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

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Received 18 September 2008; received in revised form 28 November 2008; accepted 4 December 2008

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...
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 NH4Cl, 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 isotopic 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 (Biokine 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% H2O2, and the absorbance change at 450 (ΔA450) read continuously over 3 min at room temperature. The assay was also performed in the presence of 40 μM NaN3, Myeloperoxidase activity was calculated as NaN3-inhibitable peroxidase activity and expressed in arbitrary units, where an arbitrary unit is defined as the amount of enzyme which produces a ΔA450 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 (Merek) 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 0.5 mg/ml of bacterial collagenase (high-purity collagenase, type VII Sigma) at 37°C for 48 h in digestion buffer consisting of 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% H2O2, and the absorbance change at 450 (ΔA450) measured continuously over 3 min at room temperature. The assay was also performed in the presence of 40 μM NaN3, Myeloperoxidase activity was calculated as NaN3-inhibitable peroxidase activity and expressed in arbitrary units, where an arbitrary unit is defined as the amount of enzyme which produces a ΔA450 of 0.001/min.

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(V) 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 scanner 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 retinoid 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'-ATGCGGTCCCTGTATCTGCT-3', antisense: 5'-CTCTTGGGACACCTAGTTCC-3'; COL1A2 sense: 5'-GAAAGATGTCACCCTGGAAAC-3', antisense: 5'-CAGGCTCCTCTTCAATCCATC-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 R² 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 technique as described [27]. Briefly, the ultrathin sections were initially incubated in 3% H2O2 for 10 min. Then, after blocking and incubating with specific anti-collagen I primary antibody (Calbiochem) and gold-conjugated secondary 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%.

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.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Concentrations of retinol and retinol palmitate in plasma and lungs of control, VAD and RR rats</th>
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<tr>
<td></td>
<td>Control</td>
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<tr>
<td>Plasma (µM)</td>
<td></td>
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<tr>
<td>All-trans retinol</td>
<td>1.73±0.01 (6)</td>
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<tr>
<td>All-trans retinol palmitate</td>
<td>ND</td>
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<tr>
<td>Lung (nmol/g of tissue)</td>
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<tr>
<td>All-trans retinol</td>
<td>1.13±0.01 (6)</td>
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<tr>
<td>All-trans retinol palmitate</td>
<td>2.87±0.59 (6)</td>
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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
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 \( \alpha(1) \) chains that form the molecule of collagen I using real-time RT-PCR. Each mRNA was quantified and normalized to the quantity of \( \beta2 \) microglobulin mRNA. As shown in Fig. 5, in close
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 \( \alpha_1(1) \) chain and by 75% for the \( \alpha_2(1) \) 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 \( \alpha_1(1) \) chain gene transcription or mRNA stability or both. As would be expected from the chain composition of collagen I, the \( \alpha_1(1) \) mRNA to \( \alpha_2(1) \) 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 \( \alpha(1) \) 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-1\( \alpha \), IL-1\( \beta \), and TNF-\( \alpha \), 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-\( \beta 1 \)

TGF-\( \beta 1 \) 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-\( \beta 1 \) content in the lung. VAD produced a 50% increase in the lung content of active TGF-\( \beta 1 \) (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-\( \beta 1 \) 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-\( \beta 1 \) 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 mmol/g) compared to controls (65±4 mmol/g) and after retinoic acid treatment MDA increased even further (235±38 mmol/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-
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
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.
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 pO2 [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 Δ6-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 concretely.

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. H2O2 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-β1-Smad signaling pathway. Retinoic acid receptors would function as activators of the TGF-β1/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

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**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 control group; P<.01.

**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 [33,36] suggesting them as a possible early pathogenic step and a contributor 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.

Acknowledgments

The authors thank Dra. María Gabaldón (Centro de Investigación, Hospital “La Fe”, Valencia) for her kind and skillful help in performing the whole animal perfusion and fixation.

References


