Activin-A is over-expressed in severe asthma and is implicated in angiogenic processes

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ONLINE DATA SUPPLEMENT

METHODS

Study population

All asthmatic patients had a physician confirmed diagnosis of asthma according to GINA guidelines [1] and had been attending the asthma outpatient clinic of Athens Chest Hospital "Sotiria" for at least two years prior to study enrolment. None of the participants had symptoms of upper respiratory tract infection for at least 3 months prior to bronchoscopy. None of the patients were treated with monoclonal antibodies during the study. Healthy controls (HC) had no history of asthma or any other chronic disease. Severe asthma was defined as disease that requires treatment with high-dose inhaled corticosteroids (ICS) plus a second controller to prevent it from becoming uncontrolled [2]. Asthma exacerbations were defined according to the 2012 NIH Asthma Outcomes Workshop [3] as episodes of severe symptom deterioration and elevated rescue medication usage that required the administration of systemic corticosteroids de novo or at an elevated dosage for at least three days. Those patients exhibiting an exacerbation according to the aforementioned definition during their 2 year follow-up visited our Dept. on an outpatient basis and were treated according to current guidelines (SABA, OCS and antibiotics if bacterial infection was suspected). None of the patients required hospitalization at the time. Serum sampling during exacerbations was always performed before administration of treatment. Atopy was defined according to the World Allergy Organization nomenclature [4] and assessed by means of skin-prick tests for 18 common aeroallergens (HAL Allergy Benelux, Leiden, the Netherlands).

Sample processing and storage

Blood samples were centrifuged for 15 min at 1,000 x g at room temperature (RT). BAL samples were centrifuged for 15 min at 300 x g at 4^{o}C. Serum and BAL supernatants were stored at -80^{o}C within 2 hours after collection. BAL cell pellets were resuspended in 1 mL PBS and used for total and differential cell counts. Endobronchial biopsy specimens were embedded in paraffin after being fixed in 10% neutral buffered formalin overnight at RT or in Tissue-tek® OCT™ (Thermo Fisher Scientific) and snap-frozen in isopentane (BDH Chemicals) pre-cooled in liquid nitrogen and stored at -80^{o}C until cryostat sectioning.

Fiberoptic bronchoscopy and sample collection

Bronchoscopy was performed by two experienced physicians using a flexible bronchoscope (WM-N60 mobile workstation; Olympus, Tokyo, Japan) on an outpatient basis at Athens Chest Hospital "Sotiria", as previously described [5]. After inspection of the bronchial tree a bronchial lavage was performed (120 mL total volume divided into 20 mL aliquots) according to the European Respiratory Society Task Force guidelines [6], in either the middle lobe (RB4/5) or the lingula (LB4/5) and was not guided by CT-scan. BAL samples with a recovery of ≥ 60% were kept for further analysis (HC n = 13, MMA n = 12, SA n = 19). Endobronchial biopsy specimens were obtained following BAL fluid collection from all subjects, from various sites of the subsegmental carinae of the right lower lobes or right middle lobe [7]. A minimum of three bronchial biopsy specimens were taken from each subject.

Cytokine measurements

Commercially available ELISA kits were used to determine the concentrations of human activin-A (DY338; R&D Systems, Wiesbaden, Germany), IL-17A (88-7176-

88, eBioscience, Frankfurt, Germany), IL-18 (BMS267/2, eBioscience, Frankfurt, Germany), IL-32 (DY3040-05, R&D Systems), VEGF (DY293B, R&D Systems) and sVEGFR1 (BMS268/3, eBioscience) in serum, BAL and endothelial cell culture supernatants, according to the manufacturers' instructions. The limit of detection (LOD) for each ELISA kit used was determined by adding two standard deviations to the measured mean optical density of the zero standard replicates and calculating the corresponding concentration and was measured to be 50 pg/mL for activin-A, 5 pg/mL for IL-17A, 20 pg/mL for IL-18, 30 pg/mL for IL-32, 20 pg/mL for VEGF and 30 pg/mL for sVEGFR1. When a sample was measured to be under the LOD a value equal to the measured limit of detection was arbitrarily assigned to it before further statistical processing.

Vessel number measurements

Vessels were identified with the endothelial marker CD31 (goat polyclonal anti-CD31, sc-1506, 1:200, Santa Cruz, USA), and continuous analysis of all of the lamina propria to a depth of 100 μm below the basement membrane was performed at x400 magnification. The total number of vessels was divided by the biopsy area examined to determine the number of vessels per unit area (mm^2). Sections from all subjects were observed using an Axioskop 40 microscopy system (Carl Zeiss, Jena, Germany) coupled with an AxioCam MRc digital camera, and measurements were performed using image analysis software (AxioVision v4.5.0.0, Carl Zeiss, Germany and ImageJ v1.47, NIH, US).

Immunohistochemistry

Paraffin sections 3-4 μm thick were utilised. Tissue processing and immunostaining was performed as previously described [5;8;9]. Briefly, after dewaxing and rehydration, sections were incubated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase for 20 minutes at RT. Heat-induced epitope retrieval was performed in preheated pH 6 citrate buffer (S2369; Dako, Glostrup, Denmark) using a microwave oven (600W) for 15 min. Blocking of non-specific binding sites was performed by incubating slides with 10% donkey serum for 30 min. PBS was used for washes, unless otherwise indicated. Sections were incubated in goat polyclonal anti-activin-A (AF338; 15μg/mL), anti-ALK-4 (AF222; 15 μg/mL) and anti-Act-RIIA (AF340; 30 μg/mL, all R&D Systems, USA) and goat polyclonal anti-CD31 (sc-1506, 1:200, Santa Cruz, USA). Negative controls were performed by substitution of the primary antibody with the same concentration of the corresponding isotypic IgG. All incubations with primary antibodies took place overnight at 4^{o}C. Bound primary antibodies were visualized by incubation for 45 min with biotinylated anti-goat IgG for activin-A, ALK-4 and Act-RIIA (BA-5000; 15μg/mL, Vector Laboratories, USA). Slides were incubated with avidin-biotin complex (VECTASTAIN ABC kit, Vector Laboratories, USA), then with the DAB substrate (SK1400; Vector Laboratories), counterstained with hematoxylin, dehydrated, mounted and studied by light microscopy.

All available sections from each patient were evaluated. Activin-A expression was evaluated in all subjects (n = 57), while the expression of activin-A's receptors act-RIIA and ALK-4, was evaluated in available subjects until tissue sample depletion (n = 37-39, samples representative for each group). Stained sections were coded and examined in a blinded manner independently by two investigators at the end of the study. Biopsy specimens were considered suitable for examination when there was ≥

1.0 mm of basement membrane length with intact epithelium and ≥ 0.1 mm^2 of subepithelial area. At least two suitable biopsy sections were examined per subject and data were pooled. The numbers of positively stained epithelial cells were counted along the entire basement membrane of each section and expressed as the percentage of the total number of epithelial cells, while positively stained subepithelial cells were determined by counting the whole section and expressed as cells per square millimetre, as previously described [8-10]. Image analysis software (AxioVision v4.5.0.0, Carl Zeiss, Jena, Germany and ImageJ v1.47, NIH, US) was used for the corresponding measurements.

Immunofluorescence

Double immunofluorescence staining was performed on 6 μm thick serial sections. Slides were warmed up at RT for 30 min, fixed in ice cold (4∘C) acetone for 5 min, air dried for 45 min, and washed in PBS. Antigen retrieval was performed (S2369; Dako, Denmark) and non-specific binding was blocked with 10% donkey serum. A goat polyclonal primary antibody was used against activin-A (AF338; 15 μg/mL, R&D Systems, USA) and ALK-4 (AF222; 15 μg/mL, R&D Systems, USA), while mouse monoclonal primaries were used for mast cell tryptase (M7052; 1:25, Dako, Denmark), neutrophil elastase (M0752; 1:25, Dako, Denmark), macrophage cell surface marker CD68 (M0876; 1:40, Dako, Denmark), endothelial cell surface marker CD34 (M7165; 1:30, Dako, Denmark) and α-smooth muscle actin (α-SMA) (Ab5694; 1:200, Abcam, Cambridge, UK). All incubations with primary antibodies took place overnight at 4^oC. For detection of neutrophil elastase antigen retrieval was not performed because of epitope destruction. The sections were washed in PBS and incubated for 1h at RT with donkey F(ab')2 anti-goat IgG (H+L) AF555 and donkey

F(ab')2 anti-mouse IgG (H+L) AF488 (A21432 and A21202 respectively, both at 5 μg/mL, Life Technologies, Carlsbad, CA) diluted in 1% donkey serum in PBS. Nuclear stain with DAPI (4',6-diamidino-2-phenylindole) was performed using ProLong Gold Antifade Reagent (P36931; Life Technologies, Carlsbad, CA).

Examination of sections and acquisition of fluorescent images was performed using a confocal laser scanning microscope (Confocal Leica TCS SP5) and fluorescent images were acquired using Differential Interference Contrast (DIC) optics. Hoechst was excited by the 405-nm laser diode and the fluorescence was collected using a long-pass (LP) 420 emission filter; the green (AF488) antibody was excited by the 488-nm argon ion laser line and the fluorescence was collected using a band-pass (BP) 505-530 emission filter, and the red (AF555) antibody was excited by the 555-nm green helium-neon laser line and the fluorescence was collected using an LP560 emission filter. Tissue sections were visualised using the confocal microscope at 1,024 x 1,024-pixel resolution through a HCX PL APO CS 20.0x0.70 DRY UV with eight-times averaging in sequential scanning (multi-track) mode with the pinhole set to obtain an optical section of approximately 1 mm in all channels.

Endothelial cell cultures

The human umbilical vein EC line (HUVEC) and the human pulmonary microvascular EC line (HPMEC) were purchased from Merck Millipore (Germany) and PromoCell (Germany), respectively. Both cell lines were used before passage four for experiments. HUVEC (0.5×10^6 per well) were seeded in six-well plates (Nunc A/S, Denmark) pre-coated with gelatine (0.5% Sigma-Aldrich) in complete medium (EndoGRO^{TM} – LS Complete Medium, Merck Millipore, Germany) containing EndoGRO^{TM} basal medium, 0.2% endothelial cell growth supplement,

2% FBS, 5 ng/mL recombinant human (rh)-EGF, 50 μg/mL ascorbic acid, 10 mM L-Glutamine, 1 μg hydrocortisone, 0.75 U/mL heparin sulfate, 100 U/mL penicillin, and 100 μg/mL streptomycin and grown to approximately 90% confluence in a 5% CO_2 incubator at 37 °C. HPMEC (0.5×10^6 per well) were seeded in six-well plates (Nunc A/S, Denmark) pre-coated with gelatin (0.5% Sigma-Aldrich) in complete medium (endothelial cell growth Medium MV2; PromoCel, Germany) containing basal medium, 5% FCS, 0.5 ng/ml rh-VEGFA-165, 5 ng/mL rh-EGF, 10 ng/mL rh-basic FGF, 20 ng/ml Long R3 IGF-1, 1 μg/mL ascorbic acid, 0.2 μg hydrocortisone, 100 U/mL penicillin, and 100 μg/mL streptomycin and grown to approximately 90% confluence in a 5% CO_2 incubator at 37 °C. Cellular viability was assessed by Trypan blue exclusion.

Proliferation assays

HPMEC and HUVEC proliferation was measured using a commercially available kit (WST-8, Cayman Chemicals) as previously described [11]. Briefly, 5×10^4 cells were plated in 100 μL of medium on a 96-well microplate. After 24h of incubation, recombinant human (rh)-IL-13 (10 ng/mL, 213-ILB-025, R&D Systems), rh-activin-A (10-500 ng/mL, 338-AC-01M/CF, R&D Systems), rh-VEGFA-165 (2 ng/mL, 583704, Biolegend) or a combination of activin-A and VEGF were added in concentrations similar to those in previously described studies [12;13]. A human anti-activin-A polyclonal antibody was used for blocking activin-A *in vitro* (15 μg/mL, AF338, R&D). Concentration response studies were initially performed in HPMEC and HUVEC for activin-A (at 25, 50, 100, 200 and 400 ng/mL) and VEGF (at 0.5, 1, 2, 5 and 10 ng/mL) to determine optimal concentrations. Activin-A and VEGF were used at 200 ng/ml and 2 ng/ml, respectively, concentrations that are similar to those

previously used by others [12]. Dose response and kinetic studies are presented in Figure E2 for HPMEC, with similar results being obtained for HUVEC (not shown). After incubation for 48h (app. 90% confluence), the medium was replaced with new medium containing 10% WST-8. After 2h of incubation, the formation of formazan was determined photometrically at 450 nm with a microplate reader. All experiments were repeated three times in tetraplicate wells. The results are expressed as percentages of medium control values.

Immunocytochemistry

Cytospin preparations of HUVEC and HPMEC (2-4 x 10^4 cells/slide) were fixed with 4% paraformaldehyde for 10 minutes at RT, then with 15% sucrose in PBS for 10 min and washed with PBS for 20 min. Endogenous peroxidase was blocked by incubation for 10 min in methanol containing 0.3% hydrogen peroxide and non-specific binding blocked with 10% fetal bovine serum in PBS. Slides were incubated with primary antibodies against activin-A, ALK-4, Act-RIIA (all R&D Systems) and pSmad2/3 (SantaCruz, USA). An antibody against the Von Willebrand factor (A0082; 1:50, Dako, Denmark), a well-known glycoprotein that is abundantly expressed in endothelium, was utilized as a positive control. Rabbit and goat IgGs were used as negative controls (X0903; Dako, Denmark and sc-2028, SantaCruz, USA, respectively**)**. Slides were incubated overnight, washed in PBS for 20 min and then incubated with goat anti- rabbit HRP (sc-2030; 1:200, SantaCruz, USA) and donkey anti-goat HRP (sc-2020; 1:200, SantaCruz, USA) secondary antibodies for 45 min at 37°C. After washing, slides were incubated 30 minutes at RT in DAB substrate. Slides were counterstained with hematoxylin for 2 min and observed in a blinded

fashion using an Olympus BX microscope connected with a Zeiss Vision KS300 imaging system (Carl Zeiss, Germany).

In vitro angiogenesis assay

For the *in vitro* angiogenesis assay, the kit V2a (ZHA 4000, TCS Cellworks, UK) was employed as previously described [11]. Cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO2. Concentration response studies were initially performed for activin-A (at 10, 20, 50, 100, 150, 200 and 250 ng/mL) and VEGF (at 0.5, 1, 2 and 5 ng/mL) to determine optimal concentrations. Culture medium with rh-activin-A (200 ng/mL, R&D Systems) was replenished on days 5, 7, and 9. Culture medium alone and rh-VEGFA (2 ng/mL, Biolegend) served as negative and positive controls, respectively. On day 14, cells were fixed, and vascular structures visualised (CD31 Tubule Staining Kit, ZHA-1225; TCS Cellworks) according to the manufacturer's instructions. Multiple photomicrographs (4x objective, four separate fields per well) were taken at clock points 12, 3, 6, and 9, and angiogenesis in each field was quantified using image analysis software (AngioSys; TCS Cellworks). The analysis software segmented the images using a grey-level threshold tool to select CD31-labelled cells. The resultant binary images were skeletonised, and branch points were removed to determine the total lengths of individual tubules. Branch points were counted, and the total area of CD31 labelling was determined from the original binary images, permitting overall numbers of vascular junctions, tubules, and tubule length to be determined. Pictures of vasculogenic capacity of each well were taken using an Olympus BX40 microscope connected with a Zeiss Vision KS300 imaging system. All experiments were repeated three times in tetraplicate wells.

RNA extraction and Real-Time PCR

Total RNA was isolated by HUVEC in TRI Reagent (Molecular Research Center) following stimulation with activin-A (200 ng/ml), VEGF (2 ng/ml) or control (PBS) for 96 hours, according to the manufacturer's instructions. RNA samples were treated with RQ1 RNase-Free DNase (Promega) to eliminate contaminating genomic DNA and reverse-transcribed using Superscript II (Invitrogen), according to the manufacturer's recommendations. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a house keeping gene. The expression of human membrane VEGF receptor 1 (mVEGFR1), sVEGFR1, VEGFR2 and GAPDH mRNA were analyzed using SsoFast^{TM} Eva Green Master mix (Bio-Rad) and a validated set of primers (**Table E1**). The relative expression of human genes was calculated using the 2-ΔΔCt method and normalized according to the expression of the endogenous GAPDH gene by Livak et al [14].

References

1. From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2012. Available from: http://www.ginasthma.org/.

2. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, Adcock IM, Bateman ED, Bel EH, Bleecker ER, Boulet LP, Brightling C, Chanez P, Dahlen SE, Djukanovic R, Frey U, Gaga M, Gibson P, Hamid Q, Jajour NN, Mauad T, Sorkness RL, Teague WG. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. Eur Respir J 2014; 43:343-73.

3. Fuhlbrigge A, Peden D, Apter AJ, Boushey HA, Camargo CA, Jr., Gern J, Heymann PW, Martinez FD, Mauger D, Teague WG, Blaisdell C. Asthma outcomes: exacerbations. J Allergy Clin Immunol 2012; 129:S34-S48.

4. Johansson SG, Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF, Motala C, Ortega Martell JA, Platts-Mills TA, Ring J, Thien F, Van CP, Williams HC. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. J Allergy Clin Immunol 2004; 113:832-6.

5. Samitas K, Zervas E, Vittorakis S, Semitekolou M, Alissafi T, Bossios A, Gogos H, Economidou E, Lotvall J, Xanthou G, Panoutsakopoulou V, Gaga M. Osteopontin expression and relation to disease severity in human asthma. Eur Respir J 2011; 37:331-41.

6. Haslam PL, Baughman RP. Report of ERS Task Force: guidelines for measurement of acellular components and standardization of BAL. Eur Respir J 1999; 14:245-8.

7. Jeffery P, Holgate S, Wenzel S. Methods for the assessment of endobronchial biopsies in clinical research: application to studies of pathogenesis and the effects of treatment. Am J Respir Crit Care Med 2003; 168:S1-17.

8. Kariyawasam HH, Pegorier S, Barkans J, Xanthou G, Aizen M, Ying S, Kay AB, Lloyd CM, Robinson DS. Activin and transforming growth factor-beta signaling pathways are activated after allergen challenge in mild asthma. J Allergy Clin Immunol 2009; 124:454-62.

9. Semitekolou M, Alissafi T, Aggelakopoulou M, Kourepini E, Kariyawasam HH, Kay AB, Robinson DS, Lloyd CM, Panoutsakopoulou V, Xanthou G. Activin-A induces regulatory T cells that suppress T helper cell immune responses and protect from allergic airway disease. J Exp Med 2009; 206:1769-85.

10. Nakao A, Sagara H, Setoguchi Y, Okada T, Okumura K, Ogawa H, Fukuda T. Expression of Smad7 in bronchial epithelial cells is inversely correlated to

basement membrane thickness and airway hyperresponsiveness in patients with asthma. J Allergy Clin Immunol 2002; 110:873-8.

11. Corrigan CJ, Wang W, Meng Q, Fang C, Wu H, Reay V, Lv Z, Fan Y, An Y, Wang YH, Liu YJ, Lee TH, Ying S. T-helper cell type 2 (Th2) memory T cell-potentiating cytokine IL-25 has the potential to promote angiogenesis in asthma. Proc Natl Acad Sci U S A 2011; 108:1579-84.

12. Kaneda H, Arao T, Matsumoto K, De Velasco MA, Tamura D, Aomatsu K, Kudo K, Sakai K, Nagai T, Fujita Y, Tanaka K, Yanagihara K, Yamada Y, Okamoto I, Nakagawa K, Nishio K. Activin A inhibits vascular endothelial cell growth and suppresses tumour angiogenesis in gastric cancer. Br J Cancer 2011; 105:1210-7.

13. Sironi M, Sciacca FL, Matteucci C, Conni M, Vecchi A, Bernasconi S, Minty A, Caput D, Ferrara P, Colotta F, . Regulation of endothelial and mesothelial cell function by interleukin-13: selective induction of vascular cell adhesion molecule-1 and amplification of interleukin-6 production. Blood 1994; 84:1913-21.

14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-8.

TABLE E1. Set of validated primers utilised for the analysis of the expression of human membrane VEGF receptor 1 (mVEGFR1), sVEGFR1, VEGFR2 and GAPDH mRNA.

Gene	**Forward Primer 5'→ 3'**	**Reverse Primer 5'→ 3'**
mVEGFR1	GTTCAAGGAACCTCGGACAA	GCTCACACTGCTCATCCAAA
sVEGFR1_variant1	TTGGGACTGTGGGAAGAAAC	TTGGAGATCCGAGAGAAAACA
sVEGFR1_variant2	CATGCTAATGGTGTCCCCGA	GATTGTCTTGGCTCTCCAACT
sVEGFR1_variant3	TCCGAAGCAAGGTGTGACTT	AGAGAAGCTTGTAGGTGGCAA
VEGFR2	GAGGGGAACTGAAGACAGGC	GGCCAAGAGGCTTACCTAGC
GAPDH	GCAAATTCCATGGCACCGT	TCGCCCCACTTGATTTTGG

Supplementary Figure Legends

Figure E1. Flow diagram showing patient groups and procedures emplyed in the present study. MMA: mild-to-moderate asthma; SA: severe asthma; EBB: endobronchial biopsy, BALF: bronchoalveolar lavage fluid.

Figure E2. Dose response and kinetic studies. Graphs show the effects of a) rActivin-A and b) VEGF on HPMEC proiliferation. Data are presented as mean ± SEM from three independent experiments in tetraplicate wells for dose response studies and four independent experiments in tetraplicate wells for kinetic studies. rActA/rActivin-A: recombinant activin-A, HPMEC: human pulmonary endothelial cells.

Figure E3. No associations were observed between the BMI and activin-A expression in the bronchial tissue; however, there was a trend for a negative association between BMI and serum/BALF activin-A levels that did not reach statistical significance. BMI: body-mass index, BALF: bronchoalveolar lavage fluid.

Figure E4. No significant differences were observed in the a) serum, b) BAL fluid or airway expression of c-d) activin-A and e-h) its signaling receptors with respect to atopic status in asthmatic patients. Data are presented as median ± interquartile range. BAL: bronchoalveolar lavage.

Figure E5. No significant differences were observed in the a) serum, b) BAL fluid or airway expression of c-d) activin-A, e-h) its signaling receptors and i) angiogenesis with respect to oral corticosteroid treatment in severe asthma patients. Data are

presented as median ± interquartile range. OCS: oral corticosteroids, BAL: bronchoalveolar lavage.

Figure E6. Correlations between lung function, as depicted by FEV_1, and a) angiogenesis and airway expression of b-c) ALK4 and d-e) Act-RIIA. FEV1: forced expiratory volume in one second.

Figure E7. IL-32 concentrations in HPMEC culture supernatants. No significant differences were observed between the groups studied. Data are presented as mean ± SEM from three independent experiments in tetraplicate wells. Act-A: activin-A, VEGF: vascular endothelial growth factor.

Figure E8. a) IL-17 and b) IL-32 concentrations in the BAL fluid. No significant differences were observed between the groups studied. Data are presented as median ± interquartile range. HC: healthy controls, MMA: mild/moderate asthmatics, SA: severe asthmatics.

Figure E9. Correlation between activin-A levels and a) IL-18, b) sVEGFR1 and c) VEGF in the bronchoalveolar lavage fluid (BALF) in all subjects.