Vascular Endothelial Growth Factor Expression in Nasal Polyps of Aspirin-Intolerant Patients

Kai Fruth, MD; Chengjing Zhu, MD; Eduard Schramek; Johannes Angermair; Wassim Kassem; Boris R. Haxel, MD; Astrid Schneider; Wolf J. Mann, MD, PhD; Juergen Brieger, PhD

Objective: To study differences between aspirin-tolerant patients and aspirin-intolerant patients concerning vascular endothelial growth factor (VEGF) expression. Recent publications strongly suggest the involvement of VEGF and its receptors in the pathophysiologic process of nasal polyps.

Design: We subjected 43 polyp specimens to semiquantitative immunohistochemical analysis. We quantified VEGF and its receptors (Flk, Flt, and neuropilin) in all samples. To gain insight into potential VEGF-mediated cellular responses, we determined proliferative (Ki67) and apoptotic (caspase 3) indices.

Patients: Polyp samples were obtained from 22 aspirin-intolerant patients and from 21 aspirin-tolerant patients, and control specimens were obtained from 24 subjects with healthy nasal respiratory mucosa.

Setting: Laboratory; Department of Otorhinolaryngology–Head and Neck Surgery, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany.

Main Outcome Measures: Expression levels of VEGF, VEGF receptors, and proliferative and apoptotic indices.

Results: We found higher expressed levels of VEGF and neuropilin and stronger proliferation in nasal polyps from aspirin-tolerant and aspirin-intolerant patients compared with controls. In polyps from aspirin-intolerant patients, VEGF was expressed at considerably higher levels compared with those from aspirin-tolerant subjects. Apoptotic activity remained unchanged in all 3 groups.

Conclusions: Nasal polyps from aspirin-tolerant and aspirin-intolerant patients are characterized by strong proliferation and high levels of VEGF and neuropilin expression. Nasal polyps from aspirin-intolerant patients show distinctly increased VEGF levels. The relevance of these findings for future therapeutic approaches is yet to be determined.


CHRONIC RHINOSINUSITIS (CRS) is an inflammatory disease of the upper respiratory tract and one of the most common chronic diseases worldwide with high socioeconomic costs. Nasal polyp formation in CRS is characteristic and frequently associated with an intolerance reaction after aspirin intake, such as rhinorrhea, bronchospasm, and shock symptoms related to a non-IgE-mediated pharmacological hypersensitivity reaction and bronchial asthma, termed the Samter triad. The development of the complete triad can take years. Nasal polyps related to the Samter triad are additionally characterized by high recurrence rates after sinus surgery. The etiology of nasal polyps is still under controversial debate. Staphylococcal superantigens, airborne fungi, and the dysregulation of arachidonic acid metabolism as the crucial part of polyp formation within the Samter triad are under discussion.

Vascular endothelial growth factor (VEGF) is known to play an important role during the angiogenesis and modulation of capillary permeability. Nasal polyps are characterized by remarkable edema that could be explained by an upregulated VEGF release. Several studies have reported high levels of VEGF in the epithelium and endothelium of nasal polyps. However, other basic mechanisms of tissue homeostasis, such as proliferation and apoptosis, might be involved in nasal polyp formation and potentially regulated by VEGF, as demonstrated by Coste et al. and Lee et al. These authors found evidence of a VEGF-dependent inhibition of apoptosis in nasal mucosa and observed an increased cell growth rate in nasal polyps compared with control mucosa.
The expression of VEGF receptors (VEGFRs) is an essential precondition to allow any effect of VEGF on its target cells. Several types of VEGFRs, including Flk for VEGFR2, Flt for VEGFR1, and neuropilin for NRP1, have been identified. Receptors 1 and 2 for VEGF are the major receptors important for proper embryonic vasculature. Knockouts for VEGFR2 are nonviable, with solely minimal developed vasculature, whereas knockouts for VEGFR1 show a disorganized vasculature. Neuropilin 1 acts as a VEGF coreceptor to enhance VEGFR2 binding and bioactivity.14-17

Until now, differences between VEGF and VEGFR expression in nasal polyps from patients with aspirin intolerance compared with those without remained speculative. Identification of specificities at the molecular level could lead to a better understanding of the different phenotypic growth patterns with a potential therapeutic impact. Therefore, in the present immunohistochemical study, we analyzed the expression levels of VEGF and VEGFRs in healthy nasal tissue samples and in nasal polyps from aspirin-intolerant and aspirin-tolerant patients. To gain insight into potentially regulated downstream mechanisms, we also analyzed markers of proliferation (Ki67) and apoptotic activity (caspase 3). Finally, we outlined and discussed differences at the molecular level of nasal polyps from aspirin-tolerant and aspirin-intolerant patients.

**METHODS**

**SPECIMENS**

We collected a total of 67 specimens. Forty-three specimens of nasal polyps, including 22 from clinically determined aspirin-intolerant patients and 21 from aspirin-tolerant patients, were obtained during sinus surgery (30 male and 13 female patients; mean age, 48.8 years; range, 16-59 years). Twenty-four nasal mucosa samples serving as control specimens were taken from the inferior turbinates of patients undergoing surgery for nasal obstruction without any endoscopic and radiologic signs or symptoms of CRS (13 male and 11 female patients; mean age, 37.1 years; range, 16-59 years).

Chronic rhinosinusitis with nasal polyps was diagnosed by means of nasal endoscopy and computed tomography according to the guidelines of the 2007 European position paper on rhinosinusitis and nasal polyps.18 Aspirin intolerance was diagnosed using the patients’ history and symptoms. Patients with recurrent nasal polyps after sinus surgery combined with bronchial asthma and an intolerant reaction such as bronchospasm after aspirin intake were identified as aspirin intolerant. Patients who did not have signs of aspirin intolerance underwent primary surgery. We analyzed the expression of VEGF, VEGFRs (Flk for VEGFR2, Flt for VEGFR1, and neuropilin for NRP1), Ki67 (proliferation), caspase 3 (apoptosis), and the spatial distribution (endothelium vs epithelium) of these proteins by means of immunohistochemistry.
This study was approved by the institutional review board and performed in accordance with the actual version of the Declaration of Helsinki.

**IMMUNOHISTOCHEMICAL STAINING**

Biopsy specimens were snap frozen in liquid nitrogen immediately after resection and stored at −80°C until needed for procedures. Specimens were embedded in optimal cutting temperature medium (Cryomatrix; Shandon). We prepared 6-µm sections using a cryostat (Leica) and mounted them on glass slides (Superfrost Plus; Thermo Scientific). Sections and cells were fixed using ice-cold acetone (Sigma-Aldrich Corp), and endogenous peroxidases were blocked by 2.5% methanol/hydrogen peroxide (Roth). An avidin/biotin blocking kit (Zymed Laboratories) was used to reduce background staining. After 30 minutes of preincubation with 10% normal goat or normal rabbit serum (Dako), the primary antibodies consisting of polyclonal rabbit anti-VEGF (1:150 dilution; Santa Cruz Biotechnology), polyclonal rabbit anti-Flt (1:100 dilution; Santa Cruz Biotechnology), monoclonal mouse anti-Flk (1:150 dilution; Santa Cruz Biotechnology), polyclonal goat anti-NRP1 (1:150 dilution; Santa Cruz Biotechnology), monoclonal mouse anti-Ki67 (1:100 dilution; Dako), and polyclonal rabbit anti–caspase 3 (1:100 dilution; Cell Signaling Technology) were overlaid without washing and incubated overnight at 4°C. Slides were consecutively washed twice with Tris-buffered saline (TBS)/polyborate (Twee) wash buffer (400mM sodium chloride); incubated with the secondary antibodies (Dako) consisting of biotinylated goat anti-rabbit (1:250 dilution), goat antimouse (1:250 dilution), or rabbit antigoat (1:600 dilution) for 30 minutes; washed 2 times for 5 minutes with TBS/Tween wash buffer (147mM sodium chloride); incubated with horse serum-peroxidase–labeled streptavidin (1:200 dilution; Dako) for 30 minutes; and washed 2 times for 5 minutes with TBS/Tween wash buffer. Finally, the slides were incubated with diamobenzidine tetrahydrochloride (Dako) for 1 minute and washed with TBS/Tween wash buffer. The slides were counterstained with hematoxylin (1:5 dilution; Merck KGaA) for 5 minutes and washed with tap water for 10 minutes. All slides were then treated with an increasing isopropanol series (70%, 80%, 90%, and 100%; Hedinger GmbH & Co), treated 2 times for 5 minutes with xylol (Merck), and mounted with a coverslip. Sections incubated with TBS/Tween wash buffer instead of the primary antibodies served as negative controls, and normal kidney and placenta tissue served as positive controls (data not shown).

**QUANTIFICATION OF IMMUNOHISTOCHEMICAL STAINING**

We applied a computer-based analysis method to quantify the immunohistochemical staining results as described before.6,19 In brief, 3 representative areas (original magnification ×400, corresponding to 25 × 25 µm) were documented in jpg format using an inverted microscope (Axiovvert 200; Zeiss). For quantification, the jpg documents were analyzed using commercially available software (Photoshop; Adobe Systems Inc). All images of specimens from a given experimental group (control, aspirin tolerant, and aspirin intolerant) were imported in the same master file, and the staining results were analyzed in parallel. This method allows measuring of the stained area and the labeling intensity and hence the quantification and relative comparison of expression levels. Results were expressed as arbitrary units.

To calculate the proliferative and apoptotic activities of a given sample, we counted Ki67- and caspase 3–positive cells in 3 randomly selected grids (25 × 25 µm) at original magnification and then expressed the results as a percentage of all cells within the 3 grids (approximately 300 cells/grid).

**STATISTICAL ANALYSIS**

Differences among the groups (control and nasal polyps with and without aspirin intolerance) concerning continuous variables (VEGF, Flk, Flt, neuropilin, and Ki67) were analyzed by...
means of the nonparametric Kruskal-Wallis test. In the case of
\( P < .05 \), multiple comparisons were performed using pairwise
comparisons (Mann-Whitney test).

All \( P \) values are 2 sided. Analyses are not adjusted for mul-
tiple testing, and \( P \) values serve for description only. \( P < .05 \)
demonstrates a difference between the observed groups.

**RESULTS**

**IMMUNOHISTOCHEMICAL ANALYSIS OF VEGF, VEGFR, CASPASE 3, AND Ki67 IN PATIENT BIOPSY SPECIMENS**

We observed a strongly increased level of VEGF expres-
sion in epithelial cells of nasal polyps from aspirin-
tolerant patients compared with the controls (\( P = .01 \)). When
analyzing tissue samples from aspirin-intolerant patients,
we found higher VEGF levels compared with those from
aspirin-tolerant patients (and controls) (\( P < .001 \)). In the
endothelium, the level of VEGF expression in nasal pol-
yps from aspirin-intolerant patients was remarkably higher
compared with the controls (\( P = .004 \)) and compared with
nasal polyps from aspirin-tolerant patients (\( P = .049 \))
(\textbf{Figure 1} and \textbf{Figure 2}).

A precondition for a stimulatory activity of VEGF is
the expression of VEGFR on the potential target cells.
Therefore, we analyzed the expression of Flk, Flt, and
NRP1. Expression of Flk was comparable in the epithe-
lium and endothelium in all analyzed tissue samples (data
not shown). Staining for Flt yielded lower levels in the
epithelium of nasal polyps from aspirin-intolerant pa-
tients compared with the controls (\( P = .007 \)) and lower
levels in the endothelium of nasal polyps from aspirin-
tolerant compared with those from aspirin-tolerant pa-
tients (\( P = .046 \)), but the difference did not reach statis-
tical significance compared with the controls (\( P = .30 \))
(\textbf{Figure 3} and \textbf{Figure 4}). In the endothelium and epi-
thelium of nasal polyps, NRP1 levels were increased com-
pared with those in the controls. No differences were ob-
served between nasal polyps of aspirin-intolerant and
aspirin-tolerant patients (\textbf{Figure 5} and \textbf{Figure 6}).

The percentage of Ki67-positive cells, indicating pro-
liferating cells, was clearly higher in the epithelium of
nasal polyps from aspirin-intolerant (\( P = .01 \)) and
aspirin-tolerant patients (\( P = .001 \)) compared with the
controls (\textbf{Figure 7} and \textbf{Figure 8}) but did not differ
between aspirin-intolerant and aspirin-tolerant patients.
The staining of caspase 3 as an indicator of apoptotic
cells demonstrated a generally low level and did not
reveal any differences between the groups (data not shown).

**COMMENT**

Increasing evidence supports the concept of nasal polyp
growth related to VEGF tissue levels.\(^6\)-\(^13\) However, no dif-
ferentiation concerning aspirin intolerance has yet been
reported. In accordance with these reports, we found high
levels of VEGF in epithelial and endothelial cells of na-
sal polyps compared with healthy mucosa. In nasal pol-
yps from aspirin-intolerant patients, VEGF levels were
Figure 4. Representative Flt-stained samples. Epithelium samples were obtained from control specimens from healthy mucosa (A), polyps from aspirin-tolerant patients (B), and polyps from aspirin-intolerant patients (C). Endothelium samples were obtained from controls (D), polyps from aspirin-tolerant patients (E), and polyps from aspirin-intolerant patients (F). An intense membrane staining was found in the epithelium (A) and in the endothelium (D) of the controls. Staining was less intensive in tissue samples from aspirin-intolerant patients in the epithelium compared with the controls (C). Significant changes are indicated by an arrow (original magnification ×400).

Figure 5. Neuropilin 1 (NRP1) expression in epithelium and endothelium. A, High levels of NRP1 expression in the epithelium of nasal polyps from aspirin-intolerant and aspirin-tolerant patients compared with the control specimens from healthy mucosa. B, High levels of NRP1 expression in the endothelium of nasal polyps from aspirin-intolerant and aspirin-tolerant patients compared with controls. AU indicates arbitrary units. Graph elements are described in the legend to Figure 1.

also heightened compared with those of aspirin-tolerant patients. This key observation of our study demonstrates molecular differences between nasal polyps from aspirin-tolerant and aspirin-intolerant patients.
Furthermore, it is known that leukotrienes can transcriptionally activate VEGF production.\textsuperscript{20} One might speculate that the imbalance of leukotrienes and prostaglandins with a predominance of leukotrienes in aspirin-intolerant patients might lead to the observed additionally increased VEGF levels. Vascular endothelial growth factor is a strong mitogen of endothelial cells and a prosurvival factor for VEGFR-expressing cells in general. The VEGF homodimers bind Flk and Flt, causing receptor dimerization and therefore signal transduction. Simultaneously, VEGF can bind neuropilin, thereby enhancing signal transduction through Flk and Flt. Vascular endothelial growth factor receptors transmit the VEGF growth signal from outside the cell via tyrosine kinases to the nucleus, resulting in the upregulated transcription of a multitude of growth-promoting genes and in the direct upregulation of mitosis.\textsuperscript{14,17}

High levels of VEGF in polyps might activate VEGFRs and downstream pathways, resulting in increased proliferation, thereby promoting polyp formation. Therefore, we analyzed the expression of all 3 VEGFRs. We found the major VEGFR Flk expressed at comparable levels in the analyzed tissue samples, potentially indicating a basic susceptibility for VEGF stimulation. Levels of the coreceptor NRPI were found to be increased in polyps, and levels of Flt were lowered. Neuropilin 1 is known to enhance bioactivity and binding affinity of VEGF.\textsuperscript{17} One might suggest that high VEGF levels in combination with high levels of expressed NRPI receptors as found in nasal polyps, especially in those from aspirin-intolerant patients, are part of the pathophysiology of nasal polyp formation. These data are in accordance with the functional data reported by Lee et al.,\textsuperscript{15} who demonstrated by means of in vitro inhibitor experiments that proliferation in polyps is largely regulated by neuropilin but less so by Flt. Consequently, we observed increased proliferation in nasal polyps. Our observation of a low level of Flt expression strengthens the assumption of a minor impact of Flt regarding cell proliferation in nasal polyps.\textsuperscript{15}

Furthermore, our findings suggest an impact of VEGF on both epithelial and endothelial cells. One might assume that the impact of VEGF on epithelial
cells mainly results in cell proliferation, indicated by high levels of Ki67. In endothelial cells, VEGF might lead to increased vessel permeability and finally to the well-known characteristic tissue edema of nasal polyps according to the initially described function of VEGF as a vascular permeability factor. Surprisingly, heightened VEGF levels in aspirin-intolerant patients were not associated with additionally increased proliferation compared with the aspirin-tolerant patients. The enhanced VEGF expression in aspirin-intolerant patients obviously has no additional effect on epithelial cell growth but might stimulate tissue edema by enhanced vessel permeability of the endothelium.

Nasal polyps are characterized by high proliferation, VEGF expression, and neuropilin expression. Furthermore, nasal polyps from aspirin-intolerant patients show remarkably increased levels of VEGF that might be related to increased vessel permeability and the observed tissue edema. Tissue samples of the inferior turbinates from patients without CRS are widely used as controls; however, biological specificities compared with nonpolypoid osteomeatal complex tissue cannot be definitely excluded.

In addition to sinus surgery, current options for treatment of CRS include the application of systemic and topical corticosteroids to reduce inflammation, aspirin desensitization in patients with the Samter triad, nasal saline washings, and others. As a clinical consequence of our results, we should establish novel therapeutic attempts that modulate cell proliferation and VEGF expression locally and analyze their benefit within clinical trials with emphasis on polyp growth and quality of life.

In summary, our data suggest that nasal polyp growth of aspirin-tolerant and aspirin-intolerant patients differs with respect to VEGF expression. The definite clinical relevance of this observation needs further investigation.

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Correspondence: Kai Fruth, MD, Department of Otorhinolaryngology—Head and Neck Surgery, University Medical Center of the Johannes Gutenberg University Mainz, Langenbeckstrasse 1, 55101 Mainz, Germany (kai.fruth@unimedizin-mainz.de).

Author Contributions: Drs Fruth and Zhu contributed equally to this work. Drs Fruth and Briejer and Ms Schneider had full access to all the data in the study and take full responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Fruth and Briejer. Acquisition of data: Fruth, Zhu, Schramek, Angermair, Kassem, and Mann. Analysis and interpretation of data: Fruth, Zhu, Schramek, Haxel, Schneider, and Briejer. Drafting of the manuscript: Fruth, Zhu, Angermair, and Kassem. Critical revision of the manuscript for important intellectual content: Fruth, Schramek, Haxel, Schneider, Mann, and Briejer. Statistical analysis: Fruth, Schramek, and Schneider. Obtained funding: Briejer. Administrative, technical, and material support: Fruth, Haxel, and Briejer. Study supervision: Fruth, Mann, and Briejer.

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REFERENCES


Correction

Error in Byline and Author Contributions. In the article titled “Long-term Outcome of Radiofrequency Ablation for Intraoral Microcystic Lymphatic Malformation” by Kim et al, published in the December 2011 issue of the Archives (2011;137[12]:1247-1250), the surname of the second author was misspelled in the byline on page 1247 and Author Contributions section on page 1250. The name should have read “Katie Kavanagh, MD.”