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Vitamin A deficiency alters rat lung alveolar basement membrane Reversibility by retinoic acid ☆

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Abstract

Vitamin A is essential for lung development and pulmonary cell differentiation and its deficiency results in alterations of lung structure and function. Basement membranes (BMs) are also involved in those processes, and retinoic acid, the main biologically active form of vitamin A, influences the expression of extracellular matrix macromolecules. Therefore, we have analyzed the ultrastructure and collagen content of lung alveolar BM in growing rats deficient in vitamin A and the recovering effect of all-*trans* retinoic acid. Male weanling pups were fed a retinol-adequate or -deficient diet until they were 60 days old. A group of vitamin A-deficient pups were recovered by daily intraperitoneal injections of all-*trans* retinoic acid for 10 days. Alveolar BM in vitamin A-deficient rats doubled its thickness and contained irregularly scattered collagen fibrils. Immunocytochemistry revealed that these fibrils were composed of collagen I. Total content of both collagen I protein and its mRNA was greater in vitamin-deficient lungs. In agreement with the greater size of the BM the amount of collagen IV was also increased. Proinflammatory cytokines, IL-1α, IL-1β and TNF-α, did not change, but myeloperoxidase and TGF-β1 were increased. Treatment of vitamin A-deficient rats with retinoic acid reversed all the alterations, but the BM thickness recovered only partially. Retinoic acid recovering activity occurred in the presence of increasing oxidative stress. In conclusion, vitamin A deficiency results in alterations of the structure and composition of the alveolar BM which are probably mediated by TGF-β1 and reverted by retinoic acid. These alterations could contribute to the impairment of lung function and predispose to pulmonary disease.

Keywords: Vitamin A; Retinoic acid; Basement membrane; Collagen; Oxidative stress; TGF-β1

1. Introduction

Although a global progress in the control of vitamin A deficiency (VAD)-related disorders has been seen in the last decade, VAD is still an important public health problem particularly for children in developing countries. VAD, even the asymptomatic subclinical form, increases morbidity and mortality from several childhood infections such as measles and diarrhea and also increases the incidence and morbidity of respiratory tract illnesses [1].

Retinoids, active metabolites of vitamin A, are required for normal mammalian fetal development and are involved in lung and alveolar formation during the neonatal period. They are also required for maintenance of alveolar architecture after the alveoli have been formed [2,3]. In rats, pulmonary alveoli are formed during the early postnatal life and they continue to form until about age 6–7 weeks [3]. Postnatal retinoic acid treatment increases the number of alveoli in developing rat lung, attenuates glucocorticoid-induced inhibition of postnatal alveolarization [4], increases type I collagen content in airspace walls and improves survival of newborn rats exposed to hyperoxia [5]. Similarly, studies of adult rats with elastase-induced emphysema, and rat pups exposed to hyperoxia, have shown that retinoids promote post hoc improvements in alveolar septation and lung architecture [3,6]. In humans, administration of vitamin A reduces the incidence of bronchopulmonary dysplasia in vitamin A-deficient premature infants [7]; laboratory evidence suggests it could have a protective effect on respiratory status in patients with cystic fibrosis [8], and its potential application in the prevention and treatment of emphysema, based on the results with animals, is an exciting area of research [9].

Basement membranes (BMs) are laminar structures of highly specialized extracellular matrix (ECM) which separate cells from adjacent connective tissue. In the lung, BMs are associated with the airway and alveolar epithelium, endothelium, bronchial and vascular smooth muscle cells, nerve cells and visceral pleura. In the alveoli, they are also part of the air–blood barrier and thence traversed during the gas-exchange process. Quantitatively, their main components are type IV collagens and laminins which provide a framework where other BM components and contacting cells bind. Besides their structural functions, BMs direct cell migration, influence cell

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metabolism and regulate cell differentiation, proliferation and apoptosis [10,11]. In accordance with their functions, alterations in their structure and/or composition are involved in a great variety of illnesses. Concretely, BM thickening with altered deposition of ECM proteins, appearing before detectable functional defects, is a key pathological feature in several lung and kidney diseases such as asthma and diabetes [12,13]. Accumulation of some ECM proteins such as collagen IV and laminin has also been described in chronic pulmonary obstructive disease and idiopathic pulmonary fibrosis. Deposition of these proteins inversely correlated with lung function suggesting its implication in lung deterioration [14].

It is known that retinoic acid modulates through specific nuclear receptors the expression of several ECM proteins both directly, acting on their gene promoters, and indirectly, modifying the expression of other profibrotic factors [15,16]. Consequently, a deficiency of vitamin A could induce changes in the composition and the structure of BMs resulting in alterations of organ function and protracted pathological consequences. In fact, VAD promotes bronchial hyperreactivity [17], leads to emphysematous lungs with areas of interstitial pneumonitis [9,18] and results in squamous cell metaplasia with a relative decrease in the proportion of mucous and ciliated cells in the tracheobronchial tree [1]. Moreover, surfactant synthesis and ornithine decarboxylase activity, the rate-limiting enzyme in the synthesis of polyamines, are significantly lower in type II pneumocytes isolated from VAD rats [18], indicating an impaired functional and proliferative capacity. These alterations in lung function and architecture are associated with changes in ECM protein content and distribution. It has been reported that lungs of VAD rats have less collagen in the adventitia of small arteries and arterioles and in the alveolar septa; however, its amount is increased especially in the areas of interstitial pneumonitis and remains normal in peribronchial ECM. The content of elastin is also lower in the lung parenchyma as well as in the small arteries and arterioles of VAD rats [18,19]. Retinoic acid treatment of the VAD animals restores the collagen content and the mechanical properties of the lung but not the parenchymal defects such as the reduction in alveolar surface area. This correlation between collagen content and mechanical properties evidences the strong influence of the ECM on the lung function [9]. As BMs play an important role in pneumocyte differentiation [20], regulate gap junction formation and intercellular communication between alveolar epithelial cells [21], contain apoptotic and survival signals for endothelial cells [22] and form a continuous structure throughout the entire lung, they can be involved in the functional consequences of VAD. Therefore we have analyzed the ultrastructure and composition of ABM in the lung of VAD rats and the reversibility of the alterations by treatment with retinoic acid. Since VAD could induce an inflammatory process and be associated with oxidative stress [23,24], both processes resulting in increased BM protein expression [25], we have also measured proinflammatory cytokines, inflammatory cell infiltration markers and lipid peroxidation.

2. Methods and materials

2.1. Animal treatment

Vitamin A-deficient animals were prepared as previously described [26]. Briefly, pregnant Wistar female rats (Charles River, Barcelona, Spain) were randomly assigned either to a control or to a VAD group and housed in individual cages in a room maintained at 22–25°C with a 12-h light–dark cycle. The day after delivery, the dams of the control group were fed a complete solid diet (AIN-93, ICN Biomedicals, Cleveland, OH, USA). The dams of the VAD group were fed the same diet but devoid of vitamin A. After 21 days of lactation period, male pups of each group were weaned into their corresponding dam diet until they were 60 days old. The 60-day-old male rats of the VAD group were randomly subdivided into three groups: VAD group, sacrificed that same day together with the controls; retinoic acid recovered (RR) group, treated further with 10 daily intraperitoneal injections of 100 μg of all-*trans* retinoic acid in 100 μl of sunflower seed oil; and vehicle-treated group, injected as the RR group but with sunflower oil only. All groups were pair fed. Animals were anesthetized with sodium pentobarbital (50 mg/kg) before sacrifice. The protocol was approved by the Ethic Committee for Animal Research and Welfare at the University of Valencia.

2.2. Tissue sample preparation

Blood and lung samples for biochemical studies were processed as previously described [26] and stored frozen until they were used. For ultrastructural and immunocytochemical studies, whole anesthetized animals were perfused with 0.5% glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer (pH 7.4).

2.2.1. Samples for ultrastructural studies

Small fragments of rat lung were postfixed in 1.5% glutaraldehyde and 1% formaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 60 min at 4°C, dehydrated in graded concentrations of ethanol and embedded in Epon 812 (six blocks per sample) [26,27]. Ultrathin sections were examined on a Philips CM 100 Transmission Electron Microscope.

2.2.2. Samples for immunocytochemistry

Small fragments of rat lung, previously perfused, were postfixed with the same fixative solution for 120 min at 4°C, washed in the buffer, incubated for 50 min in 50 mM NH_4Cl, dehydrated in methanol and embedded in Lowicryl K4M following the progressive lowering temperature procedure as previously described [27].

2.3. Determination of retinoids

Plasma retinol and lung retinoids were extracted as described [28], dissolved in methanol/ethanol (1:1, v/v) and measured by the isocratic HPLC method of Arnaud et al. [29]. A Nova-pak C-18 column (3.9×150 mm, Waters) and a mixture of acetonitrile/dichloromethane/methanol (70:20:10), as eluent, were used.

2.4. Determination of TNF-α, IL-1α, IL-1β and TGF-β in lung

Pieces of frozen lung (0.1 g) were homogenized in 1 ml of 50 mM Tris-HCl (pH 7.5) containing 0.1% Triton X-100 and 5 μl of protease inhibitor cocktail (Sigma), sonicated twice for 30 s with cooling and centrifuged at 450,000×*g* (Optima MAX ultracentrifuge, Beckman) for 10 min. The supernatants were analyzed for TNF-α, IL-1α, IL-1β and TGF-β contents by commercial ELISA kits (Biolink 2000, Promega in the case of TGF-β) as recommended by the manufacturer.

2.5. Determination of myeloperoxidase activity

Lung extracts obtained as described for cytokine determinations were assayed for myeloperoxidase activity by a described photometric method based on the oxidation of *o*-dianisidine [30], with some modifications. Ten microliters of extract, diluted 1/10 in 50 mM Na-citrate-phosphate buffer (pH 5.0), was added to 990 μl of dilution buffer containing 0.1 mg/ml *o*-dianisidine (Sigma) and 0.015% H_2O_2, and the absorbance change at 450 (ΔA_{450}) registered continuously over 3 min at room temperature. The assay was also performed in the presence of 40 mM NaN_3. Myeloperoxidase activity was calculated from NaN_3-inhibitable peroxidase activity and expressed in arbitrary units, where an arbitrary unit is defined as the amount of enzyme which produces a ΔA_{450} of 0.001/min.

2.6. Determination of lipid peroxidation

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) in the lung extracts obtained as described for cytokine determinations and by using HPLC for separating the MDA-thiobarbituric acid adducts as reported [31]. Standards of MDA-bis (Merck) were used for determining MDA concentration.

2.7. Solubilization of collagen I and collagen IV

Lung pieces of 0.1 g were homogenized in 2 ml of Tris-HCl 50 mM (pH 7.5) containing 0.25% Triton X-100 and 10 μl protease inhibitor cocktail (Sigma). The suspension was centrifuged and the pellet digested extensively with 0.3 mg/ml of bacterial collagenase (high-purity collagenase, type VII, Sigma) at 37°C for 48 h in digestion buffer consisting of 50 mM Hepes (pH 7.5), 10 mM $CaCl_2$, 0.05% NaN_3 and 5 μl/ml of the protease inhibitor cocktail mentioned above. The suspension was centrifuged and EDTA, to a final concentration of 25 mM, was added to the supernatant. The solubilized material was stored at −80°C until used. Digestion of the pellet was repeated to confirm that no more collagen fragments were solubilized. A time course of the solubilization process was analyzed by Western blotting to detect the collagen fragments accumulating with the incubation time. Purified collagen (Sigma) was also digested with collagenase to confirm the accumulation of the quantified fragments.

2.8. Protein quantification by Western blotting

SDS-PAGE and immunoblotting were carried out as previously described [26]. Collagenase solubilized samples were separated in 6–20% acrylamide gradient gels in reduced conditions (collagen I) or in nonreducing conditions (collagen IV), and

transferred electrophoretically to PVDF paper at 150 mA constant current for 18 h at 4°C. Specific rabbit polyclonal antibodies against collagen I (Calbiochem; diluted 1/1000) and collagen IV (Santa Cruz; diluted 1/100) were used for immunodetection. The polyclonal anti-collagen IV antibody reacted with the six α(IV) chains. Anti-rabbit IgG conjugated to alkaline phosphatase (Promega; diluted 1/7500) was used as the secondary antibody. After color development, blots were scanned with an HP scanjet 5400c, and protein bands were quantified using the Scion Image Programme. Linearity ranges were established by blotting different amounts of each sample. Control samples were included in each blot as reference for the relative quantification of the protein bands.

2.9. Total RNA extraction and quantitative polymerase chain reaction

The response to VAD and to retinoic acid was assessed by measuring the steady-state level of mRNA in lung tissue from control, VAD and RR rats as described [32]. Total RNA was isolated from lung samples using the guanidinium thiocyanate method. One microgram of RNA was reverse transcribed to cDNA using Ready-To-Go You Prime First-Strand Beads (Amersham Pharmacia Biotech) using random primers. Primer sets from each candidate gene were designed by Primer3 software and synthesized by Sigma. These were COL1A1 sense: 5-ATGGTGCTCCTGGTATTGCTG-3, antisense: 5-CTCCTTTGGCACCAGTGTCTC-3; COL1A2 sense: 5-GAAGATGGTCACCCTGGAAAAC-3, antisense: 5-CAGGCTGTCCTTTCAATCCATC-3. Measurement of gene expression was performed using the AB 7900HT Fast Real-Time PCR System and SYBR Green Master Mix (Applied Biosystems) following the manufacturer's instructions. Standard curves were generated for each gene to determine the PCR efficiency and quantification of the copies of each mRNA in the samples. Linear regression analysis of the standard curves documented in all cases an R^2 value of 0.99. Normalization of samples was performed by the reference gene, β2-microglobulin, whose expression did not change in our experimental situations. All PCR products were tested by agarose gel electrophoresis and by dissociation curves. Correct amplification was verified in all samples and in all reactions by dissociation curves.

2.10. Ultrastructural morphometric analysis

The thickness of the BM was determined in randomly selected micrographs. In each microphotograph, the thickness of the alveolar BM was measured at intervals of 1 cm with a minimum of 10 measurements per micrograph. The minimum sample size was determined by the progressive mean technique (confidence limit, ±5%) [26,27].

2.11. Ultrastructural immunolocalization of collagen I

Collagen I was located in the BM from the alveolar wall with the immunogold procedure as described [27]. Briefly, the ultrathin sections were initially incubated in 3% H_2O_2 for 10 min. Then, after blocking and incubating with specific anti-collagen I primary antibody (Calbiochem) and gold-conjugated second antibody, the ultrathin sections were counterstained with uranyl acetate and examined in a Philips CM 100 Transmission Electron Microscope.

2.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Unless otherwise specified, histograms represent means (bars)±S.D. (brackets); n=6, for the control group; n=7 for the VAD group; and n=4, for the RR group. The data from the three groups were analyzed using one-way ANOVA followed by the Bonferroni test. Differences were considered significant when the P value was <.05.

3. Results

3.1. Retinoid status in plasma and lung

The determination of plasma retinol concentration has shown that our model of VAD induces a dramatic decrease in the levels of retinol in plasma and lung and of retinol palmitate in lung (Table 1). The retinol concentration in plasma from VAD rats decreased to less than 5% of the control group and did not increase with the retinoic acid treatment. Likewise, the amounts of retinol and retinol palmitate per gram of tissue in VAD and RR lungs were reduced by more than 95%.

Unless specified, the results obtained with the vehicle-treated group did not significantly differ from the VAD group and for simplicity they will not be shown.

Table 1
Concentrations of retinol and retinol palmitate in plasma and lungs of control, VAD and RR rats

	Control	VAD	RR
Plasma (μM)			
All-*trans* retinol	1.73±0.01 (6)	0.07±0.01 (7)*	0.05±0.01 (4)*
All-*trans* retinol palmitate	ND	ND	ND
Lung (nmol/g of tissue)			
All-*trans* retinol	1.13±0.01 (6)	0.03±0.01 (7)*	0.05±0.01 (4)*
All-*trans* retinol palmitate	2.87±0.59 (6)	0.007±0.003 (7)*	0.05±0.04 (4)*

Values are expressed as mean±S.D., with the number of animals indicated in parenthesis; *significantly different from control group; P<.05.

3.2. Effect of VAD and retinoic acid treatment on the ultrastructure of lung alveolar BM

Vitamin A deficiency induces alterations in the structure of alveolar BM which are improved by retinoic acid treatment. As shown in Fig. 1, electron microscopic examination revealed that the regular and uniform structure of alveolar BM in control animals (A, B) was enlarged and showed frequent inclusions of collagen fibril bundles in the VAD group (C, D). Splitting was also seen in some segments of the VAD BM, and the caveolar compartment in capillary endothelial and alveolar epithelial cells was apparently increased. However, definitive assessment and quantification of this increment need further work. After retinoic acid treatment, the BM reduced its size approaching that of the controls, and the collagen fibrils and BM splitting mostly disappeared (E, F).

In addition, the ultrastructural analysis showed a more abundant deposition of collagen fibrils in the interstitial ECM from the VAD compared to control or RR animals (data not shown).

A morphometric analysis was performed to compare the ABM size from the different groups. In order to better define the BM limits and to obtain more accurate values, the measurements were only done on the thin part of the alveolar walls where the alveolar epithelial BM and the endothelial capillary BM fuse. Fig. 2 summarizes the quantitative results concerning the BM thickness in control, VAD and RR animals. The ABM from VAD lungs (103±17 nm) was about twofold that observed in controls (49±6 nm), and treatment of VAD animals with retinoic acid reduced its size by 25% (76±8 nm) but it did not reestablish the control value.

As we have mentioned above, the ABM in VAD lungs presented frequent and abundant fibrils of apparently collagenous material. Fig. 3A shows representative images and, as can be seen, the fibrils appear dispersed in different orientations along the BM and occasionally attached in clusters to the plasma membrane of the endothelial cells. In order to find out whether these fibrils were composed of collagen I, sections from control, VAD and RR rat lung embedded in Lowicryl K4M were used for an immunogold procedure using a polyclonal anti-collagen I antibody. Subsequent analysis allowed the detection of gold particles into the structure of VAD ABM (Fig. 3B) which were absent in the control group and very scarce after retinoic acid treatment. In agreement with the immunological results, measurements of fibril width showed no significant difference between the intra-BM (45.6±3.0 nm) and the adjacent connective tissue (47.6±4.0 nm) fibrils, mainly composed of collagen I, supporting the identification of this collagen as a component of the intra-BM fibrils.

3.3. Effect of VAD and retinoic acid treatment on collagen content

In order to elucidate whether the alterations observed in the ABM and the interstitial ECM were accompanied by changes in the amount of its constituent molecules, we determined the amounts of both

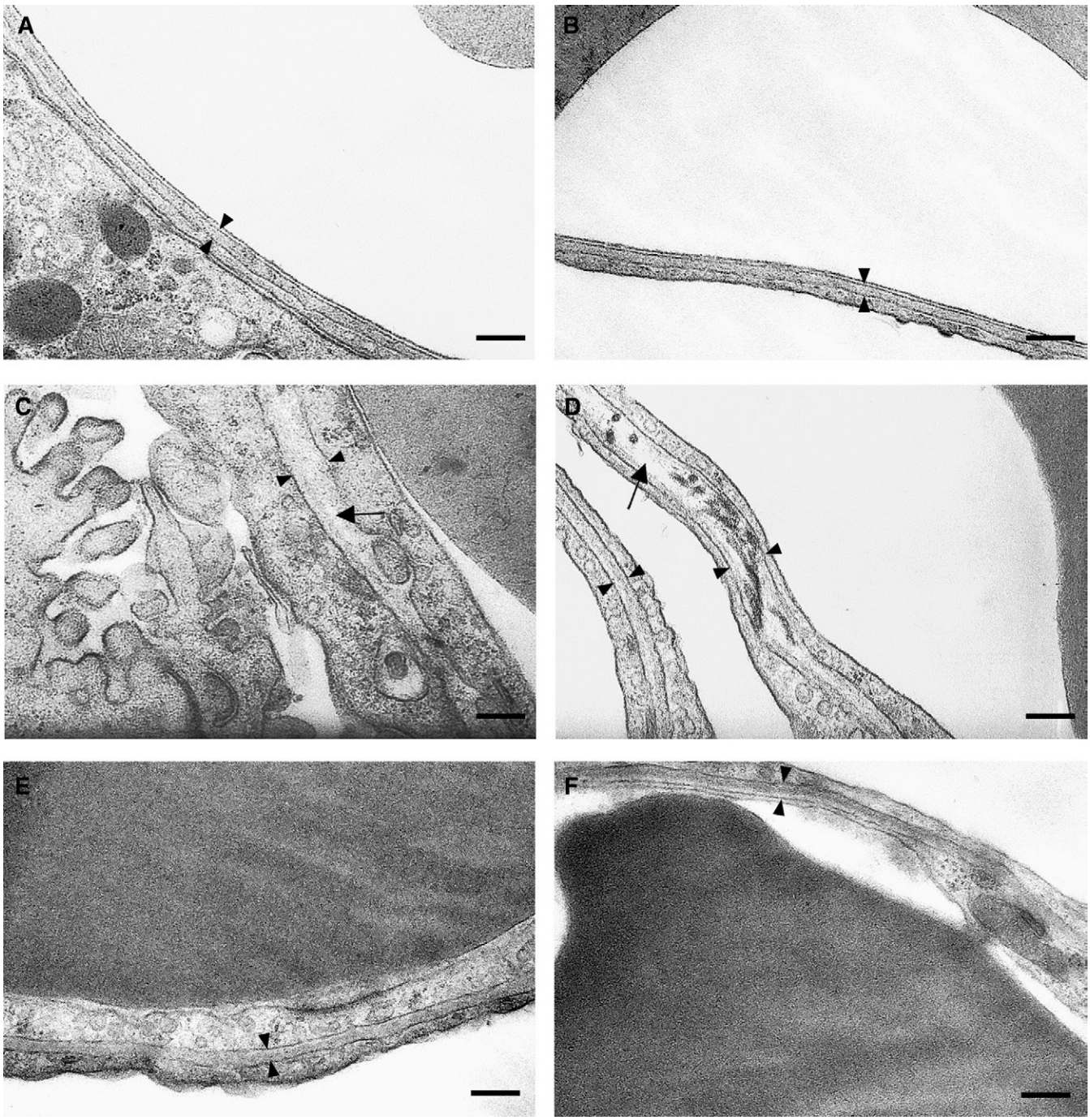

Fig. 1. Ultrastructure of the lung alveolar basement membrane from control, VAD and RR rats. The electron microphotographs show segments of the alveolar BM which results from the fusion of alveolar epithelial and capillary endothelial BMs. Control ABM (A and B) appears with a regular morphology and a normal size. VAD ABM (C and D), on the contrary, is clearly thickened and contains irregularly scattered fibrils (D) of collagenous material. Splitting of an ABM segment is also appreciated (arrows in C and D). In microphotograph D, a less altered segment of the VAD ABM can be seen. Administration of retinoic acid (E and F) reduces the size and practically eliminates the collagen fibrils from the BM. Arrowheads mark the boundaries of the fused BM. Scale bar is 200 nm.

collagen IV and collagen I, the major components of BMs and ECM, respectively. Immunoblot analysis indicated that, in agreement with the greater size of BM and the accumulation of collagen fibrils in the ECM, the amount of collagen IV was 78% greater (Fig. 4A) and that of collagen I 100% greater (Fig. 4B) in VAD animals than in controls. After intraperitoneal administration of retinoic acid, the amounts of both collagens decreased to values not significantly different from those found in control rats (Fig. 4).

3.4. Correlation between the amounts of protein and mRNA for the collagen I subunits

In addition, to check whether the changes in the amount of protein corresponded to those in mRNA, we analyzed the mRNA content for the α(I) chains that form the molecule of collagen I using real-time RT-PCR. Each mRNA was quantified and normalized to the quantity of β2 microglobulin mRNA. As shown in Fig. 5, in close

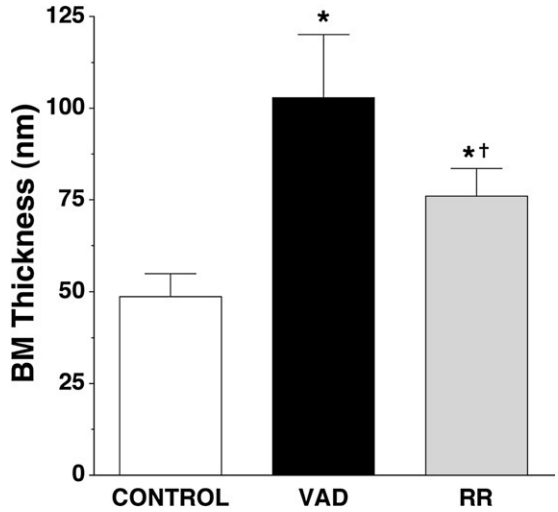

Fig. 2. Thickness of the lung alveolar basement membrane from control, VAD and RR rats. Morphometric analysis was done on electron micrographs of the fused alveolar BM as described in Methods and Materials. VAD doubles the thickness of the ABM, and treatment with retinoic acid reverses, but not totally, its size. Histograms represent means±S.D.; *significantly different from the control group; †significantly different from the VAD group; $P<.001$.

agreement with the variations in the amount of collagen I, the levels of the mRNAs for each collagen I chain were increased by about 80% for the α1(I) chain and by 75% for the α2(I) chain in VAD and returned to values not significantly different from those of the control group after retinoic acid treatment. The correlation in the changes between the proteins and its corresponding mRNAs indicates that VAD and retinoic acid regulate collagen I expression acting mainly at the mRNA level. However, it cannot be concluded whether they act by modifying α(I) chain gene transcription or mRNA stability or both. As would be expected from the chain composition of collagen I, the α1(I) mRNA to α2(I) mRNA ratio is approximately 2 for the control group. The fact that this ratio is maintained in the VAD and RR groups indicates a coordinated regulation of the expression of both α(I) chains.

3.5. Effect of VAD and retinoic acid treatment on the content of inflammatory markers

To determine whether the changes observed in ABM were related to an inflammatory process, we measured in the lung tissue the proinflammatory cytokines IL-1α, IL-1β and TNF-α, as well as the content of myeloperoxidase, a leukocyte infiltration marker. Table 2 summarizes the results concerning the amount of these molecules for control, VAD and RR groups. As shown, only the amount of myeloperoxidase was affected by VAD and retinoic acid treatment showing a moderate but significant increase in the VAD group of animals.

3.6. Effect of VAD and retinoic acid treatment on the amount of TGF-β1

TGF-β1 is a profibrotic cytokine which induces the synthesis of ECM molecules; its expression can be modulated by RARs and it has been associated with the pathogenesis of fibrosing lung diseases. We therefore assessed whether VAD and retinoic acid would modify the TGF-β1 content in the lung. VAD produced a 50% increase in the lung content of active TGF-β1 (13.7±1.3 vs. 9.2±1.1 ng/g), and treatment with retinoic acid reduced it to the control value (9.0±2.3 vs. 9.2±1.1 ng/g) (Fig. 6). This suggests that TGF-β1 could be a mediator in the effects of VAD and retinoic acid on the lung ECM.

3.7. Effect of VAD and retinoic acid treatment on oxidative stress

Reactive oxygen species are known inducers of the expression of TGF-β1 and ECM proteins, and VAD is a condition potentially leading to an imbalance between ROS production and antioxidant defenses in the lung. In consequence, we measured in lung tissue the content of MDA, a product of lipid peroxidation, as an indicator of oxidative stress. The lungs of VAD rats doubled their content of MDA (130±21 nmol/g) compared to controls (65±4 nmol/g) and after retinoic acid treatment MDA increased even further (235±38 nmol/g) to values 80% greater than those of VAD group (Fig. 7). These results show that the intraperitoneal administration of retinoic acid to VAD animals worsens their oxidative status and highlight the ability of retinoic acid to revert the alterations of the ECM even in the presence of increased oxidative stress.

4. Discussion

In a previous study [26], we have reported that VAD alters profoundly the renal BMs with modifications such as twofold and sixfold thickening of glomerular and tubular BMs, respectively; the presence of abundant collagen fibrils in the tubular BM; and a twofold increment in the content of type IV collagen. In the present work, we have analyzed the lung alveolar BM which, like the glomerular BM, contains segments formed by merging epithelial and endothelial BMs. The results indicate that VAD enlarges the lung alveolar BM, induces the ectopic appearance of collagen fibrils in this BM and increases the total amount of both type I and type IV collagens. We also show that these alterations are, at least partially, restored to the control status by treatment with all-*trans*-retinoic acid. The similarities in the modifications of renal and lung BMs suggest a common mechanism for the effects of VAD on the BMs of different tissues.

The enlargement of BMs is an early event in many diseases. Thickness increases have been described for the glomerular and alveolar BMs of both diabetic mice and humans [12,33]. Also, bronchial BM thickening occurs in various lung diseases such as bronchial asthma or chronic obstructive pulmonary disease [13,14]. In VAD animals, we report increments in alveolar BM thickness of similar or even greater magnitude with sizes double those of the controls. In addition to thickening, VAD results in splitting of some segments of the fused alveolar BM which divides it into two halves. The splits could represent dissociations of the epithelial and endothelial BMs. These alterations of the alveolar BM may contribute to impairing gas exchange, alveolo-capillary permeability and lung function [17,34].

The VAD alveolar BM also contains abundant, disordered collagen fibrils. Our immunocytochemical analysis indicates that these fibrils are composed of type I collagen. However, it cannot be excluded that other fibrillar collagens such as collagen III and V are also constituents of the fibrils. Nevertheless, the similar width between the BM fibrils and those of the underlying interstitial collagen suggests that they are mainly or exclusively composed of collagen I. The proportionality between the increments in total collagen IV content and alveolar BM thickness indicates also that there is no change in the collagen IV density of VAD lung BM.

Such alterations as alveolar BM thickening and collagen deposition both within the BM and within the interalveolar septae have been observed in the two most common subtypes of idiopathic pulmonary fibrosis [35] and in patients with bronchiolitis obliterans syndrome, the chronic graft failure which develops in lung transplant recipients [36]. The authors show data supporting the possibility that immune-

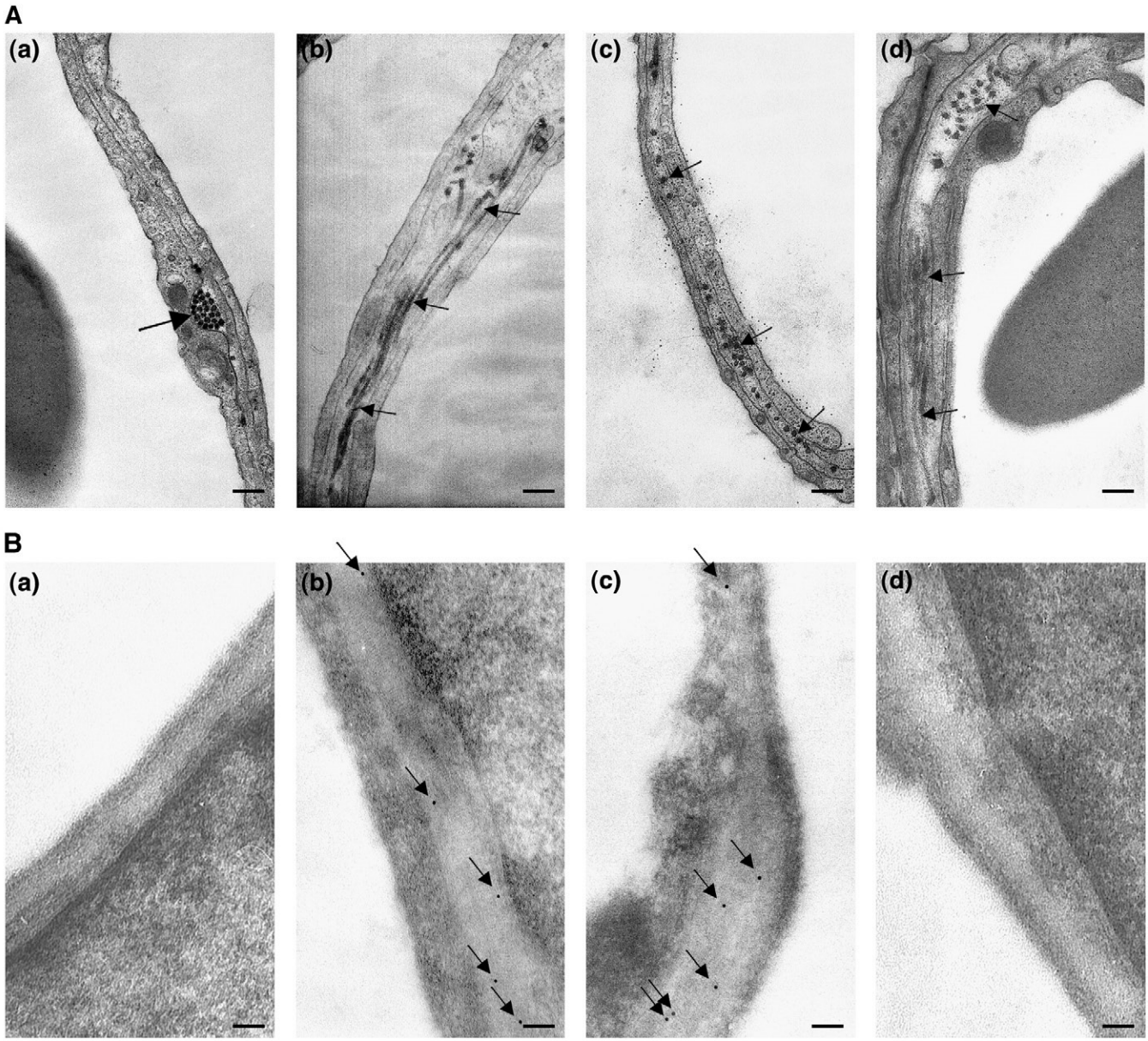

Fig. 3. Conventional electron microscopy and immunocytochemical characterization of the alveolar BM fibrils. (A) Representative segments of the conventional electron microscopy of the fused ABM from VAD lungs (a–d) showing the inclusion of fibrils along the BM (arrows). In (a), the presence of fibril bundles adjacent to the plasma membrane of capillary endothelial cells (arrow) is observed. Scale bar is 200 nm. (B) Gold immunodetection of collagen I inside the alveolar BM. Ultrathin sections of fixed lungs were embedded in Lowicryl, incubated with polyclonal anti-collagen I antibodies and immunorevealed with a gold-labeled second anti-IgG antibody. The electron microphotographs in (B) show the fused ABM from control (a), VAD (b, c), and RR (d) lungs. Scale bar is 75 nm.

based microvascular endothelial cell injury is involved in the evolution of lung BM abnormalities and of excessive collagen deposition within the terminal lung parenchyma characteristic of these pathologies. It cannot be deduced from our study whether this is also the case in VAD. However, the increase we have detected in myeloperoxidase activity, indicative of inflammatory cell infiltration of the VAD lung, could fit in this possibility. A VAD-induced oxidative insult to the endothelial cells could induce the expression of adhesion molecules and cytokines in the microvascular endothelium which would recruit and activate inflammatory cells triggering the fibrotic process [37]. In fact, we demonstrate that the lungs of the VAD rats are under oxidative stress as manifested by the increase in their MDA content. The infiltration of inflammatory cells together with the presence of areas of interstitial pneumonitis with increased collagen has already been observed in the lungs of VAD rats [18]. However, in spite of the leucocyte infiltration, the absence of increments in proinflammatory interleukins, such as IL-1α, IL-1β and TNFα, in the lungs of our VAD rats indicates that the inflammatory process is present but mild.

On the contrary, in VAD lungs there is an increase in the active form of a fibrogenic factor, TGF-β1, which decreases to the control value by retinoic acid treatment. As TGF-β1 is able to induce the synthesis of collagens I, III and IV [38,39], it could be a mediator in the effects of VAD and retinoic acid on the ECM of rat lungs. Based on the information provided in the literature, the relationship between retinoids and the TGF-β system appears quite complex and the results published on the effects of retinoic acid on the expression of TGF-β1 are conflicting. However, in line with our data, treatment with

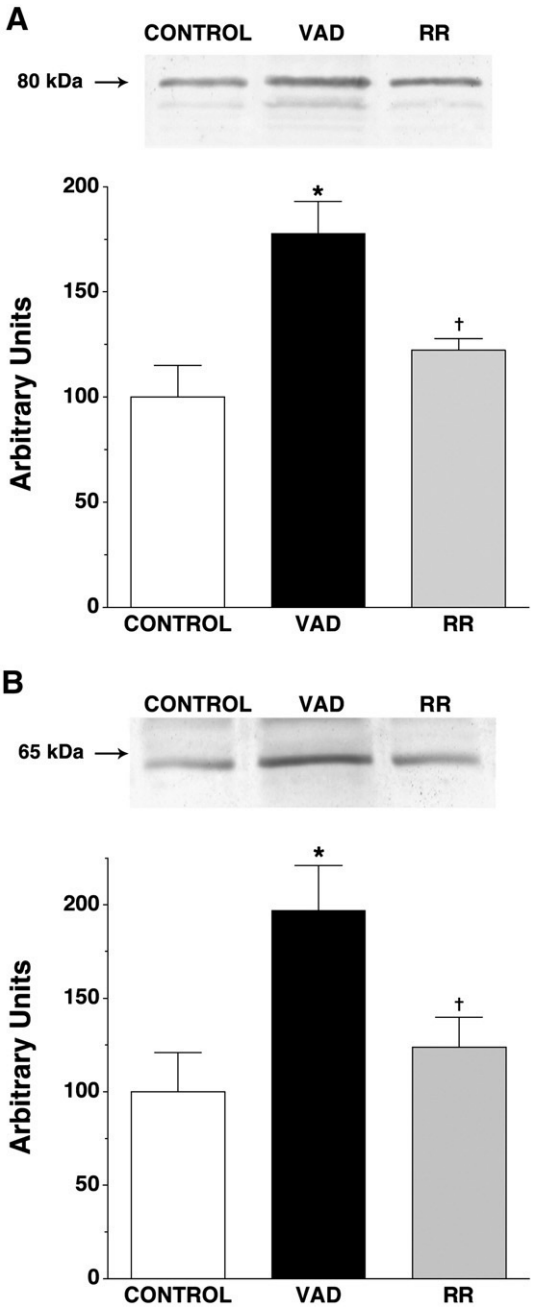

Fig. 4. Quantification of collagen IV and collagen I in the lungs of control, VAD and RR rats. Collagenase solubilized material from control, VAD and RR rat lungs was separated by SDS-PAGE and immunoblotted with polyclonal anti-collagen IV (A) or anti-collagen I (B) antibodies. (A) An accumulating band of 80 kDa was scanned for evaluating the type IV collagen content in the different lungs. (B) An accumulating band of 65 kDa was scanned for evaluating the type I collagen in the different lungs. The histograms represent the densitometric values as percentage of the control group. Data are mean±S.D.; *significantly different from control group; †significantly different from VAD group; $P<.0001$. Insets show a representative Western blot of the quantified bands.

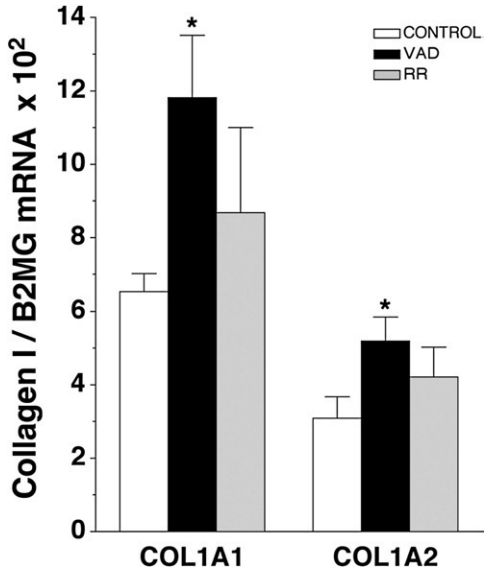

Fig. 5. Quantification of collagen I mRNA in the lungs of control, VAD and RR rats. Total RNA was extracted from the lung tissues, and the mRNAs specific for the two α chains of collagen I were quantified by real-time RT-PCR. The bars show the mRNA amount for each α(I) chain normalized to the mRNA for β2-microglobulin, determined identically in the same sample. Values are mean±S.D.; *significantly different from control group; $P<.05$.

retinoic acid reduced significantly the glomerular TGF-β1 protein content in an experimental model of glomerulonephritis with elevated TGF-β1 expression [40]. Additionally, it has been proposed that retinoic acid receptors may function as activators of the TGF-β/Smad pathway in the absence of retinoic acid or as inhibitors in its presence [41]. Although many cell types are able to synthesize TGF-β1, the close agreement in the degree of variation of MPO and TGF-β1 in both VAD and RR lungs leads to considering the inflammatory cells as its main source. Increased expression of TGF-β1 mRNA by alveolar macrophages associated with sites of active fibrosis has already been observed in lung tissue from patients with idiopathic pulmonary fibrosis [42]. In this context, it is worthy to emphasize the appearance of collagen fibril bundles attached to the plasma membrane of endothelial cells which could be indicative of an endothelial origin for these fibrils and would be in agreement with the involvement of these cells in the fibrotic process.

Alternatively or additionally, the absence of retinoic acid through transcriptional modulation could alter the expression of ECM molecules. Several retinoic acid response elements have been found in the promoter of the α2(I) collagen gene. Binding of retinoic acid-activated receptors to these sites inhibits the expression of the α2(I) gene without affecting the α1(I) gene in hepatic stellate cells [43]. Other authors, on the contrary, have described inhibition by retinoic acid of α1(I) gene transcription in human lung fibroblasts [44]. Our results also fit in these findings since VAD results in increments of collagen I and treatment with retinoic acid reverses the situation to the control values. We are not aware of any report on the interaction of retinoic acid receptors with the promoters of collagen IV. However, it is known that these receptors can directly interact with and modify the transcriptional activity of other nuclear factors such as Sp1 which are capable of binding specifically, at least, to the *col4α1–col4α2*

Table 2
Measurements of cytokines and myeloperoxidase in control, VAD and RR rat lungs

	Control	VAD	RR
IL-1α (ng/g tissue)	11.23±1.86 (5)	9.23±0.43 (5)	10.68±0.23 (4)
IL-1β (ng/g tissue)	8.37±0.79 (5)	8.83±0.96 (5)	9.57±2.12 (4)
TNFα (ng/g tissue)	0.97±0.26 (5)	1.04±0.24 (5)	0.78±0.13 (4)
Myeloperoxidase (arbitrary units/g of tissue)	371.97±59.97 (6)	503.60±96.30 (7)*	420.37±66.24 (4)

Values are expressed as mean±S.D., with the number of animals indicated in parenthesis; *significantly different from control group; $P<.05$.

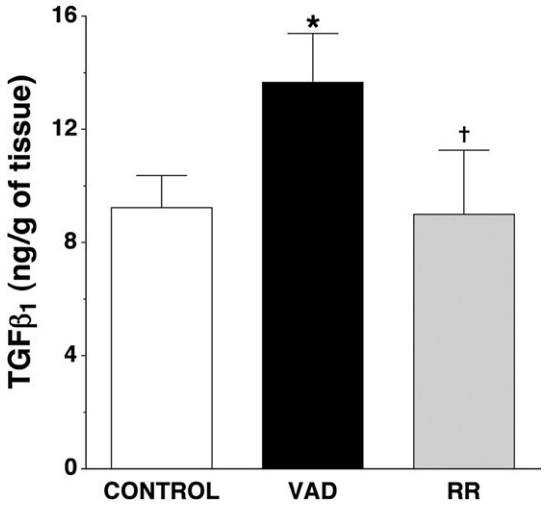

Fig. 6. TGF-β1 content in the lungs of control, VAD and RR rats. Pieces of lung tissue were homogenized in Triton X-100 with protease inhibitors, and active TGF-β1 was determined in the solubilized material by enzyme-linked immunosorbent assay. Values are presented as mean±S.D.; *significantly different from control group; †significantly different from VAD group; $P<.01$.

promoter as well as to elements located downstream [45,46]. Therefore, it is also possible that there is a direct implication of retinoic acid in the expression of collagen IV.

With the exception of ABM thickness, treatment with retinoic acid for 10 days restores the alterations induced by VAD to values not significantly different from those of controls. ABM thickness recovers only partially in spite of complete restoration of collagen IV content and disappearance of collagen I fibers from the BM. This fact suggests that other VAD-modified BM components are not recuperated by retinoic acid.

Remarkably, the correcting effect of retinoic acid occurs even in the presence of increased oxidative stress. As compared with the untreated ones, we have found in the RR lungs duplication in their MDA content indicative of increased oxidative stress. The MDA increment induced by retinoic acid seems to be a surprising result in the light of the antioxidant function shown for retinoids [47]. However, many studies in cultured cells have already described the pro-oxidant action of retinoic acid and other vitamin A derivatives [48–50]. In this sense and in agreement with our result, it has been published recently that retinoic acid induces a pro-oxidant condition in cultured Sertoli cells, leading to increased lipid peroxidation, DNA oxidative damage and cell death [50,51]. These effects occur at concentrations higher than 1 μM and appear to be mediated by the generation of reactive oxygen species [50]. The pharmacokinetics in serum and brain of intraperitoneal injections of rats with retinoic acid [52] suggest that micromolar concentrations could be achieved in the RR lungs with the retinoic acid treatment we have used. The amount injected of retinoic acid (0.4 mg/kg daily for 10 days) is similar to that used by others (0.5 mg/kg daily for 12 days) to reverse the anatomical characteristics of elastase-induced emphysema in rats and to induce septation in a rat model of pharmacologically caused failure of septation [4].

The mechanism of lipid peroxidation induced by retinoic acid is not fully clarified yet. However, based on published works several possibilities exist. Superoxide and carbon-centered radicals could be generated by autoxidation of retinoic acid, similarly to what has been observed with retinol and retinal [48]. The autoxidation could be enhanced in the lung due to its high pO_2 [53]. Additionally, retinoic acid could stimulate the VAD-initiated autoxidation of unsaturated fatty acids [54] whose concentration, in turn, could be increased due to the activation by retinoic acid of Δ^6-desaturase [55]. Finally, retinoic acid or its oxidative-cleavage products could inhibit oxidative phosphorylation leading to enhanced formation of reactive oxygen species [56]. All these mechanisms could act concertedly.

It is worthy to consider here that reactive oxygen derivatives, acting as secondary intracellular messengers, have been shown to activate transcription factors such as activated protein-1 and to induce the synthesis of the fibrogenic cytokine TGF-β1 and of various ECM proteins. H_2O_2 increases by twofold the synthesis of collagens I, III and IV in human mesangial cells through activation of the TGF-β1 system [25]. In consequence, the ability of retinoic acid to reestablish the control values for the VAD-modified ECM proteins in the presence of elevated oxidative stress indicates that retinoic acid mediates this effect through its action on nuclear receptors or another unknown mechanism and not by acting as an antioxidant.

In brief and based on our and others' results, we suggest the following events as a possible mechanism. The oxidative stress induced by VAD triggers the production of TGF-β1, probably by activated leucocytes and mediated by activated/injured epithelial/endothelial cells [25,42]. TGF-β1 in paracrine action induces the expression of type I and type IV collagens in fibroblasts and epithelial–endothelial cells [25,38,39]. This effect could be enhanced by the absence of retinoic acid. When administered, retinoic acid counteracts the action of TGF-β1 by two ways, repressing its synthesis [40] and interfering with the TGF-β/Smad signaling pathway. Retinoic acid receptors would function as activators of the TGF-β/Smad pathway in VAD lungs and as inhibitors in retinoic acid-treated ones [41]. Retinoic acid could also directly modulate the expression of the ECM proteins [43–46].

To conclude, we provide novel insights on how chronic VAD during the growing period alters the ECM and how retinoic acid administration reverses the alterations. Specifically, this work demonstrates that VAD results in abnormalities of the lung ECM such as duplication of the ABM thickness, similar increments in the total amount of collagens I and IV, and appearance of collagen fibers in the ABM. These changes are accompanied by increases in MPO activity, active TGF-β1 content and MDA production. Treatment with retinoic acid restores the alterations to the control values except for

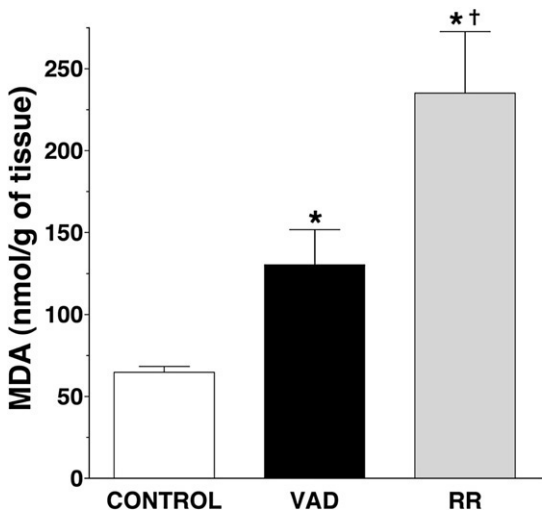

Fig. 7. Effect of VAD and retinoic acid treatment on oxidative stress in rat lung. Control, VAD and RR lungs were assayed for lipid peroxidation as a measure of tissue oxidative stress. Tissue homogenates were incubated with thiobarbituric acid, and the adduct of thiobarbituric acid with MDA was quantified by HPLC. The amount of MDA was considered as an estimation of lipid peroxidation. Data expressed as mean±S.D.; *significantly different from control group; †significantly different from VAD group; $P<.001$.

the BM thickness, which recovers only partially, and for the MDA, which increases even further. The VAD-induced abnormalities of the lung ECM are a common finding in patients with fibrotic diseases [35,36] suggesting them as a possible early pathogenic step and a contributing factor in the development of these diseases. Our results highlight the ability of retinoic acid to correct alterations of the ECM even though there is a high level of oxidative stress. However, it must be considered also that treatment with moderate amounts of retinoic acid, at least in VAD lungs, increases oxidative stress which could lead to unwanted deleterious effects.

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References

[1] Biesalski HK, Nohr D. Importance of vitamin-A for lung function and development. Mol Aspects Med 2003;24:431–40.
[2] Chytil F. Retinoids in lung development. FASEB J 1996;10:986–92.
[3] Massaro D, Massaro GD. Pulmonary alveoli: formation, "the call for oxygen", and other regulators. Am J Physiol Lung Cell Mol Physiol 2002;282:L345–58.
[4] Massaro GD, Massaro D. Retinoic acid treatment partially rescues failed septation in rats and mice. Am J Physiol Lung Cell Mol Physiol 2000;278:L955–60.
[5] Veness-Meehan KA, Bottone F, Stiles A. Effects of retinoic acid on airspace development and lung collagen in hyperoxia-exposed newborn rats. Pediatr Res 2000;48:434–44.
[6] Veness-Mehan KA, Pierce RA, Moats-Staats BM, Stiles AD. Retinoic acid attenuates O_2-induced inhibition of lung septation. Am J Physiol Lung Cell Mol Physiol 2002;283:L971–80.
[7] Shenai JP, Kennedy KA, Chytil F, Stahlman MT. Clinical trial of vitamin A supplementation in infants susceptible to bronchopulmonary dysplasia. J Pediatr 1987;111:269–77.
[8] Aird FK, Greene SA, Ogston SA, Macdonald TM, Mukhopadhyay S. Vitamin A and lung function in CF. J Cyst Fibros 2006;5:129–31.
[9] Baybutt RC, Molteni A. Vitamin A and emphysema. Vitam Horm 2007;75: 385–401.
[10] Freyer AM, Johnson SR, Hall IP. Effects of growth factors and extracellular matrix on survival of human airway smooth muscle cells. Am J Respir Cell Mol Biol 2001;25:569–76.
[11] Nguyen NM, Senior RM. Laminin isoforms and lung development: all isoforms are not equal. Dev Biol 2006;294:271–9.
[12] Carlson EC, Audette JL, Veitenheimer NJ, Risan JA, Laturnus DI, Epstein PN. Ultrastructural morphometry of capillary basement membrane thickness in normal and transgenic diabetic mice. Anat Rec Part A 2003;271A:332–41.
[13] Shahana S, Björnsson E, Lúdviksdóttir D, Janson C, Nettelbladt O, Venge P, et al. Ultrastructure of bronchial biopsies from patients with allergic and non-allergic asthma. Respir Med 2005;99:429–43.
[14] Kranenburg AR, Willems-Widyastuti A, Moori WJ, Sterk PJ, Alagappan VK, de Boer WI, et al. Enhanced bronchial expression of extracellular matrix proteins in chronic obstructive pulmonary disease. Am J Clin Pathol 2006;126:725–35.
[15] Matsui T. Differential activation of the murine laminin B1 gene promoter by RARα, RORα, and AP-1. Biochem Biophys Res Commun 1996;220:405–10.
[16] Salbert G, Fanjul A, Piedrafita FJ, Lu XP, Kim SJ, Tran P, et al. Retinoic acid receptors and retinoid X receptor-α down-regulate the transforming growth factor-β1 promoter by antagonizing AP-1 activity. Mol Endocrinol 1993;7: 1347–56.
[17] McGowan SE, Smith J, Holmes AJ, Smith LA, Businga TR, Madsen MT, et al. Vitamin A deficiency promotes bronchial hypereactivity in rats by altering muscarinic M_2 receptor function. Am J Physiol Lung Cell Mol Physiol 2002;282:L1031–9.
[18] Baybutt RC, Hu L, Molteni A. Vitamin A deficiency injures lung and liver parenchyma and impairs function of rat type II pneumocytes. J Nutr 2000;130: 1159–65.
[19] McGowan SE, Holmes AJ. Vitamin A deficiency alters pulmonary parenchymal collagen and tissue mechanics. Respir Physiol Neurobiol 2007;156:312–9.
[20] Olsen CO, Isakson BE, Seedorf GJ, Lubman RL, Boitano S. Extracellular matrix-driven alveolar epithelial cell differentiation in vitro. Exp Lung Res 2005;31: 461–82.
[21] Guo Y, Martinez-Williams C, Yellowley CE, Donahue HJ, Rannels DE. Connexin expression by alveolar epithelial cells is regulated by extracellular matrix. Am J Physiol Lung Cell Mol Physiol 2001;280:L191–202.
[22] Lima e Silva R, Kachi S, Akiyama H, Shen J, Aslam S, Yuan Gong Y, et al. Recombinant non-collagenous domain of α2(IV) collagen causes involution of choroidal neovascularization by inducing apoptosis. J Cell Physiol 2006;208: 161–6.
[23] Barber T, Borras E, Torres L, García C, Cabezuelo F, Lloret A, et al. Vitamin A deficiency causes oxidative damage to liver mitochondria in rats. Free Radic Biol Med 2000;29:1–7.
[24] Gatica L, Alvarez S, Gomez N, Zago MP, Oteiza P, Oliveros L, et al. Vitamin A deficiency induces prooxidant environment and inflammation in rat aorta. Free Radic Res 2005;39:621–8.
[25] Iglesias-De la Cruz MC, Ruiz-Torres P, Alcamí J, Diez-Marqués L, Ortega Velázquez R, Chen S, et al. Hydrogen peroxide increases extracellular matrix mRNA through TGF-β in human mesangial cells. Kidney Int 2001;59:87–95.
[26] Marín MP, Esteban-Pretel G, Alonso R, Sado Y, Barber T, Renau-Piqueras J, et al. Vitamin A deficiency alters the structure and collagen IV composition of rat renal basement membranes. J Nutr 2005;135:695–701.
[27] Renau-Piqueras J, Zaragozá R, De Paz P, Báguena-Cervellera R, Megías L, Guerri C. Effects of prolonged ethanol exposure on the glial fibrillary acidic protein-containing intermediate filaments of astrocytes in primary culture: a quantitative immunofluorescence and immunogold electron microscopic study. J Histochem Cytochem 1989;37:229–40.
[28] Podda M, Weber C, Traber MG, Milbradt R, Packer L. Sensitive high performance liquid chromatography techniques for simultaneous determination of tocopherols, tocotrienols, ubiquinols and ubiquinones in biological samples. Methods Enzymol 1999;299:330–41.
[29] Arnaud J, Fortis I, Blachier S, Kia D, Favier A. Simultaneous determination of retinol, α-tocopherol and β-carotene in serum by isocratic high-performance liquid chromatography. J Chromatogr 1991;572:103–16.
[30] Dewald B, Baggiolini M. Methods for assessing exocytosis by neutrophil leukocytes. Methods Enzymol 1986;132:267–77.
[31] Wong SH, Knight JA, Hopfer SM, Zaharia O, Leach Jr CN, Sunderman Jr FW. Lipoperoxides in plasma as measured by liquid-chromatographic separation of malondialdehyde-thiobarbituric acid adduct. Clin Chem 1987;33:214–20.
[32] Mansego ML, Blesa S, González-Albert V, Tormos MC, Saez G, Redón J, et al. Discordant response of glutathion and thioredoxin systems in human hypertension? Antioxid Redox Signal 2007;9:507–14.
[33] Weynand B, Jonckheere A, Frans A, Rahier J. Diabetes mellitus induces thickening of the pulmonary basal lamina. Respiration 1999;66:14–9.
[34] Guazzi M, Oreglia I, Guazzi MD. Insulin improves alveolar-capillary membrane gas conductance in type 2 diabetes. Diabetes Care 2002;25:1802–6.
[35] Magro CM, Waldman WJ, Knight DA, Allen JN, Nadasky T, Frambach GE, et al. Idiopathic pulmonary fibrosis related to endothelial injury and antiendothelial cell antibodies. Hum Immunol 2006;67:284–97.
[36] Magro CM, Shusterman BD, Pope-Harman AL, Ross Jr P. Ultrastructure as a diagnostic adjunct in the evaluation of lung allograft biopsies. Ultrastruct Pathol 2005;29:95–106.
[37] Jacobi J, Kristal B, Chezar J, Shaul SM, Sela S. Exogenous superoxide mediates pro-oxidative, proinflammatory, and procoagulatory changes in primary endothelial cell cultures. Free Radic Biol Med 2005;39:1238–48.
[38] Douthwaite JA, Johnson TS, Haylor JL, Watson P, El Nahas AM. Effects of transforming growth factor-β1 on renal extracellular matrix components and their regulating proteins. J Am Soc Nephrol 1999;10:2109–19.
[39] Fine A, Goldstein RH. The effect of transforming growth factor-β on cell proliferation and collagen formation by lung fibroblasts. J Biol Chem 1987;262: 3897–902.
[40] Morath C, Dechow C, Lehrke I, Haxsen V, Waldherr R, Floege J, et al. Effects of retinoids on the TGF-β system and extracellular matrix in experimental glomerulonephritis. J Am Soc Nephrol 2001;12:2300–9.
[41] Pendaries V, Verrecchia F, Michel S, Mauviel A. Retinoic acid receptors interfere with the TGF-β/Smad signaling pathway in a ligand-specific manner. Oncogene 2003;22:8212–20.
[42] Broekelmann TJ, Limper AH, Colby TV, McDonald JA. Transforming growth factor β1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. Proc Natl Acad Sci U S A 1991;88:6642–6.
[43] Wang L, Tankersley LR, Tang M, Potter JJ, Mezey E. Regulation of the murine α2(I) collagen promoter by retinoic acid and retinoid X receptors. Arch Biochem Biophys 2002;401:262–70.
[44] Krupsky M, Fine A, Berk JL, Goldstein RH. Retinoic acid-induced inhibition of type I collagen gene expression by human lung fibroblasts. Biochim Biophys Acta 1994;1219:335–41.
[45] Pollner R, Schmidt C, Fisher G, Kühn K, Pöschl E. Cooperative and competitive interaction of regulatory elements are involved in the control of divergent transcription of human COL4A1 and COL4A2 genes. FEBS Lett 1997;405:31–6.
[46] Shimada J, Suzuki Y, Kim SJ, Wang PC, Matsumura M, Kojima S. Transactivation via RAR/RXR-Sp1 interaction: characterization of binding between Sp1and GC box motif. Mol Endocrinol 2001;15:1677–92.
[47] Palace VP, Khaper N, Qin Q, Singal PK. Antioxidant potentials of vitamin A and carotenoids and their relevance to heart disease. Free Rad Biol Med 1999;26: 746–61.
[48] Murata M, Kawanishi S. Oxidative DNA damage by vitamin A ant its derivative via superoxide generation. J Biol Chem 2000;275:2003–8.
[49] Gimeno A, Zaragozá R, Vivó-Sesé I, Viña JR, Miralles VJ. Retinol, at concentrations greater than the physiological limit, induces oxidative stress and apoptosis in human dermal fibroblasts. Exp Dermatol 2004;13:45–54.
[50] Conte da Frota Jr ML, Gomes da Silva E, Behr GA, Roberto de Oliveira M, Dal-Pizzol F, Klamt F, et al. All-*trans* retinoic acid induces free radical generation and modulates antioxidant enzyme activities in rat Sertoli cells. Mol Cell Biochem 2006;285:173–9.

[51] Pasquali MAB, Gelain DP, Zanotto-Filho A, de Souza LF, de Oliveira RB, Klamt F, et al. Retinol and retinoic acid modulate catalase activity in Sertoli cells by distinct and gene expression-independent mechanisms. Toxicol in Vitro 2008;22: 1177–83.
[52] Le Doze F, Debruyne D, Albessard F, Barre L, Defer GL. Pharmacokinetics of all-*trans* retinoic acid, 13-*cis* retinoic acid, and fenretinide in plasma and brain of rat. Drug Metab Dispos 2000;28:205–8.
[53] Palozza P, Luberto C, Calviello G, Ricci P, Bartoli GM. Antioxidant and prooxidant role of β-carotene in murine normal and tumor thymocytes: effects of oxygen partial pressure. Free Radic Biol Med 1997;22:1065–73.
[54] Freyaldenhoven MA, Lehman PA, Franz TJ, Lloyd RV, Samokyszyn VM. Retinoic acid-dependent stimulation of 2,2′-azobis(2-amidinopropane)-initiated autoxidation of linoleic acid in sodium dodecyl sulfate micelles: a novel prooxidant effect of retinoic acid. Chem Res Toxicol 1998;11:102–10.
[55] Alam SQ, Alam BS, Chen TW. Activities of fatty acid desaturases and fatty acid composition of liver microsomes in rats fed β-carotene and 13-*cis* retinoic acid. Biochim Biophys Acta 1984;792:110–7.
[56] Siems W, Sommerburg O, Schild L, Augustin W, Langhans CD, Wiswedel I. β-Carotene cleavage products induce oxidative stress in vitro by impairing mitochondrial respiration. FASEB J 2002;16:1289–91.