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Title: *Pseudomonas aeruginosa* induces VEGF synthesis in airway epithelium *in vitro* and *in vivo*

Subtitle: **Possible relevance to peribronchial angiogenesis**

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Abstract **(195 words)**

Background: *Pseudomonas aeruginosa* (PA) airway infection and bronchial blood vessel proliferation are features of bronchiectasis. Because vascular endothelial growth factor-A (VEGF) regulates angiogenesis, we hypothesized that PA infection induces VEGF synthesis in epithelium and peribronchial angiogenesis. Because EGF receptors (EGFR) activation regulates VEGF synthesis in cancer, we also evaluated the roles of EGFR.

Methods: Airway epithelial cells were incubated for 24h with PA supernatants and VEGF concentrations were measured in culture medium by ELISA. C57/Bl6 mice were instilled intratracheally with sterile or with PAO1-coated ($6.10^5 \pm 3.10^5$CFU/animal) agarose beads with or without the EGFR inhibitor AG1478 (12.5 mg/kg/d, i.p.). Epithelial immunostaining for VEGF and phosphorylated EGFR, and peribronchial vascularity were quantified using morphometric analysis. VEGF expression was further assessed by western blot in mouse lung homogenates.

Results: PA supernatants induced dose-dependent VEGF synthesis in cultured airway epithelial cells, effects which were prevented by EGFR antagonists. In mice, persistent PAO1 infection increased immunostaining for VEGF and phosphorylated EGFR in airway epithelium, and resulted in increased peribronchial vascularity within 7 days. These effects were reduced by EGFR inhibition.

Conclusion: Persistent PA infection induced VEGF synthesis in airway epithelium and peribronchial angiogenesis, at least in part, via EGFR-dependent mechanisms.

Introduction

Bronchiectasis is defined as persistent or recurrent bronchial sepsis related to irreversibly damaged and dilated bronchi [1, 2]. *Pseudomonas aeruginosa* (PA) is one of the most frequently isolated pathogens from the lower respiratory tract in bronchiectasis [3]. Bronchiectasis is associated with bronchial angiogenesis (the generation of new blood vessels from existing bronchial vessels) [4, 5], but the mechanisms underlying this abnormal bronchial angiogenesis are unknown.

The vascular endothelial growth factor-A (VEGF) appears a key angiogenic mediator [5]. It is a vascular endothelial mitogen for arteries and veins and it regulates vascular permeability by inducing the fenestration in endothelial cells [6]. VEGF is synthesized by various respiratory cells, including airway epithelial cells [7], and Baluk et al. have shown that its expression in airway epithelium was sufficient to trigger tracheal angiogenesis in mice [8].Various cytokines such as interleukin-1 and TNF-alpha increased the expression of VEGF in airway epithelial cells [9], suggesting that infection and inflammation may trigger VEGF synthesis and angiogenesis.

Our aim was to study the involvement of persistent bronchial infection in the development of bronchial angiogenesis in experimental models relevant to bronchiectasis. We first studied VEGF synthesis in vitro in airway epithelial cells upon incubation with PA bacterial products. Because there is no animal model known to develop bronchiectasis, we used a mouse model of prolonged bronchial infection created by intratracheal instillation of PA-coated agarose beads [10] to study airway VEGF expression and peribronchial vasculature. Because recent evidence suggested that various infectious stimuli in the airways lead to activation of the epidermal growth factor receptor (EGFR) [11] and because EGFR has been shown to be pro-angiogenic, albeit in tumors [12], we further studied in vitro and in our mouse model if EGFR could be involved in PA-induced VEGF synthesis and angiogenesis.

Methods

In vitro experiments

NCI-H292 (human pulmonary mucoepidermoid carcinoma) and BEAS-2B (non-tumorigenic human bronchial epithelial) cells were cultured as described in the *Online Depository*. Primary cultures of airway epithelial cells, a kind gift of Pr A. Coste (Créteil, France), were obtained from three patients undergoing surgery for idiopathic nasal polyposis and were cultured as previously described [13].

For preparation of *Pseudomonas aeruginosa* (PA) supernatants, bacteria were grown in trypticone soy broth dialysate (TSBD) medium with aeration at 37°C to late log phase. The broth cultures were then centrifuged at 10,000 rpm for 50 min [14]. Supernatants were harvested and kept at –80°C until used.

To assess the dose-dependent effect of the laboratory strain PAO1 on VEGF synthesis, confluent epithelial cells were incubated in the presence of PAO1 supernatants (dilutions, 1:32 to 1:8 in cell culture medium) for 24h. Supernatants of six strains of PA (3 nonmucoid and 3 mucoid; dilution, 1:8) obtained from the sputum of CF patients were also studied. In selected studies, cells were pretreated with inhibitors or with antibodies (Ab) for 30 min before exposure to PA supernatants. The following products were used: AG1478 (10^{-5}M, Calbiochem, Nottingham, UK), a selective inhibitor of EGFR tyrosine kinase; AG9 (10^{-5}M, Calbiochem, an inactive analog of AG1478); an EGFR-neutralizing Ab (Ab-3, C225, 4 μg/ml; Calbiochem). Concentrations of inhibitors and Abs were selected based on previous literature [15]. After the 24h-incubation in the presence of PA supernatants, cell culture media were harvested and concentrations of immunoreactive VEGF were measured by ELISA (R&D Systems,

Minneapolis, MN, USA) according to the manufacturer's protocol. Each sample was measured in duplicate.

Animal studies

Preparation of sterile and PAO1-coated agarose beads

Sterile and PAO1-coated agarose beads were prepared according to slight modifications of the method described by van Heeckeren and Schluchter [10]. Briefly, 1 ml of PAO1 suspension ($5.4x10^9 \pm 1.9x10^9$ CFU) was mixed with 8.5 ml of 50°C pre-warmed 2% agarose type XII (Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS), and 0.5 ml of Indian ink (to allow bead visualization in tissue). This agarose-broth mixture was added to 150 ml of heavy mineral oil (Sigma) equilibrated at 50°C. The mixture was stirred for 6 minutes at room temperature, then cooled over at 4°C with continuous stirring for 20 minutes and finally centrifuged for 20 minutes at 3200*g*. The beads were washed three times with 10 mL of 0.5%, 0.25% and finally no sodium deoxycholate (Sigma) in PBS. The rate of stirring and cooling was determined to obtain beads with a median size of 300 μm. Bead size was further secured by filtration through 400 and 300 μm filters. Beads were diluted in sterile PBS to obtain a 20% bead concentration. To estimate the number of bacteria entrapped in beads, aliquots of bead suspensions were homogenized, and were plated on trypticase soy agar for bacterial culture. Control beads were prepared as described above without the addition of PA and these beads were found to be sterile when cultured on trypticase soy agar.

Mouse infection model

Six-week-old female C57BL/6N mice were purchased from Harlan (Gannat, France) and housed in conventional conditions. Mice were anesthetized with a ketamine-xylazine (15-2 mg/ml) solution administered intraperitoneally (0.1 ml/10 g of weight), and were intubated with a 20-g canula attached to a 1 ml syringe. A 150 µL inoculum of sterile or PAO1-coated beads (containing $6.10^5 \pm 3.10^5$ CFU) was instilled through the canula into the trachea. In selected experiments, mice were treated intraperitoneally with the selective inhibitor of EGFR tyrosine kinase AG1478 (12.5 mg/kg/d) [16] or with vehicle, 24h before instillation of PAO1-coated beads and daily until sacrifice. Mice were sacrificed at day 1 and day 7. To confirm that persistent airway infection was present at day 7 after instillation of PAO1-coated beads, animals were sacrificed and 10-fold dilutions of left lung homogenates were plated on trypticase soy agar: Mean ± SEM bacterial counts at day 7 were $8.4x10^2 \pm 3.8x10^2$ CFU/lung (n=14 animals), confirming that persistent airway infection occurred in our model. No bacteria were detected in lung homogenates of animals that were instilled with sterile beads (*not shown*). The study was performed in accordance with the European Community Guidelines and complied with the procedures of our Institut Fédératif de Recherche (Paris, France).

Immunohistochemical staining and morphometric analysis

For histological analysis, lungs were removed after flushing 4% paraformaldehyde through the right heart, and were fixed in 4% paraformaldehyde and included in paraffin. Five µm-paraffin sections were prepared and stained as described previously [17]. The following primary Abs were used: a polyclonal rabbit Ab raised against the N-terminus of VEGF-A (A-20, dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA); a polyclonal goat Ab raised against the phosphorylated (activated) EGFR (dilution 1:50, sc-16803; Santa Cruz Biotechnology); a polyclonal rabbit Ab raised against the endothelial marker von Willebrand

factor (vWF; Dako, Glostrup, Denmark) to identify peribronchial blood vessels. Antigen unmasking by incubation with protease from *Streptomyces griseus* (0.01%, Sigma) for 20 min was required to improve the quality of staining for vWF. Biotinylated anti-rabbit or anti-goat Ab (1:200; Vector Laboratories, Burlingame, CA, USA) were used for secondary Ab and bound Ab were visualized according to standard protocols for avidin-biotin-peroxidase complex method (Elite ABC kit; Vector). Tissue sections were counterstained with haematoxylin. Omission of primary antibodies and incubation with irrelevant immunoglobulins were used as negative controls.

Quantitative morphometric analysis was performed by 2 independent observers using a light microscope (Leica Microsystems, Wetzlar, Germany) connected to a computer [18]. Measurements of volumes occupied by phospho-EGFR and VEGF immunostaining in airway epithelium were performed by point counting using a grid [19, 20]. Volumes occupied by positively-stained cells were expressed per volume of epithelium (%). For each animal, 40 randomly selected high power fields (400X) of bronchial epithelium in two different tissue sections were assessed. Peribronchial blood vessels were identified using immunostaining for vWF. Volumes of blood vessels in the peribronchial compartment (located between the epithelium basement membrane and the alveolar attachments [21]) were also estimated using point counting. Numbers of small (<15 μm), medium (16-35 μm) and large (>35 μm) diameter blood vessels in the peribronchial space were counted for at least 10 bronchi (150 – 300 μm of internal diameter) at 200X magnification [21]. Results were expressed as number of vessels/bronchus and as numbers of vessels/surface area of peribronchial compartment (*not shown*) [21]. Both methods gave similar results.

Western blot for VEGF protein

***Lung homogenates were subjected to electrophoresis and were transferred to polyvinylidene difluoride membrane (Bio-Rad, France). Blots were performed using a rabbit polyclonal Ab to VEGF-A (A-20, 1:1500; Santa Cruz Biotechnology). Additional information on western blotting is provided in the* Online Depository.**

Statistical analysis

Data obtained from in vitro experiments are presented as mean ± SEM of at least 3 independent experiments performed in duplicate. Data obtained from measurements of VEGF protein concentrations in cultured airway epithelial cells were analyzed using one-way analysis of variance (ANOVA) for repeated measurements followed by the post-hoc Student-Neuman-Keuls test for multiple comparisons. For in vivo experiments, each experiment was performed in a minimum of 8 mice per condition. Data obtained from morphometric analysis were analyzed using the non-parametric Kruskal–Wallis test followed by the post-hoc Dunn's test. The interobserver coefficients of variation for morphometric measurements were less than 15%. All analyses were performed by using the Prism 5 software (GraphPad Inc., USA). Values of $P \leq 0.05$ were considered to indicate statistical significance.

Results

Pseudomonas aeruginosa supernatants increase VEGF production in human airway epithelial cells.

Under baseline conditions, NCI-H292 cells produced low concentrations of VEGF protein (**Figure 1**). Incubation of the cells with supernatant of the *Pseudomonas* laboratory strain PAO1 induced a dose-dependent increase in VEGF protein concentrations (**Figure 1A**). This increase was 3-fold at dilution 1:32 ($P<0.05$) and went up to 5-fold at dilution 1:8 ($P<0.01$). PAO1 supernatant also increased VEGF synthesis in BEAS-2B cells (+80 ± 22% compared to Baseline; n=6, $P=0.01$) and in primary cultures of airway (nasal polyp) epithelial cells at the air liquid interface (+28 ± 8% compared to Baseline; n=3, $P=0.03$; see *Online Depository*). Incubation of NCI-H292 cells with supernatants prepared from clinical strains of PA also increased VEGF concentrations ($P<0.05$ for all strains) (**Figure 1B**).

Next we examined whether secreted products from other bacteria frequently found in subjects with bronchiectasis also induced VEGF synthesis. Supernatants obtained from various strains of *S. pneumoniae* and *H. influenzae* induced VEGF synthesis in NCI-H292 cells, whereas *S. aureus* supernatants did not induce VEGF production (*see Online Depository*).

EGFR signaling pathway is involved in PA-induced VEGF production in NCI-H292 cells.

Because epidermal growth factor receptor (EGFR) activation by its ligands induces VEGF production in various cell types [12, 22], and because PAO1 supernatant has been shown to activate EGFR in airway epithelium [15], we hypothesized that PA supernatants induced VEGF synthesis *via* EGFR activation. When NCI-H292 cells were pretreated with a selective EGFR tyrosine kinase inhibitor AG1478 (10μM) that prevents EGFR phosphorylation, there was no

increase in VEGF concentrations upon incubation with PAO1 supernatant (P<0.01) (**Figure 2**). Pretreatment with AG9 (10 μM, an inactive analog of AG1478) was without effect. PAO1 supernatant-induced VEGF production was also prevented by pre-incubation with an EGFR neutralizing Ab (P<0.01) (**Figure 2**), but not with an irrelevant Ab (*data not shown*).

Persistent PAO1 airway infection induces VEGF expression in the airway epithelium in mice.

In Control mice (no instillation), immunostaining for VEGF was weak and localized to airway epithelium (**Figure 3**). In mice that have received intratracheal instillation of sterile agarose beads, a weak immunostaining for VEGF was observed in the airway epithelium at day 1 and day 7 after instillation (**Figure 3**). By contrast, in mice instilled with PAO1-coated beads, resulting in persistent airway infection at day 7 (see Methods), increase in VEGF immunostaining was observed in the airway epithelium at both day 1 and day 7 after instillation (**Figure 3**). Morphometric analysis indicated a significant 1.5 fold increase in epithelial VEGF staining at day 7 (P<0.05 as compared with Control mice, and P<0.05 as compared with mice instilled with sterile beads; **Figure 4A**). VEGF protein expression was further examined by western blot in mouse lung homogenates. Results confirmed that VEGF expression was increased at day 7 in lungs of mice instilled with PAO1-coated beads (**Figure 4B and C).**

Role of EGF receptor activation in VEGF expression in mouse airway epithelium in vivo.

As our in vitro studies have demonstrated that VEGF synthesis upon PA-supernatant stimulation was dependent on EGFR activation, we studied phosphorylated (activated) EGFR in mouse bronchial epithelium. In control mice, epithelial staining for phosphorylated EGFR was

low (**Figure 3 and 4D**). Similarly, in mice that have received intratracheal instillation of sterile beads, epithelial staining for phosphorylated EGFR was low and did not change between day 1 and day 7 after instillation (**Figure 3 and 4D**). By contrast, whereas staining for phosphorylated EGFR was low in the epithelium of mice instilled with PAO1-coated beads at day 1, a significant 2-fold increase in EGFR immunostaining was observed at day 7 ($P<0.01$ as compared with Control mice, and $P<0.01$ as compared with mice instilled with sterile beads) (**Figures 3 and 4D**).

To further examine the role of EGFR activation in VEGF airway epithelial expression in mice upon PAO1-coated beads instillation, mice were treated with the EGFR tyrosine kinase inhibitor AG1478 (12.5mg/kg/d; i.p.) or with vehicle, before intratracheal instillation of PAO1-coated beads and daily until sacrifice at day 7. There was no difference in phosphorylated EGFR expression between mice instilled with PAO1-coated beads and treated with vehicle, and mice that have only been instilled with PAO1-coated beads (**Figure 4D**). As expected, treatment with AG1478 resulted in a very low phosphorylated EGFR staining in the airway epithelium ($P<0.01$ as compared with results in mice that were instilled with PAO1-coated beads and treated with vehicle alone) (**Figure 4D**). When expression of VEGF was studied, there was no difference in VEGF expression between mice instilled with PAO1-coated beads and treated with vehicle, and mice that have only been instilled with PAO1-coated beads (**Figure 4A**). However, VEGF staining in airway epithelium was reduced by 2-fold in mice treated with the EGFR inhibitor ($P<0.001$ as compared with results in mice that have been instilled with PAO1-coated beads and treated with vehicle; **Figure 4A**).

Persistent PAO1 airway infection induces peribronchial angiogenesis in mice.

As epithelial VEGF is an angiogenic factor for airway blood vessels [8], we next examined whether instillation of PAO1-coated beads resulted in peribronchial angiogenesis. Blood vessels in the peribronchial compartment were studied using the endothelial marker von Willebrand factor (vWF). Morphometric analysis of volume occupied by vWF staining in the peribronchial compartment showed no significant difference between control mice and mice instilled with sterile or PAO1-coated beads (*data not shown*). A morphometric analysis was then performed that differentiated between small (<15 μm), medium (16-35 μm) and large (>35 μm) peribronchial blood vessels as described by Lee et al [21] (see Methods). Instillation of sterile beads had no effect on blood vessel counts according to their size. In contrast, instillation of PAO1-coated beads resulted in a marked increase in small blood vessels counts at day 7, whereas no effect was observed on medium and large vessel counts (**Figures 5A and 5B**). Treatment of mice with AG1478 reduced, but did not abolish, increase in small blood vessel numbers in mice instilled with PAO1-coated beads.

Discussion

Our results show VEGF synthesis by airway epithelial cells upon *Pseudomonas aeruginosa* stimulation both in vitro and in vivo. *Pseudomonas aeruginosa*-induced VEGF synthesis was associated in vivo with increased numbers of small peribronchial blood vessels. Administration of EGFR inhibitors reduced PA-induced VEGF synthesis in cultured airway epithelium and in mouse airway epithelium, and small peribronchial vessels in vivo.

VEGF has been shown to be expressed by alveolar type II cells, airway epithelial cells, smooth muscle cells, fibroblasts and alveolar macrophages [7]. In our study and under baseline conditions, VEGF synthesis by bronchial epithelial cells was low, both in vitro and in mice. This is in accordance with previously published studies: Lee et al have shown low levels of VEGF expression in normal human bronchial epithelial cells under basal conditions [23] and Corne et al have found low expression of VEGF in the bronchial epithelium of C57BL/6 mice [24]. However, upon incubation in the presence of PA supernatants in vitro or in the presence of persistent PAO1 infection in vivo, we observed that VEGF synthesis by airway epithelial cells markedly increased. Although VEGF was expressed in other cell types (e.g., macrophages; *not shown*), these data implicate airway epithelium as a major site of VEGF synthesis after bacterial stimulation.

Our study is the first to show in vitro and in vivo increased VEGF synthesis by airway epithelial cells upon exposure to PA. It is strengthened by the fact that both the laboratory strain PAO1 and several clinical PA strains induced VEGF expression in vitro. Our data is consistent with the report of increased VEGF synthesis by chondrocytes after a 6h exposure to PA supernatants [25]. In the latter study, the authors also reported induction of VEGF synthesis in chondrocytes exposed to *S. aureus* supernatants (but no information on bacterial strains was

provided) [25], whereas in the present studies various strains of *S. aureus* were without effect on VEGF synthesis in airway epithelium. These differences may be related to different behavior of airway epithelial cells vs. chondrocytes or to different bacterial strains. Importantly, infectious agents other than PA may also enhance VEGF synthesis in airway epithelium. We found that *S. pneumoniae* and *H. influenzae* supernatants induced VEGF synthesis in cultured airway epithelial cells (see Online Depository). Further, the respiratory syncytial virus (RSV) has been shown to stimulate VEGF synthesis in vitro in airway epithelial cells and in vivo in nasal secretions in patients [23]. Altogether, these data support the concept that airway infection may be associated with increased VEGF expression in airway epithelium.

The present study was not designed to identify specifically which products in PA supernatants were responsible for EGFR activation and VEGF synthesis. We found that PA-induced VEGF synthesis persisted when PAO1 supernatant was boiled or treated with proteases before incubation with epithelial (NCI-H292) cells (Martin C. and Burgel PR; personal communication). Although PA lipopolysaccharide (LPS) induced VEGF synthesis in NCI-H292 epithelial cells dose-dependently, we found no effect of PA-LPS on VEGF synthesis in BEAS-2B cells and in primary cultures of airway epithelial cells (see *Online Depository*). These latter findings are consistent with the report by Murphy et al., who found no effect of LPS on VEGF synthesis in primary cultures of airway epithelial cells derived from lung allograft [26]. Further studies will be necessary to identify soluble products responsible for PA-induced VEGF synthesis in airway epithelium.

We found that PA-induced VEGF synthesis in vivo and vitro was dependent, at least partly, on EGFR activation. Mechanisms leading from bacterial stimuli to EGFR activation require discussion. Airway epithelium is the first line of defense against invading pathogens and

senses bacterial signals via cognate receptors (e.g., Toll-like receptors) [11]. Koff et al. have recently shown that activation of various Toll-like receptors by their recombinant ligands induced VEGF synthesis in cultured airway epithelial cells via a cascade of event culminating in EGFR activation [27], but the role of specific bacterial stimuli was not studied [27]. Interestingly, Varoga et al. have shown that activation of TLR2 by *S. aureus* supernatant resulted in ERK1/2-dependent VEGF synthesis in cultured human chondrocytes, but the role of EGFR activation in this process was not explored [25]. We speculate that TLRs play roles in PA-induced EGFR activation and VEGF synthesis, but further studies will be required to establish these mechanisms.

The number of small peribronchial vessels had increased 7 days after instillation of PAO1-coated beads. This time-course is consistent with previous studies that showed that 7 days after intranasal inoculation of *Mycoplasma pulmonis* in various strains of mice, modifications in tracheal microvasculature and endothelial cell proliferation occurred [28, 29]. This rapid angiogenesis was also observed in the absence of bacterial stimulation. In a tet-on transgenic mouse model in which VEGF was selectively overexpressed in the airway epithelium under the control of a tissue-specific promoter, endothelial sprouting was observed 1 day after switching on VEGF expression, and a 2-fold increase in vessel density had occurred by day 7 [8]. Although these data show that angiogenesis is a rapid process, we suggest that longer studies may more closely mimic vascular abnormalities occurring in chronically infected airways in humans with bronchiectasis.

The overexpression of VEGF in airway epithelium following PA infection *in vivo* suggests that PA-induced peribronchial angiogenesis is mediated by interaction between epithelial VEGF and endothelial VEGF receptors. However, multiple proangiogenic molecules

(e.g., growth factors and cytokines) [30] may play roles in PA-induced peribronchial angiogenesis and studies using VEGF receptors antagonists will be required to confirm the role of VEGF. Additionally, although increased peribronchial vascularity induced by PA was reduced by EGFR inhibition, the exact role of EGFR in this process was not determined. This could be due to a direct effect of the EGFR antagonist on endothelial cells or to an indirect effect related to reduction of VEGF synthesis in airway epithelium.

Our report is the first to experimentally show a relationship between bronchial infection with PA and peribronchial angiogenesis. It suggests a role for chronic airway infection in the development of the abnormal bronchial circulation containing multiple tortuous and hypertrophic blood vessels, which is found in subjects with bronchiectasis. We speculate that intensive antibiotic therapy against persistent bacterial infection may help reducing abnormalities in the bronchial circulation in patients with bronchiectasis.

In conclusion, we showed that exposure of airway epithelium to PA products increased expression of VEGF synthesis in vitro and in vivo, at least in part via EGFR activation. PA-induced VEGF synthesis was associated with rapid peribronchial angiogenesis. We suggest that the effects of EGFR inhibitors in reducing bronchial angiogenesis in chronic airway inflammatory diseases (e.g. bronchiectasis) need to be further examined in humans. Ultimately, it may prove a useful approach to reduce angiogenesis triggered by persistent bacterial infection.

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Figure legends

Figure 1: Effects of *Pseudomonas aeruginosa* supernatants on VEGF production in NCI-H292 cells.

Confluent NCI-H292 cells were cultured in cell culture medium alone (Baseline), in the presence of the bacterial culture medium trypticone soy broth dialysate (TSBD, 1:8 dilution) or incubated with *Pseudomonas aeruginosa* (PA) supernatants (sup) for 24h. VEGF protein concentrations were measured by ELISA in cell culture medium at 24h. **A:** Cells were treated with serial dilutions (from 1:32 to 1:8) of PAO1 supernatant. **B:** Cells were treated with PAO1 supernatant or with supernatants prepared from six different clinical strains of PA (three nonmucoid and three mucoid, labeled 1 to 6; dilution 1:8). Data are mean ± SEM (n=3 separate experiments in duplicate). *, P <0.05 and **, P<0.01compared to Baseline.

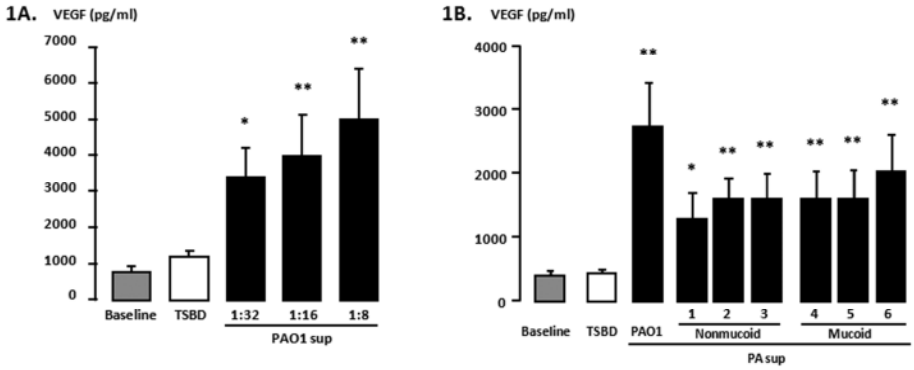

Figure 2: Role of EGFR in *Pseudomonas aeruginosa* supernatant-induced VEGF production in NCI-H292 cells.

Confluent NCI-H292 cells were incubated for 24h with PAO1 supernatant (PAO1 sup, 1:8 dilution) alone or were pre-treated with a selective EGFR tyrosine kinase inhibitor (AG1478), an inactive compound (AG9) or an EGFR neutralizing Ab (EGFR Ab). VEGF protein concentrations were measured by ELISA in cell culture medium at 24h.

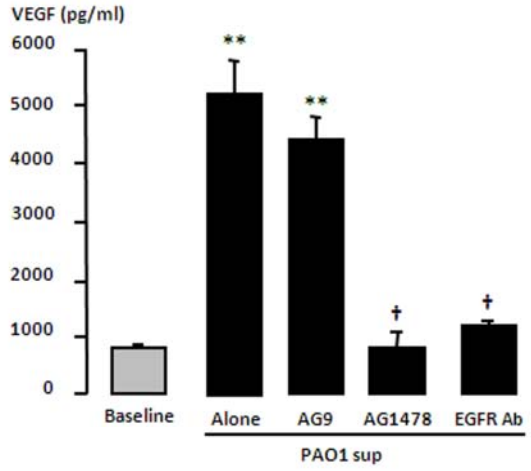

Figure 3: Representative photomicrographs of immunostaining for VEGF and phosphorylated EGFR in control mice, and in mice instilled with sterile or with PAO1-coated beads.

Airway tissue sections were obtained from mice without instillation (Control), and after1 day and 7 days in mice that received intratracheal instillation of sterile or PAO1-coated beads (B). Sections were stained with antibodies to VEGF (upper and middle panels) and to phosphorylated-EGFR (p-EGFR, lower panel) and were counterstained with hematoxylin. In Control mice, weak or no staining for VEGF and p-EGFR were present in epithelium. Instillation of sterile beads only resulted in a small increase in VEGF staining in epithelium at day 7. However, instillation of PAO1-coated beads induced a marked increase in VEGF and p-EGFR staining (*brown staining*) in epithelium. At low magnification (upper panel), airway epithelium appears as a major site of VEGF expression after PAO1 infection. Higher magnification (middle panel) shows granular staining for VEGF throughout the epithelium after PAO1 infection that also resulted in the recruitment of numerous inflammatory cells (mostly neutrophils, arrows) in the airway lumens and in the adjacent alveoli (arrowheads).Photomicrographs are representative of results obtained in at least 8 animals per condition. All bars 50 μm. Original magnifications 100X (upper panel), and 400X (middle and lower panels).

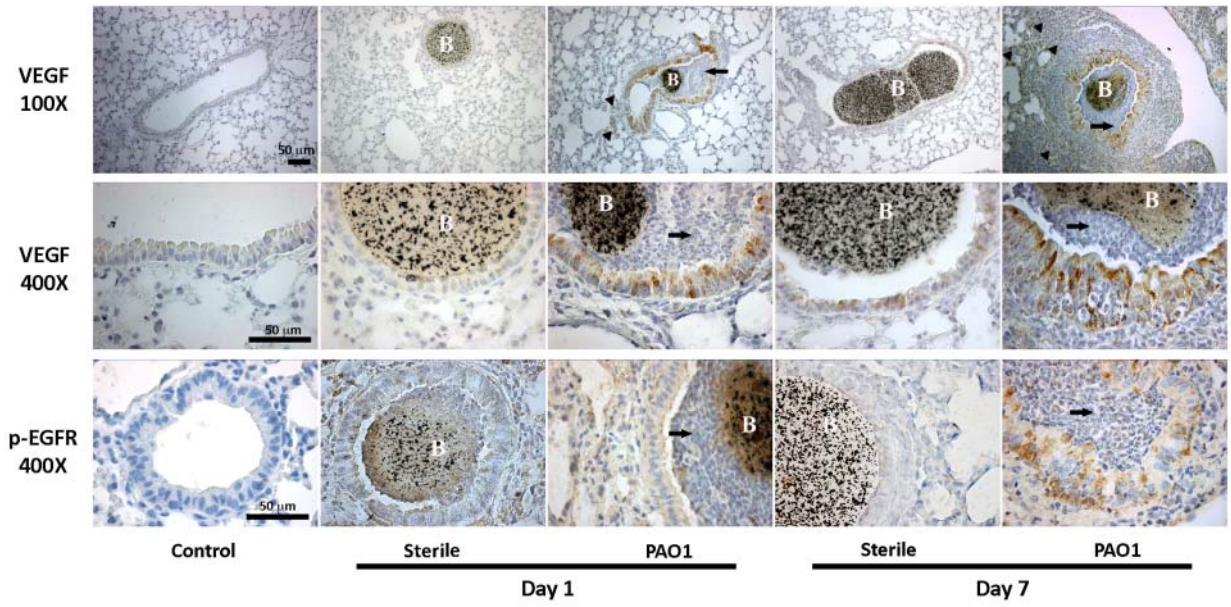

Figure 4: Effects of PAO1 infection on VEGF expression and EGFR phosphorylation in mouse airways. Mice were sacrificed without instillation (Control), and 1 day and 7 days after intratracheal instillation of sterile or PAO1-coated beads. In some experiments, mice were pretreated with AG1478 (12.5 mg/kg/d, i.p.) or with vehicle (DMSO), 24 hours before instillation and daily until sacrifice at day 7. **A:** Airway sections were immunostained for VEGF protein and morphometric analysis of VEGF immunostaining in airway epithelium was performed. **B:** Representative western blot for VEGF protein in lung homogenates. Membrane were stripped and reprobed with an Ab to β–Actin to normalize for protein loading. **C**: Quantification of relative VEGF protein levels in western blots of lung homogenates. **D:** Airway sections were immunostained for phosphorylated EGFR (p-EGFR) and morphometric analysis of p-EGFR immunostaining in airway epithelium was performed. Data are mean ± SEM from at least 8 mice per condition for morphometric analysis and 4 mice per condition for western blotting. *, $P<0.05$ and **, $P<0.01$ as compared with Control; †, $P<0.01$ as compared with sterile beads at day 7; and ‡, $P<0.01$ as compared with PAO1-coated-beads-instilled mice at day 7.

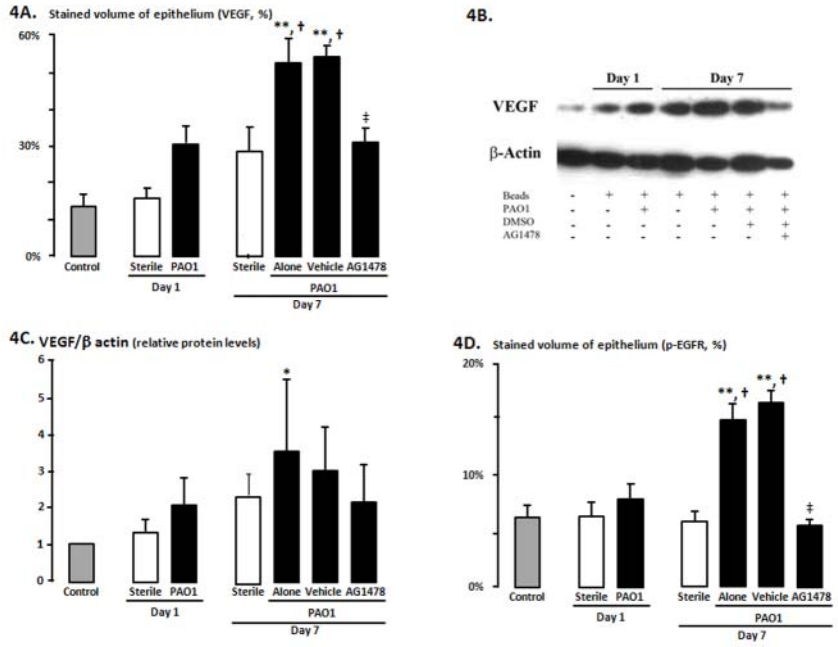

Figure 5: Effects of PAO1 airway infection on peribronchial blood vessels in mice. Airway tissue sections were obtained from mice without instillation (Control), and 1 day and 7 days after intratracheal instillation of sterile or PAO1-coated beads. In some experiments mice were pretreated with AG1478 (12.5 mg/kg/d, i.p.) or with vehicle, 24 hours before instillation and daily until sacrifice at day 7. **A**: Representative photomicrographs of immunostaining for von Willebrand factor (vWF), an endothelial marker for identification of peribronchial blood vessels (brown color). Although large (L) and medium (M) blood vessels are present adjacent to airways in Control and in instilled animals, only animals instilled with PAO1-coated beads have numerous small peribronchial blood vessels (right panel, **arrowheads**). Bar 50 μm. Original magnification 200 X. **B**: Small (<15μm), medium (16-35μm) and large (>35μm) vWF-positive peribronchial blood vessels were counted as described previously [21].Data are mean ± SEM

from at least 8 mice per condition.*, $P<0.001$as compared with Control and †, $P<0.05$ as compared with mice instilled with sterile-beads.