Induction of Vascular Remodeling in the Lung by Chronic House Dust Mite Exposure

Kristina Rydell-Törmänen[1,*], Jill R. Johnson[2,*], Ramzi Fattouh[2], Manel Jordana[2], and Jonas S. Erjefält[1]

[1]Department of Experimental Medical Science, Division of Vascular and Airway Research, Lund University, Lund, Sweden; [2]Department of Pathology and Molecular Medicine, Division of Respiratory Diseases and Allergy, Centre for Gene Therapeutics, McMaster University, Hamilton, Ontario, Canada

Structural changes to the lung are associated with chronic asthma. In addition to alterations to the airway wall, asthma is associated with vascular modifications, although this aspect of remodeling is poorly understood. We sought to evaluate the character and kinetics of vascular remodeling in response to chronic aeroallergen exposure. Because many ovalbumin-driven models used to investigate allergic airway disease do so in the absence of persistent airway inflammation, we used a protocol of chronic respiratory exposure to house dust mite extract (HDME), which has been shown to induce persistent airway inflammation consistent with that seen in humans with asthma. Mice were exposed to HDME intranasally for 7 or 20 consecutive weeks, and resolution of the inflammatory and remodeling response to allergen was investigated 4 weeks after the end of a 7-week exposure protocol. Measures of vascular remodeling, including total collagen deposition, procollagen I production, endothelial and smooth muscle cell proliferation, smooth muscle area, and presence of myofibroblasts, were investigated histologically in lung vessels of different sizes and locations. We observed an increase in total collagen content, which did not resolve upon cessation of allergen exposure. Other parameters were significantly increased after 7 and/or 20 weeks of allergen exposure but returned to baseline after allergen withdrawal. We conclude that respiratory HDME exposure induces airway remodeling and pulmonary vascular remodeling, and, in accordance with airway remodeling, some components of these structural changes may be irreversible.

Keywords: vascular remodeling; house dust mite; smooth muscle; procollagen I; myofibroblast

Allergic airway inflammation is known to be associated with persistent inflammation and tissue remodeling, affecting the airways and pulmonary vasculature (1–3). Characteristics of remodeling include subepithelial fibrosis, smooth muscle thickening, proliferative responses in epithelium and endothelium, and increased bronchial vascularity (4, 5). Most studies investigating remodeling in allergic airway inflammation have studied autopsy or biopsy material from humans with asthma, which, although important, provide only a small piece of a larger picture. Animal models are useful for studying the progression of disease; however, few truly chronic models of asthma exist. The most commonly used model, which uses respiratory ovalbumin (OVA) exposure, mimics a more acute situation. A more chronic model has been recently described in which allergic airway disease is induced by house dust mite (*Dermatophagoides pteronyssinus*) extract (HDME), which results in persistent Th2-polarized airway inflammation without the use of exogenous adjuvants (6). Respiratory HDME exposure recapitulates several of the clinical features of asthma, including airway hyperresponsiveness and structural changes to the airway wall (6). In contrast to mice exposed to OVA, animals exposed to HDME do not develop inhalation tolerance to the allergen when exposed for a prolonged period (7), which makes this model suitable for studies involving long-term allergen administration.

CLINICAL RELEVANCE

Vascular remodeling in asthma is poorly understood. We aimed to identify the nature of remodeling in different vessel types in a model of persistent allergic airway inflammation, and to determine whether these changes are reversible.

Investigations of vascular remodeling in asthma have predominantly focused on the bronchial circulation, which is easily accessed by bronchial biopsies. In clinical asthma, these vessels are known to increase in length, number, and size, and thickening of the vascular basement membrane has been observed (8–10). This increased vascularity has been related to the severity of the disease and infiltration of eosinophils (10). Furthermore, perivascular eosinophilia of bronchial-associated large blood vessels has been described as a feature in cases of sudden fatal asthma (3), and vascular remodeling without eosinophilia has been shown in a study of mild and fatal asthma (11). Vascular remodeling is a well known, and in many cases crucial, feature of several inflammatory lung conditions, such as chronic obstructive pulmonary disease, systemic sclerosis, and pulmonary arterial hypertension (12–14). In animal models of pulmonary infection, remodeling of the bronchial and tracheal vessels is well described (15, 16). However, in animal models of allergic airway inflammation, the phenomenon of remodeling of the pulmonary circulation was to our knowledge unknown until 2005, when we published findings demonstrating perivascular eosinophilia and vascular remodeling in large bronchial-associated blood vessels (17). Recently, it has been shown that respiratory OVA administration induces vascular hyperresponsiveness to serotonin, angiotensin II, endothelin-1 (18), and phenylephrine (19). The latter mechanism was found to be dependent on the vascular endothelium, highlighting the involvement of endothelial cells in inflammatory responses.

The aim of this study was to investigate any morphologic alterations in the pulmonary circulation in mice subjected to chronic allergic airway inflammation induced by HDME exposure. We investigated structural changes in blood vessels of different sizes and locations (large, medium, and small vessels; bronchial-associated or solitary) by evaluating proliferation of

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*These authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to Kristina Rydell-Törmänen, PhD, Department of Experimental Medical Science, Division of Vascular and Airway Research, BMC D12, S-221 84, Lund, Sweden. E-mail: Kristina.Rydell-Tormanen@med.lu.se

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endothelial and vascular smooth muscle cells, expression of α-smooth muscle actin and procollagen I, and perivascular collagen deposition. We investigated the long-term kinetics of these structural changes by evaluating the degree of vascular remodeling after 7 weeks of HDME exposure, a point at which eosinophilic airway inflammation and structural changes to the airway wall are evident, and after 20 weeks of HDME exposure, characterized by significant neutrophilic infiltration of the lung, a phenotype more characteristic of severe asthma. Additionally, we questioned whether the observed structural changes to the lung vasculature were reversible with cessation of allergen exposure.

Based on the results of this study, we conclude that structural changes to the lung vasculature associated with HDME-induced airway inflammation are significant but partially resolve after the cessation of allergen exposure, in contrast to structural changes to the airway wall (6). This discrepancy suggests that components of tissue remodeling involving the airways and the vasculature in the lung have different kinetics, resulting in variations in the magnitude of structural changes that are dependent on the duration and severity of disease.

MATERIALS AND METHODS

Animals

Female wild Balb/c mice (6–8 wk old) were purchased from Jackson (Bar Harbor, ME). The animals were housed under specific pathogen–free conditions following a 12-hour light-dark cycle. All experiments were approved by the Animal Ethics Board of McMaster University and followed the guidelines by the Canadian Council on Animal Care.

Antigen Administration

The mice were exposed to purified HDM whole-body extract (Greer Laboratories, Lenoir, NC) intranasally (25 μg protein in 10 μl saline) for 5 consecutive days, followed by 2 days rest for 7 or 20 weeks. In one experiment, allergen exposure was ceased, and 4 weeks of resolution were allowed to pass before the animals were killed. No additional adjuvant was given, and naive, age-matched animals were used as controls. Studies have shown no differences in inflammation or lung structure of naive animals compared with saline-treated control animals (20).

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed as previously decribed (21). Briefly, the lungs were dissected free, the trachea was cannulated, and the lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml). Total cell counts were done using a hemocytometer and followed by centrifugation and resuspension in PBS. Smears were prepared with a cytospin and stained with HEMA 3 set (Biochemical Sciences Inc., Swedesboro, NY). Differential counts were determined from 500 leukocytes using standard hematologic criteria.

Immunohistochemistry

When BAL was performed, the lungs were inflated with 10% formalin at a pressure of 20 cm H_2O, fixed in 10% formalin for 23 hours, dehydrated, and embedded in paraffin. Sections 3 μm thick were used for immunohistochemistry. For immunohistochemistry, a standard protocol was used (17). Details on the antibodies used and their visualization are given in Table 1. Briefly, the selected antibody was applied onto the section in a predetermined dilution, incubated (overnight in 4°C or 1 hour at room temperature), washed, and incubated with secondary antibody (45 min in room temperature). For double-labeling, the procedure was repeated with a second primary and secondary antibody. Double labeling was performed for quantification of proliferating smooth muscle cells and myofibroblasts (herein defined as cells co-positive for α-SMA and procollagen I). When antigen retrieval was necessary (for visualization of procollagen I positivity, enzymatic retrieval was performed using Pepsin/HCl (0.4% pepsin in 0.01M H HCl) for 30 minutes at 37°C.

Total Collagen Content

Total collagen was visualized by Picro Sirius Red (Sigma, St. Louis, MO). Briefly, paraffin sections were hydrated and incubated in Picro Sirius Red solution for 1 hour before washing, dehydration, and mounting.

Quantification and Statistics

Quantification was preformed as previously described (17). Briefly, the vascular basement membrane were measured by manual trace (on digital images, nine images per slide) and related to the number of positively labeled cells (procollagen I–positive, myofibroblasts, and proliferating cells or labeled area [smooth muscle and total collagen/Picro Sirius Red]). For statistical analysis, the Wilcoxon signed ranks test was used (Analyze It; Analyze-it Software, Ltd., Leeds, UK). Data are given as mean values ± SEM, and $P < 0.05$ was considered significant.

RESULTS

Persistent Airway Inflammation

The presence of an allergic inflammation was confirmed by BAL. Seven weeks of HDME exposure resulted in robust airway inflammation, characterized by a significant proportion of eosinophils (Table 2). After 20 weeks of exposure, total lung inflammation remained elevated compared with naive mice and

TABLE 1. OVERVIEW OF THE ANTIBODIES AND VISUALIZATION METHODS USED IN THE STUDY

Remodeling Parameter		Antibody	Visualization
Proliferating endothelial cells	Proliferation	PCNA (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA)	HRP-conjugated goat anti-rabbit + DAB (Jackson ImmunoResearch, West Grove, PA)
	Cell nucleus		HTX
Proliferating smooth muscle cells	Proliferation	PCNA	HRP-conjugated goat anti-rabbit
	Smooth muscle cells	α-SMA, AP-conjugated (1:200) (Sigma, St. Louis, MO)	New Fuchsine (DAKO A/S, Glostrup, Denmark)
Smooth muscle area	Smooth muscle	α-SMA, Cy3-conjugated (1:,5000) (Sigma)	
Procollagen I production	Procollagen I–producing cells	PINP (1:100)* Antigen retrieval: Pepsin/HCl	Alexa 488 (goat anti-rabbit) (Molecular Probes, Eugene, OR)
Actin-procollagen co-positivity	Procollagen I–positive cells	PINP (1:100)	HRP-conjugated goat anti-rabbit + DAB
	Smooth muscle cells	α-SMA, AP-conjugated (1:200)	AP + New Fuchsine (both from DAKO)

Definition of abbreviations: α-SMA, α–smooth muscle actin; AP, alkaline phosphatase; DAB, 3,3-diaminobenzidine tetrahydrochloride; HRP, horseradish peroxidase; PCNA, proliferating cell nuclear antigen; PINP, N-terminal of procollagen I..

* This antibody was received as a kind gift from Professor Juha Risteli, Oulu University, Oulu, Finland.

TABLE 2. EOSINOPHILIA AND NEUTROPHILIA IN LAVAGE FLUID AFTER HOUSE DUST MITE EXTRACT EXPOSURE

	Naive	7 wk of HDME exposure	20 wk of HDME exposure	7 + 4 wk of HDME exposure followed by a 4-wk resolution period
Eosinophilia	0.02 ± 0.02	22.8 ± 1.9*	3.7 ± 1.4†	0.025 ± 0.025‡
Neutrophilia	0.91 ± 0.55	5.9 ± 1.1*	9.4 ± 3†	0.88 ± 0.3‡

Definition of abbreviation: HDME, house dust mite extract.

Data represent percentage of cells (eosinophils or neutrophils) of the total number of cells in bronchoalveolar lavage fluid and are given as mean ± SEM.

* $P < 0.01$ compared with naive mice.

† $P < 0.05$ compared with 7 wk of HDME exposure.

‡ $P < 0.01$ compared with 7 wk of HDME exposure.

was characterized by a reduction in the proportion of eosinophils and an increase in neutrophils compared with the inflammatory response induced by 7 weeks of HDME exposure (Table 2). After the cessation of allergen exposure, lung inflammation returned to baseline in terms of total inflammation and numbers of eosinophils and neutrophils (Table 2).

Endothelial Remodeling

Endothelial proliferation was significantly elevated in bronchial-associated vessels compared with naive mice after 7 weeks of HDME exposure and increased further with continued allergen exposure (Table 3). Additionally, 4 weeks of resolution resulted in a significant decrease compared with 7 weeks of HDME exposure (Table 3). Mid-sized vessels displayed a similar pattern as bronchial-associated vessels. Seven and 20 weeks of HDME exposure induced significant endothelial cell proliferation compared with control mice, and the increase from 7 to 20 weeks was significant. Four weeks of resolution abolished the increase caused by 7 weeks of HDME (Table 3). The number of proliferating endothelial cells in small solitary vessels displayed a different pattern compared with bronchial-associated and mid-sized vessels. In small solitary vessels, the degree of proliferation did not change between 7 and 20 weeks of HDME exposure. However, in similarity to other vessel types, a 4-week resolution period abolished the increase (Table 3).

Vascular Smooth Muscle Remodeling

A significant increase in the number of proliferating smooth muscle cells in bronchial-associated vessels was present after 7 weeks of HDME exposure compared with control mice, and withdrawal of the inciting allergen resulted in a significant decrease in the number of proliferating cells. After 20 weeks of HDME exposure, a significant increase in proliferating smooth muscle cells (SMCs) compared with control mice was observed (Table 3). In mid-sized and small solitary vessels, proliferation of SMCs was significantly increased after 7 weeks of HDME exposure, and the degree of smooth muscle proliferation did not increase further in these vessels after 20 weeks of allergen exposure. After a 4-week resolution period, the number of proliferating SMCs returned to baseline (Table 3).

In all vessel types investigated in this study, the area positive for smooth muscle actin did not increase significantly after 7 weeks of HDME exposure, but after 20 weeks of HDME exposure a significant increase in smooth muscle area was observed compared with all other groups (Figures 1 and 3).

The number of myofibroblasts was significantly increased around bronchial-associated and mid-sized solitary vessels after 7 and 20 weeks of HDME exposure, and a 4-week resolution period abolished this increase (Figures 2 and 3). Small solitary vessels did not display any significant increase after 7 weeks, but after 20 weeks of exposure a significant increase compared with control mice was found (Figure 2).

Alterations in Collagen Synthesis

The total perivascular collagen content around bronchial-associated vessels increased significantly after 7 weeks of HDME exposure compared with levels observed in naive mice, and 4 weeks of resolution did not significantly abolish the increase (Figure 1). Twenty weeks of HDME exposure resulted in a tendency toward increased total collagen content around bronchial-associated vessels compared with naive mice (Figure 3). Total perivascular collagen content in mid-sized and small solitary vessels increased significantly after 7 weeks of HDME exposure and after 20 weeks had increased further (Figure 3). A 4-week resolution resulted in a slight decrease in total collagen content compared with 7 weeks, although collagen levels remained significantly elevated over controls (Figure 1).

The number of procollagen I–producing cells (fibroblasts) in all types of vessels increased significantly after 7 weeks of HDME exposure, but, in contrast to total collagen content, a 4-week resolution phase abolished the increase (Figure 2). Twenty weeks of HDME exposure also resulted in an increased number of

TABLE 3. PROLIFERATIVE RESPONSES OF ENDOTHELIAL CELLS AND SMOOTH MUSCLE CELLS TO HOUSE DUST MITE EXTRACT EXPOSURE

	Endothelial Cell Proliferation			Smooth Muscle Cell Proliferation		
Treatment	BA	MSS	SS	BA	MSS	SS
Naive	1.1 ± 0.3	0.7 ± 0.4	0 ± 0	6.1 ± 0.7	4.8 ± 0.6	5 ± 2.2
7 wk	6.3 ± 0.8*	7.1 ± 0.5*	98. ± 2.0*	33.6 ± 11.2*	15.9 ± 0.8*	20.2 ± 1.9*
20 wk	9.9 ± 1.3*	9.2 ± 0.5*	9.8 ± 0.6*	15.3 ± 1.5*	16.3 ± 1.3*	17.2 ± 1.7*
7+4 wk	1.7 ± 0.2†	1.7 ± 0.5†	1.3 ± 0.4†	5.1 ± 0.3†	3.8 ± 0.6†	5.6 ± 1.1†

Abbreviations: BA, bronchial-associated blood vessels; MSS, mid-sized solitary blood vessels; SS, small solitary blood vessels.

Cells were labeled with an antibody directed against proliferating cell nuclear antigen, counted manually on digital images, and correlated to the length of the basement membrane. The data represent number of proliferating cells per mm basement membrane, and are given as mean ± SEM.

* $P < 0.05$ compared with naive mice.

† $P < 0.05$ compared with 7 wk of HDME exposure.

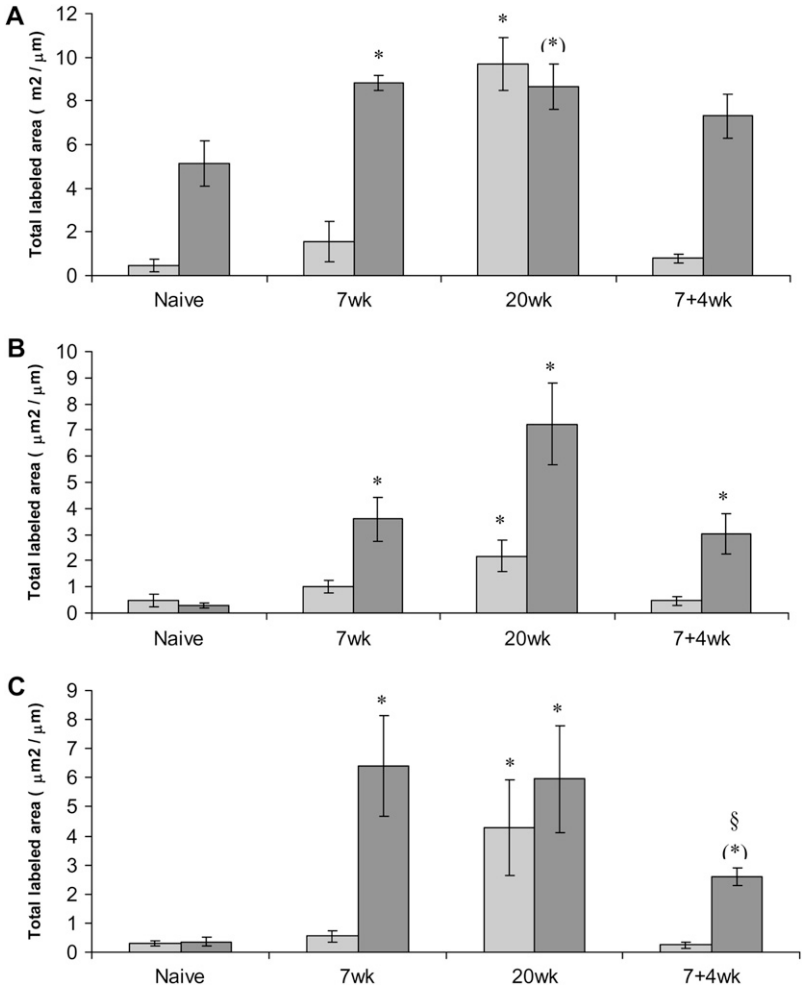

Figure 1. Total collagen content and smooth muscle area increased after house dust mite exposure. The increases were present in bronchial-associated vessels (*A*), mid-sized solitary vessels (*B*), and small solitary vessels (*C*). A resolution period did not significantly abolish the increased collagen deposition; however, tendencies were observed in small solitary vessels. Smooth muscle area increased more slowly than total collagen deposition, and no difference between 7 weeks of exposure and 7 weeks exposure + 4 weeks of resolution was found. This lack of difference is most likely due to remodeling not having been established after 7 weeks of allergen exposure, supported by the significant increase after 20 weeks of exposure. Total collagen content was visualized by Picro Sirius Red (*dark shaded bars*), and smooth muscle area was visualized by an antibody directed against α-smooth muscle actin (1:5,000, Sigma; *light shaded bars*). The Wilcoxon signed ranks test was used for statistical analysis, all groups were compared against naive mice, and the resolution group (7 + 4 wk) was also compared against 7 wk. *$P < 0.05$ compared with naive mice. §$P < 0.05$ compared with 7 weeks of exposure.

procollagen I–producing cells (Figure 2). We noted the occurrence of what seemed to be procollagen I–positive cells inside the smooth muscle lumen (Figure 3) and small foci, positive for procollagen I, located in the lung parenchyma (Figure 3).

DISSCUSSION

The present study demonstrates that allergic airway inflammation, induced by respiratory HDME exposure, results in vascular remodeling of the bronchial-associated and solitary vessels. This vascular remodeling includes several well known features similar to airway remodeling, such as increased smooth muscle area, enhanced procollagen I synthesis, total collagen deposition, and increased proliferation of endothelial and smooth muscle cells.

We have previously described the presence of remodeling of the pulmonary circulation in mice subjected to allergic airway inflammation caused by OVA (17). However, respiratory OVA exposure does not model chronic airway inflammation, and whether similar changes occur after chronic allergen-induced airway inflammation (which more closely mimics the human situation) has not been determined. To study the potential vascular remodeling effect of chronic allergic airway inflammation, a well established mouse model, using house dust mite as allergen, was used (6, 20). Animals were divided into four groups, and the study was designed to allow comparison between different allergen exposure times (allergen-naive animals compared with 7 and 20 weeks of HDME exposure) and investigation of resolution (7 wk HDME exposure followed by a 4-wk period of nonexposure).

Unlike OVA, HDME is a complex material consisting of hundreds of protein and nonprotein components, many of which are biochemically active and may play a role in enhancing Th2 immune responses. A number of dust mite allergens possess proteolytic activity (22, 23), capable of disrupting the integrity of airway epithelial cells through the degradation of the tight junction adhesion proteins occludin and ZO-1 (24, 25). Moreover, HDME proteases have direct proinflammatory effects because HDME-purified Der p 1, -3, and -9 have been shown to induce the production of granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-6, and IL-8 from airway epithelial cells (26) through the activation of protease-activated receptor-2 (27). It has also been suggested that HDME may enhance IgE synthesis and privilege the generation of a Th2-polarized response (28, 29).

The HDME exposure model results in several structural changes affecting the airways, such as goblet cell hyperplasia, subepithelial collagen deposition, and increased contractile tissue (airway smooth muscle and myofibroblasts [6]). In this study, similar changes were observed in the lung vasculature in bronchial-associated and solitary vessels. The main findings of these experiments include evidence for increased proliferation of endothelial and vascular smooth muscle cells, augmented smooth muscle area, enhanced procollagen I production, increased total collagen deposition, and increased number of myofibroblasts.

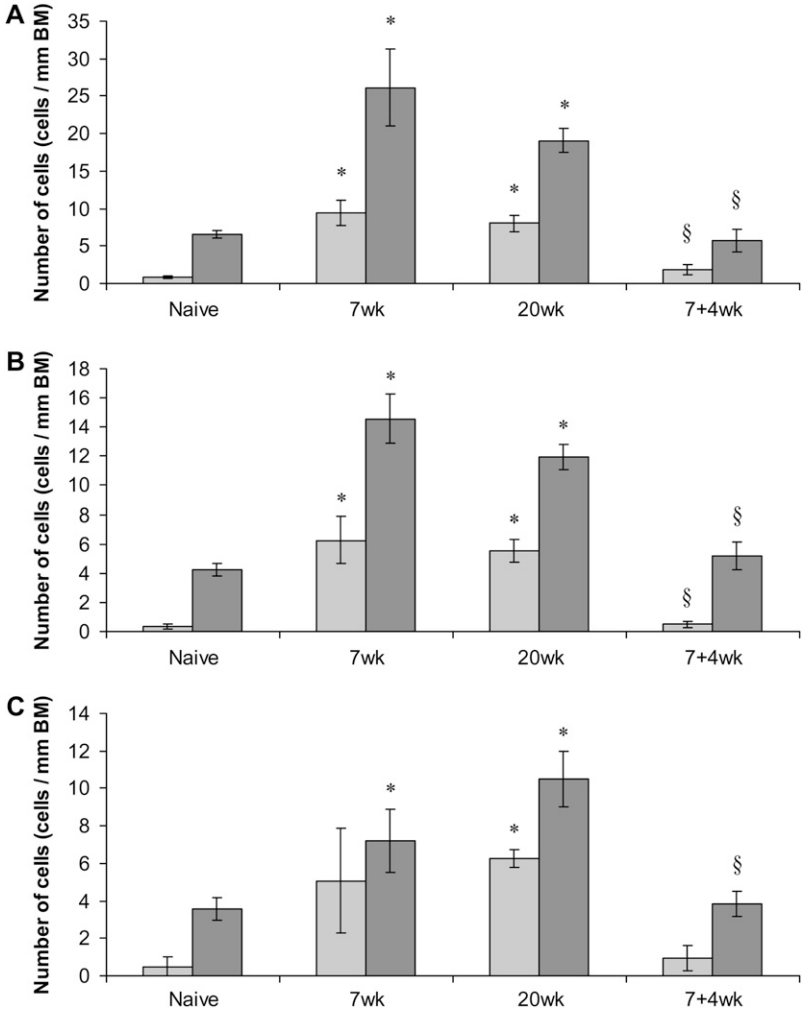

Figure 2. House dust mite extract exposure resulted in increased numbers of myofibroblasts and procollagen I production. The number of myofibroblasts increased around bronchial associated (*A*) and mid-sized solitary (*B*) vessels after only 7 weeks of allergen exposure. The increase was present at 20 weeks, when small solitary vessels (*C*) displayed a significant increase compared with naive mice. The number of procollagen I–producing cells (fibroblasts, *dark shaded bars*) increased after 7 weeks of exposure in all types of vessels and was still increased after 20 weeks of exposure. Four weeks of resolution significantly abolished the increase. The number of procollagen I–producing cells is an indication of collagen synthesis because the antibody labels intracellular procollagen I. Myofibroblasts (*light shaded bars*) were visualized by double labeling using an antibody directed against α-smooth muscle actin and procollagen I. The Wilcoxon signed ranks test was used for statistical analysis, all groups were compared against naive mice, and the resolution group (7 + 4 wk) was compared against 7 weeks. $^{*}P < 0.05$ compared with naive mice. $^{\S}P < 0.05$ compared with 7 weeks of exposure.

Our investigation of several aspects of vascular remodeling demonstrates different kinetics depending on remodeling parameter and type of vessel. Specifically, endothelial proliferation in all vessels and number of fibroblasts in bronchial-associated and mid-sized solitary vessels were maximal after 7 weeks of exposure, whereas proliferation of smooth muscle cells in all vessels and fibroblasts in small solitary vessels continued to increase to 20 weeks of allergen exposure.

Most parameters returned to baseline 4 weeks after the cessation of 7 weeks of allergen exposure; however, the total perivascular collagen deposition did not resolve in a similar way and remained significantly elevated in solitary vessels compared with allergen-naive mice after 4 weeks of resolution. A more thorough investigation into the mechanisms responsible for these changes revealed a clear return to baseline levels in endothelial and smooth muscle cell proliferation and number of myofibroblasts. This failure to reverse increased collagen deposition around pulmonary vessels after allergen withdrawal occurred despite a reduction in the activity and number of collagen-producing cells. Further investigations are warranted to determine if the collagen deposited around the vasculature persists with longer periods of allergen withdrawal, and, if so, to investigate whether these structural changes can be reversed by pharmacotherapy.

The precise mechanisms responsible for vascular remodeling induced by respiratory HDME exposure are unknown; however, enough is known about angiogenic mediators in other situations (bronchial remodeling and vascular remodeling in other organs) for us to speculate. Vascular endothelial growth factor is one of the most potent known angiogenic factors (30), and its ability to induce vascular remodeling, airway remodeling, and an increased response to methacholine has been described (31). The release of vascular endothelial growth factor from a variety of cells, including endothelial cells, eosinophils, and fibroblasts, is stimulated by TGF-β, GM-CSF, IL-6, IL-8, and TNF-α (30), mediators that have been associated with the airway inflammatory response to HDME exposure (6). In support of this, a correlation between GM-CSF and the number of eosinophils, as well as increased levels of GM-CSF in BAL, has been found in patients with systemic sclerosis (32), a disease known for the occurrence of vascular remodeling. It is therefore plausible to suggest that the vascular remodeling we observed in this study is induced by a similar mechanism.

Overall, the findings of this study suggest that the structural remodeling of the pulmonary vasculature might be resolvable if the inflammatory response to allergen is terminated. Our data suggest that aeroallergen exposure induced increased proliferation and activity of lung structural cells, leading to in increased collagen deposition and smooth muscle area around the vasculature resolves as soon as stimulation is stopped; however, the consequences of this increased activation are more persistent. This is in accordance with structural changes to

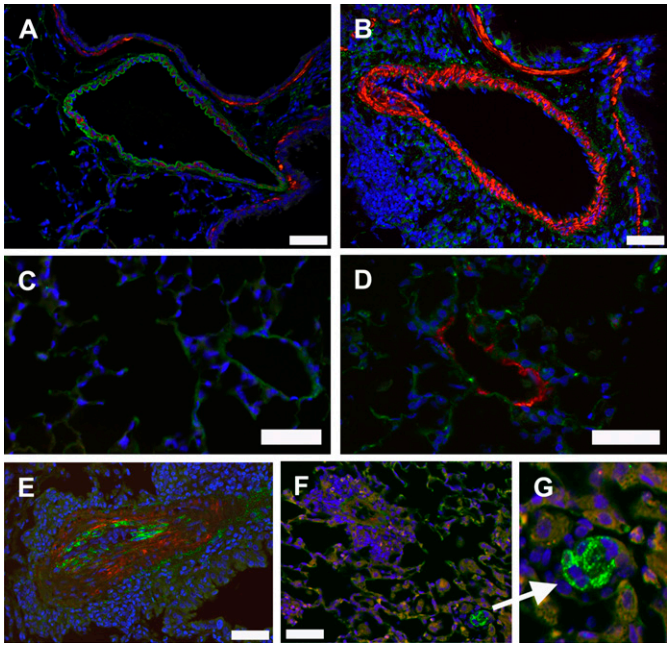

Figure 3. Images of vessels in naive animals and animals exposed to house dust mite extract (HDME) for 20 weeks. Visualization was done using antibodies directed against α-smooth muscle actin (*red*) and procollagen I (*green*). Cells co-positive for the two antibodies appear yellow, and the cell nucleus was visualized by labeling with the DNA-marker Hoechst 33342 (*blue*). Naive animals display little or no smooth muscle (*A*, bronchial associated vessel; *C*, small solitary vessel), whereas smooth muscle was enhanced in bronchial associated vessels (*B*) and appeared around small solitary vessels (*D*) after 20 weeks of HDME exposure. In the airway epithelium of animals exposed to HDME for 20 weeks, we observed what seemed to be myofibroblasts (*B*). We noted signs of vascular remodeling, such as expression of procollagen I, in some bronchial-associated vessels (*E*) and small foci of procollagen I–producing cells (*F* and *G*). These small foci were localized in the lung parenchyma and were unevenly distributed and rarely occurred but were still present in several of the sections analyzed. *Scale bars* represent 50 μm.

the airways, suggesting that if not the same, at least similar mechanisms are responsible for the remodeling of airways and blood vessels associated with chronic allergic lung inflammation.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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