Lower sensitivity of nasal polyp fibroblasts to glucocorticoid anti-proliferative effects

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KEYWORDS
Asthma;
Fibroblasts;
Glucocorticoids;
Nasal mucosa;
Nasal polyps

Summary
Background: Treatment with glucocorticoids (GCs) is the cornerstone of nasal polyp (NP) therapy, but some patients respond poorly to them. Fibroblasts are involved in both inflammation and remodelling of NP. We aimed to evaluate whether NP fibroblasts are less sensitive to GCs’ anti-proliferative and anti-inflammatory effects, compared to nasal mucosa (NM) fibroblasts.

Methods: Fibroblasts were obtained from NP (n = 8) from asthmatic patients undergoing endoscopic surgery and NM (n = 8) from patients undergoing nasal corrective surgery. Fibroblasts were stimulated with DMEM at 0.5% or 5% FBS, or TGF-β (5 ng/ml), with or without dexamethasone (10^{-11} to 10^{-5}M) for different times. Cell proliferation, collagen mRNA expression and IL-6 and IL-8 release were measured.

Results: After 3 days, dexamethasone dose-dependently inhibited proliferation of NM (p < 0.001) but not that of NP fibroblasts. Dexamethasone (10^{-6}M) reduced by 25% the proliferation of NM fibroblasts. Dexamethasone also inhibited proliferation of NM (p < 0.01) but not that of NP fibroblasts at 5 days. TGF-β induced collagen-1α1, -1α2, and -3α1 mRNA levels in both NM and NP fibroblasts (p < 0.05), and dexamethasone did not alter TGF-β-induced collagen mRNA levels in either fibroblast type at 24 h. Dexamethasone dose-dependently

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Introduction

Inflammation and remodelling, i.e., a pathological wound repair mechanism, are hallmarks of both asthma and chronic rhinosinusitis, with or without nasal polyposis. Chronic rhinosinusitis with nasal polyps (NP) reveals frequent epithelial damage, a thickened basement membrane, oedema, fibrotic foci and a reduced number of vessels and glands. Compared with control NM, the stroma of NPs contains abundant infiltration of inflammatory cells, predominantly activated eosinophils and increased fibroblast numbers. Fibroblasts are the major and nearly exclusive producers of extra-cellular matrix (ECM) proteins in airways, including collagens, fibronectin and proteoglycans. Increased collagen and fibronectin deposition has been found in NPs compared to NM. Fibroblasts also release enzymes and a variety of pro-inflammatory mediators, including growth factors, cytokines, and chemokines, such as IL-6 and IL-8, which contribute to the perpetuation of inflammatory and fibrotic processes in the airways.

Among NP patients, those with associated asthma, and especially those with aspirin-intolerant asthma, have increased fibroblast proliferation and ECM deposition, and increased fibroblast differentiation into myofibroblasts. As part of the various structural changes observed, fibrotic alterations have a great impact on the long-term outcome of NPs.

Anti-inflammatory treatment with intranasal glucocorticoids (GC) has been the cornerstone of NPs therapy. The main target of GC action is the inhibition of inflammatory cell activation and survival, although structural cells, including fibroblasts, are also targets of GC treatment. Some patients, however, do not respond, or respond poorly, to GC therapy, resulting in a need for repeated surgery to remove NPs.

GCs appear to be little effective in reducing the thickened basement membrane and airway-wall fibrosis. Indeed, long-term treatment with high doses of potent inhaled GCs is usually required in adults with mild asthma to reverse some of the changes resulting from remodelling. In vivo treatment of NPs with intranasal fluticaine propionate did not decrease collagen deposition.

In addition, contrasting effects of GCs have been reported on airway fibroblast proliferation, with some studies showing increased proliferation by GCs in fibroblasts from asthmatic patients, and others reporting decreased proliferation by GCs in NP fibroblasts. No comparative studies of the anti-proliferative potency of GCs in NM and NP fibroblasts have been reported to date.

The objective of the present study was to determine whether fibroblasts from inflamed nasal tissue, i.e. fibroblasts from patients with NPs and asthma who have a poor clinical response to GC therapy, are less sensitive to the inhibitory effects of in vitro GCs, compared to NM fibroblasts.

Methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Lonza and foetal bovine serum (FBS) from Biological Industries. Trypsin-EDTA, penicillin, streptomycin and HEPES were purchased from Invitrogen, and amphotericin B from Squibb. Dexamethasone (Fortecortin®) was obtained from MSD, and transforming growth factor (TGF)-β and DuoSet ELISAs from R&D. The cell proliferation kit II (XTT) was purchased from Roche Diagnostics, and the RNeasy Mini kit and RT-PCR reagents from Qiagen and Applied Biosystems, respectively.

Subjects

NPs tissues were obtained from 8 patients with nasal polyposis and asthma undergoing functional endoscopic sinus surgery (FESS). NM was obtained from the inferior turbinate of 8 subjects undergoing turbinectomy for turbinate hypertrophy with or without septoplasty for nasal septum deviation. All NP and NM patients included herein had been followed up in the Rhinology Unit & Smell Clinic, ENT Department of our Hospital for at least one year prior to surgery, and were assigned for surgery because they had not shown an improvement by GC treatment in nasal obstruction, rhinorrhea, and/or quality of life, as described elsewhere.

The diagnosis of severe nasal polyposis was based on criteria described in the EPPOS document, i.e., nasal symptoms, nasal endoscopic examination, and CT scan of paranasal sinuses, as reported elsewhere. The diagnosis of asthma was established on the basis of the clinical history and the demonstration of a reversible bronchial obstruction. Diagnosis of aspirin intolerance was made on the basis of a clear-cut history of asthma attacks precipitated by non-steroidal anti-inflammatory drugs (NSAIDs), and confirmed by aspirin nasal challenge in patients with an isolated episode of NSAID-induced asthma exacerbation. No subject from the NM group had a history of nasal or sinus disease (chronic rhinitis/rhinosinusitis). One of the NM subjects had been treated with intranasal GCs more than one month prior to surgery (Table 1). None of the patients had suffered from upper respiratory infection during at least the 2 weeks prior to surgery. All subjects agreed to participate in the study, which was approved by the Ethics Committee of our Institution.
Fibroblast culture

Fragments (approximately 3 × 3 mm) of the nasal tissues were placed in 6-well culture dishes in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 µg/ml amphotericin B, in a humidified atmosphere at 37 °C and 5% CO₂. Fibroblasts were isolated from the tissue fragments through adhesion and migration on the plastic surface. Once fibroblast cultures had reached 80% confluence, i.e. after a minimum of 3 weeks, the tissue fragments were removed and adhered cells were trypsinised (0.05% trypsin-0.02% EDTA) and sub-cultured in 150 cm² flasks. The purity of fibroblasts cultures was determined by immunocytochemistry of cytokeratin and vimentin. Briefly, fibroblasts cultured in 4-well culture slides were washed with PBS, fixed with 4% paraformaldehyde (15 min), permeabilised with 0.2% Triton X-100 (10 min), blocked with 1% bovine serum albumin-PBS (1 h), incubated first with primary antibodies (1 h, 37 °C) against vimentin (V5255, Sigma) or pan-cytokeratin (C2562, Sigma) and then with fluorescent secondary antibody (1 h), and stained (nuclei) with DAPI (1/10,000 dilution). Finally, slides were mounted using Prolong Gold antifade reagent (Invitrogen). The percentage of positive cells was quantified using fluorescence microscopy. After the second passage, 100% of both NM and NP cell cultures were cytokeratin-negative and vimentin-positive (Fig. 1). Fibroblast cultures were also tested for mycoplasma contamination using a PCR-based mycoplasma detection kit (VenorGeM, Minerva Biolabs). In all experiments, prior to drug incubation, cells were growth-arrested by incubation with serum-free medium for 18–24 h. Experiments were carried out between passages 4 and 8.

Cell proliferation

Fibroblasts were plated in 96-well culture plates (1 × 10³ cells/well). The following day, cells were growth-arrested as indicated above and incubated with cell medium supplemented with 0.5% FBS, in the absence or presence of dexamethasone at 10⁻¹¹ to 10⁻⁴M, for 3 and 5-days. The cell medium, with or without dexamethasone, was changed at day 3 in cells incubated with dexamethasone for 5-days. After treatment, cell proliferation was determined using the colorimetric Cell Proliferation Kit II (XTT). Absorbance was measured on a microplate spectrophotometer at 490 nm.

Collagen mRNA expression

Fibroblasts were plated in 6-well culture plates (10⁵ cells/well). Sub-confluent cell cultures were growth-arrested as indicated above, pre-incubated with dexamethasone (10⁻¹⁰M, 10⁻⁸M, and 10⁻⁶M) for 1 h, and then incubated with transforming growth factor (TGF)-β (5 ng/ml) for 24 h. Total RNA from cell cultures was extracted using the RNeasy Mini kit. One microgram of total RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit. The mRNA expression of collagens 1α1, 1α2, and 3α1 was analyzed by real-time PCR (7900HT Fast Real-Time PCR System, Applied Biosystems) using TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays (Assay ID’s: Hs00164004_m1, Hs00164099_m1, Hs00164103_m1, Applied Biosystems).

IL-6 and IL-8 production

Fibroblasts were plated in 24-well culture plates (3 × 10⁴ cells/well). Sub-confluent cell cultures were growth-arrested as indicated above, pre-incubated with dexamethasone (10⁻¹⁰M, Table 1 Demographic data and clinical characteristics of the study population.

<table>
<thead>
<tr>
<th></th>
<th>Nasal mucosa</th>
<th>Nasal polyps</th>
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<tbody>
<tr>
<td>Patients (n)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>3 (37.5)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Age, years (mean ± SEM)</td>
<td>39.4 ± 3.9</td>
<td>41.0 ± 4.0</td>
</tr>
<tr>
<td>Asthma, n (%)</td>
<td>0 (0)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Aspirin intolerance, n (%)</td>
<td>0 (0)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Intranasal glucocorticoids, n (%)</td>
<td>1 (12.5)</td>
<td>7 (87.5)</td>
</tr>
<tr>
<td>Skin prick test positivity, n (%)</td>
<td>1 (12.5)</td>
<td>3 (37.5)</td>
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Figure 1 Representative immunofluorescent staining image of control NM fibroblasts showing (A) presence of vimentin fibers (green fluorescence) in the cytoplasm of cultured cells, and (B) total absence of immunofluorescent staining for cytokeratins, confirming the absence of epithelial cells in the cultures. Original magnification ×200. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
10^{-8}M, and 10^{-6}M) for 1 h, and then stimulated with cell medium supplemented with 5% FBS. Cell culture supernatants were collected at 4 h post-stimulus. IL-6 and IL-8 production in supernatants were measured by ELISA. Cytokine production (pg/ml) was corrected by cell number using the XTT assay.

**Statistical analysis**

Data are expressed as median and 25th to 75th percentile. A non-parametric statistical analysis was performed using the Friedman test and Wilcoxon rank test for within-group comparisons, and the Kruskal–Wallis test and Mann–Whitney U test for between-group comparisons. Statistical significance was set at p < 0.05.

**Results**

**Effect of dexamethasone on fibroblast proliferation**

Dexamethasone treatment for 3-days provoked a dose-dependent inhibition of cell proliferation in fibroblasts from NM (p < 0.001) but not in fibroblasts from patients with NPs (Fig. 2A). The inhibitory effect of dexamethasone on the proliferation of NM fibroblasts was modest, thus causing a 25% inhibition of cell proliferation at the highest dexamethasone concentrations compared to control cells (Fig. 2A). As shown in Table 2, fibroblasts from NPs, and particularly those from aspirin-intolerant asthmatics (AIA), were more resistant to the anti-proliferative effect of dexamethasone. In common with the 3-day treatment, incubation of cells with dexamethasone for 2 additional days dose-dependently inhibited cell proliferation in fibroblasts from NM (p < 0.01) but not in NP fibroblasts (Fig. 2B). The inhibitory effect of dexamethasone on cell proliferation was not due to induction of cytotoxicity, as demonstrated by determining lactate dehydrogenase (LDH) activity in cell culture supernatants (data not shown).

**Effect of dexamethasone on collagen mRNA expression**

We then investigated the capacity of dexamethasone to reduce the fibrogenic function of nasal fibroblasts by measuring the effect of dexamethasone on the mRNA expression of collagen types 1α1, 1α2 and 3α1. To stimulate the mRNA expression of collagens, nasal fibroblasts were incubated with TGF-β. As shown in Fig. 3, TGF-β induced the mRNA expression of collagens 1α1 (Fig. 3A), 1α2 (Fig. 3B) and 3α1 (Fig. 3C) in fibroblasts from both NM and NPs. No significant differences in the extent of mRNA induction of the three collagen types by TGF-β were found between NM and NP fibroblasts, or between ATA and AIA NP fibroblasts (data not shown). Dexamethasone did not have any significant effect on the TGF-β-induced collagen mRNA expression either in NM or in NP fibroblasts (Fig. 3). Incubation of nasal fibroblasts with a much less potent fibrogenic stimulus, i.e. 5% FBS, did not significantly up-regulate the mRNA expression of any collagen type, and nor did dexamethasone decrease collagen mRNA expression of 5% FBS-treated fibroblasts (data not shown).

![Figure 2](image-url) **Figure 2** Effect of dexamethasone on fibroblast proliferation. Effect of (A) a 3-day and (B) a 5-day exposure with dexamethasone on the proliferation of NM (left graphs) and NP fibroblasts (right graphs) from either aspirin-tolerant (open triangles) or aspirin-intolerant (solid triangles) asthmatic patients. The median value is represented. *p < 0.05 compared to control (cells incubated with DMEM 0.5% FBS) by Wilcoxon test.
Effect of dexamethasone on IL-6 and IL-8 production

Because fibroblasts also locally release cytokines and chemokines such as IL-6 and IL-8 that contribute to the maintenance of inflammation and remodelling of the airways, we aimed to investigate the inhibitory effects of dexamethasone on IL-6 and IL-8 production in our nasal fibroblasts. As shown in Fig. 4, FBS treatment increased IL-6 (Fig. 4A) and IL-8 production (Fig. 4B) in fibroblasts from both NM and NPs compared to FBS-deprived cells. Dexamethasone dose-dependently decreased FBS-induced IL-6 and IL-8 production in fibroblasts from both NM and NPs. For both IL-6 and IL-8, NM fibroblasts appeared to be more sensitive to the inhibitory effect of dexamethasone than NP fibroblasts. Thus, a sub-maximal concentration of dexamethasone, i.e. \(10^{-8}\)M, inhibited IL-6 and IL-8 production in NM fibroblasts but not in NP fibroblasts (Fig. 4). A similar

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Comparison of dexamethasone inhibition of cell proliferation in the different fibroblast subtypes.</th>
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<tr>
<td>Dexamethasone concentration (log M)</td>
<td>(-8)</td>
</tr>
<tr>
<td>Nasal mucosa (NM)</td>
<td>81 (76–93)</td>
</tr>
<tr>
<td>Nasal polyops (NP)</td>
<td>102 (88–114)*</td>
</tr>
<tr>
<td>Aspirin-tolerant NP</td>
<td>90 (82–115)</td>
</tr>
<tr>
<td>Aspirin-intolerant NP</td>
<td>103 (102–114)*, NS</td>
</tr>
</tbody>
</table>

Results expressed as median and 25th–75th percentile of the percentage of proliferation compared to untreated cells (100%). * \(p<0.05\) compared to nasal mucosa and NS = not significant compared to aspirin-tolerant nasal polyps, at the corresponding dexamethasone concentration (Mann–Whitney U test).

Figure 3 Effect of dexamethasone on TGF-\(\beta\)-induced collagen mRNA expression. Fibroblasts were incubated with TGF-\(\beta\) and dexamethasone (DEX) for 24 h (see Methods) and the mRNA expression of collagens (A) 1\(\alpha\)1, (B) 1\(\alpha\)2, and (C) 3\(\alpha\)1 in NM (left graphs) and NP fibroblasts (right graphs) from either aspirin-tolerant (open triangles) or aspirin-intolerant (solid triangles) asthmatic patients was analyzed by real-time PCR. The median value is represented. * \(p<0.05\) and # \(p=0.058\) compared to untreated cells by Wilcoxon test.
sensitivity to dexamethasone-mediated inhibition of IL-6 and IL-8 was found between ATA and AIA NP fibroblasts (data not shown).

Discussion

Our study shows that dexamethasone exerts regulatory effects on fibroblast proliferation and the release of the pro-inflammatory cytokines IL-6 and IL-8. We also report that fibroblasts obtained from an inflamed and remodelled tissue (nasal polyp) are relatively insensitive to the inhibitory effects of dexamethasone, compared with those obtained from non-inflamed NM. To our knowledge this is the first study to compare the in vitro response of human NM and NP fibroblasts to GCs.

The anti-inflammatory and immuno-suppressive effects of GCs are partly mediated through the modulation of cell proliferation. We found that dexamethasone provoked a mild inhibition of cell proliferation in NM fibroblasts but did not significantly inhibit proliferation of fibroblasts derived from patients with NPs and asthma who had undergone surgery due to their poor response to GC therapy. This finding suggests that the presence of inflammation and remodelling with in the NP of these patients enhances resistance of nasal fibroblasts to GCs. Our findings concur with clinical observations of GCs having almost no effect on normal control fibroblasts. GCs have, however, proved to decrease proliferation induced by a variety of stimuli of fibroblasts from non-diseased peripheral lung tissue obtained from patients undergoing lung resection. Finally, GCs have been found to decrease as well as increase proliferation of human foetal lung fibroblasts.

One possible explanation to the different response to GCs between the nose and the bronchi could be ascribed to differences in the potency of the GC used. However, the same GC — dexamethasone — increased proliferation of bronchial fibroblasts from asthmatic patients, whereas, as we show here, it had no significant effect on the proliferation in NP compared to NM, might be initially explained by tissue/cell desensitisation due to the previous GC treatment of the NP patients. However, the long washout period included with the generation of the cultured fibroblast lines, which in total was at least of five weeks, together with the fact that previous studies report that in vivo GC therapy only provokes—if any21,22—a transient down regulation of the GC receptor23 would not favour this hypothesis.

In contrast to our results, two studies undertaken on NP fibroblasts show that GCs decrease proliferation.17,18 An increase in 3H-thymidine incorporation induced by dexamethasone has been reported in bronchial fibroblasts derived from mild-to-moderate asthmatics, compared with those obtained from non-asthmatic subjects.15,24 Surprisingly, dexamethasone decreased 3H-thymidine incorporation in fibroblasts from severe asthmatics.24 More recently, Fouty and co-workers16 reported that dexamethasone also increased proliferation (i.e., nearly doubled the number of cells in the S phase) of fibroblasts from patients with moderate asthma, whereas it had no effect on normal control fibroblasts. GCs have, however, proved to decrease proliferation induced by a variety of stimuli of fibroblasts from non-diseased peripheral lung tissue obtained from patients undergoing lung resection.25–27 Finally, GCs have been found to decrease as well as increase proliferation of human foetal lung fibroblasts.30

Figure 4  Effect of dexamethasone on IL-6 and IL-8 production. Fibroblasts were incubated with DMEM 5% FBS and dexamethasone (DEX) for 4 h (see Methods) and (A) IL-6 and (B) IL-8 release in cell culture supernatants from NM (left graphs) and NP fibroblasts (right graphs) from either aspirin-tolerant (open triangles) or aspirin-intolerant (solid triangles) asthmatic patients were measured by ELISA. The median value is represented. ∗p < 0.05 compared to untreated cells and †p < 0.05 compared to FBS-treated cells by Wilcoxon test.

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of fibroblasts from patients with NPs and asthma. Other methodological aspects, such as the period of analysis, the cell density or cell culture age at the time of analysis, the stimulus used to induce proliferation or the technique used to evaluate proliferation, could explain the different response between upper and lower airway fibroblasts. Finally, we cannot rule out the possibility that fibroblasts from the nose are phenotypically different, and therefore behave differently, from fibroblasts from the lower airways. Indeed, based on morphologic and functional characteristics of fibroblasts isolated from proximal airways and distal lung parenchyma, Kotaru and co-workers described at least two distinct phenotypes of fibroblast in the lung.

The involvement of airway fibroblasts in fibrogenic responses includes their capacity to produce ECM. We sought to examine the production of collagens and its regulation by GCs in nasal fibroblasts, which had not been previously investigated. We found that TGF-β, but not 5% FBS, significantly up-regulated the expression of collagens in both NM and NP fibroblasts. The increase induced by TGF-β is probably the result of the phenotypic transformation of fibroblasts to myofibroblasts, which are the major producers of ECM proteins. Dexamethasone did not exert any significant effect on TGF-β-induced collagen expression in fibroblasts from either NM or NPs, nor did it decrease collagen expression in fibroblasts incubated with a much less potent fibrogenic stimulus, i.e. 5% FBS. Our findings concur with a previous study showing that in vivo treatment of patients with NPs with topical GCs does not decrease collagen deposition. Our results also concur with in vivo studies showing no effect of GCs on collagen expression and synthesis induced by different stimuli in both primary lung fibroblasts and bronchial fibroblasts from normal and asthmatic subjects. Indeed, collagen synthesis and ECM deposition induced by incubation of fibroblasts with 5–10% foetal calf serum are increased by GC treatment.

Fibroblasts help regulate physiological functions, as well as inflammatory and immunological responses, by producing various growth factors and cytokines. Previous studies reported GCs’ ability to inhibit the release of various cytokines, including IL-6 and IL-8, by stimulated lung fibroblasts. We aimed to investigate whether there was a different sensitivity to dexamethasone inhibition of IL-6 and IL-8 in NM and NP fibroblasts. Dexamethasone dose-dependently decreased FBS-induced IL-6 and IL-8 production in both NM and NP fibroblasts. For both cytokines, however, NP fibroblasts appeared to be less sensitive to the inhibitory effect of a sub-maximal concentration of dexamethasone (10–8 M) compared to NM fibroblasts. The inhibitory effect of GCs on cytokine production is caused by various mechanisms, including repression of transcription factors such as NF-κB and AP-1, destabilization of the cytokine mRNA and transactivation of GC target genes such as mitogen-activated protein kinase phosphatase-1 and GC-induced leucine zipper.

In summary, our study shows that fibroblasts from patients with NPs and asthma who have a poor clinical response to GC treatment, display a relative resistance to the anti-proliferative effects of GCs, compared to fibroblasts from control nasal mucosa. In addition, GCs failed to inhibit collagen expression in any fibroblast type. In contrast, GCs effectively down-regulated IL-6 and IL-8 production by nasal fibroblasts, though NP fibroblasts were less sensitive to dexamethasone than NM fibroblasts. Overall, our findings reinforce the concept that GCs are less effective in reducing fibrosis and remodelling of the airways than in reducing inflammation. Our results also suggest that the impaired sensitivity of nasal polyp fibroblasts to the in vitro anti-proliferative and anti-inflammatory effects of glucocorticoids concurs in part with the poor clinical response that these nasal polypl patients show to glucocorticoid treatment.

Conflict of interest statement
None declared.

Ethics statement
All subjects included in the study agreed to participate in it. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona.

Acknowledgments
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