

Regression of polypoid nasal mucosa after systemic corticosteroid therapy: A proteomics study

Armin Farajzadeh Deroee, M.D.,^{1,3,4} Jasmin Oweinah,¹ Mohsen Naraghi, M.D.,^{3,4}
Werner Hosemann, M.D.,¹ Bahador Athari,^{3,4} Uwe Völker, Ph.D.,² and Christian Scharf, Ph.D.^{1,2}

ABSTRACT

Background: Nasal polyposis (NP) treatment and pathophysiology have always been challenging for otolaryngologists. Among the medical treatments for NP, glucocorticoids (GCs) have been always one of the mainstays but the mechanisms of their action are not well defined. Identifying and comparing the protein profile of chronic rhinosinusitis (CRS) with NP before and after treatment with GCs can help to clarify NP pathogenesis and mechanisms of corticosteroid effects on NP.

Methods: Samples of nasal polyps were taken from selected NP patients in workup for endoscopic sinus surgery. None of the patients used local or systemic GCs within 30 days before the sampling. The patients were given prednisone, 30 mg/day, for 4 days before the surgery to shrink the NP tissue. Nasal polyp samples were collected during surgery. Proteins from samples were extracted and separated by immobilized pH gradient–based two-dimensional difference gel electrophoresis (2D DIGE). Resulting 2D-gel images were statistically analyzed using Delta2D software and differently expressed protein spots were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Results: A reference map of 1963 proteins could be established. Expression of 20 proteins changed significantly (twofold, $p < 0.05$) after treatment of NP with GCs. Heat shock proteins and retinoic acid were induced by GCs. Ingenuity pathway analysis of all identified proteins indicated that apoptosis pathway is triggered in NP after GC administration.

Conclusion: Identifying the changes of NP proteome due to GCs and the biological functions of proteins can lead to new ways of treatment and open new fields in NP research.

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Two major variants of chronic rhinosinusitis (CRS) are CRS with nasal polyposis (NP) and CRS without NP.¹ NP tends to recur more often after surgery² and is more difficult to treat. During recent years much attention has been given to cellular events leading to NP.

Because glucocorticoids (GCs) have been always one of the mainstays of NP therapy, many genomics studies have been performed on their effects on the cytokines and peptides in NP: GCs reduce the levels of inflammatory agents and adhesion molecules that are needed for recruitment of inflammatory cells, mainly eosinophils, which play a pivotal role in NP pathogenesis.³ With GC administration, MHC II expression in NP is decreased and CD24 expression, which induces B-cell apoptosis, is increased. Corticosteroids induce production of anti-inflammatory mediators. Uteroglobin, IK-cytokine, transforming growth factor β (TGF β), and mammaglobin are anti-inflammatory genes that are induced by GCs. Peptides derived from uteroglobin are among the most potent anti-inflammatory agents identified.^{4–6} It was shown that expression of survivin enhanced polypogenesis.⁷ However, a drawback of genomic studies is that they are not able to provide the information regarding posttranscriptional events. Studying the proteins themselves can be a solution to overcome this shortcoming. “Proteomics” is the simultaneous analysis of complex protein mixtures such as cell lysates and tissue

extracts, and capable of recording quantitative changes in protein levels.⁸ Proteomics is also able to provide information on posttranscriptional changes and posttranslational modifications of proteins and determines the actual structures of the cells. Contemporary proteomic strategies allow assessment of protein modifications with high coverage and offer qualitative and quantitative analysis. Currently, a wide collection of gel-based proteomics or two-dimensional gel electrophoresis (2D GE) and nongel-based technologies are available for proteomic studies. Gel based technique, unlike nongel based method, enables assessment of posttranslational modifications at the protein level, but compared with the nongel-based one it identifies a narrower range of proteins⁹. Therefore, low abundant proteins like transcription factors and cytokines are not well covered by gel based techniques. However, this shortcoming can be compensated for because pathways induced by these regulatory factors can be evaluated.

One of the greatest advantages of proteomics comes from pharmaceutical research for faster new protein target identification in transformed cell lines or diseased tissues.⁸ Compared with genomic studies, 2D GE is probably more practical in evaluating the treatment effects of GCs in NP.

Based on the aforementioned facts, we present a 2D GE study to assess the changes in protein profile of NP before and after systemic treatment with GCs. To our knowledge, this is the first proteomics study about the effects of GCs on NP. Comparative analysis of the protein profile of polyposis and with that during the regression process, can lead to a better understanding of NP pathogenesis and possible mechanisms of GC effects on polyposis.

METHODS

All of the adult NP patients who came to a referral rhinology clinic within a 4-month period were evaluated to enter the study. Diagnosis of NP was given by nasal endoscopy. The NP grade had to be 3 or 4. The patients were candidates for endoscopic sinus surgery for the treatment of NP.

To minimize the confounding factors, the following exclusion criteria were set for the studied population:

From the ¹Department of Otorhinolaryngology and Head and Neck Surgery, and the ²Interfaculty Institute for Genetics, Functional Genomics Lab, Ernst-Moritz-Arndt-University, Greifswald, Germany, ³Rhinology Research Society, Tehran, Iran, and ⁴Otorhinolaryngology Research Center, Department of Otorhinolaryngology, Head and Neck Surgery, Tehran University of Medical Sciences, Tehran, Iran
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Address correspondence and reprint requests to Christian Scharf, Ph.D., Greifswald University Medical School, Department of Otorhinolaryngology–Head and Neck Surgery, Walther-Rathenau-Str. 43–45, D-17475 Greifswald, Germany
E-mail address: scharf@uni-greifswald.de
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Table 1 Nasal polyposis patients selected for the study

Case No.	Sex	Age (yr)	Nasal Polyposis Grade
1	M	29	4
2	M	31	4
3	F	38	4

1. History of smoking.
2. Asthma—excluded *via* history.
3. Aspirin intolerance—excluded *via* history.
4. Mucociliary system disorders—excluded *via* history.
5. Fungal rhinosinusitis—allergic fungal rhinosinusitis was excluded *via* skin-prick test, CT scan, and the mucosal visualization during surgery.
6. Immunodeficiency diseases—excluded *via* history.
7. Any acute rhinosinusitis within 30 days of the surgery.
8. Use of local or systemic corticosteroids within 30 days of the biopsy.

Among 46 NP patients, 4 cases were selected for the project initially but 3 entered the study (Table 1) because one of the patients was excluded after the surgery because of the positive microscopic and cultural mucosal results for fungi. All of the selected subjects were notified about the nature of the study and those who were willing to enter the study were given a written consent form. During the primary endoscopic evaluation and the biopsy, a small polyp was resected for proteomics study. The patients were instructed to take 30 mg of prednisone daily for 4 days before the surgery to shrink the NP tissue to improve the visual field and decrease bleeding during the surgery. On the day of surgery another small nasal polyp was harvested for the study. The samples were immediately frozen in liquid nitrogen and proteins were extracted from the tissue. Aliquots of cytosolic proteins of each sample were used for differential in gel electrophoresis (DIGE) and separated by 2D polyacrylamide gel electrophoresis (PAGE) in pI-ranges from 4 to 7 and 6 to 11.

Sample Preparation for Proteomics Analysis

For cell lysis, the weight of each polyp was determined and the frozen tissue was disrupted with a Bead Mill Homogenizer (B. Braun Biotech, Melsungen, Germany). Smashed frozen cell pieces were suspended in 200 μ L of sample buffer (8 mol/L of urea and 2 mol/L of thiourea) and cell debris was removed by centrifugation (20,000 $\times$ g, 60 minutes, 4°C). The supernatants were transferred into new tubes, and the protein concentrations were determined with a Bradford assay (Bio-Rad, Munich, Germany).

Two-Dimensional Difference Gel Electrophoresis

Labeling of samples for DIGE was performed according to the manufacturer's instructions (GE Health Care, Freiburg, Germany). Briefly, the required amount of protein was adjusted to pH 8.5 with 50 mmol/L of NaOH and samples were labeled with 400 pmol of dye per 50 μ g of protein for 30 minutes on ice. The reactions were stopped by adding 10 nmol of lysine followed by incubation on ice for 15 minutes. When rehydration was not performed immediately, the labeled samples were stored at -80°C . In all of the experiments the Cy2-dye was used for the labeling of an internal standard that was composed of aliquots of samples of all biopsy specimens included in the experiment. The Cy3 and Cy5 dyes were used for the labeling of samples of the individual patients. Samples were analyzed in quadruplicate for each biopsy before and after GC treatment.

Those four technical replicates were used for the implementation of a dye swap: labeling of two aliquots of each sample with Cy3 and

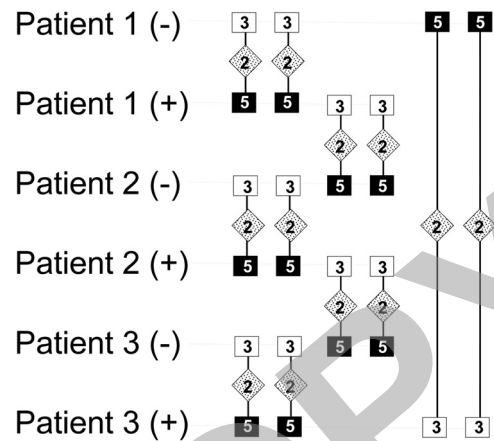


Figure 1. Two-dimensional difference gel electrophoresis (2D-DIGE) experimental design. Protein patterns of biopsy specimens of untreated (–) and corticoid-treated (+) nasal polyposis (NP) were compared using DIGE Technology (GE Healthcare). Cy2 (2) was used to label the internal standard, which was a protein mixture, and includes the proteins of all analyzed samples separated on each gel. Four technical replicates were made for each patient. Cy3 (3) and Cy5 (5) were used to label these replicates (two pairs each). This was done to exclude dye-specific artifacts during the statistical analysis. All gels then were compared *via* the internal standard on each gel labeled by Cy2.

Cy5, respectively. Variations in the intensity values between the Cy3- and Cy5-labeled technical replicates would indicate dye specificities that were subsequently excluded by the statistical tests used.

For the 2D GE, two labeled samples (Cy3 and Cy5, each 50 mg) and the corresponding internal standard (Cy2, 50 mg) were mixed and rehydrated on immobilized pH gradient strips (24 cm; GE Healthcare, Munich, Germany) with a pH range from 4 to 7. Following a standard protocol previously described by Thiele *et al.*,¹⁰ the immobilized pH gradient strips were subjected to isoelectric focusing. In the second dimension, proteins were separated on 12.5% SDS-polyacrylamide gels in low-fluorescent glass plates (GE Healthcare) with a Dodeca system (Bio-Rad). Resulting images of the three different gel channels were acquired with a laser scanner (Typhoon 9400; GE Healthcare) at the excitation/emission wavelength of 488/520 nm (Cy2), 532/670 nm (Cy3), and 633/670 nm (Cy5).

Statistical Analysis

After scanning, analysis of the 2D PAGE images was performed with software packages (Delta2D, Decodon GmbH, Greifswald, Germany; and Genespring, Agilent Technologies, Waldbronn, Germany). For identifying changes in intensity of protein spots affected by GCs, all images of the study were first matched *via* the corresponding Cy2-channel and then merged to generate a fused image with the union fuse option, which greatly improves comparison among all patients. Spot detection and spot editing were performed with the Delta2D software on the fused gel image. Subsequently, the spot map and the corresponding labels of the fused gel image were propagated to each of the gel images included in the analysis, thus ensuring uniform analysis throughout the study.

For graphical and statistical analysis of the obtained expression values, the data were transferred into the expression analysis software suite Genespring GX (Agilent Technologies). The intensity values were normalized by a two-step procedure. In the first step, intergel differences in the intensities were corrected by dividing the background-corrected values of the spot volumes by the median of all spots of the channel (Cy2, Cy3, or Cy5). Fluorescence intensities of each spot were then calibrated with the internal standard (Cy2) by

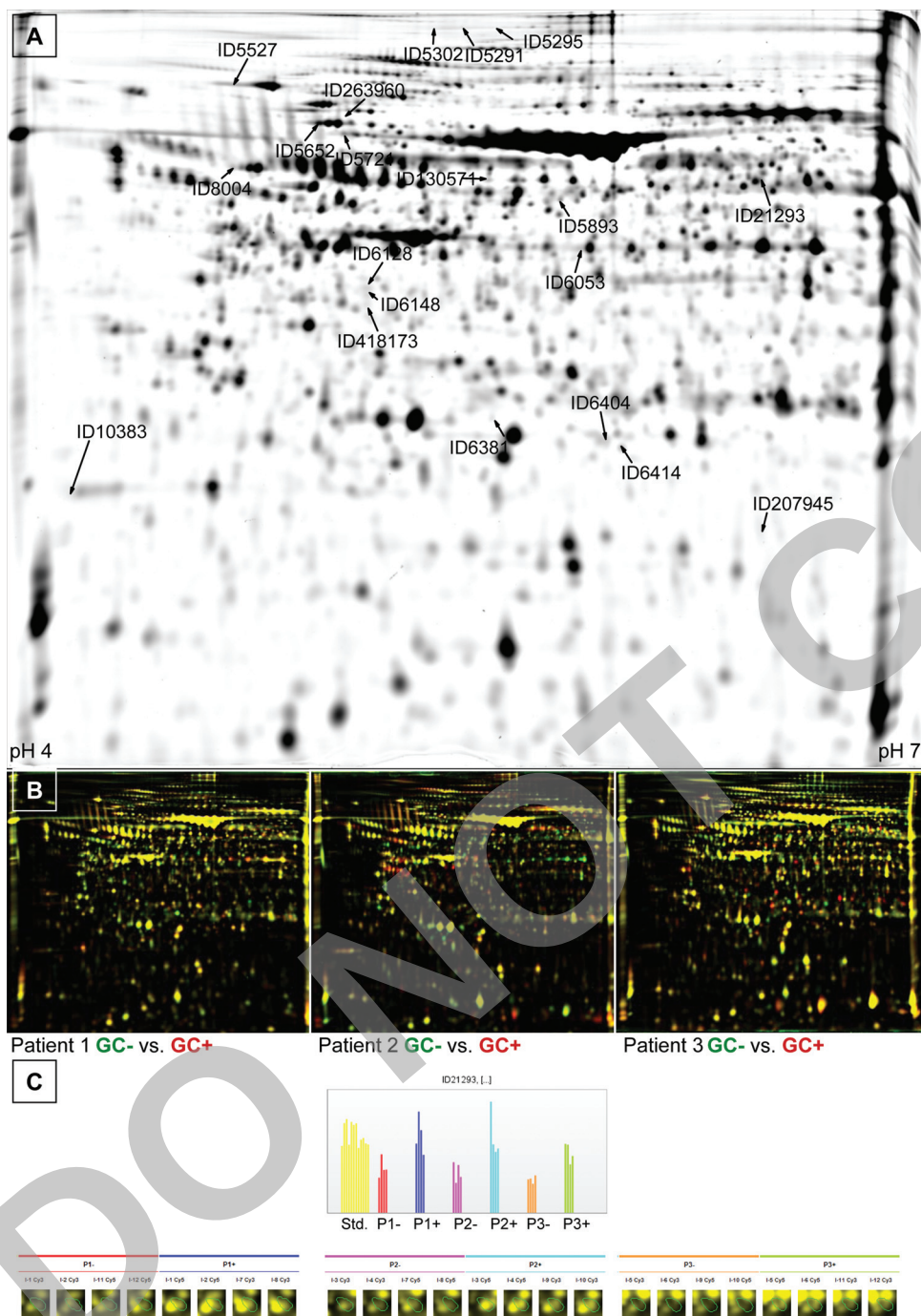


Figure 2. (A) Monitoring of changes in the protein pattern. Spots that display statistically significant changes between untreated and treated samples were labeled. (B) Dual channel overlay of difference gel electrophoresis (DIGE)-labeled gels of untreated (glucocorticoid [GC]-/green) and GC-treated (GC+/red) nasal polyposis (NP) for the three patients. Proteins in two states in similar level appear yellow, those present at higher level before the treatment are displayed in green, and proteins accumulating specifically after treatment appear red. (C) Graphical display of a selected example of a differentially expressed protein ID21293 (P00352), and corresponding dual channel overlays of all DIGE-labeled gels for spot ID21293. Expression of this spot is significantly increased in the GC-treated replicates.

dividing the median normalized spot volumes of the Cy3 and Cy5 channels by the corresponding value of the Cy2 channel. Corticoid-dependent changes in fluorescence intensities of the different protein spots were calculated by dividing the values of the treated nasal polyps by the corresponding baseline values of untreated nasal polyps (Fig. 1). We defined stringent selection criteria to minimize the number of false positive results and defined the change in the level of a protein spot as significant only when the following three criteria were fulfilled: (i) the change in the intensity ratio between the corticoid-treated and the untreated nasal polyps had to exceed 1.5; (ii) the *p* value of the corresponding analysis of variance test had to be lower than 0.05; and (iii) the raw volume values of the spot had to exceed 0.3, thus avoiding calculating ratios of spots close to background intensities.

Preparative 2D GE and Matrix-Assisted Laser Desorption—Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)

For preparative 2D GE, 600 μ g of protein was pooled from treated and untreated nasal polyps from each patient (300 μ g each) and added to the rehydration buffer. A 2D GE was performed as described previously. Preparative gels were stained with colloidal Coomassie brilliant blue according to the manufacturer's instructions (GE Healthcare). After scanning, analysis of the 2D GE images was performed also with the computer software Delta-2D (Decodon GmbH) in completion to Cy-dyes-labeled gels. Protein spots of interest were excised for further analysis. Identification of proteins by means of MALDI-TOF-MS was performed as previously described.¹¹

Table 2 The statistically significant changed proteins detected between nontreated (–) and glucocorticoid-treated (+) samples

Patient/Treatment (±)	1/–	1/+	2/–	2/+	3/–	3/+	p Value	Mean	SwissProt ID
	Normalized	Normalized	Normalized	Normalized	Normalized	Normalized			
Induced proteins									
ID207945	1	1.75	1	2.10	1	3.27	0.048	2.37	N/I
ID21293	1	1.68	1	2.37	1	1.73	0.046	1.93	P00352
ID10383	1	2.60	1	3.16	1	1.82	0.041	2.53	N/I
ID5721	1	1.22	1	2.81	1	2.50	0.036	2.18	N/I
ID8004	1	1.24	1	2.19	1	1.56	0.036	1.66	P07237
ID6381	1	3.32	1	33.24	1	4.62	0.034	13.73	P01834
ID6148	1	2.74	1	4.07	1	2.28	0.032	3.03	N/I
ID6414	1	2.16	1	2.70	1	1.25	0.032	2.04	N/I
ID5893	1	1.79	1	2.58	1	1.34	0.030	1.90	P61158
ID6053	1	1.53	1	1.32	1	1.53	0.020	1.46	P48594
ID6128	1	4.82	1	6.05	1	2.94	0.019	4.60	P05198
ID5652	1	1.70	1	1.75	1	2.13	0.018	1.86	P11021
ID5527	1	1.57	1	3.08	1	1.74	0.014	2.13	P14625
ID6404	1	1.81	1	2.60	1	1.58	0.013	2.00	Q99497
ID130571	1	2.48	1	7.12	1	3.87	0.011	4.49	P50990
ID418173	1	3.51	1	4.02	1	2.24	0.009	3.26	N/I
ID263960	1	2.07	1	2.75	1	2.22	0.009	2.35	P46940
Repressed proteins									
ID5302	1	0.30	1	0.40	1	0.44	0.035	0.38	N/I
ID5291	1	0.45	1	0.57	1	0.48	0.013	0.50	N/I
ID5295	1	0.46	1	0.59	1	0.40	0.002	0.48	N/I

Glucocorticoid-induced changes of different protein spots in fluorescence intensities were calculated by dividing the values of the treated nasal polyps by the corresponding baseline values (untreated nasal polyps).

N/I = not identified by mass spectrometry.

Table 3 The statistically significant changed proteins detected and identified in treated nasal polyposis samples

SwissProt ID	Mean	Protein Score*	Total Ion Score	Protein MW	Protein PI	Peptide Count	Function
P00352	1.9	385	271.5	54,826.9	6.30	19	Retinal dehydrogenase 1
P07237	1.7	714	432.64	57,080.7	4.76	34	Protein disulfide-isomerase precursor
P01834	13.7	260	227.88	11,601.7	5.58	4	Igκ chain C region
P61158	1.9	616	375.13	47,341.0	5.61	29	Actin-related protein 3
P48594	1.5	586	330.05	44,825.5	5.86	29	Serpin B4
P05198	4.6	143	53.24	36,089.4	5.02	15	Eukaryotic translation initiation factor 2 subunit 1
P11021	1.9	821	524.74	72,288.4	5.07	38	78-kDa glucose-regulated protein precursor
P14625	2.1	675	406.27	92,411.3	4.76	40	Endoplasmic precursor
Q99497	2.0	196	157.66	19,878.5	6.33	6	Protein DJ-1
P50990	4.5	452	185.87	59,582.5	5.42	34	T-complex protein 1 subunit θ
P46940	2.3	366	239.29	189,133.8	6.08	33	Ras GTPase-activating-like protein IQGAP1

MW = molecular weight; PI = isoelectric point.

*A protein score above 69 was considered significant ($p < 0.05$).

RESULTS

In this study, we analyzed the response of nasal polyps to GC treatment using DIGE Technology (GE Healthcare). The analysis included biopsies of three patients before and after systematic GC therapy—each biopsy specimen was analyzed in four independent technical replicates (Fig. 2). A 2D reference map of 1963 protein spots could be established. Twenty protein spots were found to be regulated (ANOVA tested, $p < 0.05$ and a factor of 1.5 compared with the corresponding untreated sample) in at least two of three patients (Table 2). Eleven of those 20 proteins were identified by mass spectrometry (MALDI-TOF-MS) in two independent analyses (Table 3). The identification revealed that these spots include both structural

and regulatory proteins. Ingenuity pathway analysis (IPA) was performed to monitor possible interactions between the identified proteins itself and regulatory elements.

DISCUSSION

This is the first 2D GE gel and proteomics study focusing on the protein profile changes of NP after receiving GCs. Because there are possibly confounding genetic and proteomic differences underlying NP and these may significantly affect the results, to minimize the bias in our study, we restricted the patients to a stringently selected group of NP patients who had no known conditions such as allergies, which could affect nasal mucosa. The differentially expressed proteins be-

fore and after treatment were categorized by functional aspects and, in the following, the most important changes of NP proteins from biological and pathological points of view are going to be discussed.

Heat shock proteins (HSPs) 70 and 90 were significantly induced after GC administration. General chaperones, which are typically HSPs, are abundant in the cytosol, endoplasmic reticulum, and mitochondria. Common features of chaperone action are prevention of aggregation and promotion of correct folding and assembly of proteins.¹² HSP70 protects the cell against proteotoxicity. Folding in the endoplasmic reticulum is subject to stringent quality control with high concentrations of chaperones such as HSP70 and HSP90.¹³ There are also complex systems for detecting and dealing with accumulation of unfolded or incorrectly processed proteins by the ubiquitin-proteasome system by autophagy or by initiating apoptosis.¹² It was interesting to see elevated levels of ubiquitin after GC treatment. GCs might also protect and reverse the pathological mucosal changes in NP by decreasing misfolding of proteins. Another possible role for HSPs is in apoptosis, which was revealed by IPA and is going to be discussed later.

The GC receptor (GR), a ligand-regulated transcription factor, binds GCs and regulates transcription of target genes after binding specific DNA sequences. GR is guided from the moment of its synthesis, through signal transduction and until its degradation by a variety of chaperones. Two main chaperones that influence GR assembly and activity are HSP70 and HSP90 systems.¹⁴ Increased levels of these chaperones after GC in our study suggest that GC may up-regulate NP tissue responsiveness to itself by inducing HSPs. Not all patients with nasal polyps respond to GCs. A possible explanation for this could be alterations in the relative expression of the two isoforms of the human GRs, GR α and GR β . Increased expression of GR β has been reported in patients with GC-resistant asthma, ulcerative colitis, and NP.¹⁵ This unresponsiveness may be caused by some dysfunctions in the HSP machine because effects of GCs are very much dependent on HSPs.

Protein disulfide-isomerase precursor was induced by GCs. The majority of disulfide-linked cytosolic proteins are thought to be enzymes that transiently form disulfide bonds while catalyzing oxidation-reduction (redox) processes. It was shown that reactive oxygen species can act as signaling molecules by promoting the formation of disulfide bonds within redox-sensitive proteins¹⁶ and since oxidative stress has been suggested as one of the mechanisms leading to NP,¹⁷ GCs seems to protect the cell from oxidative stress by increasing the expression of protein disulfide-isomerase precursor.

Retinoic acid (RA) has shown to be effective in the maintenance of the mucociliary phenotype of epithelial cells in the upper respiratory tract. It was indicated that RA stimulated ciliary regeneration, differentiation, and, in parallel, inhibited squamous cell differentiation.^{18,19}

Increased levels of actin and related proteins may be caused by the ciliary regeneration induced by GCs. However, besides a pivotal structural protein in the cilia, actin is also involved in many other cell activities. Thus, its increase may not be specifically related to ciliary regeneration alone.

To achieve a clearer picture of proteomic findings, we used IPA. This is a powerful curated database and analysis system for understanding how proteins work together to effect cellular changes.²⁰ The list of differentially expressed proteins was loaded into Ingenuity Pathway analysis and the initial results show that there is a close connection between TNF- α and some of the significantly changed proteins such as HSP90, IgG κ , and CASP3 after treatment of NP with GCs. These proteins are all regulators in the apoptosis pathways.^{21–23} Our findings about the effects of GCs on apoptosis are in accordance with the Li *et al.* study²⁴ describing the induction of the transcription factor AP-1 (especially c-Jun) after GC treatment. Previously, Zhou *et al.*²⁵ showed a c-Jun induction in the GC-evoked apoptotic pathway. In our gel-based analysis, transcription factors could not be addressed directly but we identified proteins that are affected by CASP3 (EIF-2

and DJ-1) and TNF- α (IGKC, IgM κ , HSP90, and HSP70), which are directly regulated by c-Jun. Because of the functions of these proteins, it seems that apoptosis is induced by GCs in NP. However, additional studies are mandatory to clarify apoptosis pathways.

Now that we have performed the first step of our study *via* the gel-based method, we are able to increase the number of detected proteins in future experiments by applying a gel-free (MS-based) technique.²⁶ This method is useful to detect proteins that can not be addressed by 2D gels such as low molecular weight proteins, protein complexes, low abundant proteins, and cell membrane-associated proteins.

CONCLUSION

We found significant structural and functional important changes of proteins caused by GCs in NP. HSPs, RA, and apoptosis induction may have a central role in the NP regression after GC treatment. Proteomics studies can enable us to discover that the pathways lead to NP pathogenesis and treatment. Additional studies are recommended to illuminate the field.

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