

**Dual Role of Vascular Endothelial Growth Factor in Experimental  
Obliterative Bronchiolitis**

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Online Data Supplement

## **Methods**

### **Heterotopic Rat Tracheal Transplantation**

Specific pathogen-free inbred male DA (AG-B4, RT1<sup>a</sup>) and WF (AG-B2, RT1<sup>u</sup>) rats (Harlan, Horst, The Netherlands) weighing 200-300 g and of 2-3 months of age were used. All transplantations were performed heterotopically to the recipient's bursa omentalis as described (E1). Syngeneic tracheal grafts were transplanted from DA donors to DA recipients and allografts from DA donors to WF recipients. All grafts were harvested 10 and 30 days after transplantation. Nontransplanted DA trachea served as normal control. Permission for animal experimentation was obtained from the State Provincial Office of Southern Finland. All rats received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (National Academy Press, Washington, D.C. 1996).

### **Experimental Design**

First, we investigated VEGF ligand and receptor expression during the development of OB in nonimmunosuppressed rats at mRNA and protein level by in situ hybridization and immunohistochemistry, respectively. Second, we performed gene transfer in tracheal allografts using adenoviruses encoding mouse VEGF164 (AdVEGF) or  $\beta$ -galactodidase (AdlacZ) and transplanted these allografts into recipients receiving cyclosporine A (CsA) 1.5 mg/kg/day to investigate whether VEGF enhances the development of OB. To investigate the efficiency of the adenoviral gene transfer, we infected DA tracheal grafts with  $\beta$ -galactosidase encoding adenovirus for whole-mount X-gal staining and with enhanced green

fluorescent protein (EGFP) -encoding adenovirus (AdEGFP) for fluorescent microscopy analysis. Expression was analyzed after 3 and 7 days (both groups) and 10 and 30 days (AdlacZ infected grafts only; for details, see online repository and (12)). Concomitantly with VEGF gene transfer, one group of recipients received VEGFR tyrosine kinase inhibitor PTK787/ZK222584 (PTK787; Novartis, Basle, Switzerland), a second group a PDGFR tyrosine kinase inhibitor (imatinib), and a third group N-nitro-L-arginine methyl ester (L-NAME) for the whole study period to investigate the mechanisms how VEGF regulates epithelial regeneration (L-NAME) and the development of OB (PTK787 and imatinib). Third, we gave allograft recipients PTK787 or vehicle and CsA 1 mg/kg/day to investigate the effect of inhibition of VEGFR activation on the development of OB. All analyses were performed by two independent observers in a double-blinded manner with excellent inter-observer correlation.

### **Drug Regimens**

CsA (Novartis) was diluted in Intralipid (Fresenius Kabi, Bad Homburg, Germany) and administered sc at the doses described above. PTK787 (Novartis) was diluted in polyethylene glycol (av.mol.wt 300; Sigma, St. Louis, MO) and was given 100 mg/kg/day via an orogastric tube. Imatinib (Novartis) was diluted in saline and administered 10 mg/kg/day intraperitoneally. PTK787 is a potent VEGFR inhibitor and can inhibit also other class III kinases, such as PDGFR- $\beta$  tyrosine kinase, c-Kit and c-Fms, but at higher concentrations (E2). Imatinib is a potent PDGFR inhibitor and inhibits also Bcr-Abl and c-kit but not other receptor or cytoplasmic tyrosine kinases (E3). The doses used were chosen on the basis of pharmacological data given to us by the manufacturer (Table E1). With the dosage used in this study, the plasma

concentrations of both PTK787 and imatinib remain throughout the study period above the level needed for total inhibition of VEGFR and PDGFR protein tyrosine kinases, respectively (E2, E3). L-NAME (Sigma) was administered into drinking water 1 g/l which is sufficient for effective inducible nitric oxide synthase (iNOS) inhibition (E4). Neither PTK nor imatinib altered CsA 24-hour blood trough levels in our preliminary study.

### **Adenovirus-mediated VEGF<sub>164</sub> Gene Transfer**

All adenoviral gene transfer was performed by incubating the harvested donor trachea with  $0.2 \times 10^9$  plaque-forming units of AdVEGF, AdlacZ, or AdEGFP in PBS at +4°C for 30 minutes, with 6 mmol/l EDTA to permeabilize the epithelium. After incubation, the grafts were placed into the recipient's bursa omentalis. Mouse VEGF<sub>164</sub>,  $\beta$ -galactosidase, and EGFP encoding adenoviruses were produced as previously described (E5). Briefly, cDNAs were subcloned into pAdCMV plasmid, constructed by subcloning the human CMV immediate early promoter, multiple cloning site, and the bovine growth hormone polyA signal from pcDNA3 plasmid (Invitrogen, Groningen, The Netherlands) into a pAdBgIII vector. Replication-deficient E1-E3–deleted clinical GMP-grade adenoviruses were produced in 293T cells. Adenoviruses were analyzed to be free of replication-competent viruses, lipopolysaccharide, mycoplasma, and other microbiological contaminants. The functionality of adenoviruses has been demonstrated in a recent study (E5). To investigate the efficiency of the adenoviral gene transfer, we incubated DA tracheal grafts with  $0.2 \times 10^9$  pfu of adenoviral vector encoding  $\beta$ -galactosidase for whole-mount X-gal staining or EGFP-encoding adenovirus for fluorescent microscope analysis. AdlacZ infected grafts were harvested after 3, 7, 10, and 30 days and fixed

in 0.2% glutaraldehyde in 0.1M phosphate buffer for 20 minutes. Overnight whole mount stainings were performed in 1 mg/ml X-gal staining solution. After an overnight wash, they were fixed overnight in 4% PFA in PBS, routinely processed, and embedded in paraffin. Cross sections were examined by light microscopy. AdEGFP infected grafts were removed for expression analysis after 3 and 7 days. After harvesting, they were fixed in 2% PFA in PBS at RT for 1 hour. The grafts were washed twice in 20% sucrose in PBS for 30 min and then incubated overnight in 20% sucrose in PBS at +4°C.

### **Histological Evaluation**

Tracheal allografts were excised, embedded in Tissue-Tek (Miles Inc., Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -70°C until used. For histological evaluation, frozen sections were stained with Mayer's hematoxylin-eosin (H&E). Epithelial necrosis was determined as percentage of loss of epithelium lining the tracheal lumen. Luminal occlusion was evaluated by determining the reduction in luminal area using the NIH Image program version 1.59 (National Technical Information Service, Springfield, VI).

### **Immunohistochemistry**

Serial cryostat sections (4-6 µm) were stained using the peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA), and the reaction was revealed by 3-amino-9-ethylcarbazole (AEC, Vectastain). The following antibodies were used: rabbit anti-VEGF (2 µg/ml; sc-152, Santa Cruz Biotechnology Inc., Santa Cruz, CA), goat anti-VEGF (0.4 µg/ml; sc-1836, Santa Cruz), goat anti-VEGFR-1 (1 µg/ml; sc-316-G, Santa Cruz), goat anti-VEGFR-2 (2 µg/ml; sc-315-G,

Santa Cruz), mouse anti-CD4<sup>+</sup> (5 µg/ml; #554835, Pharmingen, San Diego, CA), mouse anti-CD8<sup>+</sup> (5 µg/ml; #554854, Pharmingen), mouse anti-ED1<sup>+</sup> macrophages (5 µg/ml; #22451D, Pharmingen), and rabbit anti-Ki67 (1:2000; #NCL-Ki67p; Novocastra Laboratories, Newcastle upon Tyne, UK). The sections were counterstained with Mayer's hematoxylin and aquamounted (Aquamount; BDH Ltd., Poole, UK).

For inflammatory cells, the results are expressed as the number of positively staining cells per allograft cross section. In the case of VEGF, VEGFR-1, and VEGFR-2, the intensity of staining was scored semiquantitatively from 0 to 3 as follows: 0, no visible staining; 1, few cells with faint staining; 2, moderate intensity with multifocal staining; and 3, intense diffuse staining of the cells analyzed. VEGF expression was assessed separately from the epithelial layer and the allograft airway wall, consisting of subepithelial space and tissue surrounding the cartilage ring. Specificity controls were performed using the same immunoglobulin concentration of species- and isotype-matched antibodies or irrelevant primary antibodies. Additional specificity controls for VEGF, VEGFR-1, and VEGFR-2 stainings involved the use of a working dilution of the polyclonal antibodies after overnight incubation with a 20-molar excess of control peptide (for VEGF: sc-152-P; for VEGFR-1; sc-316-P; for VEGFR-2: sc-315-P; Santa Cruz). Analyses were done by two independent observers in a blinded fashion.

### **Microvascular Remodeling and Lymphangiogenesis**

Allograft vascularization and lymphangiogenesis were determined using immunohistochemistry as described above. Mouse anti-rat endothelial cell antigen-1

(RECA-1, 1:10; #MCA970; Serotec, Oxford, UK) was used to quantitate the number of allograft blood vessels, mouse anti-high molecular weight melanoma-associated antigen (HMW-MAA) (MAb 225.28; 1:50; a generous gift from Dr S. Ferrone, Roswell Park Cancer Institute, Buffalo, NY) for the detection of activated pericytes (E6), and rabbit anti-LYVE-1 (recognizing a receptor for hyaluronan in lymphatic endothelium; 1:1000) for the detection of lymphatic vessels. For amplification of the LYVE-1 immunohistochemic signal, the TSA Biotin system (Perkin-Elmer, Boston, MA) was used. The results are expressed as positive vessels/allograft tracheal cross section.

### **In Situ Hybridization**

The following templates were used to generate antisense and control sense probes for in situ hybridization: human VEGF (AC X62568, bp 11-592; in pGEM3z, Promega, Madison, WI), human VEGFR-1 (AC NM002019.1, bp 1-1898, in pGEM3z, Promega), human VEGFR-2 (AC X61656, bp 1-714, in pBluescript II KS, Stratagene, La Jolla, CA; all three templates were generous gifts from Dr Kari Alitalo). Probes were generated using a digoxigenin RNA labeling mix (#1277073, Roche, Basel, Switzerland). Non-radioactive in situ hybridization on tracheal sections was performed with Ventana Discovery automate (Ventana Medical Systems, Tucson, AZ). In brief, the samples were deparaffinized with heat treatment followed by post-fixation and RiboClear (Ventana) pre-treatment. Enzymatic proteinase treatment was performed for 4 min and both antisense and sense probes were hybridized at +65°C (VEGF receptors) or +58°C (VEGF) for 6 h. After hybridization, the sections were washed three times with 0.1 X SSC either at +60°C (VEGFR-2) or +65°C (VEGF and VEGFR-1), followed by the detection step, which consists of a 20 min biotinylated

anti-DIG (Jackson ImmunoResearch Lab., West Grove, PA) antibody (1:2000) incubation and either a 2h- (VEGFR-1) or 4h- (VEGF and VEGFR-2) BCIP/NBT substrate incubation for the color reaction. All reagents for the Discovery are commercial products provided by Ventana Medical Systems except for proteinase K (Roche), which was used at a concentration of 350 ng/ $\mu$ l. After hybridization in the Discovery, the sections were washed, counterstained with Nuclear Fast Red (Merck, Darmstadt, Germany), dehydrated and mounted with Mountex (HistoLab, Göteborg, Sweden).

### **Statistical Analyses**

All data are expressed as mean  $\pm$  SEM. Student *t* test and ANOVA-test were used for parametric comparisons, while Mann-Whitney and Kruskal Wallis and Dunn tests were used for non-parametric comparisons (StatView 4.1 programme; Abacus Concepts Inc., Berkeley, CA).  $P < 0.05$  was regarded as statistically significant.

Table E1a. Inhibitory activity of PTK787 against class III receptor tyrosine kinases

Kinase	IC50 of PTK787
VEGF receptor/KDR	0.037 $\mu$ M
VEGF receptor/Flt-1	0.077 $\mu$ M
VEGF receptor/Flk-1	0.27 $\mu$ M
Flt-4	0.64 $\mu$ M
c-Kit	0.73 $\mu$ M
c-Fms	1.4 $\mu$ M
PDGFR- $\beta$	0.58 $\mu$ M

In our experiment, PTK787 was administered 100 mg/kg/day, which according to the manufacturer results in a sufficient plasma concentration for inhibition of VEGF receptors for at least 12 hours after administration (i.e.  $>1\mu$ M). This may lead also to partial inhibition of other receptor signalling (discussed in the online supplement chapter on drug regimens). Modified from Wood *et al.*, *Cancer Res* 2000;60:2178-2189 (E2).

Table E1b. Inhibitory activity of imatinib against class III receptor tyrosine kinases

Kinase	IC50 of imatinib
PDGFR- $\alpha$	0.1 $\mu$ M
PDGFR- $\beta$	0.1 $\mu$ M
c-Kit	0.1 $\mu$ M
c-Fms	no effect at 10 $\mu$ M
Flt-3	no effect at 10 $\mu$ M
VEGF receptor / KDR	no or weak effect at 11 $\mu$ M
VEGF receptor / Flt-1	no or weak effect at 25 $\mu$ M
Tek	no or weak effect at 26.8 $\mu$ M
c-Met	no or weak effect at 101 $\mu$ M

Imatinib was administered 10 mg/kg/day on the basis of our previous studies and data from the manufacturer. This dose leads to a peak plasma level of approximately 2  $\mu$ M and a specific and enduring inhibition of PDGF receptor signalling. Modified from Buchdunger *et al.*, J Pharmacol Exp Ther 2000;295:139-145 (E7).

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