

Expression and regulation of osteopontin in chronic rhinosinusitis with nasal polyps

W.-L. Liu^{1,2,*}, H. Zhang^{1,*}, Y. Zheng^{3,*}, H.-T. Wang⁴, F.-H. Chen¹, L. Xu², Y. Wei¹, Y.-Q. Sun¹, J.-B. Shi¹ and H.-B. Li¹

¹Allergy and Cancer Center, Otorhinolaryngology Hospital, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, ²Department of Otolaryngology, Guangzhou Women and Children's Medical Center, Guangzhou, China, ³Department of Otolaryngology, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi, China and ⁴Department of Otolaryngology, PLA General Hospital, Beijing, China

Clinical & Experimental Allergy

Summary

Background Osteopontin (OPN) has been proposed to be associated with airway inflammation including asthma and chronic rhinosinusitis with nasal polyps (CRSwNP). This study sought to evaluate the expression and regulation of the OPN in CRSwNP patients.

Methods Nasal polyp (NP) tissues and normal tissues were collected from 30 CRSwNP patients and 16 control subjects. The expression and regulation of OPN, as well as eosinophil (EOS) accumulation and activation, were examined in nasal tissues using DNA microarray, immunohistochemical (IHC), immunofluorescent (IF) staining and qPCR analysis.

Moreover, the regulation of OPN in nasal epithelial cells and its effects on migration and activation of EOS were evaluated *in vitro* using flow cytometry, ELISA, qPCR and Western blot, etc.

Results DNA microarray analysis identified OPN as one of the 19 upregulated genes in polyp tissues. The mean number of OPN⁺ cells in polyp tissues was found to be significantly increased compared with the normal controls ($P < 0.01$), and OPN⁺ cells in polyp tissues significantly correlated with tissue eosinophilia [major basic protein (MBP)⁺ cells; $r = 0.51$, $P < 0.01$]. Accordingly, the mRNA and protein levels of OPN in NP tissues were significantly higher than those in normal controls ($P < 0.01$). Poly I:C, flagellin and TLR-9 agonist CpG ODN, as well as TNF- α , IFN- γ , IL-6, IL-17A and TGF- β , significantly increased OPN mRNA expression in cultured PECs and NECs ($P < 0.05$). Recombinant human OPN significantly promoted the migration of EOS, as well as enhanced EOS cationic protein (ECP) production, in an *in vitro* dispersed NP cells (DNPCs) culture system ($P < 0.05$).

Conclusions OPN promotes eosinophilic nasal inflammation in CRSwNP patients, which may represent a promising therapeutic target.

Keywords chronic rhinosinusitis, cytokine, eosinophil, epithelial cell, nasal polyps, osteopontin

Submitted 21 November 2013; revised 2 February 2014; accepted 25 March 2014

Correspondence:

Huabin Li, Allergy and Cancer Center, Otorhinolaryngology Hospital, The First Affiliated Hospital of Sun Yat-sen University, No. 58, Zhongshan 2nd Road, Guangzhou 510080, China.
E-mail: allergyli@163.com

Cite this as: W.-L. Liu, H. Zhang, Y. Zheng, H.-T. Wang, F.-H. Chen, L. Xu, Y. Wei, Y.-Q. Sun, J.-B. Shi and H.-B. Li, *Clinical & Experimental Allergy*, 2015 (45) 414–422.

Introduction

Chronic rhinosinusitis (CRS) is generally classified into two subtypes: CRS with nasal polyps (NP) (CRSwNP) and CRS without NP (CRSsNP) [1]. While CRSsNP has been demonstrated to be a predominantly neutrophilic inflammation, CRSwNP is characterized by Th2-skewed inflammation and excessive eosinophil (EOS) accumulation and a higher proportion of concomitant asthma

[2]. Although the pathogenic mechanisms underlying CRSwNP pathogenesis have been extensively investigated [3, 4], the key factor in triggering nasal eosinophilia and inflammation progression has not been fully understood.

Osteopontin (OPN), also known as early T lymphocyte activation 1, has originally been discovered in bone as an extracellular matrix protein [5–7]. OPN was a phosphorylated acidic glycoprotein and was later identified in many cell types in the immune system [8]. OPN is extensively produced by most cells of the immune

*These authors contributed equally to the study.

system and may be involved in the pathogenesis of inflammatory and immune responses including tissue fibrosis, tumour progression and immune regulation [9, 10]. OPN contains a variety of different functional motifs to engage different integrin receptors ($\alpha\text{v}\beta 1$, $\alpha\text{v}\beta 3$ and CD44, etc.), which in part explains its complex biological activities. Traditionally, it has been thought to be a Th1-associated cytokine involved in a wide variety of human inflammatory diseases (e.g. idiopathic pulmonary fibrosis, autoimmune uveitis, multiple sclerosis, etc.) [11, 12]. However, recent emerging evidences support an active role for OPN in the pathogenesis of Th2-linked airway allergic diseases [13]. Although previous studies have shown that OPN was significantly upregulated in both CRSsNP and CRSwNP patients [14, 15], the importance of OPN in the CRSwNP pathogenesis has not been well characterized.

This study was undertaken to determine the expression level and distribution pattern of OPN in CRSwNP patients using DNA microarray, real-time quantitative polymerase chain reaction (qPCR), enzyme-linked immunosorbent assay (ELISA), immunohistochemistic (IHC) staining and double immunofluorescent (IF) staining. Moreover, we evaluated the possible effects of environmental stimulators on OPN mRNA expression in cultured normal nasal epithelial cells (NECs) and polyp epithelial cells (PECs) *in vitro* and to evaluate the possible effects of recombinant human OPN on EOS chemotaxis and activation (EOS cationic protein [ECP] level) in cultured dispersed NP cells (DNPCs) *in vitro* using qPCR, flow cytometry, chemotaxis assay and ELISA.

Materials and methods

Patients and tissue samples

Adult CRSwNP patients ($n = 30$) were recruited from the First Affiliated Hospital of Sun Yat-sen University in Guangzhou. The diagnosis of CRSwNP was made according to the current European Position Paper on rhinosinusitis and nasal polyps [1]. All CRSwNP patients had undergone an unsuccessful standardized course of medical therapy and consented for tissue collection at the time of surgery. During endoscopic surgery, the polyp tissues were sampled. As normal controls, 16 patients undergoing septoplasty due to anatomical variations were enrolled, and the tissues from the uncinate process were sampled during septal surgery. The demographic data of all subjects enrolled in this study are listed in Table 1. This study was approved by the local ethics committee boards, and written informed consent was obtained from each subject.

Tissue samples were divided into three portions; the first portion was frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation, the second

Table 1. Subjects' characteristics

	Normal controls	CRSwNP patients
Characteristics		
Subject no.	$n = 16$	$n = 30$
Gender (M/F)	10/6	19/11
Age (range)	22–31	25–54
Atopy	NA	$n = 7$
Asthma history	NA	$n = 11$
Sinus surgery history	NA	$n = 9$
Methodologies used		
DNA microarray	$n = 2$ (2 M)	$n = 2$ (1 M)
IHC	$n = 12$ (8 M)	$n = 28$ (18 M) (NE, 12; E, 16)
qPCR	$n = 12$ (8 M)	$n = 28$ (18 M) (NE, 12; E, 16)
ELISA	$n = 12$ (8 M)	$n = 28$ (18 M) (NE, 12; E, 16)
IF	$n = 4$ (3 M)	$n = 4$ (2 M)
Epithelial cell culture	$n = 6$ (4 M)	$n = 6$ (3 M) (NE, 2; E, 4)
<i>In vitro</i> EOS chemotaxis assay		$n = 6$ (4 M) (E, 6)
<i>In vitro</i> EOS activation assay		$n = 10$ (6 M) (E, 10)

E, eosinophilic; NE, non-eosinophilic.

portion was used for protein isolation, and the third portion was fixed overnight in a freshly prepared fixative containing 4% paraformaldehyde in PBS (pH 7.4) and was embedded in paraffin wax for histological staining. In addition, a portion of fresh polyp and normal tissues was further used for isolation and culture of NECs and PECs and EOS chemotaxis and activation assay in DNPCs *in vitro* (Table 1).

DNA microarray

The expression profiles of OPN and other genes were detected using a commercially available cDNA array system (extracellular matrix and adhesion molecules microarray; SuperArray Bioscience Corp., Frederick, MD, USA) according to the manufacturer's instructions. See Data S1 for further details.

IHC staining

Paraffin sections (4 μm) were used, and IHC staining was performed by peroxidase-labelled streptavidin-biotin technique as we described elsewhere [16]. Briefly, 4- μm -thick sections were deparaffinized by serial treatment. After blocking the endogenous peroxidase in 3% hydrogen peroxide and with 1% bovine serum albumin, the sections were incubated overnight at 4°C in the presence of primary antibodies (rabbit anti-OPN antibody, Thermo Fisher Scientific, Fremont, CA, USA, 1 : 50; mouse anti-MBP antibody, 1 : 50, AbD Serotec,

Oxford, UK). Thereafter, each section was incubated with goat anti-rabbit or rabbit anti-mouse secondary antibody and then with horseradish peroxidase-labelled streptavidin complex (Zhongshanjinqiao, Beijing, China). Distribution of peroxidase was revealed by incubating the sections in a solution containing 3% 3,3-diaminobenzidine tetrahydrochloride before being counterstained with haematoxylin and coverslipped. Negative control studies were performed by replacing the primary antibodies with normal IgG in appropriate concentrations.

The sections were blindly examined and coded with no awareness of the clinical data with an Olympus CX40 Microscope (Olympus Optical Co, Hamburg, Germany). The number of positive cells (brown cells) was counted in 10 high-power fields (HPFs, 400 $\times$) and averaged. The eosinophilic and non-eosinophilic CRSwNP patients were identified according to the criteria we previously established [16]. Briefly, the cut-off value for the eosinophilic and non-eosinophilic CRSwNP was set at 8 MBP⁺ cells/HPF; thus, polyp with < 8 MBP⁺ cells/HPF was defined as non-eosinophilic CRSwNP.

qPCR

The mRNA expression level of OPN was detected by real-time qPCR analysis as we described elsewhere [17]. Briefly, total RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription was performed, and the cDNA was synthesized from 2 μ g of total RNA using an oligo (dT) 18 primer and M-MLV reverse transcriptase (TAKARA, Syuzou, Shiga, Japan) for quantitative PCR. RNA integrity and the success of the reverse transcription reaction were monitored by PCR amplification of β -actin transcripts. Expression of mRNA was determined using ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix TaqTM (TAKARA). The primer sequences were as follows: OPN forward, 5'-TTG CAG TGA TTT GCT TTT GC-3'; OPN reverse, 5'-AGA TGG GTC AGG GTT TAG CC-3'; β -actin forward, 5'-AAG ATG ACC CAG ATC ATG TTT GAG ACC-3'; and β -actin reverse, 5'-AGC CAG GTC CAG ACG CAG GAT-3'. PRISM samples contained 1 $\times$ SYBR Green Master Mix, 1.5 μ L 5 μ M primers and 25 ng synthesized cDNA in a 25- μ L volume. Reactions were heated to 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing extension at 60°C for 60 s. Melting curve analysis was used to control for amplification specificity. The mean threshold cycle (Ct) values were normalized to β -actin, and the relative mRNA levels of target genes were analysed with the $2^{-\Delta\Delta C_t}$. Experiments were performed in triplicate for each data point.

Double IF staining, flow cytometric and Western blot analysis and ELISA

The co-localization of OPN and inflammatory cells (EOS and mast cells) in polyp tissues was examined by double IF staining. The OPN protein expression was detected by flow cytometric analysis; the protein levels of phosphoinositide 3-kinase (PI3K) and phosphorylated PI3K (pPI3K) were detected by Western blot analysis. The levels of OPN in the nasal tissue extracts and supernatants of cultured DNPCs were measured by ELISA. See Data S1 for further details.

In vitro cell culture and stimulation

The detailed methods for culture and stimulation of HNEs and PECs, as well as DNPCs isolation and *in vitro* EOS chemotaxis and activation assay, are provided in the Data S1.

Statistical analysis

Data were expressed as the medians and interquartile ranges (IQRs) and analysed using the Kruskal–Wallis *H* test and nonparametric Mann–Whitney *U*-test except for additional note. For the *in vitro* assays, the data were expressed as the mean and standard errors of the mean (SEM) of three independent experiments; 1-way analysis of variance (ANOVA) and paired or unpaired Student's *t*-test were used for the statistical analysis. A *P* value < 0.05 was considered as statistically significant.

Results

Expression and cellular sources of OPN in CRSwNP patients

Using DNA microarray analysis, we identified 19 upregulated genes and eight downregulated genes in NP tissues (Fig. 1). Of them, OPN was increased for more than sixfolds in NP tissues. Consistently, the mean number of OPN⁺ cells in polyp tissues was found to be significantly increased compared with the normal controls (*P* < 0.01), and OPN⁺ cells in polyp tissues significantly correlated with tissue eosinophilia (MBP⁺ cells; *r* = 0.51, *P* < 0.01; Figs 2a–e), indicating OPN expression may be associated with tissue eosinophilia in CRSwNP patients. Accordingly, the mRNA and protein levels of OPN in NP tissues were significantly higher than those in normal controls (*P* < 0.01; Figs 3a and c). Furthermore, when subdivided the CRSwNP patients into eosinophilic (16/28) and non-eosinophilic subsets (12/28) according to the criteria we previously established [16], we found the mRNA and protein levels of

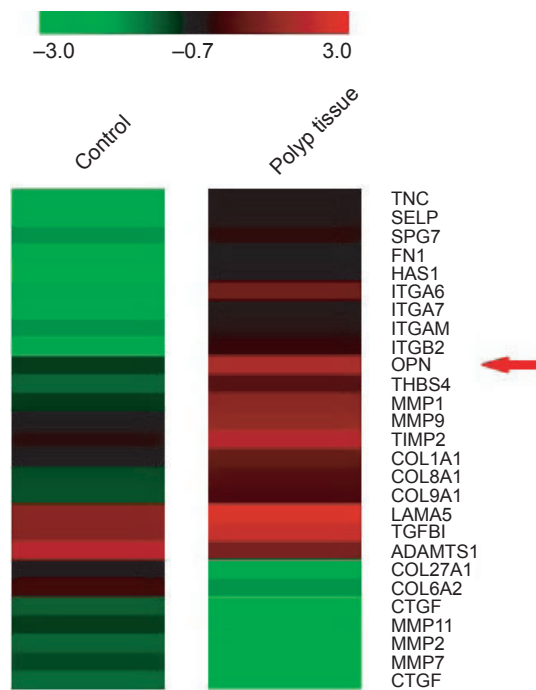


Fig. 1. DNA microarray analysis of NP tissues. Shown is the heatmap of genes upregulated or downregulated in NP tissues. Healthy uncinuate process was as normal control.

OPN in eosinophilic NP tissues were significantly higher than those in non-eosinophilic NP tissues ($P < 0.01$; Figs 3b and d). By examining the IHC results, we found OPN was highly expressed by both epithelial cells and subepithelial inflammatory cells in NP tissues (average epithelial OPN⁺ cells, 43.7/HPF; average subepithelial OPN⁺ cells, 45.9/HPF), suggesting the nasal epithelial cells were one of the main cellular sources of OPN production in CRSwNP patients. Next, we examined the distribution of OPN in subepithelial inflammatory cells using double IF staining. As a result, we found that OPN was expressed by cells positive for either MBP or tryptase (Fig. 2f), suggesting both EOS and mast cells in lamina propria of NP tissues were able to produce OPN.

Regulation of OPN in cultured PECs and NECs

Based on the finding that epithelial cells were the main cellular sources of OPN in polyp tissues as well, we thus examined the expression and regulation of OPN mRNA *in vitro*. Consequently, we found TLR-3 agonist Poly I:C, TLR-5 agonist flagellin and TLR-9 agonist CpG ODN were able to significantly increase OPN mRNA and expression in cultured PECs and NECs ($P < 0.05$; Fig. 4). Of them, Poly I:C was found to be the most prominent stimulus for OPN mRNA expression (6.4 folds in PECs and 5.1 folds in NECs after incubation for 6 h). After 0–12 h incubation of Poly I:C, OPN mRNA

levels in cultured PECs and NECs were found to be upregulated in a time-dependent manner ($P < 0.05$). When primary PECs and NECs were stimulated with proinflammatory cytokines (TNF- α , IFN- γ , IL-6, IL-17A, IL-4, TGF- β and IL-10), we found TNF- α , IFN- γ , IL-6, IL-17A and TGF- β were able to significantly increase OPN mRNA expression in cultured PECs and NECs ($P < 0.05$). Moreover, an additive effect of these cytokines (TNF- α , IFN- γ , IL-6, IL-17A and TGF- β) and Poly I:C on OPN mRNA expression in cultured PECs and NECs was observed ($P < 0.05$). In contrast, IL-4 and IL-10 were found to significantly inhibit TLR-3-mediated OPN mRNA overexpression in cultured PECs and NECs ($P < 0.05$). To further validate the regulation of TLR ligands and cytokines on OPN expression in cultured epithelial cells, we then examined the intercellular OPN expression by flow cytometric analysis. As shown in Fig. 5, in agreement with the aforementioned results, we found Poly I:C, flagellin and CpG ODN, as well as TNF- α , IFN- γ , IL-6, IL-17A and TGF- β , were able to significantly increase intercellular OPN expression in cultured PECs ($P < 0.05$).

Recombinant human OPN induced chemotaxis and activation of EOS *in vitro*

As OPN has been proposed to promote chemotaxis and activation of EOS in airway disease, we then examined the effects of recombinant human OPN on EOS chemotaxis *in vitro*. As shown in Fig. 6, we found OPN, as well as eotaxin, was able to significantly promote the migration of EOS from cultured DNPCs in an *in vitro* transwell system ($P < 0.05$). In contrast, the chemotaxis of EOS was significantly inhibited after adding anti-OPN or anti- $\alpha\text{v}\beta 3$ antibody ($P < 0.05$). By adding different concentrations of recombinant human OPN (0.1–10 $\mu\text{g/mL}$) into the transwell system, EOS migration was observed to be significantly enhanced ($P < 0.05$), indicating a dose-dependent manner. Next, we examined the effects of recombinant human OPN on EOS activation *in vitro*. By adding different concentrations of recombinant human OPN (0.1–10 $\mu\text{g/mL}$) into cultured DNPCs, we found recombinant OPN significantly enhanced ECP production in a dose- and time-dependent manner (Figs 6f and g; $P < 0.05$). To further characterize the molecular mechanisms underlying ECP production by EOS, we next examined the activation of PI3K signalling pathway after OPN incubation. Consequently, we found PI3K signalling pathway was significantly activated in cultured DNPCs in a dose- and time-dependent manner ($P < 0.05$). In contrast, the regulatory effect of OPN on ECP production was significantly inhibited in the presence of anti-OPN or anti- $\alpha\text{v}\beta 3$ antibody, or PI3K-specific inhibitor LY204002 ($P < 0.05$). These findings together suggested OPN

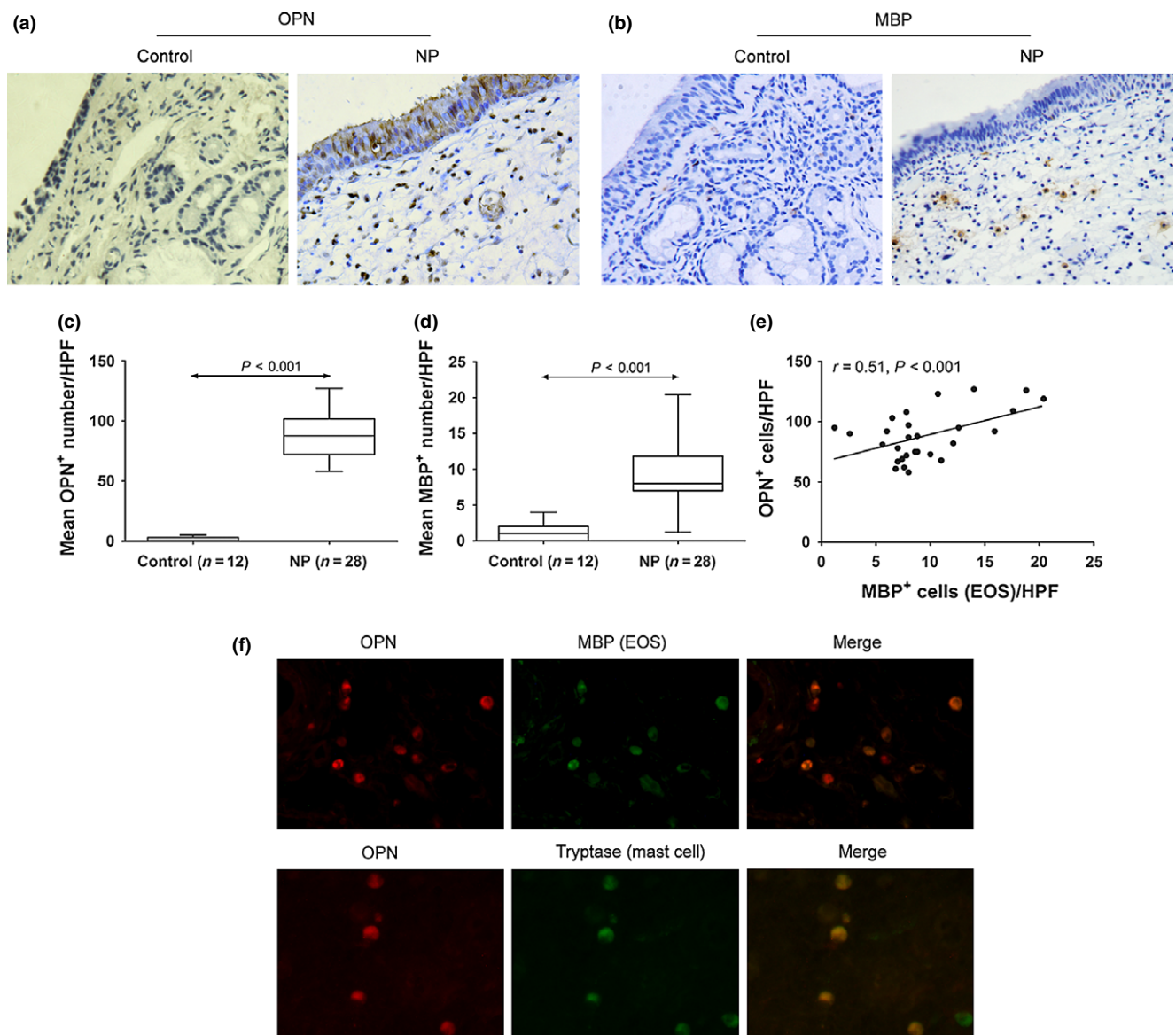


Fig. 2. The expression and cellular sources of OPN in NP tissues and normal controls. Representative IHC stainings of OPN (a) and MBP (b) in NP tissues, and normal control was shown (magnification, 200 $\times$); (c and d) The mean number of OPN-positive cells and MBP-positive cells in NP tissues and normal control; (e) the correlation of OPN-positive cells and MBP-positive cells in NP tissues; (f) representative double IF staining of OPN (red) and MBP (for EOS, green), as well as OPN (red) and tryptase (for mast cell, green), in NP tissues (magnification, 400 $\times$). Data are expressed as median (IQRs).

exerted its pro-inflammatory effect through a $\alpha v\beta 3$ and PI3K-mediated pathway.

Discussion

In the present study, we showed the expression of OPN was significantly increased in CRSwNP patients and established a clear relationship between OPN expression and EOS accumulation in NP tissues of CRSwNP patients. Moreover, we provided the first evidence that recombinant OPN was able to promote EOS migration and activation *in vitro*. These findings suggested OPN

may be regarded as a promising therapeutic target for the management of CRSwNP patients.

Osteopontin as a multifunctional protein has been investigated in allergy and asthma only recently. Several studies have also shown increased OPN expression in both CRSwNP and CRSsNP patients [14, 15], which appeared to be involved in mucosa remodelling that seen in nasal inflammatory tissues. In Lu *et al.*'s study [14], they found that mRNA and protein expression of OPN was significantly upregulated in tissues from CRS patients compared with control tissues, and CRSwNP patients showed significantly stronger mRNA and

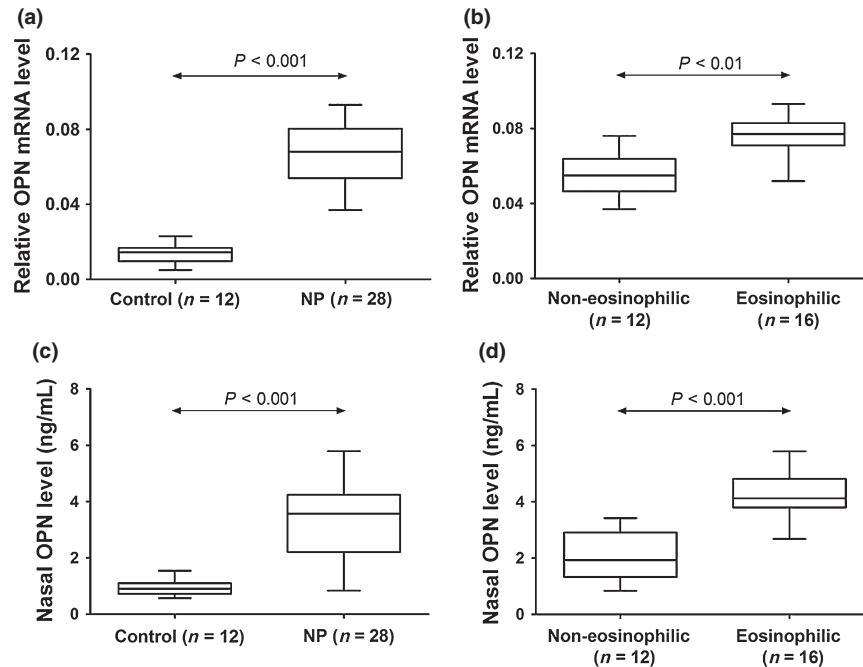


Fig. 3. The mRNA and protein expression of OPN in polyp tissues and normal control. (a) The OPN mRNA level in NP tissues and normal control; (b) The OPN mRNA level in eosinophilic and non-eosinophilic NP tissues; (c) The OPN protein level in NP tissues and normal control; (d) The OPN protein level in eosinophilic and non-eosinophilic NP tissues. Data are expressed as median (IQRs).

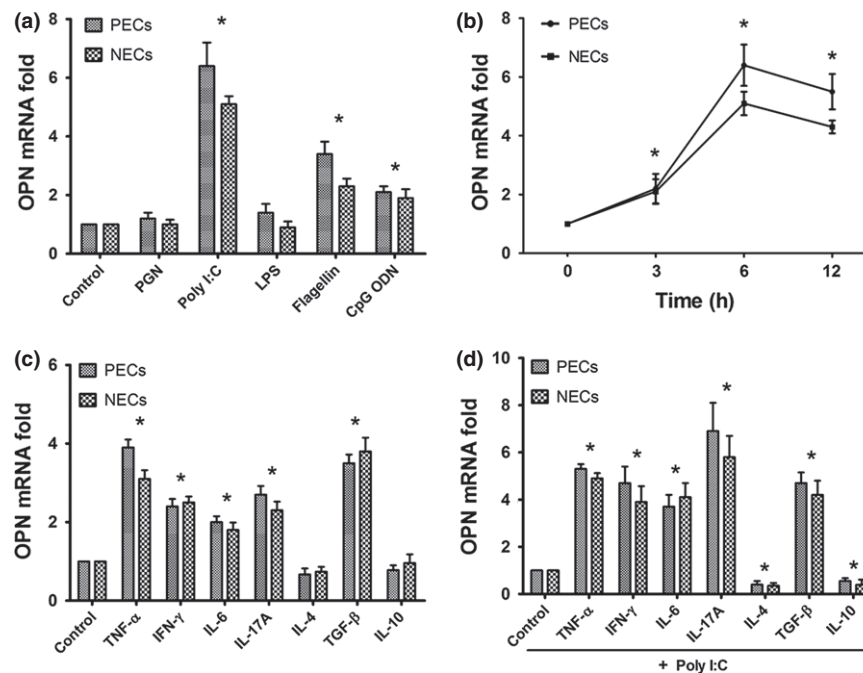


Fig. 4. The regulation of OPN mRNA expression in cultured epithelial cells. (a) The expression of OPN mRNA in PECs and NECs was cultured for 6 h in the presence of PGN (Pam₃CSK₄, TLR-2 agonist), Poly I:C (TLR-3 agonist), LPS (TLR-4 agonist), flagellin (TLR-5 agonist) and CpG ODN (TLR-9 agonist); (b) Poly I:C induced OPN mRNA expression in PECs in a time-dependent manner; (c) the expression of OPN mRNA in PECs cultured for 6 h in the presence proinflammatory cytokines (TNF- α , IFN- γ , IL-6, IL-17A, IL-4, TGF- β and IL-10); (d) the additive effect of cytokines (TNF- α , IFN- γ , IL-6, IL-17A and TGF- β) and Poly I:C on OPN mRNA expression in cultured PECs and NECs. * $P < 0.05$ compared with the unstimulated cells. The data indicated the means (SEM) of three independent experiments.

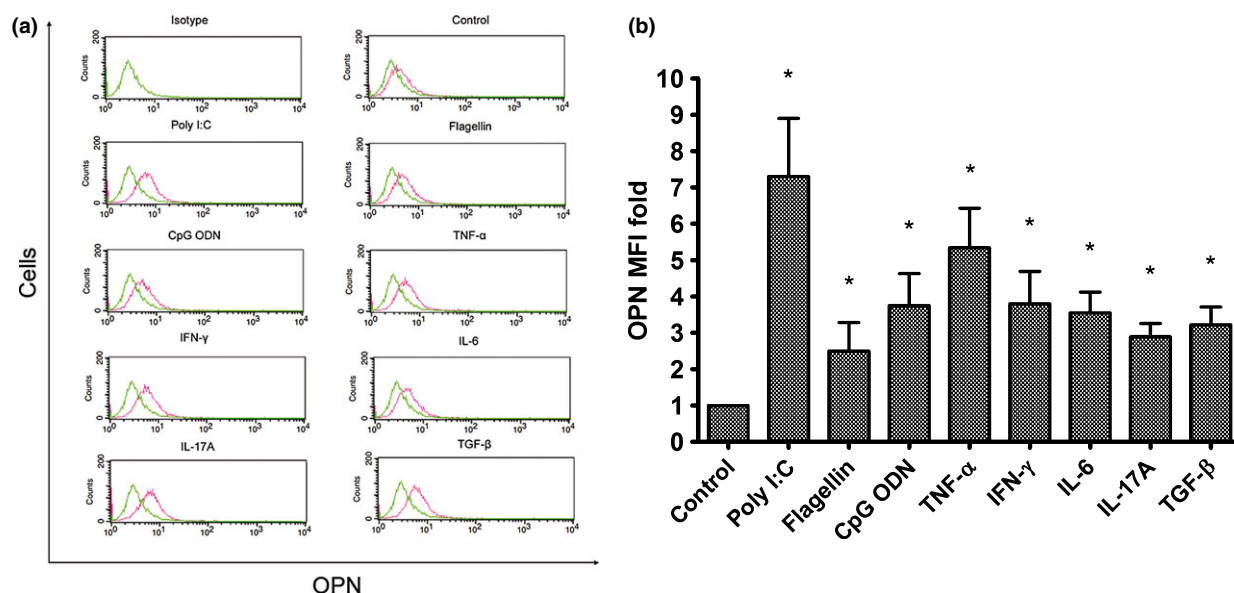


Fig. 5. The intercellular OPN expression in stimulated PECs. (a) Representative flow cytometric results of intercellular OPN expression in stimulated PECs; (b) The fold changes in mean OPN MFI in stimulated PECs. The data indicated the means (SEM) of three independent experiments. * $P < 0.05$.

protein expression in sinonasal mucosa than CRSsNP patients did, suggesting elevated OPN was more specific to Th2-dominant CRSwNP. However, the tissue OPN distribution and OPN's regulation on EOS in CRSwNP has largely been unknown. In this study, we showed the mean number of OPN⁺ cells, OPN mRNA and protein levels were significantly increased in polyp tissues, which was quite in agreement with the previous reports [14, 15]. Interestingly, we further found OPN⁺ cells in polyp tissues significantly correlated with tissue eosinophilia (MBP⁺ cells), indicating OPN expression may be associated with tissue eosinophilia in CRSwNP patients. When subdivided the CRSwNP patients into eosinophilic and non-eosinophilic subsets, we found the mRNA and protein levels of OPN in eosinophilic NP tissues were significantly higher than those in non-eosinophilic NP tissues. In Lu et al.'s study, they found most EOS in lamina propria in NP tissues were OPN⁺ cells by employing OPN immunohistochemical staining and subsequent MGG staining. In this study, we examined the co-location of OPN and MBP in polyp tissues by double immunofluorescence staining. Our results showed most EOSs, as well as mast cells, were OPN-positive cells, suggesting a self-amplified regulatory loop of EOS in NP tissues by producing OPN.

Currently, the regulation of OPN in polyp tissues remains largely unclear. In this study, we identified epithelial cells, in addition to inflammatory cells, as one of the main cellular sources of OPN in NP tissues. Considering epithelial cells constitute the first line against environmental stimulus [18], it is notable to examine

the OPN regulation in response to different inflammatory conditions. As expected, we found TLR-3, 5 and 9 agonists were able to significantly increase OPN mRNA expression in cultured PECs and NECs. TLR-3 agonist Poly I:C was a common virus mimic used for *in vitro* experiment [19]. As it was found to be the most prominent stimulator for OPN mRNA expression, we concluded airway virus infection may be essential for induction of OPN expression. On the other hand, proinflammatory cells such as TNF- α , IFN- γ , IL-6, IL-17A and TGF- β synergistically increased TLR-3-mediated OPN production in epithelial cells *in vitro*. Interestingly, no significant effect of IL-4 was observed on OPN expression in airway epithelial cells in this study. Although the inhibitory effects of Th2 cytokines (IL-4 and IL-13) on OPN levels have previously been proposed [14, 20], we only observed significant inhibitory effect of IL-4 on OPN expression in Poly I:C-conditioned epithelial cells. As OPN has been recognized as a T-bet-dependent pro-inflammatory cytokine that is produced by activated Th1 cells and other cells, which may account for antimicrobial response, thus the inhibitory effect of IL-4 on OPN levels may reflect a reciprocal inhibition of Th1/Th2 paradigm in the interface of adaptive immune response.

CRSwNP has suggested to be a Th2-skewed, eosinophilic mucosal inflammation. EOS has traditionally been considered as the primary mediators of epithelial damage [21, 22]. It mediates epithelial damage via the release of preformed effector molecules, such as MBP and ECP. In doing so, EOS plays a critical role in the

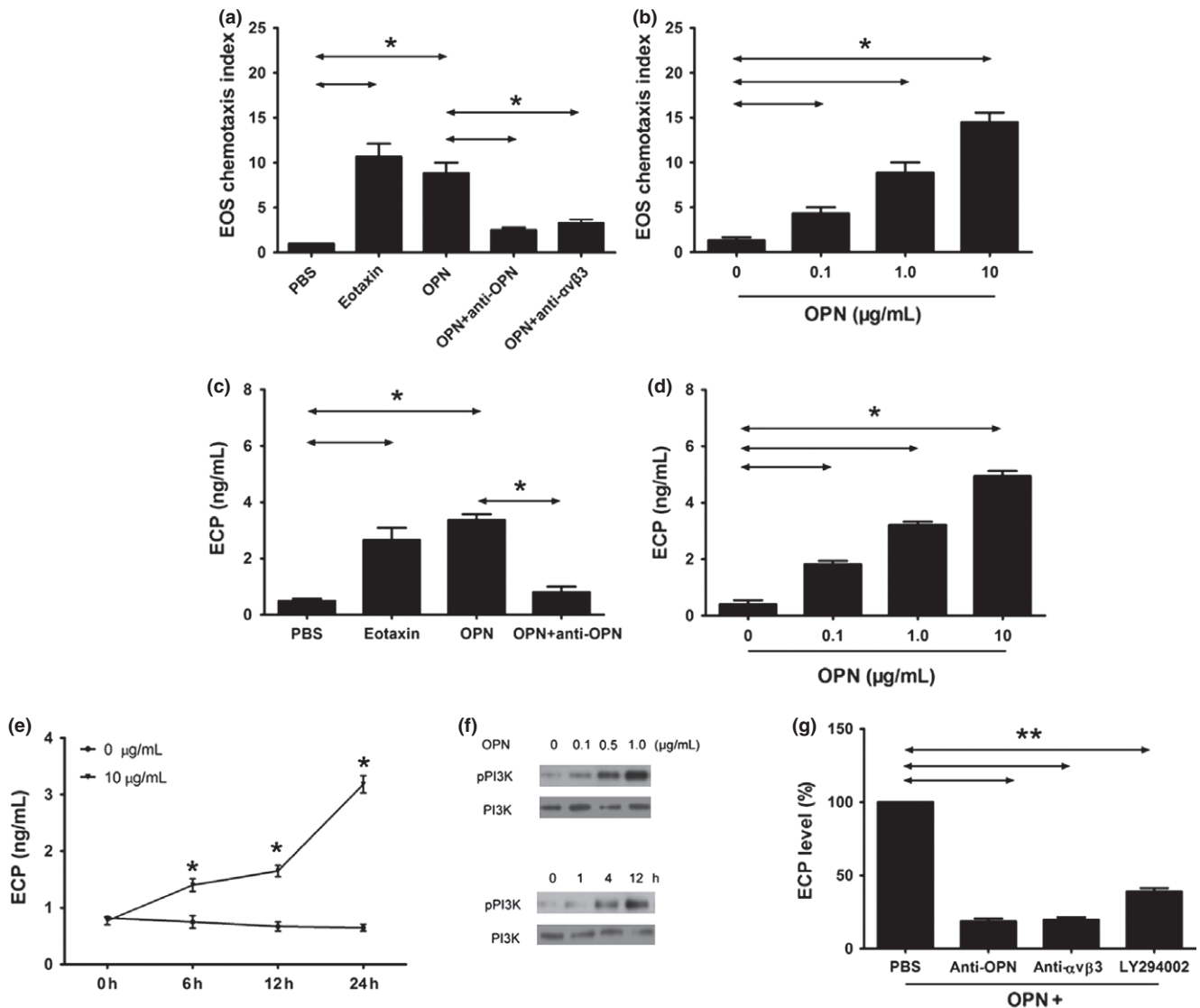


Fig. 6. Recombinant human OPN enhanced EOS chemotaxis and activation in cultured DNPCs. (a) Recombinant human OPN significantly promoted EOS migration, which was inhibited by OPN and $\alpha\beta 3$ blocking antibody; (b) Recombinant human OPN promoted EOS migration in a dose-dependent manner; (c) recombinant human OPN significantly promoted ECP production, which was inhibited by OPN-blocking antibody; (d) recombinant human OPN promoted ECP production in a dose-dependent manner; (e) recombinant human OPN promoted ECP production in a time-dependent manner; (f) representative Western blot analysis of PI3K signalling pathway in cultured DNPCs after adding recombinant human OPN; (g) OPN and $\alpha\beta 3$ blocking antibody, as well as PI3K specific inhibitor LY294002, inhibited the ECP production in response to recombinant human OPN. * $P < 0.05$, ** $P < 0.01$, compared with the unstimulated cells. The data indicated the means (SEM) of three independent experiments.

maintenance and progression of airway inflammatory response including promoting airway dysfunction and tissue remodelling. Several studies have recently addressed the role of OPN in promoting chemotaxis inflammatory cells such as EOS and mast cells in asthma and established the relationship between OPN level and disease severity [23–25]. In agreement with these studies, we have established a clear relationship between OPN expression and tissue eosinophilia in NP tissues of CRSwNP patients in the present study. To further address the importance of OPN in EOS migration and activation, we examined the effects of recombinant

human OPN on EOS migration and activation in DNPCs; we found recombinant human OPN significantly enhanced EOS migration and activation in DNPCs culture system. The molecular mechanism underlying EOS migration and activation in response to OPN is unclear. In Puxeddu et al.' report [23], the EOS chemotaxis was found to be mediated by an interaction between OPN and the $\alpha 4$ integrin expressed on the surface of EOS. In this study, our results indicated both $\alpha\beta 3$ integrin and PI3K signalling pathway act as a critical molecule for EOS chemotaxis and activation. These findings collectively expand our previous

understanding on the pathogenesis of CRSwNP by highlighting a critical role of OPN in tissue eosinophilia in CRSwNP patients.

In summary, we have shown the increased expression of OPN was associated with tissues eosinophilia in CRSwNP patient and demonstrated the importance of OPN in chemotaxis and activation of EOS *in vitro*. Our findings may be beneficial for designing a potential strategy for the optimal prevention and management of CRSwNP patients.

References

- 1 Fokkens WJ, Lund VJ, Mullol J *et al*. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology* 2012; **50**:1–12.
- 2 Hsu J, Peters AT. Pathophysiology of chronic rhinosinusitis with nasal polyp. *Am J Rhinol Allergy* 2011; **25**:285–90.
- 3 Bachert C, Zhang N. Chronic rhinosinusitis and asthma: novel understanding of the role of IgE 'above atopy'. *J Intern Med* 2012; **272**:133–43.
- 4 DeMarcantonio MA, Han JK. Nasal polyps: pathogenesis and treatment implications. *Otolaryngol Clin North Am* 2011; **44**:685–95.
- 5 Cantor H, Shinohara ML. Regulation of T-helper-cell lineage development by osteopontin: the inside story. *Nat Rev Immunol* 2009; **9**:137–41.
- 6 Reinholt FP, Hulthén K, Oldberg A *et al*. Osteopontin—a possible anchor of osteoclasts to bone. *Proc Natl Acad Sci U S A* 1990; **87**:4473–5.
- 7 Chen Y, Bal BS, Gorski JP. Calcium and collagen binding properties of osteopontin, bone sialoprotein, and bone acidic glycoprotein-75 from bone. *J Biol Chem* 1992; **267**:24871–8.
- 8 Shinohara ML, Lu L, Bu J *et al*. Osteopontin expression is essential for interferon- α production by plasmacytoid dendritic cells. *Nat Immunol* 2006; **7**:498–506.
- 9 Scatena M, Liaw L, Giachelli CM. Osteopontin: a multifunctional molecule regulating chronic inflammation and vascular disease. *Arterioscler Thromb Vasc Biol* 2007; **27**:2302–9.
- 10 Chakraborty G, Jain S, Behera R *et al*. The multifaceted roles of osteopontin in cell signaling, tumor progression and angiogenesis. *Curr Mol Med* 2006; **6**:819–30.
- 11 Cho HJ, Cho HJ, Kim HS. Osteopontin: a multifunctional protein at the crossroads of inflammation, atherosclerosis, and vascular calcification. *Curr Atheroscler Rep* 2009; **11**:206–13.
- 12 Hur EM, Youssef S, Haws ME *et al*. Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. *Nat Immunol* 2007; **8**:74–83.
- 13 Konno S, Kurokawa M, Uede T *et al*. Role of osteopontin, a multifunctional protein, in allergy and asthma. *Clin Exp Allergy* 2011; **41**:1360–6.
- 14 Lu X, Zhang XH, Wang H *et al*. Expression of osteopontin in chronic rhinosinusitis with and without nasal polyps. *Allergy* 2009; **64**:104–11.
- 15 Daines SM, Wang Y, Orlandi RR. Periostin and osteopontin are overexpressed in chronically inflamed sinuses. *Int Forum Allergy Rhinol* 2011; **1**:101–5.
- 16 Wen W, Liu W, Zhang L *et al*. Increased neutrophilia in nasal polyps reduces the response to oral corticosteroid therapy. *J Allergy Clin Immunol* 2012; **129**:1522–8.
- 17 Wei Y, Xia W, Ye X *et al*. The antimicrobial protein short palate, lung, and nasal epithelium clone 1 (SPLUNC1) is differentially modulated in eosinophilic and noneosinophilic chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2014; **133**:420–8.
- 18 Schleimer RP, Kato A, Kern R, Kuperman D, Avila PC. Epithelium: at the interface of innate and adaptive immune responses. *J Allergy Clin Immunol* 2007; **120**:1279–84.
- 19 Sha Q, Truong-Tran AQ, Plitt JR *et al*. Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Cell Mol Biol* 2004; **31**:358–64.
- 20 Konno S, Eckman JA, Plunkett B *et al*. Interleukin-10 and Th2 cytokines differentially regulate osteopontin expression in human monocytes and dendritic cells. *J Interferon Cytokine Res* 2006; **26**:562–7.
- 21 Poposki JA, Uzzaman A, Nagarkar DR *et al*. Increased expression of the chemokine CCL23 in eosinophilic chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2011; **128**:73–81.e4.
- 22 Fulkerson PC, Rothenberg ME. Targeting eosinophils in allergy, inflammation and beyond. *Nat Rev Drug Discov* 2013; **12**:117–29.
- 23 Puxeddu I, Berkman N, Ribatti D *et al*. Osteopontin is expressed and functional in human eosinophils. *Allergy* 2010; **65**:168–74.
- 24 Takahashi A, Kurokawa M, Konno S *et al*. Osteopontin is involved in migration of eosinophils in asthma. *Clin Exp Allergy* 2009; **39**:1152–9.
- 25 Samitas K, Zervas E, Vittorakis S *et al*. Osteopontin expression and relation to disease severity in human asthma. *Eur Respir J* 2011; **37**:331–41.

Acknowledgements

This study was supported by the National Natural Science Grant of China (No. 81271054, 81371074) and a grants from the Ministry of Hygiene/Education (No. 201202005, No.20130171110064).

Conflict of interests

The authors declare no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplementary materials.

Supplementary materials

Expression and regulation of osteopontin in chronic rhinosinusitis with nasal polyps

W.-L. Liu^{1,2,†}, H. Zhang^{1,†}, Y. Zheng^{3,†}, H.-T. Wang⁴, F.-H. Chen¹, L. Xu², Y. Wei¹,
Y.-Q. Sun¹, J.-B. Shi¹, H.-B. Li¹

¹Allergy and Cancer Center, Otorhinolaryngology Hospital, The First Affiliated Hospital of Sun Yat-sen University, ²Department of Otolaryngology, Guangzhou Women and Children's Medical Center, Guangzhou, ³Department of Otolaryngology, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi, ⁴Department of Otolaryngology, PLA General Hospital, Beijing, China

[†] These authors contributed equally to the study.

Correspondence: Huabin Li, Allergy and Cancer Center, Otorhinolaryngology Hospital, The First Affiliated Hospital of Sun Yat-sen University, No. 58, Zhongshan 2nd Road, Guangzhou, 510080, China. Email: allergyli@163.com

Materials and methods

DNA microarray

The expression profiles of OPN and other genes were detected using a commercially available cDNA array system (Extracellular matrix and adhesion molecules microarray; SuperArray Bioscience Corp., Frederick, MD, USA) according to the manufacturer's instructions. Briefly, nasal biopsies were taken from 2 CRSwNP patients and 2 controls. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). 3 µg of total RNA was reverse transcribed into biotin-16-deoxy-UTP-labeled single-strand cDNA by Moloney murine leukemia virus (M-MLV) reverse transcriptase. After prehybridization, membranes were hybridized with biotin-labeled sample cDNA and incubated with alkaline phosphatase-conjugated streptavidin. Chemiluminescence was visualized by autoradiography. The relative expression of different genes was estimated by comparing the average signal intensity with that of average intensity of the internal control genes. A quantitative estimate of the difference in gene expression is given by the signal log ratio with 2 as the base of the log scale. Thus, a signal log ratio of 1.0 indicates an increase in the transcript level by twofold and -1.0 indicates a decrease by 2-fold. The genes were admitted to the list of genes differentially expressed in CRSwNP patients compared with normal controls by the following criteria: (1) the difference of gene expression level between polyp tissues and normal controls was statistically significant, with a *P* value of less than 0.05; (2) the expression difference was larger than 2-fold.

Double IF staining

Double IF staining was performed to determine the co-localization of OPN and mast cell in nasal tissues as we previously described [1]. The primary antibodies, including OPN (1:50), MBP (1:50) and tryptase (1:100, Dako, Glostrup, Denmark), were incubated overnight at 4°C. After washing, the sections were further incubated with Fluor 568-conjugated goat IgG (1:1000) and Fluor 488-conjugated goat IgG (1:1000, Invitrogen) for 2 hours at room temperature. Sections stained by isotype-matched IgG instead of the primary antibody were used as negative controls. After washing with PBS and nuclear staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Santa Cruz, Santa Cruz, CA, USA), the slides were mounted with antifade glycerol. Negative control slides were prepared by staining with isotype-matched IgG instead of

primary antibody. For quantification, nasal subepithelial areas excluding glands below the basement membrane were viewed with a microscope (Olympus Optical Co) and appropriate filter sets by 2 blinded investigators. Positive cells and merged double-positive cells were observed under 400× magnification.

Flow cytometric analysis

Collected PECs were washed twice in PBS. PE-labeled anti-OPN mAb (R&D systems) was used for intercellular staining. For fixation and permeabilization of PECs, the BD Cytotfix/Cytoperm kit was used according to the manufacturer's instructions, and subsequently, collected PECs were incubated in blocking solution for 10 min at room temperature. Anti-OPN mAb or IgG isotype control (R&D systems) was added for 20 min at room temperature. After washing with PBS containing 0.5% BSA, a total of 10^5 cells were analyzed for fluorescence by flow cytometer (FACS Calibur, Becton Dickinson, Sunnyvale, CA, USA). Intercellular expression of OPN was determined by subtracting the mean fluorescence intensity (MIF) measured with the IgG isotype control from that measured with the OPN mAb. The level of OPN expression was expressed as relative ratio against OPN MIF in control (unstimulated) PECs.

Western blot analysis

The protein levels of PI3K and pPI3K were detected by western blot analysis as we previously described [2]. Briefly, the tissues or cells were collected, and total cellular protein was extracted in 100 μ L of RIPA lysis buffer at 4 °C for 30 min. The protein concentration was determined by the Bradford method. Samples containing 10 μ g of protein were boiled, subjected to SDS-PAGE in 10% Tris-glycine gels and transferred electrophoretically to a polyvinylidene fluoride membrane. The membrane was incubated with 5% fat-free skim milk in Tris-buffered solution (TBS) containing 0.05% Tween 20 (1 h, room temperature) and then incubated with primary antibodies (PI3K and pPI3K, 1:1000, Cell Signalling, Danvers, MA, USA) overnight at 4°C. The membrane was then incubated with horseradish peroxidase-linked secondary antibody and finally processed using the ECL chemiluminescence reaction kit (Cell Signalling), followed by exposure on medical film. The relative band density of phosphorylated protein was quantified with the Bio-Rad Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA, USA).

ELISA

The levels of OPN and ECP in the nasal tissue extracts, nasal secretions and supernatants of cultured DNPCs were measured using ELISA. For nasal tissue, one milliliter of PBS containing a protease-inhibitor cocktail (Keygentec Inc., Nanjing, China) was added for every 100 mg of tissue. The sample was homogenized for 1 min on ice. After homogenization, the suspension was centrifuged at 4000 rpm for 20 min at 4°C, and the supernatants were stored at –80°C for ELISA assay. Commercially available ELISA kits of OPN (R&D Systems, Minneapolis, MN) and ECP (EIAab, Wuhan, China) were used according to the manufacturer's protocols. The detection limit for OPN and ECP were 0.312 ng/mL and 0.156 ng/mL. For convenient analysis, all values below the detectable limit were considered to be 0.

Cell culture and stimulation

For *in vitro* cell isolation and culture, primary PECs and NECs were randomly collected from 6 CRSwNP patients and 6 normal controls by means of enzymatic digestion as we described elsewhere. Briefly, Fresh nasal tissues were treated with Protease (1.4 mg/ml) and Dnase (1:100) in Dulbecco's modified Eagle's medium and Ham's nutrient F12 supplemented with penicillin G sodium (50 IU/mL) and streptomycin sulfate (50 mg/mL), incubator 37 °C, 5% CO₂, 1h. After repeated centrifugation, the cells were rinsed in containing 5 ml Dulbecco's modified Eagle's medium/F12 and then transfer into BEGM medium (Lonza, Walkersville, MD, USA) and pour into the plastic flask, incubator 37 °C, 5% CO₂ overnight. The NECs and PECs can be collected after 5-7 days.

Collected PECs and NECs were cultured in submersion cultures in BEGM medium (Lonza, Walkersville, MD, USA) until passaged. When 80–90% confluence was reached, the epithelial cells were washed with PBS (37°C, pH 7.4), and fresh media without hydrocortisone was added in the presence of different stimulators or PBS (control) for different time period. These stimulators were as follows: Recombinant cytokines: human TNF- α (100 ng/mL), IFN- γ (100 ng/mL), IL-4 (100 ng/mL), IL-6 (100 ng/mL), IL-17A (100 ng/mL), TGF- β (10 ng/mL), IL-10 (100 ng/mL) (all were purchased from R&D systems); TLR agonists: 500 ng/mL PGN(Pam₃CSK₄); 500 ng/mL Poly I:C; 500 ng/mL LPS from Escherichia coli serotype 0111:B4 (Sigma-Aldrich, St Louis, MO,

USA); 100 ng/mL Flagellin and 5 μ mol/L human CpG ODN (InvivoGen, San Diego, CA, USA). After then, cell pellets were collected for qPCR and flow cytometry by following the protocol that mentioned above.

In vitro EOS chemotaxis and activation assay

DNPCs were randomly prepared from 16 fresh polyp tissues by means of enzymatic digestion. Briefly, minced polyp tissues were incubated for 2 hours at 37°C in RPMI 1640 containing 2.0 mg/mL protease, 1.5 mg/mL collagenase, 0.75 mg/mL hyaluronidase, and 0.05 mg/mL DNase. The cell suspension was then filtered through a 70-mm cell strainer (BD Falcon, Bedford, MA, USA) to remove any undigested tissue and washed 2 times with washing medium (RPMI 1640 supplemented with 2% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin). The cell pellet was resuspended in erythrocyte lysis buffer and washed with washing medium. After washing, DNPCs were suspended in culture medium (RPMI 1640 supplemented with 10% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin). Viability was assessed through the exclusion of trypan blue. Different concentrations of recombinant OPN (R&D systems, 0.1-10 μ g/mL) were incubated with DNPCs (500 μ L, 10⁵/mL) treated in the absence or presence of anti-OPN or anti- α v β 3 antibody (R&D systems) in a 24-well transwell system with 5- μ m polycarbonate filters. PBS and recombinant eotaxin (R&D systems) were used as negative and positive controls, respectively. For EOS chemotaxis assay, migrated EOS were examined using Giemsa staining and were counted in 10 HPFs using light microscopy (magnification, 400 $\times$) and averaged, and the chemotaxis index was calculated by comparing the numbers of migrated EOS in response to recombinant human OPN and PBS alone. For EOS activation assay, the ECP level in supernatants was examined by ELISA and DNPCs were collected for western blot analysis by following the protocol that mentioned above. LY294002 (10 μ M) (Sigma-Aldrich) was used as a specific inhibitor of PI3K signaling pathway.

References

- 1 Li HB, Cai KM, Liu Z, *et al.* Foxp3⁺ T regulatory cells (Tregs) are increased in nasal polyps (NP) after treatment with intranasal steroid. *Clin Immunol* 2008;**129**:394-400.

- 2 Wen W, Liu W, Zhang L, *et al.* Increased neutrophilia in nasal polyps reduces the response to oral corticosteroid therapy. *J Allergy Clin Immunol* 2012;**129**:1522-8. e5.