Vascular Endothelial Growth Factor Induction by Rhinovirus Infection

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Vascular participation manifested by a runny nose (rhinorrhea) is a prominent feature of the acute consequences of rhinovirus infection. Vascular endothelial growth factor (VEGF) is an angiogenic factor that also induces potent increases in vascular permeability; it is a candidate mediator of rhinorrhea in response to rhinovirus infection as well as contributing to enhanced vascular leakage in rhinovirus-linked asthma exacerbations. It has been shown that rhinovirus induces significant increases in both VEGF protein and mRNA in primary airway fibroblasts [Ghildyal et al. (2005): J Med Virol 75:608–615]. The current studies assessed VEGF responses to rhinovirus in primary culture airway epithelium, in epithelial and fibroblast cell lines and in rhinovirus-infected nasal secretions. Epithelial and fibroblast cells were infected with rhinovirus serotype 16 and VEGF protein and isoforms assessed by ELISA and RT-PCR, respectively. VEGF protein was released by both epithelial and fibroblast cell lines and primary airway epithelial cells in culture but was not increased following rhinovirus infection. PCR products coding for four or five of the six known VEGF isoforms were produced (121, 145, 165 and 183, and/or 189 amino acids) in cell lines and primary culture cells, but no specific isoform was linked to rhinovirus infection. Nasal VEGF was also measured in a cohort of asthmatics with verified rhinovirus and respiratory syncytial virus (RSV) infection. VEGF was not raised following rhinovirus infection alone, but was increased significantly if concomitant RSV infection was present. The data suggest that fibroblasts rather than the epithelium may play a key role in VEGF mediated vascular responses after rhinovirus infection. This may aid recruitment of inflammatory cells and contribute to airway inflammation and bronchial obstruction. J. Med. Virol. 78: 666–672, 2006.

KEY WORDS: rhinovirus; VEGF; fibroblast; epithelium; isoform; nasal aspirates

INTRODUCTION

Rhinovirus causes the majority of common colds and has been linked to respiratory conditions such as sinusitis and exacerbations of asthma and chronic obstructive pulmonary disease [Gern and Busse, 1999]. The genesis of classical cold symptoms such as rhinorrhea, nasal blockage, sore throat and cough following infection is not defined but may be the result of kinin release and other vasoactive substances induced by rhinovirus [Naciero et al., 1988]. However, initial rhinovirus infection occurs in the airway mucosa and release of other permeability factors from epithelial and fibroblast cells may explain the development of rhinorrhea at an early stage, usually within 48 hr [Grunberg and Sterk, 1999]. This is feasible because early innate responses to rhinovirus are characterized by release of a variety of mediators including the neutrophil chemokines interleukin 8 (IL-8) [Johnston et al., 1998] and epithelium neutrophil-activating peptide-78 (ENA-78) [Donninger et al., 2003; Ghildyal et al., 2005], and cytokines IL-6 and IL-11 [Zhu et al., 1996; Wang et al., 1999]. Other investigations have demonstrated that the eosinophil chemokine regulated upon activation, normal T-cell expressed and secreted

Abbreviations used: VEGF, vascular endothelial growth factor; MOI, multiplicity of infection; PCR, polymerase chain reaction; RSV, respiratory syncytial virus.

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(RANTES) is produced by airway epithelium following rhinovirus infection [Konno et al., 2002]. However, release and the role of mediators of vascular leakage are still unclear [Ghildyal et al., 2005].

One of the potent proteins involved in vascular growth and remodeling is vascular endothelial growth factor (VEGF). VEGF induces endothelial cell proliferation, augments cell migration, and reduces apoptosis [Gerhardt and Betsholtz, 2005; Ribatti, 2005]. VEGF also increases the permeability of endothelial cells, a response now known to be mediated via induction of blood vessel fenestration and the formation of channels through which blood products can extravasate [Feng et al., 1999]. VEGF can be produced by a variety of cells that include epithelial cells, macrophages, fibroblasts, granulocytes, and smooth muscle cells [Horiiuchi and Weller, 1997; Neufeld et al., 1999]. In highly vascular tissues such as lung, VEGF appears to be produced in significant quantities [Maniscalco et al., 1995]. VEGF has also been linked to airway diseases including asthma [Choi et al., 2004] and chronic obstructive pulmonary disease [Kranenburg et al., 2005]. Lee et al. [2000] demonstrated production of VEGF in epithelial cells by respiratory syncytial virus (RSV) infection but the investigators were unable to elicit VEGF responses from epithelial cells following rhinovirus infection. However, alternative cellular sources of VEGF such as mucosal fibroblasts may also contribute to VEGF production following rhinovirus infection and we have recently demonstrated its induction by rhinovirus in airway fibroblasts [Ghildyal et al., 2005].

The present study evaluated VEGF responses to infection with a major group rhinovirus in epithelial and fibroblast cell lines as well as in primary culture airway epithelial and fibroblast cells. Investigations also assessed expression of VEGF isoforms and measured VEGF in clinical samples with verified rhinovirus and RSV infection.

**MATERIALS AND METHODS**

**Cells and Virus**

Ohio-HeLa cells (termed HeLa cells in the rest of this report) were a gift from Rachel Cameron (Biota Holdings Ltd, Melbourne, Australia) and were propagated in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS). MRC5 cells were provided by Irene Peroulis (Victorian Institute of Animal Sciences, Attwood, Australia) and propagated in MEM containing 10% FBS, supplemented with 1% non-essential amino acids and 0.07% sodium bicarbonate.

Primary culture bronchial epithelial cells were obtained from Clonetics (Baltimore, MD). Primary culture airway fibroblasts were grown from lobar lung resections for lung cancer as previously described [Ghildyal et al., 2005]. Sample specimens were reviewed by an experienced pathologist to ensure absence of malignant cells in tissue used for culture. Airway samples were minced and suspended in MEM supplemented with 10% FBS, distributed into 35 mm petri dishes and grown to confluence. Cells were used at third passage and confirmed as fibroblasts (>95% purity) by phenotype and immunofluorescent staining for vimentin.

Human rhinovirus serotype 16, a major group rhinovirus (a gift from Elliot Dick and Bill Busse, Madison, WI) was used throughout this study and was propagated in HeLa cells as previously described. Rhinovirus serotype 16 was inactivated by exposure to ultraviolet light (UV-RV16) as described [Donninger et al., 2003] and used as non-infectious control.

**Time Course Experiments**

Overnight cultures of cells were infected with rhinovirus 16 at multiplicity of infection (MOI) = 0.01 (HeLa and MRC5) or MOI = 10 (primary epithelial cells and fibroblasts) for 1 hr. Previous experiments had established that these MOIs were optimal to induce infection and to assess cellular responses. After 1 hr of rhinovirus 16 incubation, cells were washed twice and fresh MEM containing 2% FBS added. Culture supernatant and cell lysate samples were collected at this time and were referred to as 0 hr; beginning of infection. Cells were lysed in Tri reagent and RNA extracted according to the manufacturer's recommendations (Amresco, Melbourne, Australia). Further samples were collected at the indicated times post-infection (p.i.). Cells incubated with non-infected cell culture supernatant (mock) or UV-RV16 were used as controls. Cell necrosis was measured after 72 hr in all supernatants using a lactate dehydrogenase (LDH) assay (Calbiochem, Merck Pty, Sydney, Australia) to exclude excessive cell death of infected cells.

**Quantification of VEGF Release From Cells Using ELISA and Detection of Protein Isoforms**

VEGF protein in supernatants was quantified by sandwich ELISA using paired antibody reagents (R&D Systems, Minneapolis, MN) as described previously [Ghildyal et al., 2005]. The ELISA has been shown to recognize human VEGF isoforms 121 and 165 [Koyama et al., 2002]. Cell associated VEGF isoforms were also studied using Western blotting of whole cell extract followed by detection with a polyclonal antibody to VEGF (Chemicon, Australia).

**Detection of VEGF Isoforms**

Total cellular RNA was used for reverse transcription (RT) in a volume of 20 µl as described [Dagher et al., 2004]. The cDNA generated was used in polymerase chain reaction (PCR). Primers were designed to detect all the known VEGF isoforms, forward primer 5'- ATG AAC TTT CTG CTG TCT TGG GT -3' and reverse primer 5'- TCA CGG CCT CTT GCT TGC AC -3' [Stimpfl et al., 2002]. These primers yield six products in PCR, namely 446 base pairs (bp), 516, 576, 630, 648 and 699 bp, corresponding to the known VEGF isoforms of 121, 145, 165, 183, 189, and 206 amino acids (aa), respectively.

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The PCR reaction contained 1 times PCR buffer, 0.2 mM dNTP, 1.5 mM MgCl₂, 0.4 pmol/μl primers, 0.8U Taq polymerase 2 μl cDNA and water to a final volume of 25 μl. The cDNA was denatured for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C, annealing at 55°C, and extension at 72°C.

RT-PCR products were fractionated on 3% ethidium bromide agarose gel and visualized using UV light. The housekeeping gene β-actin was used as internal control as previously described [Ghildyal et al., 2005]. The densities of the bands produced on the gel were quantitated using the ImageQuant™ software (Amersham Biosciences Pty Ltd, Sydney, Australia), the VEGF data being normalised to β-actin. Amounts of the various isoforms were also quantified with reference to the most abundant (121 aa) isoform.

**Measurement of VEGF in Nasal Aspirates**

Nasal aspirates were obtained from a cohort of asthmatic patients presenting to a hospital emergency department with acute severe asthma. Their characteristics have been reported [Kling et al., 2005]. Briefly, nasal aspirates were taken by nasal aspiration with a soft-tipped cannula connected to negative pressure suction. Secretions (approximately 1.5 ml) were mixed with virus transport medium and stored at −20°C [Johnston et al., 1995]. The presence of rhinovirus RNA and RSV RNA was detected using validated PCR assays and has been previously reported [Ghildyal et al., 1997; Kling et al., 2005]. For VEGF ELISA nasal aspirates were selected and analysed as follows: (i) nasal aspirates negative for both rhinovirus and RSV RNA (n = 9), (ii) nasal aspirates with rhinovirus RNA but not RSV RNA detected (n = 9), (iii) all nasal aspirates with RSV RNA detected (n = 6), and (iv) nasal aspirates with both RSV and rhinovirus RNA detected (n = 5). Only one patient in the cohort had RSV RNA detected without rhinovirus RNA being present. VEGF in nasal aspirates was measured using ELISA as noted above after treatment for 15 min with 0.1% dithiothreitol (10% sputalysin) at room temperature [Efthimiadis et al., 2002].

**Analysis of Data**

Unless otherwise stated, values are expressed as mean ± standard error (SE). Comparisons were done using one-way ANOVA with Tukey’s post-test or Student’s paired t-test as appropriate. The levels of VEGF in nasal aspirates were compared using unpaired t-tests. Statistical software Data Analysis and Data Analysis Plus (Microsoft® Office 97 Professional edition) and GraphPad Prism were used for all calculations. Statistical significance was accepted at P ≤ 0.05.

**RESULTS**

**Infection by Rhinovirus of Epithelial and Fibroblast Cells**

Previous studies in primary epithelial cells and fibroblasts have verified rhinovirus infection of these cell types using virus culture, immunofluorescence and PCR techniques [Donninger et al., 2003; Ghildyal et al., 2005]. The effects of infection could not be attributed to virus adherence or internalization alone because viral replication and production of progeny virions could be demonstrated [Donninger et al., 2003; Ghildyal et al., 2005].

**Epithelial Cell Infection and VEGF Responses**

To assess whether rhinovirus induces VEGF in epithelium, studies were conducted in both an epithelial cell line (HeLa) and in primary culture bronchial epithelial cells. HeLa cells were infected with rhinovirus 16 at MOI = 0.01 and subsequent release of VEGF was measured using ELISA. Significant amounts of VEGF (>5000 pg/ml) were measured in supernatants from mock, UV-RV16 treated and rhinovirus-infected cells 24–72 hr after infection (Fig. 1A). However, there was no significant difference in VEGF production by the rhinovirus infected cells as compared to the controls. A similar pattern was observed in supernatants taken from primary bronchial epithelial cells infected at MOI = 10 (Fig. 1B). Cell necrosis associated with rhinovirus was not increased as measured by LDH assays (data not shown).

**Fibroblast Cell Responses to Rhinovirus Infection**

To assess whether rhinovirus induced VEGF in fibroblasts, responses were studied using the fetal fibroblast cell line MRC5. At the start of the experiment (hr) a basal level of VEGF was detectable (mean = 350 pg/ml) and after 24 hr this had almost doubled. Further increases were noted after 48 and 72 hr. Again, rhinovirus did not induce VEGF above the control values for mock and UV-RV16 treated cells (Fig. 2A) and LDH assays were similar after 72 hr. Previous results in primary culture airway fibroblasts have demonstrated significant increases in VEGF protein and mRNA above controls, 48 hr after infection [Ghildyal et al., 2005]. The current observations in fetal fibroblasts are thus in contrast to the findings in primary culture airway fibroblasts.

**VEGF Isoforms Produced by Fibroblasts**

It is possible that although the overall VEGF produced is not changed, a particular isoform may be preferably induced by rhinovirus infection. To determine if this was the case in fibroblast cells, we performed RT-PCR with primers capable of identifying all six VEGF isoforms. It was possible to detect four or possibly five of the VEGF isoforms in both MRC5 and primary fibroblast cells. Based on the sizes of the final PCR products [Stimpfl et al., 2002], the VEGF isoforms produced following rhinovirus infection of primary airway fibroblasts were 121, 145, 165, 186/189 aa (Fig. 2B). Protein isoforms could not be successfully detected in Western blots (data not shown). This may be
due to non-specific reactions of the VEGF antibody (protein bands around 45 kDa were observed) or expression of VEGF below the threshold of detection. Additionally, the VEGF isoforms may be secreted too rapidly for successful detection in cell lysates.

Semi-quantitative analysis of different isoforms did not reveal induction of any particular isoform following infection (data not shown). Normalization of template mRNAs to mRNA coding for the 121 aa isoform yielded similar results (not shown). When RT-PCR was performed in MRC5 cells the results were similar overall, with the exception that the fetal cells appeared to produce considerably lower quantities of the different VEGF isoforms than primary culture airway fibroblasts.

To determine whether respiratory virus stimulation of VEGF can be further correlated with human infection, studies were undertaken to determine whether VEGF could be detected in nasal aspirates obtained from patients with rhinovirus or RSV infection.

**Fig. 1.** VEGF responses in epithelial cells after RV16 infection. 
A: The epithelial cell line Ohio-HeLa was infected with RV16 at MOI = 0.01, treated with UV inactivated RV16 (UV-RV16) or untreated (mock). Culture supernatants were collected at the indicated times and release of VEGF measured using ELISA. Significant amounts of VEGF were found after 24–72 hr that were not influenced by rhinovirus infection. 
B: Primary bronchial epithelial cell release of VEGF was measured after infection with rhinovirus at MOI = 10 as described in ‘A’ for Ohio-HeLa cells. Overall, significant increases were noted in all groups after 24 hr but this was not enhanced by rhinovirus infection. Open columns, mock-infected cells; closed columns, UV-RV16 treated cells; hatched columns, rhinovirus infected cells.

**Fig. 2.** Fibroblast VEGF responses to rhinovirus infection. 
A: Cells of the fetal fibroblast cell line MRC5 were treated similarly to the Ohio-HeLa cells in Figure 1A. Culture supernatants were collected at indicated times p.i. and level of secreted VEGF measured by ELISA. Levels of VEGF protein increased linearly and reached levels >2,500 pg/ml after 72 hr. Infection with rhinovirus did not affect VEGF release compared to mock and UV-RV16 treatment. Open columns, mock cells; closed columns, UV-RV16 treated cells; hatched columns, rhinovirus infected cells. 
B: VEGF isoforms visualized by agarose gel electrophoresis. Total cellular RNA was extracted from mock, UV-RV16 and rhinovirus 16 treated cells and used to perform RT-PCR using primers capable of detecting all VEGF isoforms. There appeared to be at least four or possibly five VEGF isoforms produced by primary fibroblasts. Molecular weight markers (in bp) are indicated on the left and the predicted isoform of each band observed is indicated on the right of the gel. Lane 1, 100 kb DNA marker; Lane 2, mock-infected cells at 0 h; Lane 3, mock-infected cells; Lane 4, UV-RV16 treated cells; Lane 5, rhinovirus infected cells; Lanes 3–5, samples collected at 48 hr.
patients with known respiratory tract infection. We obtained nasal aspirates from patients during acute exacerbations of asthma and identified rhinovirus RNA and RSV RNA or a combination of both [Kling et al., 2005]. No differences in VEGF protein were noted between patients without detectable rhinovirus/RSV RNA and patients with rhinovirus RNA only; both groups had low levels of VEGF (Fig. 3). However, the presence of RSV RNA in combination with rhinovirus induced significant increases in VEGF. In the single patient who had RSV RNA only, VEGF levels were not increased appreciably relative to those with neither rhinovirus nor RSV RNA detectable.

**DISCUSSION**

It was postulated that VEGF release by airway wall cells following rhinovirus infection might explain acute increases in vascular permeability that characterize clinical disease. VEGF production in epithelial and fibroblast cells was assessed, VEGF isoforms were identified and nasal VEGF was measured to examine human in vivo responses to rhinovirus infection. The results confirm that mucosal fibroblasts rather than epithelial cells release VEGF in response to rhinovirus and that four or five mRNA isoforms are produced, although no one isoform is specifically induced by rhinovirus. VEGF in clinical nasal samples was increased after dual RSV and rhinovirus infection, but not rhinovirus infection alone.

Respiratory viral infections caused by rhinovirus, RSV, coronavirus, adenovirus, and a variety of other 'common cold' viruses induce characteristic symptoms. Increases in nasal secretions are universal manifestations of infection, often accompanied by nasal blockage and sore throat and less often by constitutional symptoms. The genesis of a 'runny nose' is poorly understood and has been ascribed to the release of kinins [Naclerio et al., 1988] and other vasoactive substances such as leukotriene C4 [Skoner et al., 1995; Gentile et al., 2003]. The potent effects of the angiogenic factor VEGF on vascular permeability have been characterized and found to be more than a 1000-fold greater than histamine [Senger et al., 1990]. If produced during viral infections it may explain some of the explosive vascular leakage found 24–72 hr after initial exposure to the causative agent. The present studies first evaluated VEGF responses in epithelial cells, both in a cell line (HeLa) and in primary bronchial epithelial cells. Significant amounts of VEGF protein were produced in both types of cells in culture (2,500–10,000 pg/ml) but this was not increased after infection with rhinovirus. These findings are similar to studies by Lee et al. [2000] who could not detect rhinovirus induced VEGF release by epithelial cells. However, the data pertaining to rhinovirus infection was not shown in the report and it is not clear whether VEGF increased in non-infected and infected cells over time as shown in the current study. An important issue to consider is whether necrosis of rhinovirus-infected cells may reduce cell numbers precluding measurable increases in VEGF, a possibility that was investigated by measuring LDH release into the culture supernatant. However, levels of LDH were not increased in rhinovirus infection. In contrast, infection with rhinovirus serotype 2 (a minor group rhinovirus) caused substantial cell death with an increase in LDH and a late decrease of VEGF release (data not shown), a finding not observed after rhinovirus 16 infections. The difference noted in the same experimental context provides additional evidence to verify that cell death did not cause lack of VEGF release. Taken together, the study of Lee et al. [2000] and data presented now suggest that rhinovirus does not induce release of VEGF from epithelial cells.

There is now a considerable body of evidence indicating that the cellular components of the airway wall respond to infection and other stimuli in a co-ordinated fashion [Holgate et al., 2001; Richter et al., 2001]. Fibroblasts act as sentinel cells and provide early innate immune responses following infections [Smith et al., 1997]. Based on these considerations studies have previously investigated and reported the role of fibroblasts in tissue responses to rhinovirus; these studies detected release of the key neutrophil chemokine ENA-78. In tandem with ENA-78 release and over similar time courses there was production of VEGF following rhinovirus infection [Ghildyal et al., 2005]. In the current studies, these investigations were extended.

![Fig. 3. VEGF measurements in nasal aspirates.](image)
and VEGF responses to rhinovirus infection in a fibroblast cell line, the fetal cell line MRC5 were compared. Infection with rhinovirus did not induce VEGF to levels above a time-dependent increase of VEGF in mock and UV-RV16 treated cells (Fig. 2A). It suggests that VEGF responses in primary culture fibroblasts and in a fibroblast cell line are discordant and that human primary airway cells (although difficult to obtain and culture) are required to assess initial cellular activities following rhinovirus infection in a comprehensive fashion. The disparity is not unusual and there are several reports noting differences in the reaction of cell lines and primary culture cells following infection [Vitkovic et al., 1995; Richardson et al., 1996] and in response to other stimuli [Hermanns et al., 2004; Kartsogiannis and Ng, 2004; Shapiro, 2004].

At least six VEGF isoforms may be produced from the native VEGF gene by alternative splicing of RNA [Stimpfl et al., 2002]. The isoforms may differ in their biologic functions and expression following infection and hence we examined production of isoforms following rhinovirus infection of primary fibroblasts. Using RT PCR and primers designed to detect all isoforms we could identify assay products on gel electrophoresis that were consistent with production of isoforms 121, 145, 165, and 186/189 aa. Detection of the protein isoforms was also attempted but not successful. Our data suggest that similar isoforms are produced (121 and 165) as reported in response to RSV [Lee et al., 2000] but that RT-PCR identifies also the 145 and 183/189 proteins. This is most likely because these isoforms remain cell and/or matrix associated in vitro and in vivo [Dvorak, 2000]. Rhinovirus induces additional isoforms in fibroblasts implying that detection of protein moieties only may not provide information on all the isoforms. However, it remains to be established that these mRNA isoforms are translated to produce VEGF protein. Finally, it was assessed whether rhinovirus infection may induce a specific isoform but no evidence was found to support this notion. There are no comparable studies of virus infection, but studies in neoplastic disease have also suggested absence of any VEGF isoform pattern associated with disease characteristics such as invasion and progression of cancer [Stimpfl et al., 2002].

To assess whether rhinovirus infection of airway cells is relevant to events in human rhinovirus disease, studies were undertaken to determine nasal VEGF levels in a cohort of children with confirmed rhinovirus associated asthma exacerbation [Kling et al., 2005]. RSV infection was studied as a positive control because RSV is known to increase nasal VEGF [Lee et al., 2000] and children without rhinovirus or RSV were included as a control group for comparison. VEGF levels were low in patients with absent rhinovirus or RSV and also in those in whom rhinovirus RNA only was detected. In the latter group only two patients had a slight increase in VEGF detectable. It is possible that VEGF proteins had been degraded in the nasal aspirates containing rhinovirus. However, elevated levels of VEGF were observed in aspirates containing both rhinovirus and RSV; aspirates obtained from the same cohort of asthmatics with handling and storage in an identical fashion. Patients with RSV had significantly higher levels of VEGF in the nose; others have reported comparable findings in patients with RSV infection, but not with influenza infection or when either virus was absent [Lee et al., 2000]. Of note was that 5/6 patients with RSV had both RSV/rhinovirus present; unfortunately the cohort contained only one patient with RSV infection alone; thus it is not possible to judge the contribution of dual infection to increases in VEGF. This data implies that complex interactive events influence human responses to virus infection; aspects that merit investigation in future employing in vitro models. Interestingly, a study of acute asthma found elevated levels of sputum VEGF during asthma exacerbations though the role of viruses as a cause of the increases was not investigated [Lee and Lee, 2001]. Overall results suggest that measurable nasal VEGF is induced by RSV infections but not by other viruses such as rhinovirus or influenza.

The in vitro studies suggest that rhinovirus induces VEGF in airway fibroblast cells but not in epithelial cells. However, the in vivo data presented indicate that measurable VEGF is not present in the airway lumen (of the nose) following rhinovirus infection and hence there is some discrepancy between these results. One explanation may involve the anatomical position of fibroblasts relative to epithelium because if most of the VEGF following rhinovirus infection is generated in submucosal fibroblasts, mediators such as VEGF may not penetrate the basement membrane and epithelial layers to appear in luminal fluid. In contrast, if VEGF is generated mostly in epithelium, as with RSV infection, detection may be possible. Finally, considerable dilution of mediators occurs when nasal aspirates and washes are obtained, this may vary as much as 50 times [Hendley and Gwaltney, 2004] and would influence measurements if low levels of VEGF are present.

In summary, rhinovirus induces VEGF in primary fibroblast cells, but not in airway epithelium. VEGF release by fibroblasts may enhance vascular permeability in blood vessels situated in deeper tissue layers and so may amplify the recruitment of neutrophils and other blood-borne pro-inflammatory cell populations.

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