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Angiogenesis and Airway Reactivity in Asthmatic Brown Norway Rats

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Abstract

Expanded and aberrant bronchial vascularity, a prominent feature of the chronic asthmatic airway, might explain persistent airway wall edema and sustained leukocyte recruitment. Since it is well established that there are causal relationships between exposure to house dust mite (HDM) and the development of asthma, determining the effects of HDM in rats, mammals with a bronchial vasculature similar to humans, provides an opportunity to study the effects of bronchial angiogenesis on airway function directly. We studied rats exposed bi-weekly to HDM (Der p 1; 50µg/challenge by intranasal aspiration, 1,2,3 wks) and measured the time course of appearance of increased blood vessels within the airway wall. Results demonstrated that within 3 wks of HDM exposure, the number of vessels counted within airway walls of bronchial airways (0.5–3 mm perimeter) increased significantly. These vascular changes were accompanied by increased airway responsiveness to methacholine. A shorter exposure regimen (2 wks of bi-weekly exposure) was insufficient to cause a significant increase in functional vessels or reactivity. Yet, ¹⁹F/¹H MR imaging at 3T following α_vβ₃-targeted perfluorocarbon nanoparticle infusion revealed a significant increase in ¹⁹F signal in rat airways after 2 wks of bi-weekly HDM, suggesting earlier activation of the process of neovascularization. Although many antigen-induced mouse models exist, mice lack a bronchial vasculature, and consequently lack the requisite human parallels to study bronchial edema. Overall our results provide an important new model to study the impact of bronchial angiogenesis on chronic inflammation and airways hyperreactivity.

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INTRODUCTION

Increased bronchial vascularity is a prominent feature of the asthmatic airway. The systemic bronchial angiogenesis that occurs with this chronic inflammatory condition might explain persistent airway wall edema and sustained leukocyte recruitment [1,2,3]. In addition, vascular engorgement and airway edema contribute to the narrowing in small airways and play a mechanistic role in pathological airflow obstruction and hyperreactivity [4]. However, there exists little information to link directly the enhanced airway reactivity of allergic asthma with bronchial vascular permeability. Studies of acute volume loading are unable to separate out the effects of gross pulmonary edema from bronchial vascular edema on airway narrowing [5,6]. Since the bronchial vasculature in human subjects is very difficult to study in a reproducible and noninvasive manner, small animal models have been frequently employed.

Although angiogenesis in the mouse tracheal circulation has been extensively studied during bacterial inflammation [7], the tracheal C-ring cartilage prevents substantive changes in airway diameter with smooth muscle contraction in humans or animal models, thus obscuring the potential effects of increased vascularity on the responsiveness of airways in asthma. Others have studied lung vessels in general after ovalbumin [8] or house dust mite (HDM) [9] sensitization in mice and shown increased pulmonary vascularity. The relevance and veracity of these findings remain questionable since asthmatic subjects do not demonstrate an increase in pulmonary arteries but rather angiogenesis of the systemic bronchial vessels within the airway walls [1]. Since it is well established that there are causal relationships between exposure to HDM and the development of asthma in susceptible children and asthma exacerbations in sensitized individuals [10], understanding the effects of HDM in rats provides a direct parallel to human disease.

We have previously shown the effectiveness of simultaneous $^{19}\text{F}/^1\text{H}$ MR molecular imaging with $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles to quantitatively assess neovascular expansion of the bronchial arteries following pulmonary artery ligation [11]. In that study $^{19}\text{F}/^1\text{H}$ MR molecular imaging performed with a clinical 3T scanner provided the earliest noninvasive assessments of angiogenesis, 3 days after the induction of ischemia. Beyond the high sensitivity of the technique, homing was found to be very specific based upon comparisons to the contralateral normal lung, nontargeted nanoparticle signal in the ischemic lung, and in vivo competitive inhibition studies. Further, fluorescent microscopy of the bound nanoparticles confirmed that neovascular signal derived after 3 days of ischemia originated prominently from tissues near the ligation site adjacent to large airways and large systemic vessels. These data suggested that $^{19}\text{F}/^1\text{H}$ MR molecular imaging with $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles offered a unique noninvasive means to study bronchial neovessel expansion, which could be used as a biomarker to quantify the early progression of several lung pathologies, such as asthma, with prominent angiogenic features.

Although others have defined the time course of development of airways hyperreactivity and inflammation in the Brown Norway rat exposed to HDM [12], little is known about bronchial angiogenesis in this model. In the present study we describe the time course of increased vascularity and changes in airway function with HDM challenge. Our results

demonstrate early endothelial cell activation and subsequent increased vascularity, accompanied by enhanced airway reactivity. The results provide an important new model to study the mechanisms and impact of bronchial angiogenesis during chronic inflammation as well as suggest the potential clinical use of $^{19}\text{F}/^1\text{H}$ MR molecular imaging in the context of asthma.

METHODS

Rat sensitization

All protocols were approved by the Johns Hopkins and Washington University Animal Care and Use Committees. Male Brown Norway rats (BN, Charles River, 100 g) were given house dust mite allergen (HDM, Der p 1; 50 $\mu\text{g}/\text{challenge}$, Greer Laboratories) by intranasal aspiration twice/week for 1, 2, or 3 wks duration. Vehicle control rats were given an equivalent volume of PBS over the same duration. Rats were studied two days after the last intranasal challenge.

Lung Histology and Morphometry

After rats were euthanized, lungs were immediately inflated with Z-fix (Anatech, Battle Creek, MI) to a pressure of 12 cmH_2O for 24 hr. Subsequently, the left lung was cut along its long axis into three sections (medial, intermediate, and lateral) and was embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E). All complete airways cut in cross section (max/min diameter ratio <1.5) were sized by measuring the airway basement membrane perimeter (ImageJ) and the number of blood vessels within the associated airway wall were counted. Results were expressed as vessel number normalized to airway perimeter or airway perimeter², which is proportional to airway area and used previously by others [13]. Airways without bronchial vessels were not included. Morphometry was determined in lung sections from PBS control rats ($n=4$ PBS rats), after 2 wks of HDM exposure ($n=4$ HDM4 rats), and after 3 wks of HDM exposure ($n=6$ HDM6 rats).

Following the MR imaging of HDM or PBS treated rats ($n=3/\text{group}$ at 1 and 2 wks) with rhodamine-labeled, $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles, described below, the deeply anesthetized animals underwent thoracotomy and were infused through the right ventricle with 20ml of saline to remove unbound nanoparticles from the vascular bed. The euthanized animal airway passages were infused with OCT at 25 cmH_2O to preserve lung architecture. Tissues were snap frozen in OCT media and cryosections (8 μm) were produced for histological analysis. Multiple (at least 3/animal) sections were obtained from the central trachea and bronchi and from the right lung to corroborate the MR nanoparticle imaging results. Multiple digital images were acquired with an Olympus BX61 fluorescence microscopy imaging system with Biological Suite software for instrument control and image processing (Olympus America, Inc, Center Valley, PA), using Color View II camera for optical image digitization, and F-View II B&W CCD camera for fluorescent images.

MICROFIL Casting of Bronchial Artery

In anesthetized rats (2–3 animals/group), a 20G radiopaque catheter (1 ") was placed retrograde in the descending aorta with the tip placed at the level of the diaphragm and a ligature secured immediately below the diaphragm. Rats were exsanguinated, the IVC was cut, and the vasculature was perfused retrograde with 5 ml of heparinized saline. Red MICROFIL was prepared according to the manufacturers directions and 3 ml were infused at a rate of 0.5 ml/min. White silicone rubber (Xiameter RTV-4230-E; 0.7 ml) was infused through a tracheal cannula. After 24 hr, the heart and lungs were dissected free, and the tissue was cleared with glycerol of increasing daily concentrations (50%, 85%, 100%) until vessels were visible. The bronchial vasculature was visualized using an Olympus dissecting microscope (~10x magnification) using SPOT Imaging software, version 5.1.

Lung resistance and airway smooth muscle responsiveness measurements

In a separate group of anesthetized (ketamine /xylazine, 75/5 mg/kg), intubated, and paralyzed (0.6 mg succinylcholine chloride) rats (n=5 PBS rats; n=8 HDM6 rats), dynamic respiratory mechanics were determined. Rats were ventilated (90 breaths/min; 8 ml/kg) with a Scireq Flexivent system that allows measurement of lung resistance. Baseline measurements were made 1 min after a deep inspiration (30 cmH₂O). Subsequently, cumulative dose-response relationships to methacholine chloride (MCh) aerosol (15 sec with an Aeroneb nebulizer) were obtained (PBS vehicle, 0.1, 1.0, 3.0, 5 mg/ml MCh).

RT-PCR of endothelial specific genes

Total RNA was isolated from homogenized tracheal, bronchial and lung tissue by using RNeasy mini kit (Qiagen), according to the manufacturer's protocol. cDNA was generated with QuantiTect Reverse Transcription kit (Qiagen; n=5 PBS rats, n=7 HDM6 rats). The CFX96 Real-Time PCR Detection System (Bio-Rad) was used with the following settings: 50 cycles of 15 s of denaturation at 95°C, and 1 min of primer annealing and elongation at 60°C. Quantitative PCR was performed using SYBR Green gene-specific primers (Table 1). FABP4 primers were obtained from RealTimePrimers (cat # is VRPS-1669) and used according to the company protocol. Results were normalized to GAPDH levels. The fold change of gene expression was calculated using the reference sample (naïve rat tissue; n=3). The gene expression of four endothelial cell surface markers (CD31, FABP4, CD34, and CD146) was determined. Protein expression of CD31 and CD146 was confirmed by immunostaining paraffin and frozen serial sections cut as described above. Sections were stained with anti-CD146 (eBioscience, San Diego, CA) followed by the secondary antibody anti-mouse Alexa Fluor 488, and anti-CD31-Alexa Fluor 647 (Thermo-scientific, Rockford, IL). Sections were mounted with Prolong Gold antifade reagent with DAPI. Images were obtained using an Olympus IX51 microscope.

Preparation of $\alpha_v\beta_3$ -Targetted Perfluorocarbon Nanoparticles

Phospholipid-encapsulated perfluorocarbon (PFC) nanoparticles (NP) were prepared as a microfluidized suspension of 20% (v/v) perfluorooctylbromide, 2.0% (w/v) of a surfactant co-mixture, and 1.7% (w/v) glycerin in pH 6.5 carbonate buffer in distilled deionized water (76.3% v/v). $\alpha_v\beta_3$ -Peptidomimetic antagonist conjugated to PEG₂₀₀₀-

phosphatidylethanolamine (Kereos, St. Louis, MO) was used for homing angiogenesis. The surfactant co-mixture of nanoparticles included: ~ 98.5 mol% lecithin and 0.15 mol% of $\alpha_v\beta_3$ -ligand conjugated lipid. Non-targeted nanoparticles excluded the homing ligand. For fluorescent imaging studies, rhodamine-phosphatidylethanolamine (0.1 mol%) was included in the surfactant co-mixture at the equimolar expense of lecithin. The surfactant co-mixture was dissolved in chloroform, evaporated under reduced pressure, dried in a 40°C vacuum oven overnight, and dispersed into water by probe sonication. The suspensions were combined with the perfluorocarbon, buffer, and glycerin with pH adjusted to 6.5, and homogenized with an S110 Microfluidics emulsifier (Microfluidics, Newton, MA, USA) at 20,000 p.s.i. for 4 min. The nanoparticles were aliquoted under a biohood into sterile vials and sealed under inert gas until use.

The $\alpha_v\beta_3$ -integrin antagonist was a quinalone nonpeptide developed by Lantheus Medical Imaging (Billerica, MA) and synthesized by Kereos (U.S. Patent 6,511,648 and related patents). The vitronectin antagonist was reported and characterized as the ^{111}In -DOTA conjugate RP478 and cyan 5.5 homologue TA145 [14]. The homing specificity of the ligand was demonstrated and characterized with the Matrigel plug implanted in *Rag1^{tm1Mom}* Tg(Tie-2-lacZ)182-Sato and C57Bl/6 mice. [15] Nanoparticles have an IC_{50} of 50 pM for the Mn^{2+} activated $\alpha_v\beta_3$ -integrin [16].

The nominal hydrodynamic diameter (D_h) of the $\alpha_v\beta_3$ -targeted PFC nanoparticle and control particles based on dynamic light scattering measurements (Brookhaven ZetaPlus, Brookhaven Instruments Corporation) in aqueous solution was 252 nm, polydispersity of 0.14, and zeta potential of -18 mV.

Magnetic Resonance Imaging

To noninvasively evaluate the time course of bronchial neovascular proliferation, in vivo MR (3T) simultaneous dual $^{19}\text{F}/^1\text{H}$ molecular imaging with $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles ($\alpha_v\beta_3$ -PFC NP) was performed as previously reported for rats following left pulmonary artery ligation (LPAL) [11]. The endothelial cell adhesion molecule integrin $\alpha_v\beta_3$ has been shown to be differentially upregulated in proliferating compared to quiescent endothelium [17,18]. At 1, 2, or 3 wks after the initiation of HDM or PBS treatment, rats ($n=3-4/\text{group}$) were administered $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles (PFOB NP, 1.0 ml/kg) via tail vein catheter. After allowing the NPs to circulate for 2 hr, the rats were anesthetized (ketamine/xylazine; 85/13 mg/kg) and imaged with high-resolution simultaneous $^{19}\text{F}/^1\text{H}$ magnetic resonance (MR) at 3T (Philips Achieva) using an in-house, custom dual-tuned solenoid transmit-receive coil [19]

Simultaneous 3D $^{19}\text{F}/^1\text{H}$ imaging was used employing a novel steady state ultrashort echo time technique ($\text{TE}/\text{TR}=0.1\text{ms}/1.96\text{ms}$) with the frequencies set to the resonance of ^1H and the CF_2 groups of the PFOB spectrum (-representing 12 of 17 total ^{19}F nuclei per PFOB molecule) [20]. Using a highly oversampled 3D radial readout scheme, the $^{19}\text{F}/^1\text{H}$ image datasets were reconstructed, *post facto*, by modifying the Nyquist weighting factor applied to samples in k-space to obtain an optimal balance between the signal-to-noise ratio and image resolution for the in vivo ^{19}F signal levels [21]. The signal-rich ^1H datasets were always reconstructed at the proper Nyquist factor for highest native resolution. For

interstudy comparisons, a uniform ^{19}F Nyquist weighting factor was applied to all images from all animals, thereby retaining a consistent and comparable SNR level across reconstructed images. In this anesthetized rat model, neither mathematical correction nor respiratory gating were required to adjust for motion, as the breathing motion was of the same order of magnitude or less than the pixel resolution. Typical total scan time was 28 min.

Image analysis

MR data sets were imported into ImageJ (NIH) for quantification of the ^{19}F signal. The method for measuring asthma/inflammation burden is based upon the integral of ^{19}F signal intensity from $\alpha_v\beta_3$ -targeted PFOB NPs. Lung regions of interest (ROIs) were defined from anatomical matching of multislice ^1H and ^{19}F MR data sets. These ROIs consistently included the heart in order to include angiogenesis located near the trachea and bronchi. To determine the background noise level, these same ROIs were then positioned in an area outside the animal (i.e., air). For each 2D image slice, the total lung fluorine signal was normalized by subtracting the total background signal of an equivalent area. The normalized lung ^{19}F signal was then averaged for all lung ROI slices.

Statistics

All data are presented as the mean $\pm$ the standard error. One-way ANOVA was used to compare vascularity across groups followed by Dunnett's Multiple Comparison Test. Two-way ANOVA was used to assess differences in airway responses to methacholine followed by the Bonferroni test of multiple comparisons. Two sample comparisons (PBS vs HDM6) were made with unpaired t-tests. Overall MRI data were analyzed with two-way ANOVA to assess and weekly sub-analysis utilized student's t-test. A p value ≤ 0.05 was accepted as significant.

RESULTS

Critical to studying the effect of airway angiogenesis in the Brown Norway rat asthma model was the confirmation that the HDM exposure regimen resulted in airways hyperresponsiveness, a hallmark of asthma. Titrated concentrations of methacholine, a synthetic non-selective muscarinic receptor agonist, were used to challenge the rats following serial HDM exposure to demonstrate airways constriction, as reflected by respiratory system resistance (Rrs). Figure 1 shows the Rrs dose-response relationships to methacholine after 3 wks of twice weekly PBS or HDM treatment. No differences between PBS and HDM rats were observed in baseline Rrs, vehicle controls, or low doses of methacholine. However, significant differences in resistance between the two groups were observed at methacholine concentrations of 3 and 5 mg/ml ($p < 0.01$).

In the second cohort of rats, airway vascular morphometry was assessed after 2 or 3 wks PBS or HDM treatment. Gross anatomy was assessed concurrently with the quantitative histological measurement. Figure 2 presents representative airway and vascular casts demonstrating increased vascularity in an HDM rat compared to a control PBS rat. Airways were filled with white silicone rubber and the aorta was injected with red MICROFIL.

Vascular expansion of the bronchial circulatory system after 3 wks of HDM sensitization was readily apparent (red arrows), whereas in the PBS exposed rats, the bronchial vessels were fewer and very small, often failing to fill with the MICROFIL. In addition, a greatly enlarged lymphatic vessel crossing the right mainstem bronchus in the HDM rat was observed (green arrow), yet a corresponding lymphatic is barely visible in the control PBS rat, suggesting compensatory lymphangiogenesis and increased lymphatic flow perhaps to counterbalance asthmatic bronchial edema.

Representative H&E stained histologic sections from PBS and HDM treated rats 3 wks after the start of sensitization are shown in Figure 3. A paucity of bronchial vessels within the airway wall in the PBS rat compared to the abundance of blood vessels observed after exposure to HDM was easily appreciated. Lower power images also displayed a striking difference between the PBS and HDM rats with regard to an associated influx of inflammatory cells, airway wall thickening, and smooth muscle contraction. Similar sections (3 sections/rat) from medial, intermediate, and peripheral longitudinal cuts of the left lung were evaluated in PBS and HDM rats. Figure 4A shows the correlation of airway size (perimeter in mm) counted for all airways evaluated in PBS rats (56 airways) and rats exposed to HDM (84 airways). The slopes are very different from each other ($p=0.002$) and indicate that there are significantly increased numbers of vessels in HDM rats compared to PBS rats over an equivalent size range of airways studied (0.5–3 mm). A third group of rats was studied concurrently and, processed similarly, was exposed to twice weekly HDM for 2 wks. The airway vascularity for each of the three groups of rats is shown in Figure 4B where the average number of bronchial blood vessels normalized to perimeter² was calculated for each rat and then averaged for the group. Only the group of HDM6 rats (3 wks of twice weekly HDM) was significantly increased compared with PBS rats ($p<0.001$). HDM4 rats (2 wks of twice weekly HDM) were not different from PBS control rats. Furthermore, when airways reactivity was assessed in a sub-group of these rats using a single dose of methacholine (10 mg/ml), only HDM6 rats demonstrated a different increase (average 3.3-fold) in respiratory system resistance compared to PBS rats ($p<0.05$).

To corroborate the increased airway vascularity observed by quantitative histology, gene expression of three common endothelial cell surface markers was determined in homogenized aliquots of tracheal, bronchial and lung tissue. Bronchial tissue was isolated from lung parenchyma but could not be dissected completely free of parenchymal attachments. Thus those samples represented a mixed population of endothelial cells from bronchial airways and pulmonary parenchyma. Figure 5A shows the results of the fold induction of CD31 and FABP4 (left axis), and CD34, CD146 (right axis) in tracheal homogenates from PBS and HDM (3 wks of twice weekly HDM) relative to tracheal tissue from naïve rats. A significant increase in the gene expression of both CD31 and CD146 was observed in tracheal tissue ($p<0.05$). There were no significant differences in gene expression between the PBS and HDM groups in the bronchial tissue or lung parenchyma. Figure 5B provides histologic verification of increased CD31 and CD146 expression in HDM6 rats compared to PBS control rats, where CD31 was stained red, CD146 was green, and all cell nuclei stained blue with DAPI. Because of the limited bronchial vessels seen in the very large airways of PBS rats, little staining is seen. However in the HDM6 rats, co-

localization of the two surface markers was abundantly evident by the yellow coloring of airway wall vessels in large bronchi as well as in tracheal adventitial vessels (Figure 5C).

Utilizing dual $^{19}\text{F}/^1\text{H}$ MR molecular imaging of angiogenesis, as previously validated in the rat LPAL model, the temporal and spatial distribution of neovascularity in response to twice weekly exposures to PBS or HDM over 1, 2, and 3 wks of treatment were assessed [11]. Figures 6A,B present the representative $^{19}\text{F}/^1\text{H}$ MR simultaneous images of PBS and HDM4 two hours following delivery of $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles. In this example, the ^{19}F signal from PFC nanoparticle in the control mediastinum was likely dominated by circulating particles within the heart chambers. In the HDM4 animals, there was markedly enhanced signal from the central thoracic region encompassing the mediastinal structures as well as the proximal lung regions. In general, less ^{19}F neovascular signal was observed in the more distal peripheral pulmonary parenchyma. The contiguity of ^{19}F voxel enhancement in the proximal pulmonary parenchyma varied with some slices from HDM treated animals presenting a more heterogeneous pattern than others. As discussed in the methods, the $^{19}\text{F}/^1\text{H}$ image datasets were reconstructed, *post facto*, to obtain an optimal balance between the signal-to-noise ratio and image resolution for the different ^{19}F and ^1H signal levels. A uniform ^{19}F weighting was chosen and applied to all images.

Quantification of in vivo ^{19}F MR molecular imaging following $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticle infusion revealed a consistent increase in ^{19}F signal in rat airways exposed to HDM compared to PBS controls over the 3 week study (Figure 6C, $p=0.007$). Weekly sub-analyses showed an early increase in the ^{19}F signal in the HDM2 rats compared to the PBS controls, but the animal-to-animal response was variable (Figure 6E, $p=0.14$). At 2 wks, the neovascular response of HDM4 animals was more uniform and greater ($p<0.05$) than PBS control rats. Three wks after the onset of HDM sensitization treatments, ^{19}F neovascular signal declined relative to the PBS control ($p=0.36$). These observations suggested that the intensity of new angiogenic vessel formation was diminished, likely in favor of increased microvessel expansion as seen microscopically.

A cohort of HDM4 and PBS exposed animals, which corresponded to the maximal increase in neovascular pulmonary signal in the HDM group, was administered rhodamine-labeled $\alpha_v\beta_3$ -targeted or nontargeted perfluorocarbon nanoparticle. As shown in Figure 6D, fluorescent imaging of frozen sections revealed dense accumulations of nanoparticles in the bronchi of HDM rats with a paucity of nanoparticles accumulating in the PBS control animals, which is consistent with the MR findings at this time point. Within the lung parenchyma, rhodamine nanoparticle accumulation was heterogeneously but prominently distributed generally around the larger and intermediate airways in the HDM animals (Supplemental data: Figure 1).

DISCUSSION

The current work was undertaken to characterize the temporal and spatial relationship of angiogenesis, vascular expansion, and airway reactivity in the Brown Norway rat model of asthma using a clinically common allergen. House dust mites are microscopic, insect-like

pests that frequently trigger allergic reactions and asthma in sensitive individuals. An HDM sensitization protocol in rats was developed to elicit hyperreactive airways, which were substantially associated with increased airway vascularity. Histologic sections were evaluated and blood vessels within the airway wall were counted. Bronchial vessel number, normalized to airway size, was greater ($p < 0.05$) in rats that received 3 wks of bi-weekly HDM exposure than PBS control rats. Gene expression of endothelial cell markers was increased in tracheal tissue samples of HDM rats that contained only systemic airway blood vessels. $^{19}\text{F}/^1\text{H}$ MR molecular imaging of angiogenesis with highly characterized $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles, demonstrated an overall increase in neovascular expansion over the 3 week study, which was noted after one week (2 HDM treatments) and greatest relative to the PBS control after 2 wks (4 HDM treatments). The magnitude of neovascular expansion determined with MRI declined during the third week, particularly relative to the controls. This decline in neovascularization was associated with an increase in vascular density and airway hyperreactivity, which were most prominent at 3 wks (6 HDM treatments). Rhodamine-labeled $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles were richly identified around the larger more central bronchi and bronchioles in the lung parenchyma. Collectively, these results suggest that bronchial angiogenesis begins rapidly in the course of HDM sensitization in rats followed by increased vascularization, particularly around the larger airways, and increased airway hypersensitivity to methacholine challenge.

The observation that asthmatic subjects have increased airway vascularity has been documented by a number of investigators primarily through evaluation of histologic sections [1,22]. Direct visualization by bronchoscopic imaging confirmed increased airway vascularity in vivo [23] as did specialized gas washout techniques [24]. However, attempts to delve further into the mechanisms of airway angiogenesis and the pathophysiology of angiogenic vessels in asthmatic subjects are limited. Culture medium from airway smooth muscle biopsied from asthmatics and grown in vitro has been shown to cause endothelial cell tube formation in assay systems [25,26]. These studies suggest that a number of common growth factors such as VEGF-A, angiopoietin, IL-8 are all capable of stimulating microvessel growth in vitro. However, understanding the 3-dimensional changes to airway structure and function are impossible to predict from in vitro assessments.

There exist an abundance of animal models to study the mechanisms of airways hyperreactivity. Furthermore, several studies have shown that allergen sensitization leads to increased airway vascularity concomitant with enhanced reactivity and inflammation [13,27]. Both ovalbumin and house dust mite have been used to sensitize mice where investigators have reported increased lung vascularity of relatively large pulmonary vessels associated with large airways [8,9,28,29]. Whether these large pulmonary vessels represent new vessels or whether tissue consolidation due to inflammatory cell burden lead to an increased vessel count is not clear. Casting further doubt on these mouse models of allergen-driven angiogenesis, mice have been shown to lack a subcarinal bronchial vasculature even under conditions of chronic and complete pulmonary ischemia [30]. Thus, the use of mice to study allergen-induced angiogenesis as a model for human airway vascular remodeling requires further validation. However, using Brown Norway rats, mammals with an airway vasculature similar to the human, Karmouty-Quintana and colleagues have shown that bronchial angiogenesis develops concomitantly with airway hyperresponsiveness after

ovalbumin sensitization [13]. This model provides an important opportunity to further determine the pathophysiology of the angiogenic vasculature. Yet the use of house dust mite to sensitize these susceptible animals comes closer to the human condition because of the similarity in antigen susceptibility. To our knowledge, enhanced airway vascularity and its relationship to airway hyperreactivity in the Brown Norway rat sensitized to house dust mite has not been evaluated.

Critically important to establish this model was initial confirmation that the HDM exposure regimen caused an increase in airways reactivity to a non-specific smooth muscle agonist. Methacholine is frequently used for this purpose and our results demonstrated that after 3 wks of intranasal HDM sensitization, rats showed a difference in responsiveness to this muscarinic receptor agonist (Figure 1). Airway reactivity was not increased after 2 wks of sensitization, although MRI molecular imaging revealed progressively increased neovascular expansion during wks 1 and 2 of allergen exposure. Although our sensitization regimen differed somewhat from the work of others, the range of hyperresponsiveness seen after 3 wks sensitization was comparable to that previously reported in the Brown Norway rat after HDM sensitization [12,31]. Furthermore, baseline airway resistance and the level of reactivity after allergen sensitization was similar to that observed by Karmouty-Quintana who showed concomitant increases in airway vascularity after ovalbumin sensitization [13].

Having established this model of HDM sensitization and reactivity, we examined changes in airway vascularity in histologic sections of bronchial airways. Using a strict morphometric approach, we counted the number of bronchial blood vessels within the airway wall. As seen in Figure 3, clear differences in airway vascularity were obvious. Vessel number was normalized to airway size based on measurements of the non-distensible basement membrane perimeter [32]. Within the range of airways studied (0.5 - 3.0 mm), a significant increase in vessel numbers was observed throughout this size range of intraparenchymal airways. No significant increase in vessel number was observed after 2 wks of sensitization (Figure 5), although MR molecular imaging of angiogenesis was peaking. Casting of blood vessels after 3 wks of sensitization (Figure 2) demonstrated an expanded network of visible airway vessels compared to a PBS control, which corresponded to a time when neovascular expansion measured with noninvasive molecular imaging was declining. Interestingly, this gross anatomical evaluation also showed large lymphatic vessels crossing the trachea only in HDM rats.

To provide another measure of airway vascularity in this model, gene expression of several endothelial cell-specific genes was evaluated in additional rats after 3 wks of sensitization. No differences were discerned between PBS control and HDM rats in lung homogenate or large bronchi with parenchymal attachments. We interpreted these results to reflect our inability to completely dissect pulmonary tissue from large bronchi and the number of pulmonary endothelial cells relative to systemic bronchial vessels in the lung. Thus the signal of pulmonary parenchymal endothelium predominated and obscured the much smaller systemic bronchial endothelium. However, within tracheal tissue, where only systemic vessels reside, enhanced expression of both CD31 and CD146 was seen. A trend toward increased FABP4 was observed and suggests another marker of systemic angiogenesis [33]. Validation of CD31 and CD146 was made in histologic sections of large airways (Figure

5B/5C). This molecular approach provided an unbiased estimate of systemic angiogenesis in the largest airways.

Finally, this paper presents the first report of quantitative simultaneous dual $^{19}\text{F}/^1\text{H}$ MR molecular imaging of asthmatic angiogenesis using a clinical scanner at 3T. Simultaneous transmit and receive radiofrequency energy at both 120 MHz (^{19}F) and 128 MHz (^1H) has many benefits over executing sequential ^{19}F and ^1H acquisitions in a clinical setting, such as scan time efficiency, ensuring identical image geometry, and optimizing system setting (like power settings) on the ^1H signal for the sparse ^{19}F signal, and motion correction. In the present study, ^{19}F molecular imaging of angiogenesis identified and mapped neovascular expansive changes in the lung induced by HDM inhalation weeks before pathological and clinical assessments of asthmatic changes were appreciated. While further studies will be required to corroborate the physiological relationship of airway reactivity and microvascular expansion, these data present a potential role for quantitative ^{19}F MR angiogenesis imaging as an early biomarker for the clinical management of asthma in complicated susceptible patients.

CONCLUSION

This study characterizes the temporal and spatial distribution of angiogenesis, vascularity, and airway reactivity in the Brown Norway rat following serial allergen challenges with house dust mite. Early neovascular expansion was mapped to the larger central airways using simultaneous $^{19}\text{F}/^1\text{H}$ MR molecular imaging with $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles. While overall bronchial neovascular expansion was appreciated over the entire 3-week interval, progressive ramping to maximal signal response was noted over weeks 1 and 2 then followed by a decrease in angiogenesis in week 3. Concomitant with the decline in angiogenesis in week 3 was a significant increase in histological and RT-PCR quantified vascularity. Concurrent with the prominent increase in vascularity at 3 weeks, airway hyperreactivity to methacholine challenge was significantly increased in the HDM sensitized rats, while no significant difference from control was appreciated at an earlier time point. Collectively these data demonstrate the temporal relationship of angiogenesis, as assessed by ^{19}F MR molecular imaging and the onset of increased airway reactivity and mature vascular expansion in the Brown Norway rat following dust mite exposure. The data suggest that increased central airway angiogenesis in ectopic individuals could be measured noninvasively with MRI and used as an early biomarker of asthma. Furthermore, characterization of angiogenesis and airway reactivity in the Brown Norway rat model of asthma presents the opportunity to study the role that antiangiogenesis nanotherapy may have in disrupting the progression of airway inflammation and hyperreactivity in asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Respiratory system resistance (Rrs) dose-response relationships to methacholine after 3 wks of PBS (n=5 rats) or HDM (n=8 rats) treatment. Significant differences between the two groups were observed at methacholine concentrations of 3 and 5 mg/ml (2-way ANOVA $p < 0.0001$; Bonferroni Multiple Comparison, at 3 mg/ml $p < 0.01$, at 5 mg/ml $p < 0.0001$).

Figure 2.

Airway and vascular casts showing evidence of angiogenesis in an HDM rat compared to a PBS control rat. Airways were filled with white silicone rubber and the aorta was injected with red Microfil. Angiogenesis of the bronchial vasculature after 3 wks of HDM sensitization is clearly observed (red arrows), whereas in PBS rats, the bronchial vessels were so small they often failed to fill with the Microfil. A large lymphatic (green arrow) is observed in the HDM rat. Distance bar indicates 2 mm.

Figure 3. Representative histologic sections from PBS and HDM treated mice 3 wks after the start of sensitization. Note the paucity of bronchial vessels within the airway wall in the PBS rat compared to the abundance of blood vessels observed after exposure to HDM (100x and 400x magnification). Arrows indicate some of the bronchial vessels within the airway wall.

4A.

4B.

Figure 4.

A. Relationship between airway size and airway vascularity in PBS control rats (black symbols) and rats sensitized with 3 wks of bi-weekly HDM (HDM6; blue symbols). The number of airway blood vessels was significantly increased in HDM rats over the studied size range of airways ($p < 0.002$). **B:** Average number of bronchial blood vessels normalized to perimeter² (mm²; proportional to airway area) in rats exposed to PBS, HDM4 (twice weekly for 2 wks) and HDM6 (twice weekly for 3 wks). Only HDM6 was significantly

increased compared with PBS rats (ANOVA; $p=0.0002$; Dunnett's Multiple Comparison Test $*p<0.001$). HDM4 was not different from PBS control.

5A.

5B.

5C.

Figure 5.

A. Gene expression of endothelial cell surface markers in tracheal homogenate. Fold induction of CD31 and FABP4 (left axis), CD34, and CD146 (right axis) relative to naïve rats showed increases only in HDM sensitized rats. CD31 and CD146: $p < 0.05$, one-tailed; $n=5$ PBS, 7HDM rats. **B:** Histological sections demonstrating increased CD31/CD146 expression in HDM rats compared to PBS control rats. CD31: red, CD146: green, DAPI: blue. White arrows indicate systemic airway vessels. Bar=0.1mm; 100x original magnification. **C:** High power image of frozen cross section of HDM tracheal wall shows co-localization of anti-CD31/CD146 of adventitial vessels. Bar=10 μ m; 400x original magnification.

Figure 6.

A,B. $^{19}\text{F}/^1\text{H}$ dual simultaneous MR molecular imaging scans of PBS and HDM rats 2 wks after the onset of sensitization using $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles. Note uniform ^{19}F weighting was applied to all images from all animals. **C.** The overall 3-week effect of HDM sensitization on total ^{19}F signal in arbitrary units compared to PBS controls (*p=0.007). **D.** Rhodamine labeled $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles in frozen sections of large bronchi of PBS and HDM rats 2 wks after the onset of sensitization. **E.** Sub-analysis weekly comparisons of the effect of HDM sensitization versus the PBS controls showing early induction of angiogenesis by week 1 with a statistical peak difference observed after 2 wks between PBS and HDM treatment (p=0.05), which was followed by a neovascular decline in the magnitude and treatment difference at 3 wks.

TABLE 1

Quantitative PCR was performed using the following gene-specific primers. Results were normalized to GAPDH levels.

Gene		Sequence 5' to 3'
CD31 rat	Forward	AGCACAGTGGACACTACACG
	Reverse	GCTTCGGAGACTGGTCACAA
FABP4	Forward	AAGAAGTGGGAGTTGGCTTC
	Reverse	CATCCAGGGTTATGATGCTC
CD34 rat	Forward	GGGCATCTGCCTGGAATAA
	Reverse	GGCCAAGACCATCAGCAAAC
CD146 rat	Forward	GCTCAGGAAAACAGGAGATGGA
	Reverse	CCACTGGCTGTGTAAGGGAG
GAPDH rat	Forward	AGACAGCCGCATCTTCTTGTGC
	Reverse	CTCCTGGAAGATGGTGATGG