )

from patients undergoing polypectomy who had taken no steroids for at least one month before surgery. Minced tissues were digested in 190 U/mL collagenase type II in Hanks balanced salt solution without calcium ions and magnesium ions for 1 h at 37°C under gentle shaking (21). After centrifugation, dispersed cells were resuspended and cultured. All NP fibroblast cell lines were expanded and kept under cryocon-



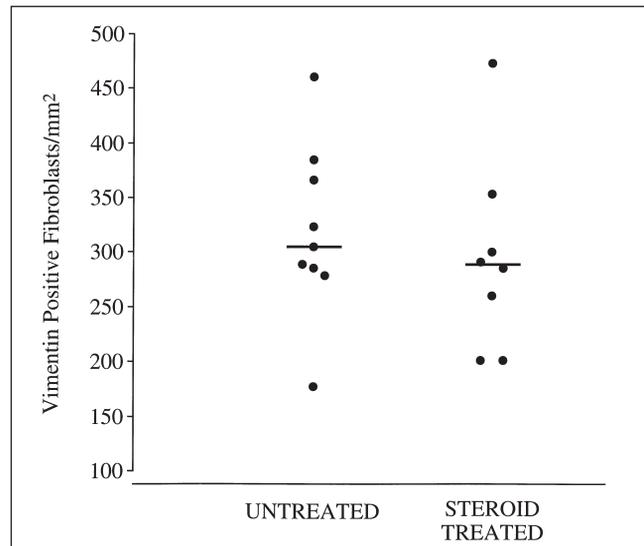
**Figure 2)** Alpha-smooth muscle actin (alpha-SMA) expression in nasal polyp tissues from untreated and steroid-treated subjects expressed as a percentage of alpha-SMA positive area/subepithelial area. Horizontal bars indicate medians. \*Nasal polyp tissues from steroid-treated subjects had a lower alpha-SMA positive area compared with tissues from untreated subjects ( $P < 0.005$ , Mann-Whitney U test)

servation for future use. Fibroblasts used in the present work were always used at a passage earlier than eight.

**Effect of budesonide on alpha-SMA expression in cultured nasal polyp fibroblasts:** NP fibroblasts were plated in four-well Lab-Tek chamber slides (Nunc Inc, Illinois) in minimum essential medium (MEM) supplemented with L-glutamine, HEPES, 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin, 0.6  $\mu$ g/mL fungizone and 10% fetal bovine serum (FBS) at a density of 4500 cells/cm<sup>2</sup>. This was referred to as day - 3. Twenty-four hours later (day - 2), the FBS concentration was reduced to 0.4%, this concentration of FBS being used for the remaining of the experiment. Forty-eight hours later (day 0) and every other day (eg, day 2 and day 4), the culture medium was changed for medium containing either budesonide at 10<sup>-10</sup>, 10<sup>-8</sup> or 10<sup>-6</sup> M concentrations or diluent only. All slides were fixed in PLP for 10 mins at day 6 and then washed in 15% sucrose in phosphate-buffer (0.1 M, pH 7.4) for 10 mins. After air drying, slides were stored at -70°C until immunocytochemistry was carried out.

Immunocytochemistry for alpha-SMA of cultured NP fibroblasts was performed using the same APAAP method described above. The concentration of the mouse monoclonal anti-human alpha-SMA was 20  $\mu$ g/mL. The number of alpha-SMA positive fibroblasts was expressed as a percentage of total fibroblasts.

**Statistical analysis:** Results are shown as either median and range or as mean and standard deviation. The Mann-Whitney U test was used for variables which were not normally distributed. The effect of budesonide on cultured fibroblasts was assessed by ANOVA followed by Scheffe test.  $P < 0.05$  was taken as significant. The coefficient of variance for three repeat counts of a single slide was less than 5%.



**Figure 3)** Vimentin-positive fibroblast counts in nasal polyp tissues from untreated and steroid-treated subjects expressed as cells/mm<sup>2</sup> of subepithelial area. Horizontal bars indicate medians. Counts were similar in both groups

## RESULTS

**Immunohistochemistry of nasal polyp tissues:** Nasal polyp tissues from untreated subjects were characterized by a substantial number of myofibroblasts (alpha-SMA positive fibroblasts) (Figure 1). In contrast, NP tissues from steroid-treated subjects had less positively stained cells (Figure 1). Quantification of alpha-SMA staining by image analysis showed that the median percentage of alpha-SMA positive area per total area (blood vessels excluded) in tissues from untreated subjects was significantly higher than that in tissues from steroid-treated subjects, 6.4% (0.5% to 11.5%) and 1.3% (0.1% to 4.7%), respectively ( $P < 0.005$ , Mann-Whitney U test) (Figure 2).

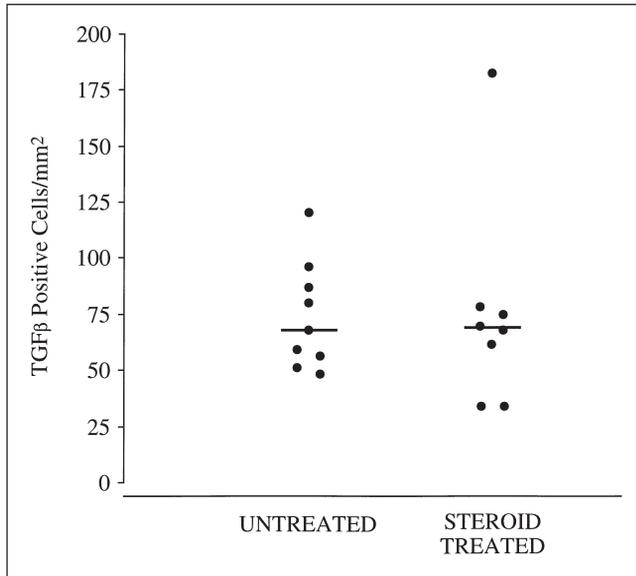
This difference in alpha-SMA staining between the two groups was not due to a decrease in fibroblast numbers because NP tissues from both untreated and ST-treated subjects contained similar number of spindle-shape, vimentin-positive cells (median 305, range 177 to 461 cells/mm<sup>2</sup> and median 289, range 201 to 473 cells/mm<sup>2</sup>, respectively) (Figure 3).

Figure 4 shows that the median number of TGF-beta positive cells in NP tissues was similar between the two groups of subjects, 68 cells/mm<sup>2</sup> (range 49 to 121) and 69 cells/mm<sup>2</sup> (range 34 to 183), respectively.

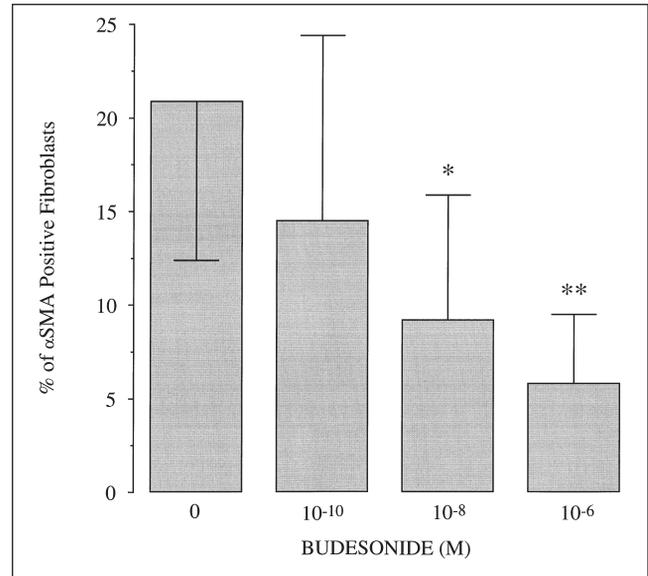
**Effect of budesonide on alpha-SMA expression in cultured nasal polyp fibroblasts:** In culture, 20.9 $\pm$ 8.6% of NP fibroblasts (n=4) showed a positive staining for alpha-SMA. Budesonide treatment decreased the number of myofibroblasts (alpha-SMA positive fibroblasts) in a dose dependent manner (Figure 5).

## DISCUSSION

Myofibroblasts, a phenotype of fibroblasts expressing alpha-SMA, are present in chronic inflammatory processes including idiopathic pulmonary fibrosis, hypersensitivity



**Figure 4** Transforming growth factor-beta (TGF- $\beta$ ) positive cell counts in nasal polyp tissues from untreated and steroid-treated subjects expressed as cells/mm<sup>2</sup> of subepithelial area. Horizontal bars indicate medians. Counts were similar in both groups



**Figure 5** In vitro effect of budesonide on alpha-smooth muscle actin ( $\alpha$ SMA) expression in four nasal polyp fibroblast lines. Results are expressed as means and standard deviations. \*Significantly different from baseline. \*\*Significantly different from baseline and budesonide 10<sup>-10</sup>M. ANOVA followed by Scheffe test ( $P < 0.05$ )

pneumonitis and asthma (6,7). Recently, their presence has been highlighted in nasal polyposis (1,13). Observations in human tissues as well as in experimental models suggest that myofibroblasts represent an aggressive phenotype of fibroblasts that could contribute to the chronicity of the inflammatory process through the disordered production of both extracellular matrix proteins (6,7,22) and cytokines (23,24). The effect of different cytokines on the phenotypic conversion of fibroblasts to myofibroblasts has been extensively studied (10-12,15). Therapeutic interventions able to reverse this process may be beneficial as shown for interferon-gamma treatment in Dupuytren contracture (25). In the present study, we have confirmed the presence of myofibroblasts in NP tissue. Moreover, we report that the number of myofibroblasts is significantly lower in NP tissues from subjects treated with nasal topical corticosteroids compared with NP tissues from untreated subjects.

Several aspects of fibroblast metabolism are modulated by corticosteroids (19). In the case of proliferation, data in the literature conflict. However, most studies conclude that corticosteroids inhibit fibroblast proliferation (19). To assess whether the observed difference in alpha-SMA expression in NP tissues between the two groups of subjects was due to a difference in total fibroblast numbers induced by the drug, we immunolocalized vimentin, a cytoskeletal marker present in all fibroblastic cells (26). Total fibroblast numbers, defined as vimentin-positive spindle-shaped cells, were similar in both groups suggesting that the down-regulatory effect of corticosteroids on alpha-SMA expression in NP tissue fibroblasts is not associated with a major effect on fibroblast numbers.

The emergence of myofibroblasts in NP can be seen either as a permanent or a transient phenotypic change in fibroblast behaviour (3). Cytokines such as TGF-beta and GM-CSF

that can be produced by both inflammatory and structural cells (8,9,27) have been shown to be capable of regulating alpha-SMA expression in fibroblasts (10-12). TGF-beta can directly induce alpha-SMA expression in fibroblasts both in vivo and in vitro (10,11). However, we did not find differences in the density of TGF-beta positive cells in NP tissues from untreated and corticosteroid-treated subjects; this is not a very surprising finding because whether corticosteroids can downregulate TGF-beta expression in vivo is unclear. In contrast with TGF-beta, GM-CSF has been shown to be involved in the emergence of myofibroblasts in vivo but not in vitro (12), thus suggesting the requirement for an additional signal present in the in vivo microenvironment during inflammation. Corticosteroids decrease the density of cells localizing GM-CSF in NP tissue (unpublished results) and, more recently, Mullol et al (27) have shown that dexamethasone inhibits GM-CSF production by NP epithelial cells in culture. The mechanisms underlying the decrease in myofibroblast density in NP tissues from corticosteroid-treated patients remain to be fully elucidated, but we suggest that GM-CSF is likely to play a more central role than TGF-beta. There is no doubt that corticosteroids can downregulate GM-CSF production and, as we show here, they can also directly downregulate alpha-SMA expression in myofibroblasts. It is then plausible that in addition to their well recognized anti-inflammatory effects, corticosteroids are positively involved in tissue remodelling.

The potential of myofibroblasts to contribute to the fibrotic process associated with chronic inflammation has been shown in several different pathologies. For example, the newly reactive alpha-SMA positive fibroblasts in experimental pulmonary fibrosis express procollagen mRNA (7); myofibroblasts from scleroderma skin synthesize elevated

levels of collagen and tissue inhibitor metalloproteinase-1 (22); and myofibroblasts from postradiation fibrosis in breast cancer patients produce interleukin-2 in contrast with normal skin fibroblasts (23). This and other studies (1,13) have described the presence of myofibroblasts in nasal polyposis. Their contribution to the fibrotic process in this disease remains to be fully determined, but a recent study has shown a high rate of proteoglycan synthesis by NP fibroblasts (14), which we believe could be due to myofibroblasts.

In summary, the difference in myofibroblast numbers, as shown by immunolocalization of alpha-SMA, between NP tissues from untreated and corticosteroid-treated subjects suggests a new therapeutic effect of nasal topical corticosteroids in nasal polyposis. This could be a direct effect of drug on myofibroblasts or an effect on the synthesis of molecules,

such as GM-CSF, mediating the emergence of this particular phenotype. Studies are currently under way to characterize further NP myofibroblasts and to understand better the mechanisms by which corticosteroids downregulate alpha-SMA expression in fibroblasts.

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