

Vascular endothelial growth factor-mediated induction of angiogenesis by human rhinoviruses

Stelios Psarras, PhD,^a Eleni Volonaki, MD,^a Chrysanthi L. Skevaki, MD,^a Maria Xatzipsalti, MD,^a Apostolos Bossios, PhD,^a Harris Pratsinis, PhD,^b Stelios Tsigkos, MD,^c Dimitrios Gourgiotis, PhD,^a Andreas G. Constantopoulos, MD,^a Andreas Papapetropoulos, PhD,^{c,d} Photini Saxoni-Papageorgiou, PhD,^a and Nikolaos G. Papadopoulos, PhD^a Athens and Patros, Greece

Background: Human rhinoviruses, major precipitants of asthma exacerbations, infect the lower airway epithelium inducing inflammation. The possibility that viral infection may mediate angiogenesis, thus contributing to airway remodeling, has not been evaluated.

Objective: To investigate whether epithelial infection with rhinovirus mediates angiogenesis *in vitro*, evaluate possible modulation by an atopic environment, and confirm angiogenic factor induction after *in vivo* rhinovirus infection.

Methods: Bronchial epithelial cells were infected with rhinovirus and levels of vascular endothelial growth factor (VEGF), and angiopoietins were measured. The angiogenic effect of epithelial products was assessed in *in vitro* models of angiogenesis. PBMCs, obtained from patients with atopic asthma and normal controls, were exposed to rhinovirus; the ability of supernatants from these cultures differentially to affect rhinovirus-mediated epithelial VEGF production was evaluated. VEGF levels were measured in respiratory secretions from patients with asthma, before and during rhinovirus-induced exacerbations.

Results: Epithelial infection with rhinovirus specifically stimulated mRNA expression and release of VEGF, but not angiopoietins, in a time-dependent and dose-dependent manner. Supernatants from these cultures were able to induce angiogenesis *in vitro*, significantly inhibited by a neutralizing anti-VEGF antibody. When bronchial cells were exposed to supernatants of rhinovirus-infected mononuclear cells from normal subjects or atopic patients with asthma, VEGF induction was significantly higher under the influence of the

atopic environment. VEGF was elevated during rhinovirus-associated asthma exacerbations.

Conclusion: Rhinovirus infection, a frequent event, induces VEGF production in bronchial epithelial cells and human airways, an effect enhanced in an atopic environment. Rhinovirus-associated, VEGF-mediated angiogenesis may contribute to airway remodeling in asthma. (J Allergy Clin Immunol 2006;117:291-7.)

Key words: Asthma, bronchial epithelial cells, airway remodeling, endothelial cells, viral infection

Although chronic inflammation is considered the hallmark of asthma, airway remodeling is being increasingly recognized as a key feature of the disease. Being poorly responsive to current asthma treatments and probably leading to irreversible abnormalities in the lung,^{1,2} airway remodeling represents an important target for future therapeutic interventions.

Alterations of the vascular compartment, including increased vascularity accompanied by vasodilatation and microvascular leakage, are also observed, and angiogenesis is acknowledged as an important component of the airway remodeling in asthma.^{1,3,4} The vessel number, occupied area, and size have all been reported to be increased in cohorts of patients with asthma compared with control subjects, but also over patients with chronic obstructive pulmonary disease.⁵⁻⁷ In addition, the degree of vascularity significantly correlates with bronchial hyperresponsiveness in asthma.⁸

Vascular endothelial growth factor (VEGF), the major angiogenic growth factor mediating vascular remodeling, promotes proliferation and differentiation of endothelial cells and induces vascular leakage and increased permeability, and alterations in its expression may lead to abnormal vessel formation.⁹ VEGF is involved in vascular remodeling in asthma, correlating to disease outcomes: in the airway mucosa of patients with asthma, VEGF-expressing cells are significantly more abundant than in control subjects,¹⁰ and the degree of vascularity in asthma correlates to the number of VEGF-positive cells and inversely correlates to airway caliber.^{4,10}

Human rhinoviruses, the precipitants of the majority of common colds, are implicated in the induction of acute asthma exacerbations.¹¹ Rhinoviruses are able to reach

From ^athe Allergy Department, 2nd Pediatric Clinic, University of Athens;

^bthe Laboratory of Cell Proliferation and Ageing, National Centre for Scientific Research "Demokritos", Athens; ^cthe George P. Livanos Laboratory, Department of Critical Care and Pulmonary Services, University of Athens; and ^dthe Laboratory for Molecular Pharmacology, Department of Pharmacy, University of Patras.

Disclosure of potential conflict of interest: P. Saxoni-Papageorgiou has received grants from Novartis and Schering-Plough. N. Papadopoulos has received grants from GlaxoSmithKline and AstraZeneca. The rest of the authors have no conflict of interest to disclose.

Supported by the Hellenic Ministry of Education, Heraklitos Program, and the University of Athens.

Received for publication May 10, 2005; revised October 31, 2005; accepted for publication November 1, 2005.

Reprint requests: Stelios Psarras, PhD, Laboratory of Cell Biology, Center of Basic Research, Foundation for Biomedical Research Academy of Athens IIBEA, Athens, Greece. E-mail: spsarras@otenet.gr.

0091-6749/\$32.00

© 2006 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2005.11.005

Abbreviations used

CM: Conditioned medium
 DAPI: 4',6-Diamidino-2-phenylindole, dihydrochloride
 HUVEC: Human umbilical vein endothelial cell
 MOI: Multiplicity of infection
 RV: Rhinovirus
 VEGF: Vascular endothelial growth factor

and replicate in the bronchial epithelium,¹² inducing release of proinflammatory mediators,¹³⁻¹⁵ thereby mediating local inflammation. Furthermore, the rate of lower respiratory infection is similar to that of rhinovirus infection of the upper airways,^{12,16} suggesting that bronchial rhinovirus infection, and subsequent inflammatory response, is probably a frequently occurring event.

On this basis, we hypothesized that rhinovirus infection may be able to elicit angiogenic responses in the human airways, thus contributing to the vascular component of airway remodeling. We evaluated the ability of RV to induce angiogenesis by stimulating production of VEGF by bronchial epithelium *in vitro*. To establish further the relevance of this concept in atopic asthma, the effect of an atopic environment on rhinovirus-mediated VEGF production and the *in vivo* production of VEGF on rhinovirus-associated asthma exacerbations were also assessed.

METHODS

Virus infection and collection of conditioned media

Ohio-HeLa cells were used for propagation and titration of minor (RV1b) and major (RV9, RV16) rhinoviruses, and low passage (8-15) BEAS-2B cells were grown and infected by rhinovirus as described,^{12,13} at multiplicity of infection (MOI) of 1, unless otherwise specified. Primary human bronchial epithelial cells were derived from an adult volunteer without asthma after informed consent in the course of another ongoing study approved by the hospital's ethics committee. They were isolated and used at passage 2 for rhinovirus infection ($n = 6$) as described.¹²

Supernatants (conditioned media [CMs]) were collected 48 hours after infection (unless otherwise specified), clarified by centrifugation (10 minutes/3000g/4°C), and stored at -70°C until used in immunoassays. Control CMs were collected from parallel cultures exposed to heat-inactivated (for 1 hour at 58°C) or noninfected HeLa cell lysates. In some experiments ($n = 6$), rhinovirus-infected BEAS-2B were treated with 10 $\mu\text{g/mL}$ cycloheximide in comparison with diluent control (DMSO). Cycloheximide was not cytotoxic to epithelial cells, assessed microscopically.

Endothelial cell proliferation and angiogenesis assays

Human umbilical vein endothelial cells (HUVECs; American Type Culture Collection, Manassas, Va) were grown on rat tail collagen type I-coated plastic surfaces in M199 medium (Gibco Invitrogen, Carlsbad, Calif), containing 15% FCS, 30 $\mu\text{g/mL}$ endothelial cell growth supplement, and 0.1 mg/mL heparin and used between passages 3-6.

To assess angiogenic activity, CMs obtained as described were further clarified (60 minutes/12,000g/4°C) to remove residual virus partially. Indeed, titration of the clarified supernatants revealed the presence of only limited amounts of rhinovirus (titer ≤ 1). HUVECs grown to 80% to 90% confluence in complete growth medium (15% FCS) were serum-starved (5% FCS) for 16-18 hours and subsequently exposed to a 1:1 mixture of BEAS-2B CM with M199-starvation medium. Exposure to complete or starvation medium served as positive and negative control, respectively. Forty-eight hours later, cell numbers were determined. Six experiments in duplicates were used.

For the VEGF neutralization experiments, BEAS-2B CM or medium containing 15 ng/mL human recombinant VEGF₁₆₅ (R&D Systems, Abingdon, United Kingdom), serving as a positive control, were incubated with 5 $\mu\text{g/mL}$ antihuman VEGF polyclonal antibody (R&D Systems) for 45 minutes at 37°C before their addition to HUVEC monolayers ($n = 6$).

For the tube formation assay ($n = 4$; modification of that described in Montesano and Orci¹⁷), confluent HUVEC monolayers were replated on collagen type I gels preformed as described,¹⁷ and 24 hours later, 50% of the medium was replaced with either BEAS-2B CM or M199 complete medium containing increasing concentrations of VEGF (positive control). On formation of tube-like structures, the collagen gels were fixed, stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; a DNA-binding dye), and transferred onto glass slides, and phase-contrast and UV images were captured under a fluorescent microscope using a Charged Coupled Device camera connected to a PC with the ImagePro plus software (Media Cybernetics; Silver Spring, Md). Slides were blinded to the nature of the sample, and at least 8 microscopic fields per gel slide were randomly scanned in a manual manner to measure the number of DAPI-stained nuclei and the length of the tubular structures.

Infection of epithelial cells under exposure to PBMC supernatants

Venous blood was drawn from 7 healthy donors and 7 patients with asthma, diagnosed according to the Global Initiative for Asthma guidelines. The study was approved by the hospital's Ethics Committee, and written informed consent was obtained in each case. Inclusion criteria for the patient group consisted of a positive skin prick test to at least 1 allergen, absence of symptoms of common cold, and abstinence from any inhaled corticosteroids or antileukotrienes as preventive asthma therapy during the last 4 weeks before enrollment. The 2 groups had comparable age and sex distribution (atopic/asthmatic group: 6 male subjects, mean age 32.1 years, range 29-37; control group: 5 male subjects, mean age 33.1, range 27-41). Blood sampling was conducted in parallel for 1 normal and 1 atopic subject with asthma.

PBMCs were isolated from whole blood as described,¹⁸ resuspended in RPMI medium supplemented with 2.5% (vol/vol) FCS and 2.5% (vol/vol) human AB serum (Sigma, St Louis, Mo), and plated at a concentration of 1.5×10^6 cells/mL. Twenty-four hours later, the cells were infected by rhinovirus at MOI 1 as described.¹⁸ After a 24-hour incubation at 33°C , supernatants were harvested and clarified by centrifugation. PBMC supernatants did not contain any titratable rhinovirus.

PBMC supernatant (0.6 mL per well) was added to BEAS-2B cells prepared for rhinovirus infection in 24-well plates as described. After 24 hours at 37°C , PBMC supernatants were removed, and BEAS-2B were infected with rhinovirus at MOI 1. Forty-eight hours later, the BEAS-2B supernatants were removed, and VEGF levels were determined.

Nasopharyngeal wash samples

Samples from 9 atopic children with asthma (4 boys, mean age 10.2 years, range 7-16), obtained in the course of a prospective clinical study conducted at the P&A Kyriakou Children's Hospital and approved by the hospital's Ethics Committee, were collected at a symptom-free period (baseline) and subsequently during a rhinovirus-associated asthma exacerbation; informed consent was obtained in all cases. Subjects with intermittent or mild persistent asthma according to the GINA guidelines and positive skin prick tests to at least 1 of a panel of locally relevant allergens were included. The presence of rhinovirus in the nasopharyngeal washes during the exacerbations (and absence at baseline) was identified by rhinovirus-specific RT-PCR.¹⁹

ELISA assays

Levels of VEGF, angiopoietin-2, and IL-6 were measured by use of commercially available ELISA assays (R&D Systems). Angiopoietin-1 levels were measured with a previously described ELISA.²⁰

RT-PCR analysis

cDNA was prepared from rhinovirus-infected and noninfected BEAS-2B cells and subjected to semiquantitative RT-PCR against β -actin as described.¹³ VEGF-specific PCR primers used were able to discriminate the VEGF 121, 145, 165, 189, and 206 transcripts (predicted amplicon length of 516, 588, 648, 720, and 771 bp, respectively).²¹

Statistical analysis

Data are expressed as means $\pm$ SEMs. Statistical analysis was conducted with the SPSS 10.0 for Windows software (SPSS Inc, Chicago, Ill). Distribution normality was assessed by the Kolmogorov-Smirnov test. Means were compared with the *t* test. For multiple comparisons, ANOVA was used, and subsequent between-group effects were evaluated with the least significant difference test. *P* values less than 5% were considered significant.

RESULTS

Rhinovirus infection stimulates VEGF production by human bronchial epithelial cells

BEAS-2B cells, a human bronchial epithelial cell line, were infected with major (RV9 and RV16) and minor (RV1b) rhinovirus serotypes. Control cells, exposed to HeLa cell extract alone, produced considerable amounts of VEGF (450.1 ± 35.4 pg/mL corresponding to 3592.8 ± 285.2 pg of VEGF per 10^6 cells at 48 hours). Infection by RV1b and RV9 induced a moderate yet significant upregulation of VEGF production ($P < .0001$ and $P = .002$, respectively) by 48 hours. RV1b infection resulted in higher induction than both RV9 (Fig 1, A) and RV16 (not shown), and thus, this serotype was used for further experimentation. Exposure of BEAS-2B cells to heat-inactivated rhinovirus resulted in complete abrogation of VEGF induction, suggesting that the effect is virus-specific (Fig 1, A; $P = .019$). Furthermore, RV1b was shown to upregulate VEGF production significantly in a dose-dependent manner at a range of 0.2-5 MOI (Fig 1, B). Time-course experiments showed that VEGF upregulation appears 24 hours after rhinovirus exposure,

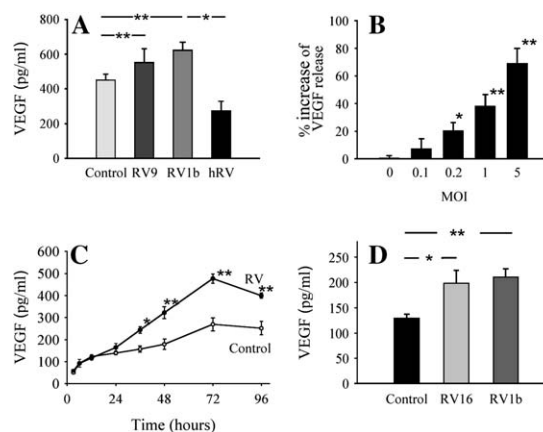


FIG 1. Rhinovirus infection induces VEGF release by human bronchial epithelial cells. **A**, VEGF production by BEAS-2B supernatants exposed to RV9 ($n = 6$), RV1b ($n = 12$), heat-inactivated RV1b (hRV; $n = 5$) or HeLa lysate (control). ** $P < .01$; * $P < .05$. **B**, VEGF production by BEAS-2B infected by RV1b at different MOIs. * $P < .05$; ** $P < .001$. **C**, Time course of VEGF production on infection by RV1b (RV). * $P < .01$; ** $P < .001$. **D**, VEGF production by primary bronchial epithelial cells infected by RV16 or RV1b. ** $P < .01$; * $P < .05$.

reaching its peak by 72 hours (Fig 1, C). Similar kinetics was observed for IL-6, measured to control for the model's compatibility with rhinovirus-mediated proinflammatory cytokine induction¹³ (not shown).

Primary human bronchial epithelial cells were also infected at MOI 1 with RV1b and RV16, and in both cases, a statistically significant induction of VEGF release was triggered compared with control (Fig 1, D; $P = .034$ for RV16 and .0011 for RV1b). The levels of induction were almost identical to that observed with BEAS-2B cells, suggesting that the response is not restricted to this continuous cell line.

In contrast, there were no detectable levels of the endothelial-selective receptor tyrosine kinase ligands angiopoietin-1 and angiopoietin-2²² in control and rhinovirus-infected BEAS-2B cells (data not shown).

Rhinovirus infection upregulates VEGF mRNA

Treatment of BEAS-2B cells with cycloheximide, a protein synthesis inhibitor, resulted in almost complete abrogation of VEGF levels in both rhinovirus-infected and uninfected cells (not shown), showing that VEGF induction depends on newly formed proteins in this system.

Using a PCR primer pair able to distinguish between the 5 known alternative transcripts of VEGF, we showed that all but the VEGF-145 variable are constitutively expressed (Fig 2, A). Rhinovirus infection induced an upregulation of total VEGF mRNA at both early (6 hours) and later (24 hours) time points (Fig 2, B). Rhinovirus-mediated upregulation was observed for all 4 transcripts present, whereas the relative proportion of transcripts did not change by rhinovirus infection (not shown). These findings indicate that VEGF induction in BEAS-2B cells by rhinovirus reflects upregulation of VEGF mRNA.

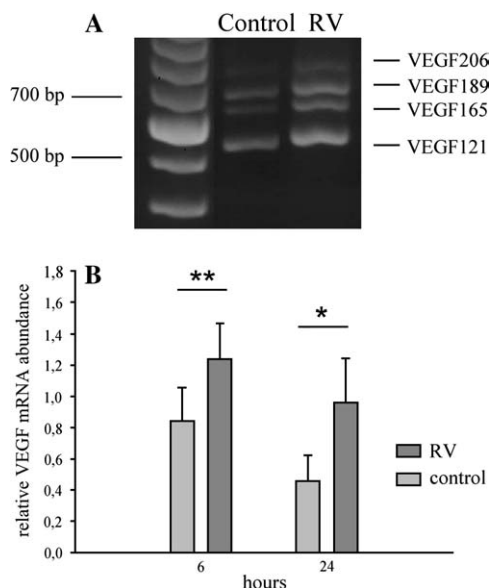


FIG 2. RV-mediated induction of VEGF reflects mRNA upregulation. **A**, RT-PCR of VEGF mRNA in BEAS-2B cells infected or not with RV. **B**, Relative abundance of VEGF mRNA in comparison to β -actin after RV infection. * $P < .05$; ** $P < .01$.

Rhinovirus epithelial infection induces endothelial cell proliferation, partly mediated by VEGF

Conditioned media from rhinovirus-infected and control BEAS-2B cultures were applied to serum-starved HUVECs. Control CMs were able to stimulate proliferation of endothelial cells significantly ($157.0 \pm 23.9\%$ of control quiescent cultures; $P = .049$), in accordance with previous data.²³ Supernatants from rhinovirus-infected cultures induced a further increase of endothelial number ($214.2 \pm 24.4\%$ over negative control), to levels significantly higher than those obtained by control CM and similar to those achieved by the positive control (Fig 3, A), thus indicating that products of rhinovirus-infected epithelial cells are able to stimulate endothelial cell proliferation.

To evaluate the contribution of VEGF in the rhinovirus-mediated angiogenic activity, the CMs were incubated with neutralizing concentrations of an anti-VEGF antibody. Antibody treatment partially reduced the noninfected epithelium-mediated proliferation of endothelial cells, and completely inhibited endothelial proliferation induced by recombinant VEGF (Fig 3, B). Similarly, pretreatment with this antibody significantly reduced rhinovirus-mediated endothelial cell proliferation by $42.4 \pm 12.9\%$ ($P = .028$; Fig 3, B).

Rhinovirus-infected epithelial cells induce tube-like structure formation by endothelial cells

To confirm functionally the angiogenic activity of rhinovirus-infected epithelial cells, we used an *in vitro*

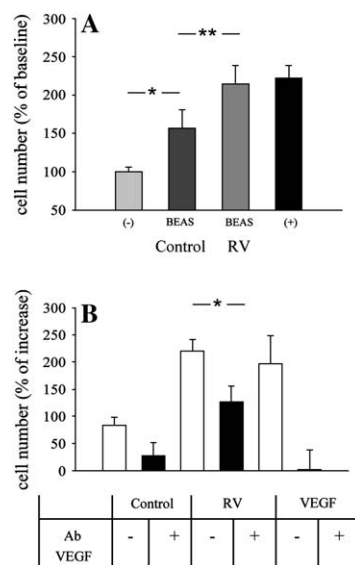


FIG 3. RV-mediated induction of endothelial cell proliferation. HUVEC cell counts after exposure to supernatants from **(A)** control BEAS-2B, RV-infected BEAS-2B (BEAS RV), complete growth medium (+), or control starvation medium (-) set at 100%. * $P = .05$; ** $P < .05$; **(B)** control BEAS-2B (Control), RV-infected BEAS-2B (RV), or 15 ng/mL VEGF, pretreated or not with neutralizing antibody (AbVEGF). Cell counts obtained by HUVEC maintained in starvation medium were set at 100% (* $P < .05$).

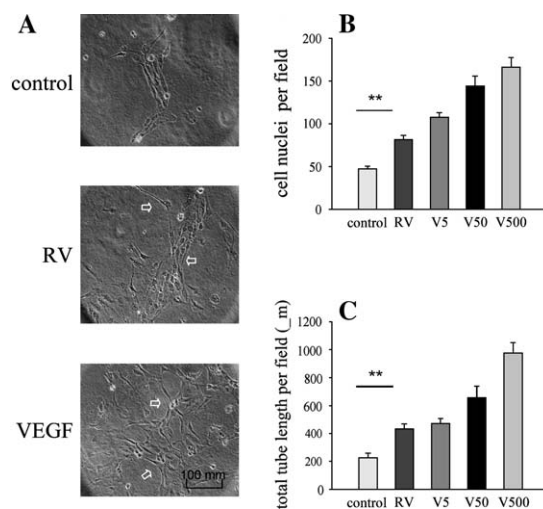


FIG 4. RV infection is able to induce angiogenesis *in vitro*. **A**, Tube-like structure formation (arrows) in HUVEC cultures exposed to supernatants from control BEAS-2B, RV-infected BEAS-2B (RV), 5, 50, or 500 ng/mL VEGF. Original magnification 500 $\times$. **B**, DAPI-stained nuclei counts per microscopic field. ** $P < .01$. **C**, Total length of tubular structures per field, calculated on the basis of bar length as shown in A. ** $P < .01$.

model of angiogenesis in which capillary-like structure formation after exposure to the stimulus in question is quantified.¹⁷ Extensive endothelial cell network formation was observed in cultures exposed to the rhinovirus-infected BEAS-2B CM, as well as to VEGF containing media (positive controls; Fig 4, A). Tube-like structures

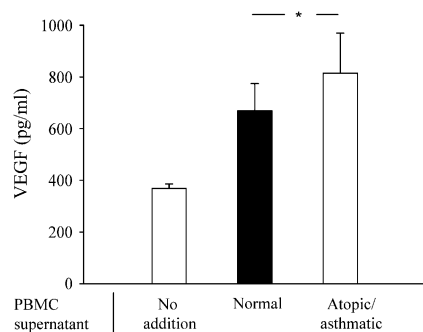


FIG 5. Exposure to PBMC supernatant affects VEGF production by bronchial epithelial cells. VEGF release by BEAS-2B cells pre-exposed to supernatants from RV-infected PBMCs isolated from atopic/asthmatic or normal subjects or left untreated (*no addition*) and subsequently infected with RV at MOI1. **P* < .05.

were also present in HUVEC cultures exposed to uninfected CM; however, these were limited. Rhinovirus-infected BEAS-2B CM resulted in a significantly increased number of endothelial cells in comparison with CM from uninfected control cultures (*P* < .01; Fig 4, B). Using suitable software, we were also able to calculate the total length of the endothelial network per microscopic field. Rhinovirus infection triggered the formation of capillary-like tubular structures of significantly greater total length than that obtained by CM from control, uninfected BEAS-2B (*P* < .01; Fig 4, C).

Epithelial VEGF release is stimulated by rhinovirus-induced PBMC products and is more pronounced in the context of an atopic environment

To model rhinovirus-mediated epithelial VEGF induction in an atopic asthmatic context, we isolated PBMCs from atopic patients with asthma and matched normal subjects, and stimulated them with rhinovirus. Supernatants from PBMCs derived from atopic subjects have reduced concentrations of T_H1 cytokines^{18,24} and were added to BEAS-2B cells before infection of the latter with rhinovirus to evaluate their effect on VEGF release, compared with supernatants from normal control PBMCs. PBMCs secreted only limited amounts of VEGF after exposure to rhinovirus (detectable in only 6/14 of rhinovirus-infected PBMC supernatants; median, 23.5 pg/mL). However, the addition of PBMC supernatants to BEAS-2B cells before infection with rhinovirus stimulated the epithelial cells, resulting in additional upregulation of VEGF production (Fig 5). Importantly, PBMCs from subjects with asthma induced the release of significantly higher amounts of VEGF by BEAS-2B than PBMCs from normal controls (813.7 ± 156.4 pg/mL vs 669.0 ± 104.8 pg/mL; *P* = .038; Fig 5), suggesting that exposure to an atopic environment may lead to more pronounced rhinovirus-mediated induction of VEGF release by bronchial epithelial cells.

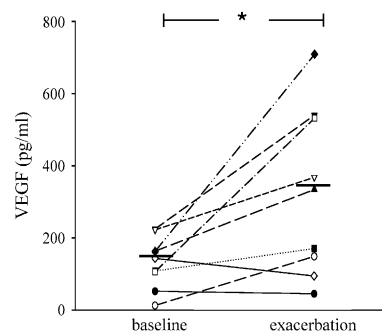


FIG 6. VEGF levels in human airways during RV-associated asthma exacerbations. Nasal aspirates were collected from 9 patients with asthma at a stable period (baseline) and subsequently, during a virologically confirmed RV-associated asthma exacerbation. VEGF levels were measured with ELISA. Horizontal bars represent mean values. **P* < .05.

VEGF levels are upregulated in the airways during rhinovirus-associated asthma exacerbations

To establish the relevance of these findings *in vivo*, we measured VEGF levels in nasopharyngeal secretions of children with atopic asthma at baseline and during a subsequent, virologically confirmed rhinovirus-associated asthma exacerbation. VEGF levels significantly increased in these patients during exacerbations (327.6 ± 76.8 pg/mL vs 132.3 ± 23.8 pg/mL at baseline; *P* = .018; Fig 6), suggesting that VEGF production is indeed associated with rhinovirus infection in atopic individuals with asthma.

DISCUSSION

We report here for the first time that infection of bronchial epithelial cells with human rhinoviruses promotes proliferation and differentiation of endothelial cells through the increased production of VEGF. Although rhinovirus-mediated upregulation of VEGF in this system was moderate, it did lead to the release of biologically significant amounts of this growth factor. Indeed, almost half of the rhinovirus-mediated endothelial cell proliferation was blocked by an antibody against VEGF. Several growth factors, such as TGF- β s, which are secreted by bronchial epithelial cells,²⁵ or fibroblast growth factor-2, which is induced on rhinovirus infection,²⁶ may account for the remaining angiogenic activity, a hypothesis currently under investigation.

Both major and minor rhinoviruses significantly induced VEGF release, and the rates were almost identical between primary bronchial epithelial cells and the continuous cell line BEAS-2B, as was the case in previous reports comparing *in vitro* VEGF expression patterns.²³ Differences in culture conditions and/or infection protocols as well as the modest degree of the induction may be responsible for the failure of previous reports to detect rhinovirus-induced VEGF production in primary

epithelial cells.^{27,28} Higher induction of VEGF by RV1b is in agreement with previous studies showing variability among rhinovirus serotypes in inducing epithelial responses *in vitro*¹³ and *in vivo*,^{29,30} although there is currently no evidence correlating the *in vitro* characteristics with the *in vivo* infectivity and asthmagenic capacity of rhinoviruses.

Vascular endothelial growth factor production seems to be induced also on rhinovirus infection of airway fibroblasts,²⁷ as well as respiratory syncytial virus infection of bronchial epithelial cells.²⁸ Although those studies focused on vascular permeability and did not assess angiogenesis, in combination with our data, they suggest that VEGF production, and possibly subsequent angiogenic effects, may characterize infection with respiratory viruses in general.

In addition to viruses, allergens, bacterial lipopolysaccharide, smoke, and hypoxia have also been shown to trigger VEGF upregulation in bronchial epithelial cells.^{23,31,32} However, exposure to such stimuli is not confined to patients with asthma; therefore, additional factors should be involved in the case of a causal relationship between any such exposure and vascular remodeling in asthma. Rhinovirus-induced PBMC responses correlate well with lung function measures in asthma,³³ and accordingly, exposure of epithelial cells to rhinovirus-infected PBMC supernatants was used to model the effect of the host environment on VEGF release during the course of an infection. The augmented induction of VEGF production under the influence of "atopic" PBMCs can be attributed to the altered content of these supernatants that have reduced concentrations of T_H1 cytokines on rhinovirus infection,^{18,24} and is in agreement with previous studies showing that T_H2 cytokines may alter the rates of production of remodeling-related growth factors by bronchial epithelial cells^{25,34} and a proposed model of remodeling pathogenesis, in which functional disturbance of the epithelial-mesenchymal trophic unit may be augmented by atopy.³ Vice versa, lung-specific VEGF overexpression in mice leads to a response skewed toward T_H2 cytokines after ovalbumin challenge,³⁵ suggesting that the interaction between disturbed T_H1/T_H2 balance and VEGF expression may establish a vicious circle in the airways, further intensifying the effect of VEGF in lung physiology.

Although this study has not focused on the potential effects of rhinovirus-mediated VEGF on vascular permeability, our findings support the suggestion that viral infection is able to mediate vascular permeability, resulting in edema characteristic of acute asthma exacerbations. In this respect, when cytokines known to be generated in response to rhinovirus infection were added to an endothelial cell model, vascular permeability was increased.³⁶ In our study, rhinovirus-mediated upregulation of VEGF was not accompanied by a similar increase in angiopoietins. It has been shown that in the absence of an active angiopoietin system, VEGF overexpression may lead to the formation of leaky vessels.³⁷ Formation of such vessels may have both acute (edema) and chronic (vascular remodeling) effects.

In further support of these ideas, VEGF seems to be considerably elevated within the first days of an asthma exacerbation (commonly virus-associated), slowly declining with time,³⁸ but still persisting 1 month later and remaining elevated in stable asthma in comparison with normal controls.^{38,39} Our data demonstrate that this may also be the case in virologically proven rhinovirus-associated exacerbations. It should be noted here that whereas virus-induced exacerbations are relatively acute events, there is considerable viral persistence, especially in atopic subjects with asthma.⁴⁰ Because the inflammatory events elicited by rhinovirus infection may persist well into convalescence in atopic subjects with asthma,⁴¹ although the kinetics of rhinovirus-mediated induction in cytokine release *in vitro* is of transient nature resembling that of VEGF (eg, IL-6 measurement in this report and reference⁴²), it is plausible to assume that persistence may be also a characteristic of rhinovirus-associated VEGF induction, especially in an atopic environment, a hypothesis that needs to be verified. Certainly the described VEGF response may be a repair mechanism that could occur with various stimuli, whether or not they induce asthma exacerbations. The importance of such a sustained VEGF overexpression is underlined and extended by the recent report demonstrating extensive angiogenesis, but also mucus metaplasia, subepithelial fibrosis, and inflammation in mice overexpressing VEGF in their lungs.³⁵

In conclusion, this study is the first to show that rhinovirus infection promotes endothelial cell proliferation and differentiation; these angiogenic responses are mediated by epithelial production of VEGF and may be augmented by the atopic host response to the virus. VEGF has also a pivotal role in regulating permeability of the endothelial compartment of the airways. In this respect, repeated infections with rhinovirus, or other respiratory viruses, may simultaneously induce acute exacerbations of asthma and contribute to vascular remodeling and a chronic asthma phenotype.

We thank Nikos Bournousouzis for excellent technical assistance.

REFERENCES

1. Vignola AM, Mirabella F, Costanzo G, Di Giorgi R, Gjornakaj M, Bellia V, et al. Airway remodeling in asthma. *Chest* 2003;123:417S-22S.
2. Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. *J Clin Invest* 1999;104:1001-6.
3. Davies DE, Wicks J, Powell RM, Puddicombe SM, Holgate ST. Airway remodeling in asthma: new insights. *J Allergy Clin Immunol* 2003;111:215-25; quiz 26.
4. Hoshino M, Takahashi M, Aoike N. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. *J Allergy Clin Immunol* 2001;107:295-301.
5. Hoshino M, Aoike N, Takahashi M, Nakamura Y, Nakagawa T. Increased immunoreactivity of stromal cell-derived factor-1 and angiogenesis in asthma. *Eur Respir J* 2003;21:804-9.
6. Chu HW, Kraft M, Rex MD, Martin RJ. Evaluation of blood vessels and edema in the airways of asthma patients: regulation with clarithromycin treatment. *Chest* 2001;120:416-22.
7. Tanaka H, Yamada G, Saikai T, Hashimoto M, Tanaka S, Suzuki K, et al. Increased airway vascularity in newly diagnosed asthma using a

- high-magnification bronchovideoscope. *Am J Respir Crit Care Med* 2003;168:1495-9.
8. Orsida BE, Li X, Hickey B, Thien F, Wilson JW, Walters EH. Vascular-ity in asthmatic airways: relation to inhaled steroid dose. *Thorax* 1999; 54:289-95.
9. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. *Nature* 2000;407:242-8.
10. Hoshino M, Nakamura Y, Hamid QA. Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma. *J Allergy Clin Immunol* 2001;107:1034-8.
11. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, et al. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* 1995;310:1225-8.
12. Papadopoulos NG, Bates PJ, Bardin PG, Papi A, Leir SH, Fraenkel DJ, et al. Rhinoviruses infect the lower airways. *J Infect Dis* 2000;181: 1875-84.
13. Papadopoulos NG, Papi A, Meyer J, Stanciu LA, Salvi S, Holgate ST, et al. Rhinovirus infection up-regulates eotaxin and eotaxin-2 expression in bronchial epithelial cells. *Clin Exp Allergy* 2001;31:1060-6.
14. Schroth MK, Grimm E, Frindt P, Galagan DM, Konno SI, Love R, et al. Rhinovirus replication causes RANTES production in primary bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1999;20:1220-8.
15. Zhu Z, Tang W, Gwaltney JMJ, Wu Y, Elias JA. Rhinovirus stimulation of interleukin-8 in vivo and in vitro: role of NF-kappaB. *Am J Physiol* 1997;273:L814-24.
16. Mosser AG, Brockman-Schneider R, Amineva S, Burchell L, Sedgwick JB, Busse WW, et al. Similar frequency of rhinovirus-infectible cells in upper and lower airway epithelium. *J Infect Dis* 2002;185:734-43.
17. Montesano R, Orci L. Tumor-promoting phorbol esters induce angiogenesis in vitro. *Cell* 1985;42:469-77.
18. Papadopoulos NG, Stanciu LA, Papi A, Holgate ST, Johnston SL. A defective type 1 response to rhinovirus in atopic asthma. *Thorax* 2002;57:328-32.
19. Papadopoulos NG, Hunter J, Sanderson G, Meyer J, Johnston SL. Rhinovirus identification by BglII digestion of picornavirus RT-PCR amplicons. *J Virol Methods* 1999;80:179-85.
20. Caine GJ, Blann AD, Stonelake PS, Ryan P, Lip GY. Plasma angiopoietin-1, angiopoietin-2 and Tie-2 in breast and prostate cancer: a comparison with VEGF and Flt-1. *Eur J Clin Invest* 2003;33:883-90.
21. Fiedler W, Graeven U, Ergun S, Verago S, Kilic N, Stockschrader M, et al. Vascular endothelial growth factor, a possible paracrine growth factor in human acute myeloid leukemia. *Blood* 1997;89:1870-5.
22. Papapetropoulos A, Garcia-Cardena G, Dengler TJ, Maisonnier PC, Yancopoulos GD, Sessa WC. Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. *Lab Invest* 1999;79:213-23.
23. Boussat S, Eddahibi S, Coste A, Fatacciolli V, Gouge M, Housset B, et al. Expression and regulation of vascular endothelial growth factor in human pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2000; 279:L371-8.
24. Xatzipsalti M, Psarras F, Konstantinou G, Gaga M, Gourgiotis D, Saxoni-Papageorgiou F, et al. Effect of the immune response to rhinovirus on rhinovirus-induced bronchial epithelial inflammation and cytotoxicity. *Allergy Clin Immunol Int* 2005;(suppl 1):547 [abstract n 1532].
25. Richter A, Puddicombe SM, Lordan JL, Bucchieri F, Wilson SJ, Djukanovic R, et al. The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am J Respir Cell Mol Biol* 2001;25:385-91.
26. Psarras S, Xatzipsalti M, Volonaki E, Bossios A, Gourgiotis D, Saxoni-Papageorgiou F, et al. Rhinovirus infection stimulates production of fibroblast growth factor-2 by airway epithelial and stromal cells. *Allergy* 2002;57(suppl 73):296 [abstract].
27. Ghildyal R, Dagher H, Donninger H, de Silva D, Li X, Freezer NJ, et al. Rhinovirus infects primary human airway fibroblasts and induces a neutrophil chemokine and a permeability factor. *J Med Virol* 2005;75: 608-15.
28. Lee CG, Yoon HJ, Zhu Z, Link H, Wang Z, Gwaltney JM, et al. Respiratory syncytial virus stimulation of vascular endothelial cell growth factor/vascular permeability factor. *Am J Respir Cell Mol Biol* 2000;23:662-9.
29. Skoner D, Doyle W, Seroky J, Van Deusen M, Fireman P. Lower airway responses to rhinovirus 39 in healthy allergic and nonallergic subjects. *Eur Respir J* 1996;9:1402-6.
30. Cheung D, Dick EC, Timmers MC, de Klerk EP, Spaan WJ, Sterk PJ. Rhinovirus inhalation causes long-lasting excessive airway narrowing in response to methacholine in asthmatic subjects in vivo. *Am J Respir Crit Care Med* 1995;152:1490-6.
31. Antony AB, Tepper RS, Mohammed KA. Cockroach extract antigen increases bronchial airway epithelial permeability. *J Allergy Clin Immunol* 2002;110:589-95.
32. Koyama S, Sato E, Tsukadaira A, Haniuda M, Numanami H, Kurai M, et al. Vascular endothelial growth factor mRNA and protein expression in airway epithelial cell lines in vitro. *Eur Respir J* 2002;20:1449-56.
33. Parry DE, Busse WW, Sukow KA, Dick CR, Swenson C, Gern JE. Rhinovirus-induced PBMC responses and outcome of experimental infection in allergic subjects. *J Allergy Clin Immunol* 2000;105:692-8.
34. Lordan JL, Bucchieri F, Richter A, Konstantinidis A, Holloway JW, Thorner M, et al. Cooperative effects of Th2 cytokines and allergen on normal and asthmatic bronchial epithelial cells. *J Immunol* 2002; 169:407-14.
35. Lee CG, Link H, Baluk P, Homer RJ, Chapoval S, Bhandari V, et al. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. *Nat Med* 2004;10:1095-103.
36. Sedgwick JB, Menon I, Gern JE, Busse WW. Effects of inflammatory cytokines on the permeability of human lung microvascular endothelial cell monolayers and differential eosinophil transmigration. *J Allergy Clin Immunol* 2002;110:752-6.
37. Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, et al. Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med* 2000;6:460-3.
38. Lee YC, Lee HK. Vascular endothelial growth factor in patients with acute asthma. *J Allergy Clin Immunol* 2001;107:1106.
39. Asai K, Kanazawa H, Kamoi H, Shiraishi S, Hirata K, Yoshikawa J. Increased levels of vascular endothelial growth factor in induced sputum in asthmatic patients. *Clin Exp Allergy* 2003;33:595-9.
40. Gern JE, Vrtis R, Grindle KA, Swenson C, Busse WW. Relationship of upper and lower airway cytokines to outcome of experimental rhinovirus infection. *Am J Respir Crit Care Med* 2000;162:2226-31.
41. Fraenkel DJ, Bardin PG, Sanderson G, Lampe F, Johnston SL, Holgate ST. Lower airways inflammation during rhinovirus colds in normal and in asthmatic subjects. *Am J Respir Crit Care Med* 1995; 151:879-86.
42. Griego SD, Weston CB, Adams JL, Tal-Singer R, Dillon SB. Role of p38 mitogen-activated protein kinase in rhinovirus-induced cytokine production by bronchial epithelial cells. *J Immunol* 2000;165: 5211-20.