当科における好酸球性副鼻腔炎手術症例の臨床背景と治療成績の変遷について

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I. 緒　　言

慢性副鼻腔炎（いわゆる蓄膿症）とはかぜ症候群などを契機に副鼻腔に生じた炎症が遷延化し，3カ月以上継続した病態と定義される。副鼻腔の炎症が遷延化する要因として従来から指摘されている二大要因として，反復するウィルス・細菌感染による粘液線毛輸送機能の障害と，鼻腔の形態学的構造異常（鼻中隔彎曲，中鼻甲介や鈎状突起の形態異常など）が挙げられている[1]。一方で近年，このプロセスとは異なる副鼻腔炎の新たな難治化因子として好酸球浸潤の存在が注目を集めており，今世紀に入ってから好酸球性副鼻腔炎という新しい疾患概念がわが国においても提唱されるようになった[2),3)]。

好酸球性副鼻腔炎とは，副鼻腔粘膜または鼻ポリープに著明な好酸球浸潤を伴う易再発性の慢性副鼻腔炎の総称であるが，疫学的にも本疾患の増加傾向が各施設より報告されている。これに対して，広島大学は平成 22 年度より厚生労働省の難治性疾患克服研究事業（藤枝班）の「好酸球性副鼻腔炎の疫学，診断基準作成等に関する研究」に参加して，副鼻腔炎手術症例の組織学的解析やデータベースの作成事業などを行っている。今回その研究の一環として，過去 10 年間における副鼻腔炎手術症例における組織学的特徴の経年的割合の変遷を検討したので報告する。

II. 対 象 と 方 法

広島大学病院ならびに中国労災病院にて慢性副鼻腔炎の診断にて内視鏡下鼻内手術（endoscopic sinus surgery, ESS）を施行し，病理組織学的に検索可能であった 2000 年〜2003 年までの 133 例と，2007 年〜2010 年までの 118 例を対象とした。定形的に HE 染色標本を作成して前篩骨洞粘膜における好酸球浸潤密度を複数視野にて計測し，好酸球性副鼻腔炎の

診断基準のベースとなるヒストグラムを作成した（図1）。そして強拡大（×400）視野での観察で平均好酸球密度が 20 個以上と未満を境界として，好酸球性副鼻腔炎と非好酸球性（化膿性）副鼻腔炎に分類した。それぞれの症例群において，年齢と性別，末梢血好酸球比率などの臨床検査結果，鼻アレルギー合併の有無，術前の CT 画像所見および術後経過の状態について比較した。

CT 画像診断の評価は Lund & Mackay が提唱した CT Score sheet をもとに行った[4]。すなわち陰影なしを 0 点，軽度の陰影を 1 点，完全な陰影を 2 点とし，上顎洞，前篩骨洞，後篩骨洞，前頭洞，蝶形骨洞，ostiomeatal complex（OMC）それぞれの陰影スコアを合計した。ESS 術後の臨床経過の評価には島田が提唱した内視鏡的鼻内所見の重症度分類を用い，上顎洞と篩骨洞の状態を記録した[5]。すなわち粘膜の状態に応じて，ポリープおよび副鼻腔貯留液がなく洞との交通路も良好なものを 1 点，単発性のポリープか粘膜浮腫，もしくは時折の貯留液が存在するものを 2 点，多発性ポリープか著明な浮腫，もしくは常時の貯留液，もしくは洞との交通路の著明な狭窄や広範囲の癒着が存在するものを 3 点とした。

なお CT 画像陰影スコアの群間の統計学的有意差の検定には Mann-Whitney の U 検定を，鼻内内視鏡所見に基づく術後改善の群間比較にはフィッシャーの両側検定を用いた。いずれも p<.05 を有意と判定

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した。

Ⅲ. 結 果

図1にそれぞれの観察期間における対象症例の好酸球浸潤密度のヒストグラムを示している。いずれの期間においてもヒストグラムは二峰性の分布を示しており，副鼻腔炎病態が組織学的に単一でなく 20 個／×400 視野以上を境にして，好酸球性副鼻腔炎の概念に一致した疾患群が存在していることが明らかとなった。またその割合は 2000 年～2003 年では 22.5％（30/133 例），2007 年～2010 年では 30.5％（36/118 例）であり，手術症例における本疾患は増加傾向にあった。図2に示したように対象症例の臨床背景について検討してみると，鼻アレルギー合併率は両期間ともに約 42％と同程度であった。また好酸球性副鼻腔炎症例の中でも気管支喘息合併例は著明な好酸球浸潤（それぞれ 8/30 例で平均 40 個と，16/37 例で平均 56.1 個／×400 視野）を伴っていた。また，術前の末梢血中好酸球数比率（％）と組織中の好酸球浸潤密度との相関を 2007 年～2010 年の症例で検討したところ，相関係数 0.34（ピアソン，p<.01）と弱いながらも正の相関が認められた。

図3と図4には術前の副鼻腔 CT 画像の陰影スコアを比較したグラフを示している。全副鼻腔の陰影スコアの合計（正常が 0，最重症が 12）では，いずれの期間においても好酸球性副鼻腔炎群の方が有意差をもってスコアが高値（重症）であるとの結果が得られた。さらに副鼻腔各洞の罹患状態の比較のために，上顎洞と篩骨漏斗の合計スコアと，前・後篩骨洞のスコアの差について比較したところ，いずれの期間においても好酸球性副鼻腔炎群の方が有意差をもって篩骨洞の陰影スコアが高値であるとの結果が得られた。

最後に治療成績について述べる。当科での術後薬物治療は，非好酸球性（化膿性）副鼻腔炎に対してはマクロライド製剤と粘液去痰剤を中心に，好酸球性副鼻腔炎に対してはロイコトリエン受容体拮抗剤と局所ステロイド製剤を中心に最低 6 カ月を目安に行っている[6),7)]。図5には鼻内内視鏡所見をもとにスコア 3 以下を良好とした術後の改善率を示している。
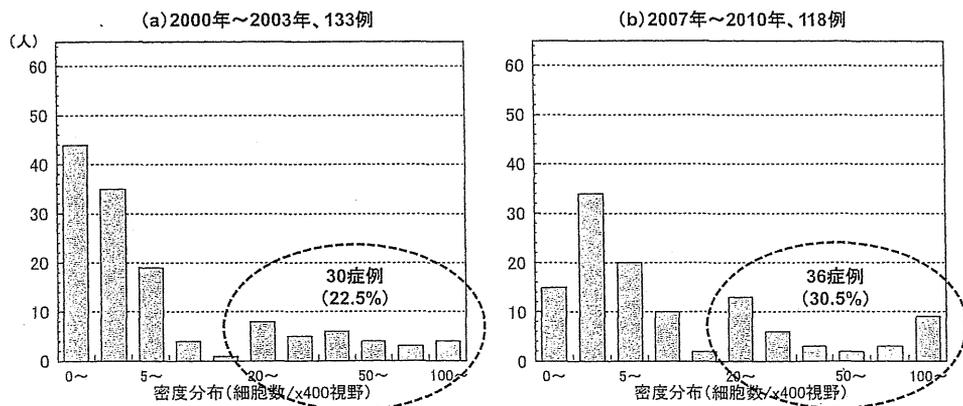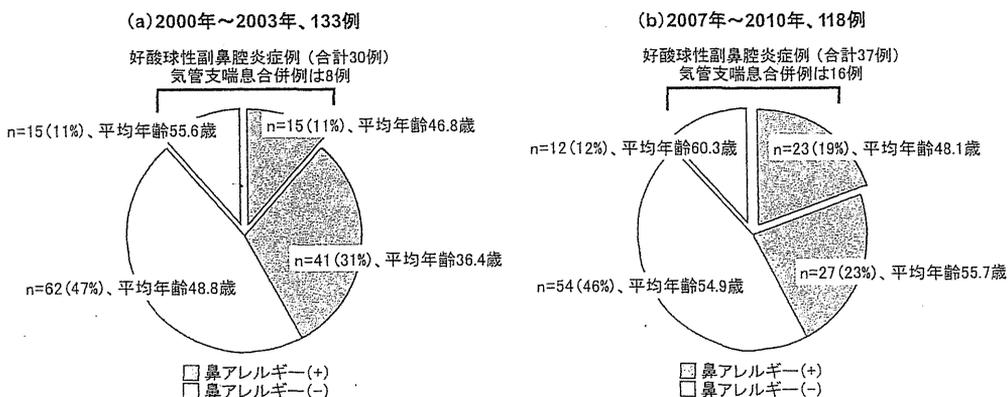

図1 対象症例における副鼻腔粘膜組織中の好酸球浸潤密度の分布
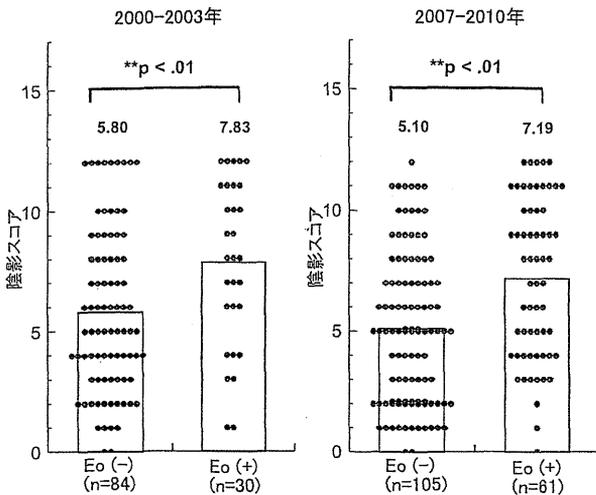

図2 対象症例の臨床背景

好酸球性副鼻腔炎の判定基準は浸潤密度が 20 個／×400 視野による。

図3　化膿性副鼻腔炎（Eo（−））と好酸球性副
　　　鼻腔炎（Eo（+））のそれぞれ各群における
　　　術前のCT画像陰影の重症度の比較
　　　全副鼻腔の合計スコアの比較グラフ。
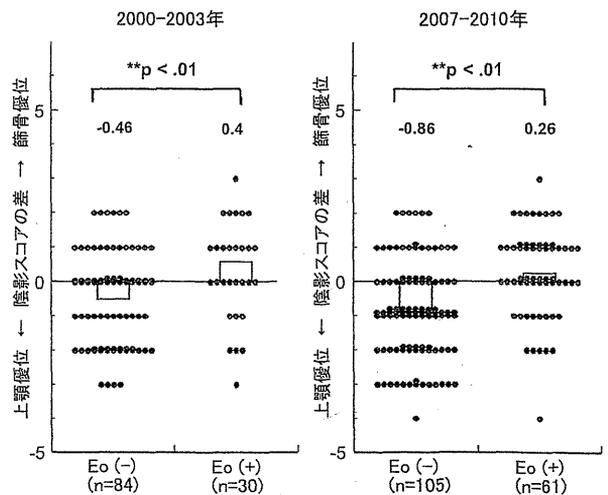

図4　化膿性副鼻腔炎（Eo（−））と好酸球性副
　　　鼻腔炎（Eo（+））のそれぞれ各群における
　　　術前のCT画像陰影の特徴
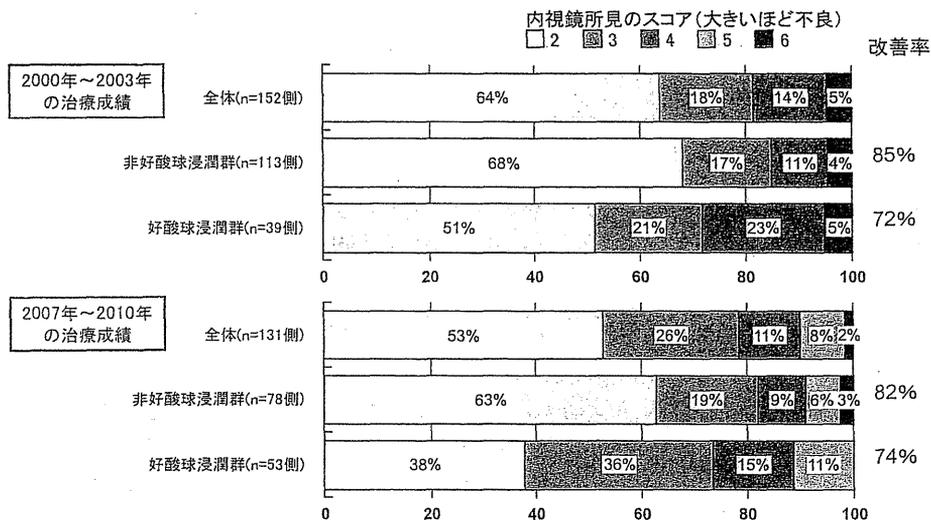　　　グラフの縦軸は，各症例における（前・後篩骨洞スコア）
　　　−（上顎洞と篩骨漏斗スコア）をプロットしている。

図5　内視鏡所見をもとに6カ月以上経過例を対象としたESS術後の改善効果
　　　上顎洞と篩骨洞の合計スコア3以下を改善とした。

非好酸球性副鼻腔炎の改善率が82～85％，好酸球性副鼻腔炎の改善率が72～74％と，いずれの期間においてもほぼ同等の改善率を示した。同時に好酸球性副鼻腔炎の方がスコア4以上の粘膜不良状態が再燃するケースが多い傾向を認めたが，群間比較ではいずれの期間においても統計学的有意差は認めなかった。

Ⅳ. 考　　　察

　本邦における好酸球性副鼻腔炎の臨床像としては，1) 組織学的に著明な副鼻腔粘膜への好酸球浸潤を呈する，2) 浮腫状の鼻茸の多発と粘稠（にかわ状）な鼻汁の存在，3) 鼻アレルギー（アトピー素因）の関与は問わない，4) 成人発症で両側罹患例が多い，5) 副鼻腔各洞のうちで上顎洞・篩骨漏斗病変に比較して，篩骨洞病変が主体である，6) 気管支喘息などの下気道病変の合併が多い，7) 病状早期より嗅覚障害が出現，8) 治療に抵抗性で病状が再燃しやすい，などといった点が挙げられている[2]。

　わが国においても好酸球性副鼻腔炎の数は徐々に増加しているとされているが，これまでに全国的な大規模調査などは行われておらず，実際の有病率や気管支喘息合併率などはっきりしたことは不明のままである。昨年度より実施されている班研究で，広

島県を含む 3,000 例以上の手術症例を対象とした全国疫学調査でも，各施設で臨床的に好酸球性副鼻腔炎と診断した症例は 27.6％の割合で存在していた。そして臨床背景の解析の結果，本疾患における有意に異なる臨床因子として，副鼻腔罹患洞が両側性である点，鼻茸の存在，嗅覚障害の合併，血液中好酸球率，画像陰影の重症度，などが現時点では挙げられている[8]。また合併疾患として好酸球性中耳炎，気管支喘息（アスピリン不耐症を含む），アレルギー性鼻炎，薬物アレルギーの増加が観察されている。これらの臨床背景と画像所見の特徴は，今回のわれわれの施設における過去 10 年間にわたる期間の検討でも同様なものであり，好酸球性副鼻腔炎という新たなタイプの鼻副鼻腔炎の増加は全国的な現象であることが確認された。現時点で好酸球性副鼻腔炎の確定診断には組織好酸球数の測定が必須であるが，その具体的数値の設定はいまだなされていない。今回の検討ではこれまでの研究の一環として，篩骨洞粘膜における 20 個／×400 視野以上を暫定的に採用し，clear cut な結果を得ることができた。この組織学的診断基準に関しては評価可能な施設の制約もあり，末梢血データで代用可能かどうかなど引き続き検討が必要性と考えられる。

　最後に好酸球性副鼻腔炎の薬物治療としては，難治化因子の主役である好酸球浸潤の軽減を直接的な標的とすることを基本とすべきであると考えられている[9]。また保存的な薬物療法単独では中長期的に安定した寛解状態を維持するのが困難な場合も多く，手術療法が選択される場合も多い。手術療法（ESS）の位置づけとしては，副鼻腔各洞の病巣を手術的に清掃し，副鼻腔が生理的に有する排泄機能をより発揮しやすい状態にもっていく強力な手段と考えられる。手術操作により骨壁を削開し，不良粘膜を截除することにより治癒過程は促進され，術直後には良好な状態が回復される。その場合においても，術後における薬物療法の併用は病状の再燃防止に重要であり，これらの治療により概ね 70％台の改善効果の維持が期待できることがあることが今回の検討からも明らかとなった。今後は患者負担が少なく効率的な治療プロトコルの確立が望まれている。

V．結　　　語

　当科において 2000 年〜2003 年までと 2007 年〜2010 年までの期間に手術を施行し，組織学的検討が可能であった症例について，好酸球浸潤の程度と臨床背景について検討した。そして両期間における好酸球性副鼻腔炎の割合の変遷などをもとに，本疾患の診断基準と治療法について考察した。

　その結果，1）この 10 年間で手術症例に占める割合は約 10％増加しており，2）診断基準には組織学的評価が重要であり，3）好酸球性副鼻腔炎の臨床像には変化は認められず，4）術後経過不良例（全体の約 1/4）に対する治療法の検討が今後も必要，であることが判明した。

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Poly(I:C) reduces expression of JAM-A and induces secretion of IL-8 and TNF-α via distinct NF-κB pathways in human nasal epithelial cells

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ABSTRACT

Human nasal epithelium is an important physical barrier and innate immune defense protecting against inhaled substances and pathogens. Toll-like receptor (TLR) signaling, which plays a key role in the innate immune response, has not been well characterized in human nasal epithelial cells (HNECs), including the epithelial tight junctional barrier. In the present study, mRNAs of TLR1–10 were detected in hTERT-transfected HNECs, which can be used as an indispensable and stable model of normal HNECs, similar to primary cultured HNECs. To investigate the changes of tight junction proteins and the signal transduction pathways via TLRs in HNECs in vitro, hTERT-transfected HNECs were treated with TLR2 ligand P3CSK4, TLR3 ligand poly(I:C), TLR4 ligand LPS, TLR7/8 ligand CL097, TLR8 ligand ssRNA40/LyoVec, and TLR9 ligand ODN2006. In hTERT-transfected HNECs, treatment with poly(I:C) significantly reduced expression of the tight junction protein JAM-A and induced secretion of proinflammatory cytokines IL-8 and TNF-α. Both the reduction of JAM-A expression and the induction of secretion of IL-8 and TNF-α after treatment with poly(I:C) were modulated by distinct signal transduction pathways via EGFR, PI3K, and p38 MAPK and finally regulated by a TLR3-mediated NF-κB pathway. The control of TLR3-mediated signaling pathways in HNECs may be important not only in infection by viral dsRNA but also in autoimmune diseases caused by endogenous dsRNA released from necrotic cells.

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Introduction

The epithelium of human nasal mucosa acts as a physical barrier that protects against inhaled substances and pathogens (Holgate, 2007; Schleimer et al., 2007). The epithelium is a major source of cytokines, chemokines, and other inflammatory mediators that affect the adaptive and innate immune responses. Furthermore, the epithelium is a highly regulated and impermeable barrier exclusively formed by the apicalmost intercellular junctions, tight junctions (Takano et al., 2005; Holgate, 2007).

The pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptor (NLR) play a key role in pathogen recognition and induction as well as regulation of innate and adaptive immune responses (Bals and Hiemstra, 2004; Kawai and Akira, 2006; Mayer and Dalpke, 2007). In addition, TLR3 recognizes viral double-stranded (dsRNA) and its synthetic analogue polyinosinic–polycytidylic acid [poly(I:C)] and

stimulates innate immune responses (Alexopoulou et al., 2001). Recognizing dsRNA, the intracellular domain of TLR3 interacts with adaptor protein TRIF (Toll/interleukin-1 receptor domain-containing adaptor-inducing IFN-β), inducing antiviral interferons and proinflammatory cytokines via transcription factor IRF3 and NF-κB (Vercammen et al., 2008; Bérubé et al., 2009; Lim et al., 2009). TLR3 is also activated by endogenous dsRNA released from necrotic cells (Kariko et al., 2004; Cavassani et al., 2008).

Stimulation of airway epithelial cells with poly(I:C) elicits the secretion of multiple proinflammatory cytokines, chemokines, together with the induction of transcription factors (Matsukura et al., 2006; Koarai et al., 2010). It is reported that mRNAs for all 10 known human TLRs are detected in cultured nasal epithelial cells (Lin et al., 2007). Upregulation of TLR2, TLR3, and TLR4 has been reported in the nasal mucosa of patients with allergic rhinitis (Fransson et al., 2005). It is also known that TLR3 expression increases in more differentiated nasal epithelial cells in vitro (Lin et al., 2007).

Tight junctions are formed by not only the integral membrane proteins claudins, occludin, and junctional adhesion molecules (JAMs) but also many peripheral membrane proteins, including the scaffold PDZ-expression proteins zonula occludens (ZO)-1, ZO-2, ZO-3, multi-PDZ domain protein-1 (MUPP1), and membrane-associated guanylate

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kinase with inverted orientation-1 (MAGI)-1, MAGI-2, MAGI-3, and cell polarity molecules ASIP/PAR-3, PAR-6, PALS-1, and PALS-1-associated tight junction (PATJ), as well as the non-PDZ-expressing proteins, cingulin, symplekin, ZONAB, GEF-H1, aPKC, PP2A, Rab3b, Rab13, PTEN, and 7H6 (Tsukita et al., 2001; Sawada et al., 2003; Schneeberger and Lynch, 2004; Kojima et al., 2009). More recently, tricellulin was identified at tricellular contacts where there were three epithelial cells and was shown to have a barrier function (Ikenouchi et al., 2005). It is known that TLR2 controls the tight junctional barrier in intestinal epithelial cells (Cario et al., 2007).

JAM-A was first identified as a platelet membrane glycoprotein and now is classified as a member of immunoglobulin superfamily molecules, which include JAM-B, -C, -4, and JAM-L (Kornecki et al., 1990; Martin-Padura et al., 1998). JAM-A is expressed at tight junctions of endothelial and epithelial cells (Martin-Padura et al., 1998; Bazzoni et al., 2000; Ebnet et al., 2000; Liu et al., 2000; Ebnet et al., 2001; Itoh et al., 2001; Mandell et al., 2004; Severson et al., 2008, 2009) and is associated with several disease condition such as hypertension (Ong et al., 2009), ischemia, and atherosclerosis (Cavusoglu et al., 2007) through the significant function of JAM-A, which has been linked with regulation of cell migration (Martin-Padura et al., 1998; Cera et al., 2004, 2009; Severson et al., 2008, 2009), platelet activation (Kornecki et al., 1990; Martin-Padura et al., 1998), and epithelial barrier function (Liu et al., 2000; Mandell et al., 2004). Interestingly, JAM-A is also a receptor for each of the three known reovirus serotypes, and the reovirus attachment protein σ1 interacts with the JAM-A extracellular D1 loop (Barton et al., 2001; Prota et al., 2003; Guglielmi et al., 2007).

We previously reported that, in human nasal epithelial cells (HNECs) in vivo and in vitro, occludin, JAM-A, ZO-1, ZO-2, claudin-1, -4, -7, -8, -12, -13, -14, and tricellulin were detected together with well-developed tight junction strands (Takano et al., 2005; Koizumi et al., 2008; Ohkuni et al., 2009). Furthermore, the hTERT-transfected HNECs that we previously established can be used as an indispensable and stable model for studying the regulation of tight junction proteins in human nasal epithelium (Kurose et al., 2007; Kamekura et al., 2009; Ohkuni et al., 2009; Ogasawara et al., 2010). We also found that poly(I:C) induced the secretion of thymic stromal lymphopoietin (TSLP), which triggers dendritic cell-mediated activation of Th2 inflammatory responses, from HNECs by using this culture system (Kamekura et al., 2009). However, the mechanisms, including the signal transduction pathways, were unclear.

In the present study, mRNAs of TLR1–10 detected in hTERT-transfected HNECs were similar to primary cultured HNECs. To investigate changes of tight junction proteins via TLRs in HNECs, hTERT-transfected HNECs were treated with TLR2 ligand P_3CSK_4, TLR3 ligand poly(I:C), TLR4 ligand LPS, TLR7/8 ligand CL097, TLR8 ligand ssRNA40/LyoVec, and TLR9 ligand ODN2006. In hTERT-transfected HNECs, treatment with poly(I:C) significantly reduced expression of the tight junction protein JAM-A and induced secretion of proinflammatory cytokines IL-8 and TNF-α. Both the reduction of JAM-A expression and the induction of secretion of IL-8 and TNF-α after treatment with poly(I:C) were modulated via distinct signal transduction pathways and finally regulated by a TLR3-mediated NF-κB pathway.

Materials and methods

Reagents and antibodies. Recombinant human IL-8 and TNF-α were purchased from PeproTech EC (London, UK). Pam$_3$Cys-Ser-(Lys)$_4$ (P3CSK4) was purchased from EMC microcollections (Tübingen, Germany). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO). Polyinosine–polycytidylic acid [Poly (I:C)], CL097, ssRNA40/LyoVec, and ODN2006 were purchased from InvivoGene (San Diego, CA). Phospho-extracellular signal-regulated kinase (ERK) 1/2 (MAPK; Thr202/Tyr204) inhibitor PD98059, p38 MAPK inhibitor SB203580, phosphatidylinositol 3 kinase (PI3K)

inhibitor LY294002, and epidermal growth factor receptor (EGFR) inhibitor AG1478 were purchased from Calbiochem Novabiochem Corporation (San Diego, CA). JNK inhibitor SP600125 and NF-κB inhibitor IMD-0354 were purchased from Sigma-Aldrich. Polyclonal rabbit anti-JAM-A, anti-claudin-1, -4, and -7, anti-occludin, and anti-tricellulin antibodies were obtained from Zymed Laboratories (San Francisco, CA). Rabbit polyclonal anti-phospho-IκB-α (Ser32) and anti-IκB-α antibodies were obtained from Cell Signaling (Danvers, MA). Rabbit polyclonal Rap1 was purchased from Millipore (Billerica, MA). Monoclonal rabbit anti-β1 integrin was purchased from Novus Biologicals (Littleton, CO). Polyclonal rabbit anti-actin antibody was obtained from Sigma-Aldrich. Alexa 488 (green)- and Alexa 594 (red)-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Molecular Probes Inc. (Eugene, OR). HRP-conjugated polyclonal goat anti-rabbit immunoglobulins were purchased from Dako A/S (Glostrup, Denmark). The ECL Western blotting system was obtained from GE Healthcare UK, Ltd. (Buckinghamshire, UK).

Cell culture and treatments. The cultured HNECs were derived from the mucosal tissues of patients with hypertrophic rhinitis or chronic sinusitis who underwent inferior turbinectomy at Sapporo Medical University, the Sapporo Hospital of Hokkaido Railway Company, or the KKR Sapporo Medical Center Tonan Hospital. Informed consent was obtained from all patients, and this study was approved by the ethics committees of Sapporo Medical University, the Sapporo Hospital of Hokkaido Railway Company, and the KKR Sapporo Medical Center Tonan Hospital.

The methods for primary culture of human nasal epithelial cells were as reported previously (Koizumi et al., 2008). Some primary cultured HNECs were transfected with the catalytic component of telomerase, the human catalytic subunit of the telomerase reverse transcriptase (hTERT) gene, as described previously (Kurose et al., 2007; Kamekura et al., 2009; Ohkuni et al., 2009; Ogasawara et al., 2010). The cells were plated on 35- or 60-mm culture dishes (Corning Glass Works, Corning, NY), which were coated with rat tail collagen (500 μg of dried tendon/ml 0.1% acetic acid). The cells were cultured in serum-free bronchial epithelial cell basal medium (BEBM, Lonza Walkersville, Inc., Walkersville, MD) supplemented with bovine pituitary extract (1% v/v), 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamycin, 50 μg/ml amphotericin B, 0.1 ng/ml retinoic acid, 10 μg/ml transferrin, 6.5 μg/ml triiodothyronine, 0.5 μg/ml epinephrine, 0.5 ng/ml epidermal growth factor (Lonza Walkersville, Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich) and incubated in a humidified, 5% CO_2:95% air incubator at 37 °C. In this experiment, 2nd and 3rd passaged cells were used.

The HNECs were treated with 1 μg/ml P3CSK4, 1–25 μg/ml Poly(I:C), 100 ng/ml LPS, 5 μg/ml CL097, 1 μg/ml ssRNA40/LyoVec, and 5 μM ODN2006. Furthermore, some cells were pretreated with 10 μM AG1478, 5 μM ODN2006, 10 μM LY294002, 10 μM PD98059, 10 μM SB203580, 10 μM SP600125, and 1–10 μM IMD-0354 at 30 min before treatment with 25 μg/ml Poly(I:C).

MTT assay. The cells plated on 24-well tissue culture plates (BD Labware, Franklin Lakes, NJ) were treated with 1–25 μg/ml Poly(I:C) for 24 h. The cell survival was evaluated with the colorimetric assay using an MTT Cell Growth Assay Kit (Millipore, Billerica, MA) according to the manufacturer's recommendations. The ratio of absorbance was calculated and presented as the mean ± SD of triplicate experiments.

RNA isolation and RT-PCR. Total RNA was extracted and purified from hTERT-transfected HNECs using TRIzol reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed into cDNA using a mixture of oligo(dT) and Superscript II RTase under the recommended conditions (Invitrogen). Each cDNA synthesis was performed in a

total volume of 20 µl for 50 min at 42 °C and terminated by incubation for 15 min at 70 °C. PCR containing 10 pM primer pairs and 1.0 µl of the 20 µl total RT reaction was performed in 20 µl of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.4 mM dNTPs, and 0.5 U of Taq DNA polymerase (Takara Bio, Inc.; Shiga, Japan), employing 25, 30, or 35 cycles with cycle times of 15 s at 96 °C, 30 s at 55 °C, and 60 s at 72 °C. Final elongation time was 7 min at 72 °C. Nine microliters of the 20 µl total PCR reaction was analyzed in 2% agarose gel after staining with ethidium bromide. To provide a quantitative control for reaction efficiency, PCR reactions were performed with primers coding for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Primers used to detect G3PDH, TLR1–10 and tumor necrosis factor receptor 1 (TNFR1) are indicated in Table 1.

Enzyme-linked immunosorbent (ELISA) assay. The concentrations of human IL-8 and TNF-α in cell culture supernatants of hTERT-transfected HNECs at 24 h after treatment were measured using ELISA kits for human IL-8 and TNF-α (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Western blot analysis. The hTERT-transfected HNECs were scraped from a 60-mm dish containing 300 µl of buffer (1 mM NaHCO₃ and 2 mM phenylmethylsulfonyl fluoride), collected in microcentrifuge tubes, and then sonicated for 10 s. The protein concentrations of the samples were determined using a BCA protein assay reagent kit (Pierce Chemical Co.; Rockford, IL). Aliquots of 15 µg of protein/lane for each sample were separated by electrophoresis in 4–20% SDS polyacrylamide gels (Daiichi Pure Chemicals Co., Tokyo, Japan), and electrophoretic transfer to a nitrocellulose membrane (Immobilon, Millipore Co., Bedford, UK) was performed.

The membrane was saturated for 30 min at room temperature with blocking buffer (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1% Tween 20, and 4% skim milk) and incubated with anti-JAM-A, anti-occludin, anti-tricellulin, anti-claudin-1, -4, -7, anti-Rap1, anti-β1 integrin, and anti-actin antibodies at room temperature for 1 h. The membrane was incubated with HRP-conjugated anti-mouse and anti-rabbit IgG antibodies at room temperature for 1 h. The immunoreactive bands were detected using an ECL Western blotting system.

Immunocytochemistry. hTERT-transfected HNECs grown in 35-mm glass-coated wells (Iwaki, Chiba, Japan), were fixed with cold acetone and ethanol (1:1) at −20 °C for 10 min. After rinsing in PBS, the cells were incubated with anti-JAM-A, anti-occludin, and anti-claudin-1, -4, and -7 antibodies overnight at 4 °C. Alexa Fluor 488 (green)-conjugated anti-rabbit IgG and Alexa Fluor 592 (red)-conjugated anti-mouse IgG (Invitrogen) were used as secondary antibodies. The specimens were examined and photographed with an Olympus IX 71 inverted microscope (Olympus Co., Tokyo, Japan).

Measurement of transepithelial electrical resistance (TER). hTERT-transfected HNECs were cultured to confluence on inner chambers of 12-mm Transwell inserts with 0.4-µm pore-size filters (Corning Life Sciences). TER was measured using an EVOM voltameter with an ENDOHM-12 (World Precision Instruments, Sarasota, FL) on a heating plate (Fine, Tokyo, Japan) adjusted to 37 °C. The values were expressed in standard units of ohms per square centimeter and presented as the mean ± SD. For calculation, the resistance of blank filters was subtracted from that of filters covered with cells.

Data analysis. Signals were quantified using Scion Image Beta 4.02 Win (Scion Co., Frederick, MA). Each set of results shown is representative of at least three separate experiments. Results are given as means ± SE. Differences between groups were tested by ANOVA followed by a post hoc test and an unpaired two-tailed Student's *t*-test and considered to be significant when $p < 0.05$.

Results

Expression of Toll-like receptors in hTERT-transfected HNECs

To investigate expression patterns of TLR1–10 in hTERT-transfected HNECs, RT-PCR was carried out compared to primary cultured HNECs. As shown in Fig. 1, mRNAs of all TLRs were detected in hTERT-transfected HNECs and the expression patterns were similar to primary cultured HNECs.

Induction of secretion of IL-8 and TNF-α from hTERT-transfected HNECs after treatment with TLR ligands

It is reported that TLR ligands induce several proinflammatory cytokines from human bronchial epithelial cells (Sha et al., 2004; Koff et al., 2008) and nasal polyp epithelial cells (Wang et al., 2007). To investigate which TLR ligands induce secretion of proinflammatory cytokines from hTERT-transfected HNECs, we measured secretion of IL-8 and TNF-α in the cultured medium at 24 h after treatment with 1 µg/ml TLR2 ligand P₃CSK₄, 25 µg/ml TLR3 ligand poly(I:C), 100 ng/ml TLR4 ligand LPS, 5 µg/ml TLR7/8 ligand CL097, 1 µg/ml TLR8 ligand ssRNA40/LyoVec, and 5 µM TLR9 ligand ODN2006.

Treatment with P₃CSK₄, poly(I:C) and ODN2006 significantly induced secretion of IL-8 (P₃CSK₄: 29.33 ± 1.12 ng/ml; poly(I:C): 87.06 ± 7.84 ng/ml; ODN2006: 10.94 ± 0.39 ng/ml) compared to the control (4.47 ± 0.46 ng/ml) (Fig. 2A). Treatment with only poly(I:C) significantly induced secretion of TNF-α (268.61 ± 20.47 pg/ml) compared to the control (9.94 ± 5.62 pg/ml) (Fig. 2A).

Reduction of protein expression of JAM-A in hTERT-transfected HNECs after treatment with poly(I:C)

It is known that intestinal epithelial barrier function and expression of tight junction molecules are changed after treatment

Table 1
Primers for RT-PCR.

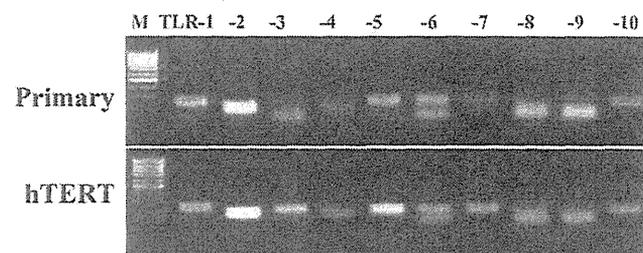| Gene	Forward primer	Reverse primer	Product size (bp)
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452
TLR1	GGGTCAGCTGGACTTCAGAG	AAAATCCAAATGCAGGAACG	214
TLR2	TGATGCTGCCATTCTCATTC	CGCAGCTCTCAGATTTACCC	157
TLR3	AGCCTTCAACGACTGATGCT	TTTCCAGAGCCGTGCTAAGT	201
TLR4	CCATAAAAGCCGAAAGGTGA	CTGAGCAGGGTCTTCTCCAC	159
TLR5	GGAACCAGCTCCTAGCTCCT	AAGAGGGAAACCCCAGAGAA	196
TLR6	AGGGCTGGCCTGATTCTTAT	TGGCACACCATCCTGAGATA	202
TLR7	CCTTGAGGCCAACAACATCT	GTAGGGACGGCTGTGACATT	201
TLR8	TCCTTCAGTCGTCAATGCTG	CGTTTGGGGAACTTCCTGTA	167
TLR9	CAGCAGCTCTGCAGTACGTC	AAGGCCAGGTAATTGTCACG	224
TLR10	ACTTTGCCCACCACAATCTC	CCCAGAAAAGCCCACATTTA	174
TNFR1	TCACCGCTTCAGAAAACCACC	TCACTCCAATAATGCCGGTACT	148

Fig. 1. RT-PCR for Toll-like receptors (TLRs) in primary cultured HNECs and hTERT-transfected HNECs. The mRNAs of TLR1–10 are detected in both types of cells. Primary: primary cultured HNECs, hTERT: hTERT-transfected HNECs. M, 100-bp ladder DNA marker.
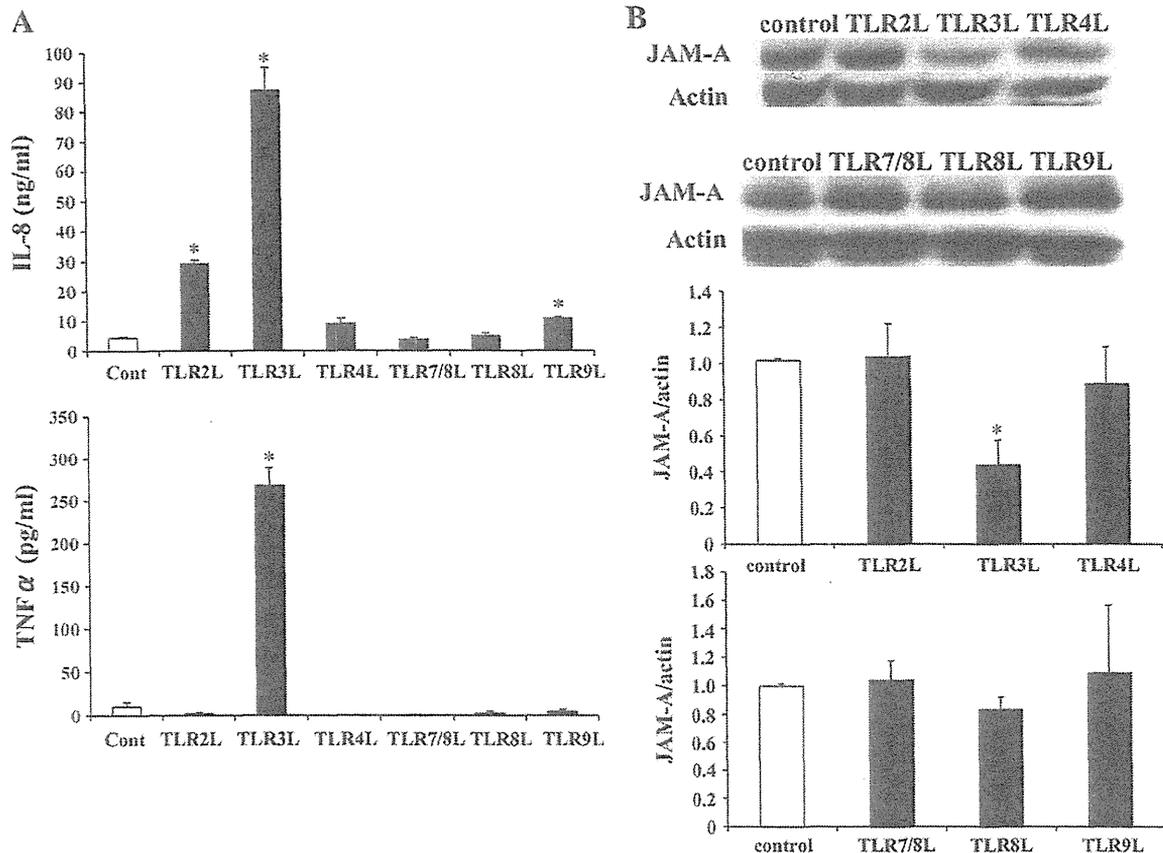
Fig. 2. ELISA (A) for IL-8 and TNF-α in the culture medium and Western blotting (B) for JAM-A in hTERT-transfected HNECs at 24 h after treatment with 1 µg/ml TLR2 ligand (TLR2L) P3CSK4, 25 µg/ml TLR3L poly(I:C), 100 ng/ml TLR4L LPS, 5 µg/ml TLR7/8L CL097, 1 µg/ml TLR8L ssRNA40/LyoVec, and 5 µM TLR9L ODN2006. The corresponding expression levels of JAM-A are shown as a bar graph. $n = 3$, *$p < 0.05$ versus control. Results are given as means ± SE. Differences between groups were tested by an unpaired two-tailed Student's t-test.

with the TLR2 ligand (Cario et al., 2004, 2007). To investigate which TLR ligands affected expression of tight junction molecules in hTERT-transfected HNECs, we treated the TLR ligands for 24 h and examined changes in expression of tight junction molecules by Western blotting. Protein expression of JAM-A but not claudin-1, -4, -7, occludin, or tricellulin was significantly decreased after treatment with poly(I:C) (Fig. 2B). No changes in protein expression of other tight junction molecules after treatment with P3CSK4, poly(I:C), LPS, CL097, ssRNA40/LyoVec, and ODN2006 were observed (Supplement data 1).

Induction of secretion of IL-8 and TNF-α and reduction of protein expression of JAM-A in hTERT-transfected HNECs after treatment with poly(I:C) in dose- and time-dependent manners

To investigate the detailed changes of secretion of IL-8 and TNF-α and protein expression of JAM-A after treatment with poly(I:C), the hTERT-transfected HNECs were treated with 1–25 µg/ml poly(I:C) from 2 to 24 h. The cytotoxicity at 24 h after treatment with 1–25 µg/ml poly(I:C) was not observed (Supplement data 2). The secretion of IL-8 was significantly increased from 1 µg/ml and production of TNF-α was significantly increased from 1 µg/ml in a dose-related manner (Fig. 3A). The protein expression of JAM-A was significantly decreased from 1 µg/ml in a dose-related manner (Fig. 3A). By treatment with 25 µg/ml poly(I:C), the secretion of IL-8 and TNF-α was significantly increased from 4 h and thereafter at 24 h, respectively (Fig. 3B). The

protein expression of JAM-A was significantly decreased at 24 h after treatment with 25 µg/ml poly(I:C) (Fig. 3B).

Change in distribution of JAM-A and barrier function in hTERT-transfected HNECs after treatment with poly(I:C)

To investigate changes in localization of tight junction molecules in hTERT-transfected HNECs at 24 h after treatment with 25 µg/ml poly(I:C), we performed immunocytochemistry for JAM-A, claudin-1, -4, -7, and occludin. Expression of JAM-A and occludin in part disappeared at cell borders of some cells after treatment with poly(I:C) (Fig. 4). No changes in distribution of claudin-1, -4, and -7 were observed compared to the control (Supplemental data 1).

To investigate the effects of poly(I:C) on the tight junction barrier function of hTERT-transfected HNECs, the cells were treated with treatment with 5 and 25 µg/ml poly(I:C) and were examined for TER. No change in the barrier function measured as TER was observed until 24 h after treatment with poly(I:C) compared to the control (Fig. 5).

Inhibition by ssDNA ODN2006 on changes in production of IL-8 and TNF-α and JAM-A expression in hTERT-transfected HNECs after treatment with poly(I:C)

It is reported that ssDNAs, including ODN2006, inhibit cytokine production induced by TLR3 activation (Ranjith-Kumar et al.,
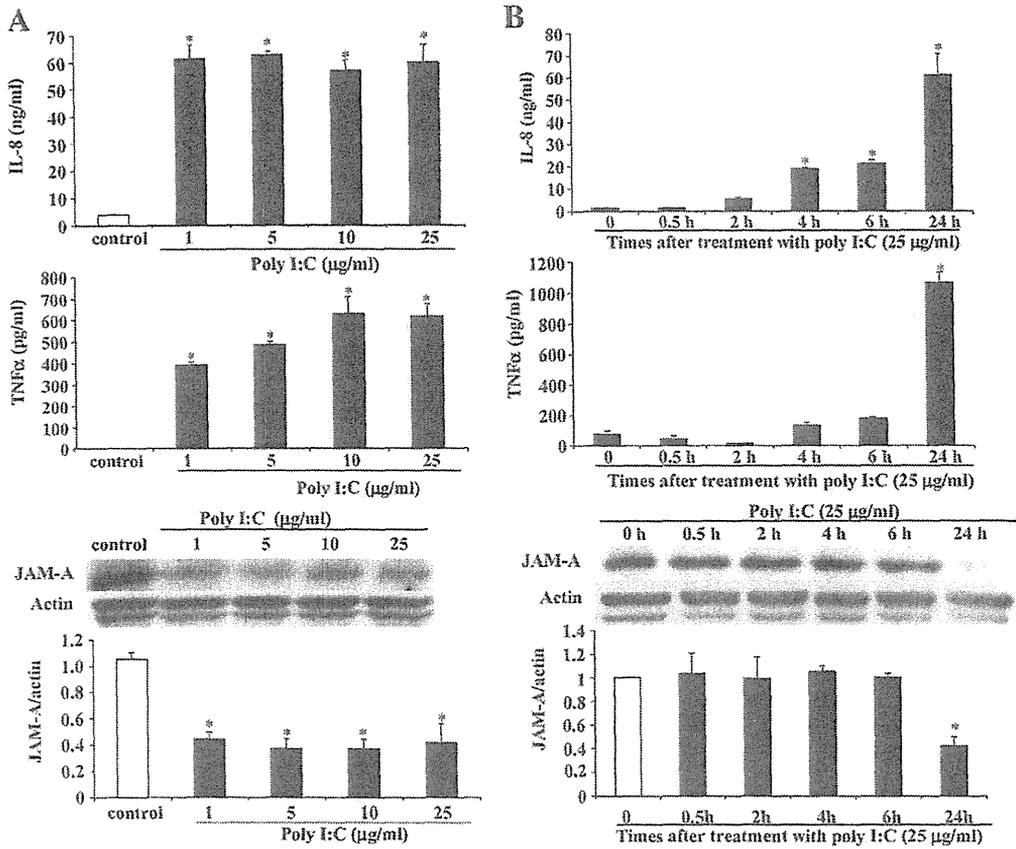
Fig. 3. (A) ELISA for IL-8 and TNF-α and Western blotting for JAM-A in hTERT-transfected HNECs at 24 h after treatment with 1–25 μg/ml poly(I:C). (B) ELISA for IL-8 and TNF-α and Western blotting for JAM-A in hTERT-transfected HNECs from 2 to 24 h after treatment with 25 μg/ml poly(I:C). The corresponding expression levels of JAM-A are shown as a bar graph. $n = 3$, *$p < 0.05$ versus control. Results are given as means ± SE. Differences between groups were tested by an unpaired two-tailed Student's t-test.
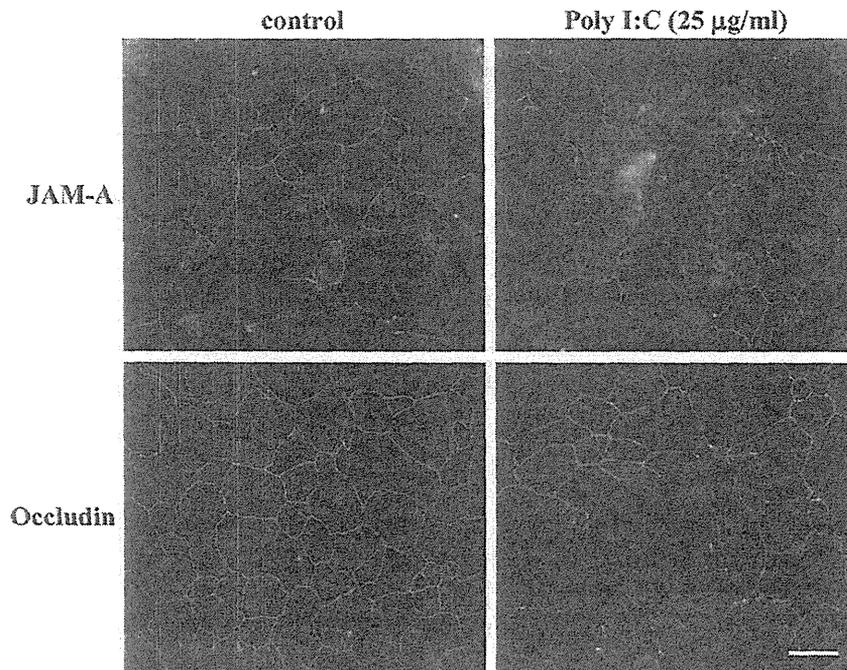

Fig. 4. Immunostaining for JAM-A and occludin in hTERT-transfected HNECs at 24 h after treatment with 25 μg/ml poly(I:C). Scale bar = 20 μm.
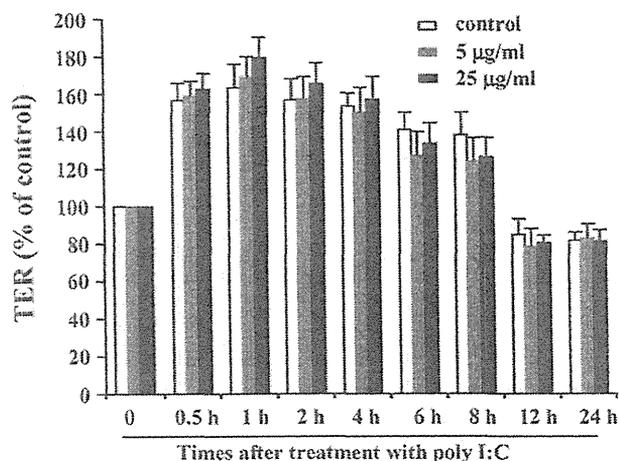
Fig. 5. Barrier function measured as TER in hTERT-transfected HNECs treated with 5 and 25 μg/ml poly(I:C). $n = 3$. Results are given as means ± SE. Differences between groups were tested by ANOVA followed by a post hoc test.

2008). To investigate whether ssDNAs inhibited changes in secretion of IL-8 and TNF-α and expression of JAM-A in hTERT-transfected HNECs after treatment with poly(I:C), the cells were pretreated with 5 μM ODN2006 at 30 min before treatment with 25 μg/ml poly(I:C). The pretreatment with ODN2006 completely inhibited induction of secretion of IL-8 and TNF-α at 24 h after

treatment with poly(I:C) (Fig. 6A). The reduction of protein expression of JAM-A at 24 h after treatment with poly(I:C) was significantly inhibited by pretreatment with ODN2006 (Fig. 6A).

Changes in secretion of IL-8 and TNF-α, and expression of JAM-A in hTERT-transfected HNECs after treatment with poly(I:C) via EGFR

It has been reported that TLR activation induces proinflammatory cytokines through activation of EGFR in human bronchial epithelial cells (Koff et al., 2008). To investigate whether changes in secretion of IL-8 and TNF-α and expression of JAM-A in hTERT-transfected HNECs after treatment with poly(I:C) are regulated via EGFR, the cells were pretreated with 10 μM EGFR inhibitor AG1478 at 30 min before treatment with 25 μg/ml poly(I:C). Pretreatment with AG1478 significantly inhibited induction of secretion of IL-8 and of TNF-α and reduction of protein expression of JAM-A after treatment with poly(I:C) (Fig. 6B).

Effects of inhibitors of various signal transduction pathways on secretion of IL-8 and TNF-α and expression of JAM-A in hTERT-transfected HNECs after treatment with poly(I:C)

TLR3 ligand stimulation is known to induce multiple signal transduction pathways, including PI3K and MAPK (Sarkar et al., 2004; Bérubé et al., 2009). To investigate which signal transduction pathways affected the changes in secretion of IL-8 and TNF-α and expression of JAM-A in hTERT-transfected HNECs after treatment with poly(I:C), the cells were pretreated with 10 μM
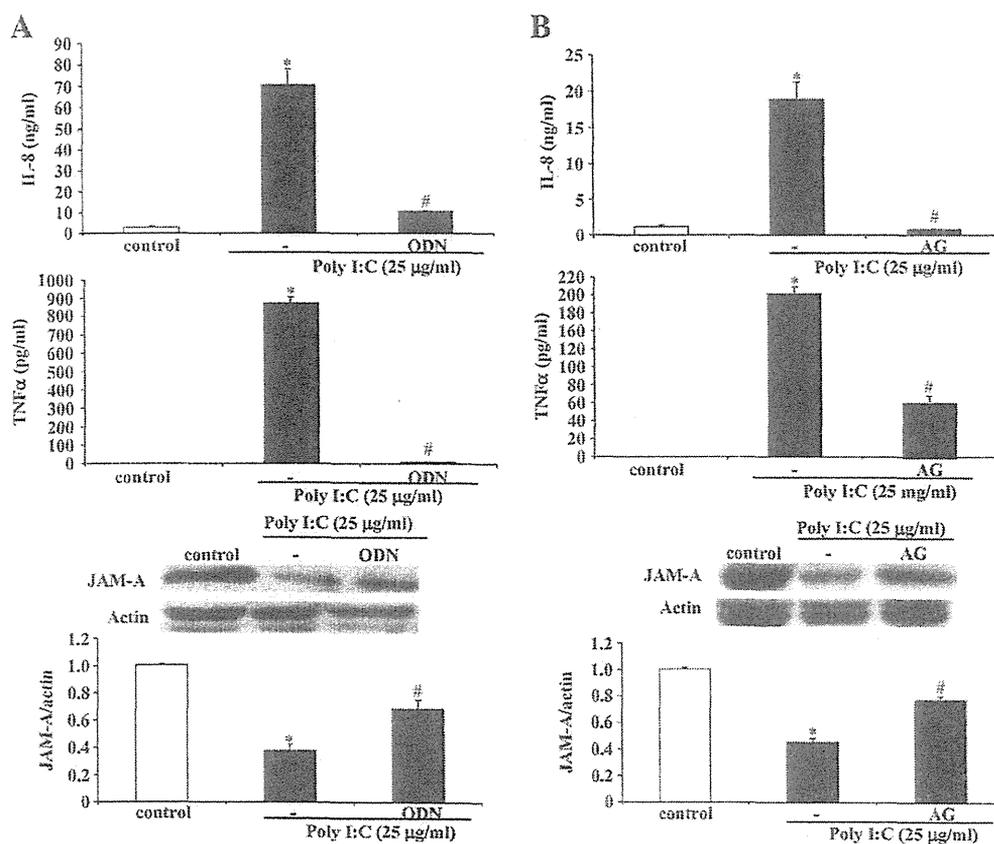
Fig. 6. (A) ELISA for IL-8 and TNF-α and Western blotting for JAM-A in hTERT-transfected HNECs pretreated with 5 μM ODN2006 at 30 min before treatment with 25 μg/ml poly(I:C) for 24 h. (B) ELISA for IL-8 and TNF-α and Western blotting for JAM-A in hTERT-transfected HNECs pretreated with 10 μM AG1478 at 30 min before treatment with 25 μg/ml poly(I:C) for 24 h. The corresponding expression levels of JAM-A are shown as a bar graph. $n = 3$. $^*p < 0.05$ versus control, $^\#p < 0.05$ versus poly(I:C). Results are given as means ± SE. Differences between groups were tested by an unpaired two-tailed Student's t-test. ODN: ODN2006, AG: AG1478.

PI3K inhibitor LY294002, 10 μM ERK1/2 inhibitor PD98059, 10 μM p38MAPK inhibitor SB203580, 10 μM JNK inhibitor SP600125 at 30 min before treatment with 25 μg/ml poly(I:C). The induction of secretion of IL-8 and TNF-α in hTERT-transfected HNECs after treatment with poly(I:C) was significantly prevented by all the inhibitors (Fig. 7A). The reduction of protein expression of JAM-A was significantly prevented by LY294002 and SB203580 but not PD98059 or SP600125 (Fig. 7A).

Changes in secretion of IL-8 and TNF-α and expression of JAM-A in hTERT-transfected HNECs after treatment with poly(I:C) via NF-κB

TLR3-mediated NF-κB signaling plays an important regulatory role in esophageal epithelial homeostasis (Lim et al., 2009). To investigate whether changes in secretion of IL-8 and TNF-α and expression of JAM-A in hTERT-transfected HNECs after treatment with poly(I:C) were regulated via NF-κB, the cells were pretreated with 1–10 μM NF-κB inhibitor IMD-0354 at 30 min before treatment with 25 μg/ml poly(I:C). As shown in Fig. 6B, the induction of secretion of IL-8 and TNF-α, expression of phospho-IκB-α, and reduction of expression of JAM-A after treatment with poly(I:C) were completely inhibited by pretreatment from 1 μM IMD-0354 (Fig. 7B).

Discussion

TLRs play an important role in innate immunity by recognizing pathogens and initiating controlled immune responses to eliminate

harmful microorganisms in airway epithelium (Bals and Hiemstra, 2004; Mayer and Dalpke, 2007). TLR3 recognizes viral dsRNA and its synthetic analogue poly(I:C) and stimulates innate immune responses in airway. In the present study, we first found that in upper airway epithelial cells (HNECs), treatment with poly(I:C) significantly reduced expression of the tight junction protein JAM-A and induced secretion of proinflammatory cytokines IL-8 and TNF-α via distinct NF-κB pathways.

Poly(I:C), a synthetic analog of viral dsRNA and a well-characterized ligand for TLR3, induces interferon and inflammatory cytokine/chemokine production in the epithelial cells (Matsumoto and Seya, 2008). However, the effects on epithelial tight junctions of treatment with poly(I:C) remain unclear. In the present study, hTERT-transfected HNECs, which detected mRNAs of TLR1–10, were treated with TLR2 ligand P_3CSK_4, TLR3 ligand poly(I:C), TLR4 ligand LPS, TLR7/8 ligand CL097, TLR8 ligand ssRNA40/LyoVec, and TLR9 ligand ODN2006. Treatment with only poly(I:C) significantly reduced expression of the tight junction protein JAM-A together with induction of secretion of proinflammatory cytokines IL-8 and TNF-α. No changes in expression of the tight junction proteins occludin, claudin-1, -4, -7, and tricellulin and in barrier function measured as TER were observed in hTERT-transfected HNECs treated with poly(I:C) (Fig. 5, Supplemental data 1).

JAM-A is expressed at tight junctions of endothelial and epithelial cells (Martin-Padura et al., 1998; Bazzoni et al., 2000; Ebnet et al., 2000; Liu et al., 2000; Ebnet et al., 2001; Itoh et al., 2001; Mandell et al., 2004; Severson et al., 2008, 2009). Although an important role
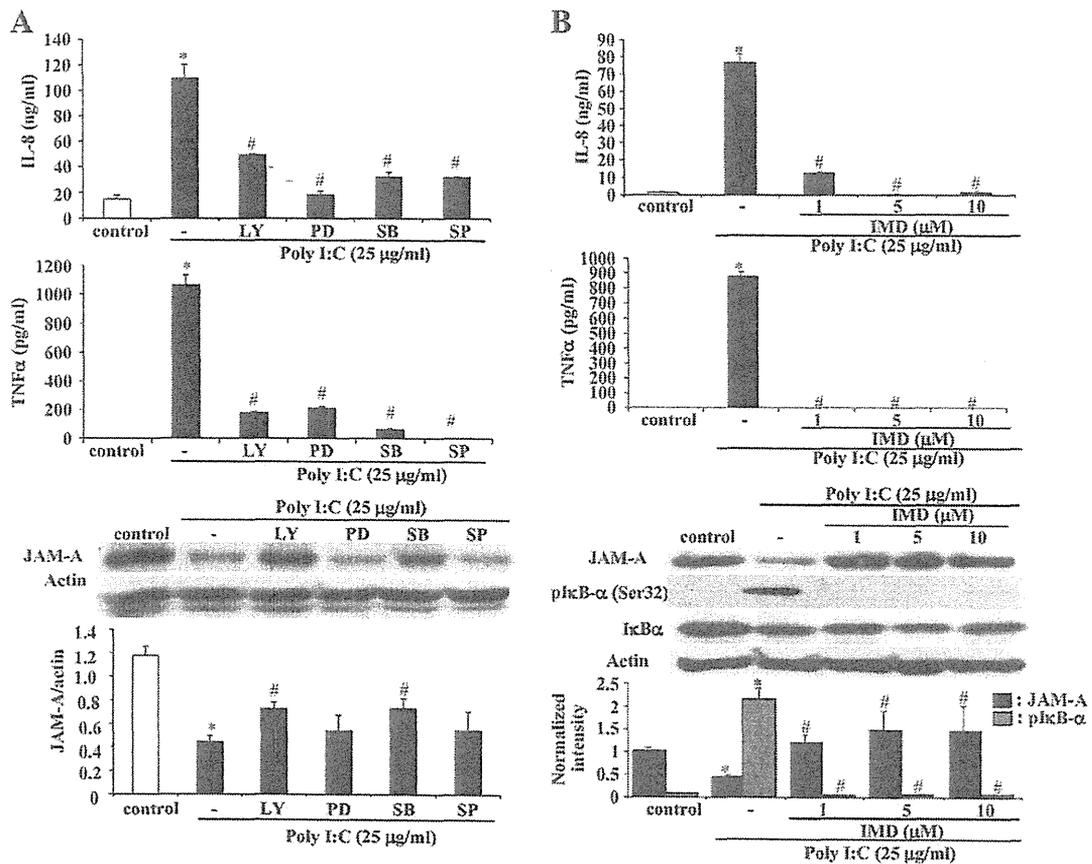
Fig. 7. (A) ELISA for IL-8 and TNF-α and Western blotting for JAM-A in hTERT-transfected HNECs pretreated with 10 μM LY294002, 10 μM PD98059, 10 μM SB203580, and 10 μM SP600125 at 30 min before treatment with 25 μg/ml poly(I:C) for 24 h. (B) ELISA for IL-8 and TNF-α and Western blotting for JAM-A and phospho-IκB-α in hTERT-transfected HNECs pretreated with 1–10 μM IMD-0354 at 30 min before treatment with 25 μg/ml poly(I:C) for 24 h. The corresponding expression levels of JAM-A and phospho-IκB-α are shown as a bar graph. $n = 3$, *$p < 0.05$ versus control, #$p < 0.05$ versus poly(I:C). Results are given as means ± SE. Differences between groups were tested by an unpaired two-tailed Student's *t*-test. LY: LY294002, PD: PD98059, SB: SB203580, SP: SP600125.

for JAM-A in vivo and in vitro is the regulation of barrier function (Liu et al., 2000; Mandell et al., 2004), the mechanisms by which this occurs are as yet unknown compared to other integral tight junction proteins, including claudins.

On the other hand, it is thought that JAM-A is a multifunctional surface protein-regulating colonic mucosal homeostasis (Severson and Parkos, 2009). In JAM-A knockout mice, not only intestinal permeability but also inflammation and cellular proliferation are increased, compared to wild-type controls (Laukoetter et al., 2007). JAM-A regulates cell migration by dimerization, and facilitates formation of a complex with Afadin and PDZ-GEF2 that activates Rap1A, which regulates the beta1 integrin level (Severson et al., 2009). More recent data have indicated that JAM-A function is mediated by outside-in signaling through its ability to dimerize (Severson and Parkos, 2009). In the present study, treatment with poly(I:C) reduced expression of JAM-A in HNECs without a change of barrier function. However, the reduction of JAM-A by poly(I:C) did not affect expression of Rap1 and b1 integrin in HNECs (Supplemental data 3). This result suggested that in upper airway epithelial cells HNECs, JAM-A may be mediated through activation of distinct scaffolding and signaling molecules to maintain mucosal homeostasis.

It is well known that poly(I:C) induces proinflammatory cytokine/chemokine production via distinct complex signal transduction pathways in airway epithelial cells. (1) Treatment with ssDNAs, including ODN2006, inhibits production of proinflammatory cytokines after stimulation with poly(I:C) via competition between ssDNAs and poly (I:C) for interaction with the same binding site in the extracellular domain of TLR3 (Ranjith-Kumar et al., 2008). (2) TLR3 ligands produce IL-8 in airway epithelial cells via a DUOX1/

ROS/TACE/TGFα/EGFR phosphorylation pathway (Koff et al., 2008). (3) The PI3K–Akt pathway plays an essential role in TLR3-mediated gene induction (Sarkar et al., 2004). More recently, it was reported that the increase of IL-8 induced by poly (I:C) in human airway epithelial cells was regulated via p38 MAPK and MAPK (Bérubé et al., 2009). (4) TLR3 triggering activates NF-κB and IRF3 transcription factors to initiate innate immune response through the induction of proinflammatory cytokines/chemokines (Vercammen et al., 2008). TLR3-induced innate immune responses in airway epithelial cells are also dependent on the NF-κB pathway (Bérubé et al., 2009).

In the present study, to investigate which signal transduction pathways affected the reduction of expression of JAM-A in hTERT-transfected HNECs after treatment with poly(I:C), we used various inhibitors considering (1–4) above and compared the changes of secretion of the proinflammatory cytokines IL-8 and TNF-α (Fig. 8).

The reduction of expression of JAM-A and induction of secretion of IL-8 and TNF-α in hTERT-transfected HNECs after treatment with poly (I:C) were prevented by ssDNA ODN2006, EGFR inhibitor AG1478, PI3K inhibitor LY24009, p38 MAPK inhibitor SB203580, and NF-κB inhibitor IMD-0354 (Fig. 8). The induction of secretion of IL-8 and TNF-α by poly (I:C) was also inhibited by MAPK inhibitor PD98059 and JNK inhibitor SP600125 (Fig. 8). These results indicated that the reduction of expression of JAM-A in HNECs after treatment with poly (I:C) was partly dependent on distinct signal transduction pathways as well as the mechanisms for the induction of proinflammatory cytokines.

Epithelial tight junctions are regulated by various proinflammatory cytokines (Coyne et al., 2002; Al-Sadi et al., 2009). In the present study, treatment with IL-8 and TNF-α induced by poly(I:C) did not
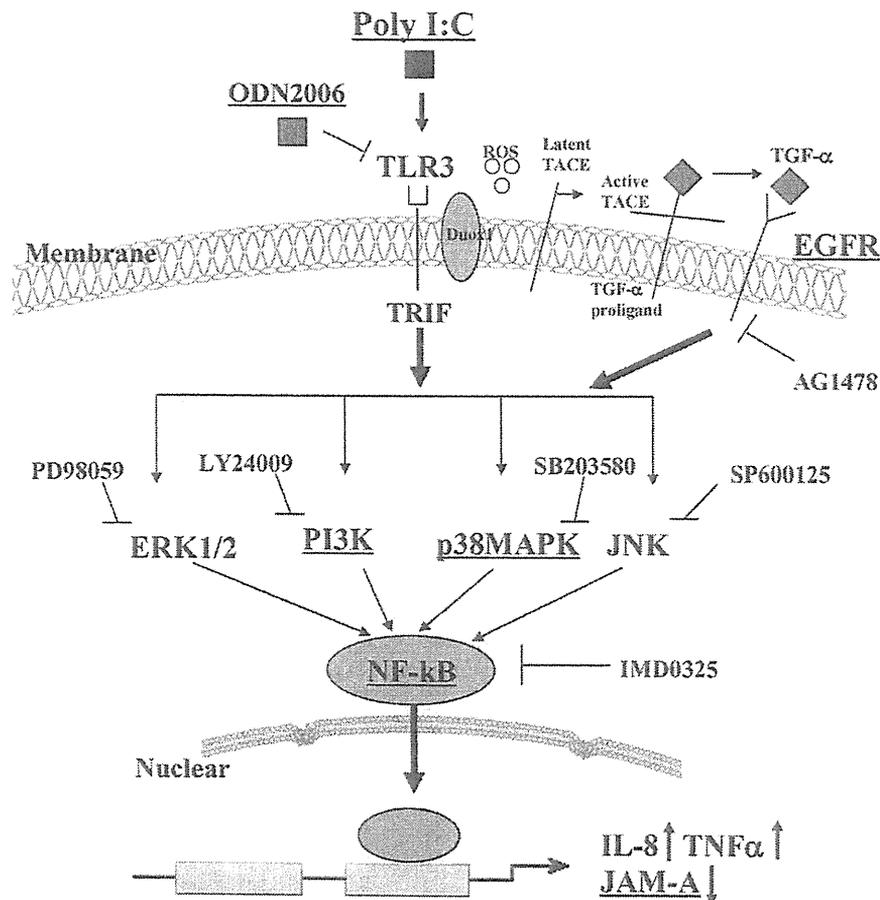

Fig. 8. Summary of upstream events in poly(I:C)-induced changes of IL-8, TNF-α, and JAN-A in hTERT-transfected HNECs.

affect the expression of JAM-A in hTERT-transfected HNECs, which expressed TNFR1 (Supplemental data 4). This indicated that the reduction of JAM-A by poly(I:C) was directly caused by the TLR3-mediated signal transduction pathway and not by the local elevation of IL-8 and TNF-α in an autocrine manner.

Rhinovirus dsRNA is recognized by TLR3 (Wang et al., 2009). Rhinoviruses induce mucin production via a TLR3-EGFR-dependent pathway in the airway (Zhu et al., 2009). On the other hand, rhinoviruses disrupt the barrier function of polarized airway epithelial cells (Sajjan et al., 2008). Furthermore, rhinovirus infection decreases the expression of ZO-1, occludin, claudin-1, and E-cadherin and reduces TER in primary cultured HNECs (Yeo and Jang, 2010). It is possible that there are different responses to viral dsRNA and the synthetic analogue poly(I:C) in regulation of tight junctions in HNECs.

In conclusion, in upper airway HNECs, the TLR3 ligand poly(I:C) reduces expression of the tight junction protein tight junction protein JAM-A via distinct signal transduction pathways and finally regulates it via the TLR3-mediated NF-κB pathway. The control of TLR3-mediated signaling pathways in HNECs may be important not only in infection by viral dsRNA but also in autoimmune diseases caused by endogenous dsRNA released from necrotic cells.

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Conflict of interest statement

No conflicts of interest are to be made.

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Role of fungal antigens in eosinophilia–associated cellular responses in nasal polyps: a comparison with enterotoxin

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Summary

Background Fungi and/or *Staphylococcus aureus* enterotoxins (SEs) may participate in the pathogenesis of eosinophilic inflammation in cases of chronic rhinosinusitis with nasal polyps (CRSwNP).
Objective We sought to determine the effects of fungal antigens on eosinophilia-associated cellular responses in nasal polyps.
Methods Dispersed nasal polyp cells (DNPCs) were prepared from 13 patients with CRSwNP. DNPCs were cultured with fungal extracts (*Aspergillus, Alternaria* and *Candida*) or SEB for 72 h, after which the levels of IL-5, IL-13 and RANTES were measured within the supernatant. Responses to β-D-glucan, mannan and chitin were also examined.
Results 38.5%, 69.2% and 30.8% of DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to 200 µg/mL of *Aspergillus*. 53.8%, 53.8% and 7.7% of DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to 200 µg/mL of *Alternaria*. 53.8%, 38.5% and 15.4% of DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to 200 µg/mL of *Candida*. All DNPCs produced these cytokines in response to 0.1 µg/mL of SEB. SEB induced significantly greater cytokine levels than the fungal extracts. No correlation between cytokine production following exposure to each of the fungal extracts or SEB and various clinical features, including nasal polyp eosinophilia and radiological severity of sinusitis was observed. Neither sensitization to fungus nor comorbidity with bronchial asthma was correlated with the fungal extract-induced cytokine production by DNPCs. β-D-glucan, mannan and chitin did not induce significant cytokine production.
Conclusions These results suggest that, although DNPCs produce IL-5, IL-13 and RANTES in response to fungal extracts, fungal antigens including major carbohydrates are less capable of inducing eosinophilia-associated cellular responses in nasal polyps than SEB.

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a major eosinophilic airway disease often associated with asthma and aspirin sensitivity [1]. The aetiology and pathophysiology of nasal polyps remain poorly understood and appears to be multifactorial; however, there is recent evidence to suggest that *Staphylococcus aureus* enterotoxins (SEs) and/or ubiquitous airborne fungi may play a role in the pathogenesis of eosinophilic inflammation [2, 3].

In CRSwNP, SE and SE-producing *S. aureus* are detected at high levels in nasal polyp specimens [4]. Lym-

phocytes in nasal polyps have altered Vβ-domains that are strongly associated with SE [5]. Sensitization to SE is observed in local tissue samples and serum samples from patients with CRSwNP, especially in asthmatic patients [6]. Patients demonstrating local SE sensitization display severe pathophysiological features, such as marked eosinophilia and high levels of eosinophilic cationic protein (ECP), IL-5, $LTC_4/D_4/E_4$, LTB_4 and LXA_4, compared with patients without local sensitization [7]. In addition, SEB induces the release of Th2-mediated cytokines in nasal polyps [8]. For example, we have recently demonstrated that dispersed nasal polyp cells (DNPCs) produce comparable amounts of IL-5, IL-13 and RANTES, three

eosinophilia-associated cytokines, in response to SEB [8]. Peripheral blood mononuclear cells (PBMCs) from patients with CRS also display a modest increase in IL-5 mRNA expression after SEB stimulation [9]. *In vivo* we have demonstrated that nasal exposure to SEB augments eosinophilic inflammation, IgE production and Th2-skewed cytokine production [10].

Using a novel collection and culture method, fungi are also highly prevalent in nasal mucus from patients with CRS, as well as healthy subjects [11, 12]. Airborne fungi can induce Th2 responses and eosinophilic inflammation in the airway both *in vivo* and *in vitro* [13–15]. For example, we have demonstrated that nasal exposure to *Aspergillus* without an adjuvant induces specific IgE/IgG1 production and local eosinophilia in mice [13]. *Alternaria* displays potent Th2-like adjuvant effects within airways, possibly by regulating the activity of dendritic cells in mice [14]. Furthermore, fungi and *Alternaria* in particular can directly induce eosinophil activation and degranulation [15]. Although the clinical effectiveness of topical antifungal agents, such as amphotericin B, on CRS is controversial, fungi may participate in the pathogenesis of eosinophilic inflammation in CRS [16–19]. Shin et al. [20] have shown that PBMCs from patients with CRS, but not healthy controls, produce IL-5 and IL-13, as well as IFN-γ, in response to fungal extracts, particularly *Alternaria*. On the contrary, Douglas et al. [9] have demonstrated that extracts of *Aspergillus* and *Alternaria* result in minimal changes in IL-5 and IFN-γ mRNA expression in PBMC. However, little is known about the role of fungal antigens in local eosinophilia-associated cellular responses.

The present study was designed to compare the effect of fungal antigens and SEB on *ex vivo* cellular responses that are closely associated with eosinophilic inflammation in DNPCs. Because DNPCs contain not only constitutive cells such as epithelial cells and fibroblasts but also infiltrating inflammatory cells, such as eosinophils, lymphocytes, mast cells and macrophages, this culture system ensures an intensive interplay between the different cell populations in nasal polyps [8]. In addition, effects of the major fungal carbohydrates contained within the fungal extracts (β-D-glucan, mannan and chitin) on the overall inflammatory responses were examined because these carbohydrates can act as adjuvants to induce eosinophilia-associated cellular responses [13, 21]. We believe that the findings presented here provide a further insight into the pathogenic role of colonizing *Staphylococcal aureus* vs. ubiquitous airborne fungi in CRSwNP.

Methods

Patients

Thirteen Japanese patients (age range: 22–65 years; mean age: 42.8 years; eight men and five women) with CRSwNP

were studied. CRSwNP was defined using the diagnostic criteria of Benninger et al. [22]. All patients were refractory to medical treatment, including macrolide therapy, and thus had endonasal sinus surgery. Three patients were asthmatic, and none was thought to have aspirin sensitivity based on a history of asthma attacks precipitated by NSAIDs. None of the participants received systemic steroids for a period of at least 8 weeks before surgery, and none received pharmacotherapy for sinusitis, such as macrolide antibiotics or intranasal steroids, for a period of at least 3 weeks before surgery. Before surgery, each patient's blood eosinophil count was measured. A radiological assessment of the severity of sinusitis was also performed in each patient according to the Lund–Mackay system [23]. Serum samples were analysed for their IgE specificity to *Aspergillus*, *Alternaria* and *Candida* using the CAP system (Phadia, Uppsala, Sweden). Sensitization to the fungi was defined as positive when the titre for either the three antigens was more than 0.7 kU allergen/L. Informed consent for participation in the study was obtained from each patient, and the study was approved by the Human Research Committee of the Okayama University Graduate School of Medicine and Dentistry.

Antigen and reagents

We purchased the following study materials: SEB (Toxin Technology, Sarasota, FL, USA), β-D-glucan, mannan, chitin, RPMI-1640, L-glutamine–penicillin–streptomycin solution, protease, collagenase, hyaluronidase and DNase I (Sigma, St Louis, MO, USA), as well as diclofenac sodium (Wako Pure Chemicals, Osaka, Japan), FCS (Invitrogen, Carlsbad, CA, USA) and red blood cell lysis buffer (Roche, Indianapolis, IN, USA). The crude extracts of *Aspergillus fumigatus*, *Alternaria alternaria* and *Candida albicans* were provided by Torii Co. (Tokyo, Japan). In brief, stationary liquid cultures in center mold medium from the fungi were left to grow for 1 week at room temperature. After the incubation, the liquid medium was filtered, concentrated and dialysed by ultrafiltration. Afterwards, the extract was subjected to sterilizing filtration and lyophilization. All the antigens were dissolved with phosphate buffered saline (Sigma), and the solubility was confirmed at inspection.

Cell cultures

DNPC were prepared from nasal polyps by enzymatic digestion, as described previously [8]. Briefly, the minced nasal polyps were incubated for 2 h at 37 °C in RPMI 1640 (Sigma) (1 g tissue per 4 mL) 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 μm cell strainer (BD Falcon, Bedford, MA, USA) to remove any undigested tissue and washed

two times with washing medium (RPMI 1640 supplemen-
ted with 2% FCS, 2 mM glutamine, 100 U/mL penicillin and
100 µg/mL streptomycin). The cell pellet was resuspended
in erythrocyte lysis buffer and washed with washing
medium. After washing, DNPC were suspended in culture
medium (RPMI 1640 supplemented with 10% FCS, 2 mM
glutamine, 100 U/mL penicillin and 100 µg/mL streptomy-
cin). 8.5±5.3%, 11.7±8.9%, 8.9±8.2%, 8.5±6.8%, 7.8±
11.1%, 10.9±10.5%, 15.5±6.7% and 21.6±7.7% cells in
DNPC express c-kit, ECP/EPX, CD79α, CD68, CD4, CD8,
cytokeratin and vimentin, respectively [8]. In flat-bot-
tomed 48-well culture plates (Asahi Techno Glass, Tokyo,
Japan), 500 µL of 1×10^6/mL DNPC were stimulated with
serial concentrations (2, 20 and 200 µg/mL) of *Aspergillus,
Alternaria, Candida* or SEB at 0.001, 0.01 and 0.1 µg/mL in
the presence of 10^{-5} M diclofenac at 37 °C in a 5% CO_2/air
mixture. Alternatively, cells were stimulated with serial
concentrations (1, 10 and 100 µg/mL) of β-D-glucan, man-
nan or chitin. As a control, DNPC were cultured without
antigen stimulation. In order to abrogate the inhibitory
effect of intrinsic PGE2 on cytokine production, DNPC
were pretreated with 10^{-5} M diclofenac because we have
demonstrated previously that the treatment with diclofe-
nac completely abolished spontaneous PGE2 production
by DNPC [8]. The culture supernatant was collected after
72 h and stored at −80 °C, after which the levels of IL-5, IL-
13 and RANTES were determined by ELISA [8]. Our
preparatory experiments revealed that fungal antigen-
specific cytokine production was increased over 72 h
incubation and maximal at 72 h in responders. Viability
was assessed by the exclusion of trypan blue stain.

Histological examination

Sections from surgically excised nasal polyps were stained
by haematoxylin/eosin solution to detect tissue eosino-
philia. Eosinophils were counted at high power (10×40) in
a blinded manner in the five fields with the greatest
cellular infiltration, after which the average number of
positive cells was determined [8].

Statistical analysis

Values are given as means±standard errors. Nonpara-
metric Mann–Whitney's U-test and Fisher's exact prob-
ability test were used for comparing data between groups,
and the Wilcoxon's signed-ranks test was used for analy-
sis within the group. If more than two groups were
compared, the Kruskal Wallis test were examined before
the use of Mann–Whitney's U-test. A correlation analysis
was performed using Spearman's correlation coefficient
by rank. P-values <0.05 were considered statistically
significant. Statistical analyses were performed with Stat-
View software (version 4.5, Abacus Concepts, Berkeley,
CA, USA).

Results

Aspergillus extract induced eosinophilia-associated cytokine production by DNPCs

Aspergillus stimulation of DNPCs induced significant IL-5
($P = 0.013$ and $P = 0.015$ at 20 and 200 µg/mL, respec-
tively), IL-13 ($P = 0.005$ and $P = 0.002$ at 20 and 200 µg/
mL, respectively) and RANTES ($P = 0.041$ at 200 µg/mL)
production (Fig. 1). Concentrations more than double the
control values were tentatively considered significant,
such that five (38.5%), nine (69.2%) and four (30.8%) out
of 13 DNPCs demonstrated significant production of IL-5,
IL-13 and RANTES, respectively, in response to 200 µg/mL
Aspergillus (Fig. 2). The dose response to *Aspergillus*
extract was relatively flat with peak production at either
20 or 200 µg/mL.

Alternaria extract induced eosinophilia-associated cytokine production by DNPCs

Seven (53.8%), seven (53.8%) and one (7.7%) out of 13
DNPCs produced IL-5, IL-13 and RANTES, respectively, in
response to 200 µg/mL of *Alternaria* (Fig. 2). Overall,
Alternaria stimulation of DNPCs-induced significant IL-5
($P = 0.009$ at 200 µg/mL) and IL-13 ($P = 0.003$ at 200 µg/
mL) production. However, *Alternaria* did not induce
RANTES production at 2 or 20 µg/mL, and inhibited
RANTES production at 200 µg/mL ($P = 0.019$) (Fig. 1).

Candida extract induced eosinophilia-associated cytokine production by DNPCs

Seven (53.8%), five (38.5%) and two (15.4%) out of 13
DNPCs produced IL-5, IL-13 and RANTES, respectively, in
response to *Candida* (Fig. 2). Overall, *Candida* stimulation
of DNPCs-induced significant IL-5 ($P = 0.023$ at 200 µg/
mL) and IL-13 ($P = 0.017$ at 200 µg/mL), but not RANTES,
production (Fig. 1).

Pathophysiological significance of increased IL-5, IL-13 and RANTES production in nasal polyps in response to fungal extracts

Eosinophils were detected in all of the nasal polyps,
ranging from 73 to 487 (mean 202.2) cells per field. The
amount of IL-5, IL-13 or RANTES in response to *Asper-
gillus* did not correlate either with the degree of eosino-
philia within nasal polyps or the radiological severity of
sinusitis or blood eosinophil counts (Table 1). In addition,
the degree of these pathophysiological parameters was
similar between patients with or without positive re-
sponses to *Aspergillus* (Fig. 3). Similarly, significant
correlations were not observed between cytokine produc-
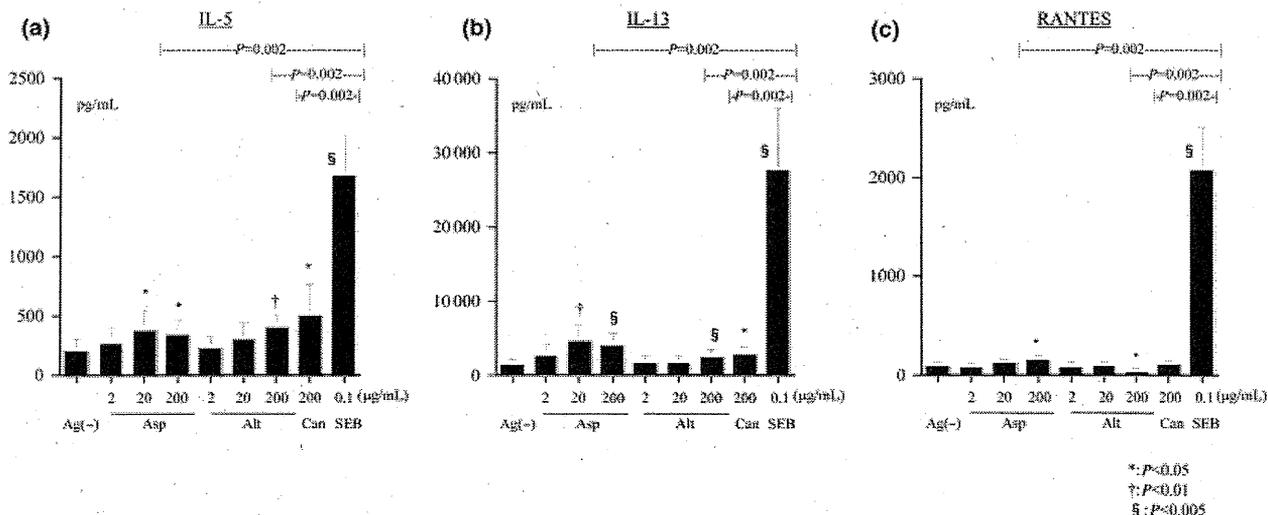tions following exposure to *Alternaria* or *Candida* and the

Fig. 1. Effect of fungal extracts on IL-5 (a), IL-13 (b) and RANTES (c) production by dispersed nasal polyp cells (DNPCs). Five hundred microlitres of 1×10^6/mL DNPCs were stimulated with *Aspergillus, Alternaria, Candida* or SEB in the presence of diclofenac. The culture supernatant was collected after 72 h, and then levels of the cytokines were determined by ELISA. Results were shown as means±standard errors of 13 cell cultures. *P*-values were determined by the Wilcoxon's signed-ranks test. *$P<0.05$, †$P<0.01$, §$P<0.005$.

clinical characteristics are outlined in Table 1. Patients with positive and negative responses to *Alternaria/Candida* demonstrated similar pathophysiological features (data not shown). Four out of 13 patients (30.8%) showed a sensitization to fungus (*Aspergillus, Alternaria* and/or *Candida*). Presence or absence of sensitization to fungus did not affect the cytokine production by DNPC (data not shown). In addition, comorbidity with bronchial asthma did not affect the production (data not shown).

SEB induced eosinophilia-associated cytokine production by DNPCs

DNPCs produced substantial amounts of IL-5, IL-13 and RANTES in response to 0.001 μg/mL of SEB, and the productions were dose dependently increased. Peak productions were archived at 0.1 μg/mL of SEB. All of 13 DNPCs responded to 0.1 μg/mL of SEB to produce significant amounts of IL-5, IL-13 and RANTES. Kruskal–Wallis test (*P*=0.005 for IL-5, *P*=0.002 for IL-13 and *P*<0.001 for RANTES) followed by the Mann–Whitney's *U*-test revealed that cytokine production was significantly greater in response to SEB compared with the fungal extracts (Figs 1 and 2). Like fungal antigens, responses to SEB did not correlate clinical parameters (data not shown).

Eosinophilia-associated cytokine production by DNPCs in response to carbohydrate on the fungal surface

Thirteen DNPCs were stimulated with serial concentrations (1, 10 and 100 μg/mL) of β-D-glucan, mannan and chitin. None of the carbohydrates induced a significant production of IL-5, IL-13 or RANTES. In fact, concentra-

tions of 1 μg/mL (*P* = 0.019) and 10 μg/mL (*P* = 0.041) of β-D-glucan inhibited RANTES production. Tentatively, taking concentrations more than double controls the levels to indicate significant production, only three (23.1%), two (15.4%) and one (7.7%) of the DNPCS-produced IL-5, IL-13 and RANTES, respectively, in response to β-D-glucan. Seven (53.8%), seven (53.8%) and two (15.4%) of the DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to mannan. Only five (38.5%), two (15.4%) and three (23.1%) of the DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to chitin.

Discussion

In the present study, we examined the effect of fungal antigens and SEB, the major candidate antigens involved in the pathogenesis of CRS, on *ex vivo* cellular responses closely associated with eosinophilic inflammation in DNPCs. Although several groups have investigated the role of fungal antigens and/or SEB in the pathogenesis of CRS using PBMCs, this is the first report comparing the effect of fungal antigens and SEB on local immune responses [9, 20].

Aspergillus, Alternaria and *Candida* induced IL-5 and IL-13 production by DNPCs. Both cytokines are involved in eosinophilic inflammation by mediating eosinophil differentiation, recruitment and survival [24]. This result is consistent with a report by Shin et al. [20] showing that PBMCs from patients with CRS, but not healthy controls, produce IL-5 and IL-13 in response to *Alternaria*. In nasal polyps, the majority of IL-5 producing cells are T cells, and mast cells/eosinophils can also express IL-5 [25]. Mononuclear cells and eosinophils in nasal polyps express IL-13
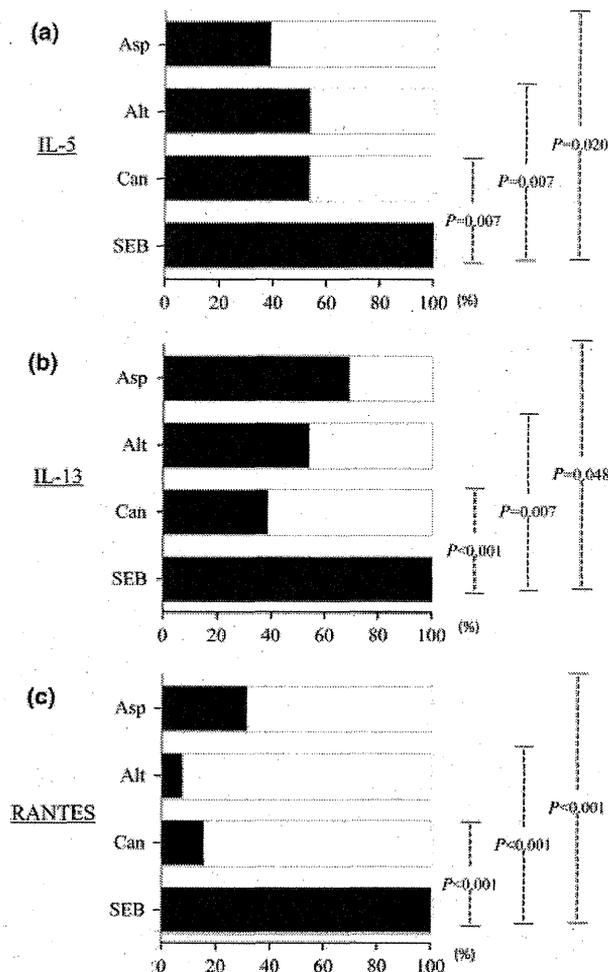
(a)

(b)

(c)

Fig. 2. Rates of positive responses of IL-5 (a), IL-13 (b) and RANTES (c) production to fungal extracts and SEB in DNPCs. Thirteen dispersed nasal polyp cells (DNPCs) were stimulated with 200 µg/mL of *Aspergillus*, 200 µg/mL of *Alternaria*, 200 µg/mL of *Candida* and 0.1 µg/mL of SEB. The response was considered as positive when the amount of cytokines produced by the antigens was over the twice of control. Black bars represent as a rate of positive response. Open bars represent as a rate of negative response. *P*-value was obtained through the use of Fisher's exact probability test.

[26]. Thus, fungal antigens may stimulate these inflammatory cells in nasal polyps directly and/or indirectly to produce IL-5 and IL-13.

On the other hand, *Aspergillus*, but not *Alternaria* or *Candida*, was observed to induce RANTES production by DNPCs. RANTES has also been associated with tissue eosinophilia in CRS [27]. In nasal polyps, RANTES is primarily detected in inflammatory cells and the epithelium [27]. *Aspergillus* contains both pan-allergens, including superoxide dismutase (Asp f6), and *Aspergillus*-specific allergens [28]. This suggests that molecules unique to *Aspergillus*, such as mitogillin (Asp f1), may participate in RANTES production by DNPCs [29].

As we demonstrated previously, DNPCs produced significant amounts of IL-5, IL-13 and RANTES in response to SEB [8]. In the present study, we compared cytokine production by DNPCs following exposure to fungal antigens and SEB. Fungal extracts at a concentration of 200 µg/mL produced significantly less IL-5, IL-13 and RANTES, than SEB at 0.1 µg/mL. This result is consistent with the results of a report by Douglas demonstrating that, in PBMCs from patients with CRS, stimulation with the fungal extracts *Aspergillus* and *Alternaria* at 100 PNU induced less expression of IL-5 mRNA than stimulation with SEB at a concentration of 0.1 µg/mL [9].

In addition, when concentrations more than double the control values were tentatively considered significant, a significant increase in cytokine production following exposure to each of the fungal extracts occurred significantly less frequently than following exposure to SEB, whereas SEB-induced cytokine production in 100% of DNPCs. These results suggest that fungal extracts are less potent at inducing eosinophilia-associated cytokine production by DNPCs compared with SEB. The major reason why we set double the control values as a cut-off point is that the spontaneous production of cytokines was observed in most diclofenac-treated DNPCs. In fact, DNPCs spontaneously produced 65.0±39.3 (1–514) pg/mL, 398.8±216.6 (0–2930) pg/mL and 58.3±11.5 (0–122) pg/mL of IL-5, IL-13 and RANTES, respectively, without antigen stimulation. This criterion might be severe, and values just below this cut-off point are considered negative. However, we may overestimate the results with no biological significance when we just analyse the observed values.

There are several explanations why responses to fungi might be lower than SEB. The responses to fungal extracts are likely to be antigen-specific responses, whereas responses to SEB represent superantigen responses. Although antigen-specific T lymphocytes are expected to be enriched in nasal polyps, they are still likely to be at a lower frequency than the frequency of T cells with Vβ elements responding to SEB. It is known that there is a skewing towards Vβ elements that recognize staphylococcal superantigens in nasal polyp tissue [5]. It has recently been demonstrated that ICAM-1 responds to SEB in DNPCs to induce cytokine production, while the interaction between HLA-DR and SEB in DNPCs has a limited effect on cytokine production [8]. It is well known that ICAM-1 is widely expressed in nasal polyps, including epithelial cells [30]. ICAM-1 can provide costimulation for SEB-induced cellular responses [31]. Recently, we demonstrated that intrinsic PGE2 inhibits SEB-induced cytokine production via the EP2-mediated pathway in nasal polyps [8]. In addition, we demonstrated that the treatment of diclofenac completely abolished the production of PGE2 by DNPC [8]. Thus, treatment with diclofenac negated the inhibitory role of PGE2, resulting in more cytokine

Table 1. Correlation of fungal extracts-induced cytokine productions and pathophysiological characterizations including nasal polyp eosinophilia, radiological severity of sinusitis, and blood eosinophil count

		Eosinophilia into nasal polyps	Radiological severity	Blood eosinophil count
Aspergillus				
IL-5	Correlation coefficient (ρ)	−0.087	0.201	0.106
	P-value	0.760	0.502	0.717
IL-13	Correlation coefficient (ρ)	−0.549	0.504	0.143
	P-value	0.057	0.083	0.621
RANTES	Correlation coefficient (ρ)	−0.144	0.187	0.323
	P-value	0.614	0.533	0.265
IL-5	Correlation coefficient (ρ)	−0.011	−0.364	0.429
	P-value	0.970	0.196	0.138
Alternaria				
IL-13	correlation coefficient (ρ)	−0.473	0.202	0.264
	P-value	0.102	0.496	0.361
RANTES	correlation coefficient (ρ)	0.302	−0.188	−0.038
	P-value	0.295	0.496	0.894
Candida				
IL-5	Correlation coefficient (ρ)	0.005	0.087	0.044
	P-value	0.985	0.781	0.879
IL-13	Correlation coefficient (ρ)	−0.247	0.188	0.154
	P-value	0.379	0.537	0.605
RANTES	Correlation coefficient (ρ)	0.246	−0.214	0.221
	P-value	0.396	0.437	0.446

Correlation coefficient (upper column) and P-value (lower column) were determined by Spearman's correlation coefficient by rank.

production. One more possible reason for lower responses to fungal extracts than SEB is that the relevant antigens in the fungal extracts may be a less concentrated stimulus than purified SEB.

No significant correlations were detected with regard to the amount of IL-5, IL-13 or RANTES produced following exposure to fungal extracts and various pathophysiological features, including nasal polyp eosinophilia, peripheral blood eosinophilia or radiological severity of sinusitis. Moreover, patients in whom fungal extracts induced cytokine responses showed similar pathophysiologic features compared with those with negative reactions. These results are consistent with recent clinical reports demonstrating that topical antifungal therapy has little effect on the pathophysiology of CRS [18, 19]. Together with the finding that fungi are detected in nasal mucus from almost all patients with CRS, as well as healthy subjects, these results suggest that fungi may not play as broad a role in the pathogenesis of CRS as suggested previously. However, our findings do not exclude the possibility that fungi may impact on the pathogenesis of particular subtypes of CRS, including allergic fungal rhinosinusitis in which fungi are known to cause type I and III allergic reactions, for which antifungal treatments may be more effective [32].

It is well known that fungal extracts contain abundant carbohydrate, including β-D-glucan, mannan and chitin [33]. Carbohydrates on the surface of fungi can participate in immune responses [12, 33–35]. For example, several carbohydrate moieties from the *Aspergillus* extract react with IgE or IgG. [33, 34]. They can bind to carbohydrate-specific receptors, including the mannose receptor and CR3 on antigen-presenting cells, resulting in efficient antigen presentation [35]. In addition, we have demonstrated that carbohydrates on *A. fumigatus* act as internal adjuvants leading to Th2 allergic inflammation, including nasal eosinophilia, in a murine model of allergic rhinitis [13]. However, none of the carbohydrates tested (β-D-glucan, mannan or chitin) induced significant production of IL-5, IL-13 or RANTES in DNPCs. These results suggest that, although fungal carbohydrates can participate in immune responses, they are not major triggers of eosinophilia-associated cytokine production in nasal polyps.

The present study has several limitations. First, the results presented in this study may not be observed in nonrefractory nasal polyps because all samples were taken from patients refractory to standard medical treatment. Second, although we have reported that IL-5, IL-13 and RANTES were produced by DNPCs in response to SEB even when diclofenac was not added [8], we do not examine whether IL-5, IL-13 and RANTES are produced in response to fungal antigens in the absence of diclofenac. The addition of diclofenac influences the results in such a way that results may not be extrapolated to an *in vivo* situation. Third, there were no healthy controls in this study. However, we cannot excise normal sinonasal tissues from healthy individuals because of ethical reasons. Fourth, only one concentration (200 µg/mL) of *Candida*