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Vitamin A deficiency disturbs collagen IV and laminin composition and decreases matrix metalloproteinase concentrations in rat lung. Partial reversibility by retinoic acid☆

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Abstract

Vitamin A is essential for lung development and pulmonary cell differentiation. Its deficiency leads to altered lung structure and function and to basement membrane architecture and composition disturbances. Previously, we showed that lack of retinoids thickens the alveolar basement membrane and increases collagen IV, which are reversed by retinoic acid, the main biologically active vitamin A form. This study analyzed how vitamin A deficiency affects the subunit composition of collagen IV and laminin of lung basement membranes and pulmonary matrix metalloproteinase content, plus the recovering effect of all-*trans*-retinoic acid. Male weanling pups were fed a retinol-adequate/-deficient diet until 60 days old. A subgroup of vitamin-A-deficient pups received daily intraperitoneal all-*trans*-retinoic acid injections for 10 days. Collagen IV and laminin chain composition were modified in vitamin-A-deficient rats. The protein and mRNA contents of chains α1(IV), α3(IV) and α4(IV) increased; those of chains α2(IV) and α5(IV) remained unchanged; and the protein and mRNA contents of laminin chains α5, β1 and γ1 decreased. The mRNA of laminin chains α2 and α4 also decreased. Matrix metalloproteinases 2 and 9 decreased, but the tissue inhibitors of metalloproteinases 1 and 2 did not change. Treating vitamin-A-deficient rats with retinoic acid reversed all alterations, but laminin chains α2, α4 and α5 and matrix metalloproteinase 2 remained low. In conclusion, vitamin A deficiency alters the subunit composition of collagen IV and laminin and the lung's proteolytic potential, which are partly reverted by retinoic acid. These alterations could contribute to impaired lung function and predispose to pulmonary disease.

Keywords: Vitamin A; Retinoic acid; Basement membrane; Collagen IV; Laminin; Matrix metalloproteinase

1. Introduction

Vitamin A and its natural bioactive derivatives, the most potent of which is all-*trans*-retinoic acid (RA), are necessary for normal mammalian foetal development and play an important role in cell proliferation, differentiation, survival and death [1–3]. In the lung, they are also involved in alveolar formation during the neonatal period and are required for alveolar architecture maintenance after the alveoli have been formed [4–6]. Consequently, vitamin A deficiency (VAD) results in congenital malformations and many significant health outcomes. At the molecular level, RA exerts its functions largely by regulating the gene expression through specific interactions with nuclear receptors which are ligand-activated transcription factors. Among the proteins whose synthesis is influenced by RA, we find the macromolecules of the extracellular matrix (ECM), including those of the basement membrane (BM) [7].

Basement membranes are specialized laminar structures of the ECM which several cell types come into contact with. They comprise large multidomain glycoproteins from different protein families, of which collagen IV, laminin, nidogen/entactin and perlecan are major components [8]. Both collagen IV and laminins are capable of polymerization and of producing lattice-type supramolecular networks. These networks interact directly or indirectly through other BM macromolecules, such as nidogen or perlecan, to provide the BMs' structural scaffold. In mammals, collagen IV is a triple-helical molecule resulting from the association of six genetically distinct α chains encoded by genes arranged in three pairs with a head-to-head orientation, a unique feature not shared by other collagen families. In spite of the many potential combinations, only three specific heterotrimers, α1α2α1(IV), α3α4α5(IV) and α5α6α5(IV), have so

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far been found in all tissues studied [9]. Laminins are also heterotrimeric glycoproteins composed of one α, one β and one γ chain for which there are currently five α, three β and three γ chain genes described in mammals. Once again, the number of possible combinations exceeds the number of laminins identified since only 18 different heterotrimers of the possible 45 have been described [10]. The isoforms of both macromolecules are distributed in a developmental and tissue-specific pattern within an organism. In a normal human lung, the α1(IV) and α2(IV) collagen chains are expressed in all BMs, whereas the others show a restricted expression. The α3(IV) and α4(IV) chains have been detected in alveolar BM but not in that of the capillary, bronchial epithelium or smooth muscle cells; the α5(IV) chain has been reported in the BMs of alveoli, bronchial epithelium and smooth muscle cells but not in capillary BM; and the α6(IV) chain has been detected in BMs of the bronchial epithelium and smooth muscle cells but not in the BMs of alveoli and capillaries [11]. Regarding the laminins in the mouse lung, the α1 chain expression is greater during lung morphogenesis, whereas the α2 chain remains in the adult lung and appears in the smooth muscle cells of terminal bronchioles and alveoli, veins and arteries. Both laminin chains are present in the BM between epithelial and endothelial cells of the blood–air barrier [12]. Similarly, adult rat alveolar epithelial cells express laminin α3 and α5, whereas adult lung fibroblasts express laminin α4 [13]. Cell surface receptors, including integrins and nonintegrin molecules, recognise particular sequences in the laminin and collagen IV chains which confer specificity to the cell–macromolecule isoform interaction and selectively activate various signal transduction pathways [14]. Therefore, changes in cell receptors or in BM composition could alter cell behaviour and metabolism.

Several studies have shown that VAD results in alterations of both the lung architecture and function and can even lead to lung pathology in rats. For example, VAD animals exhibit sharper increases in airway resistance and lung elastance after exposure to aerosolized methacholine [15], develop emphysemic lungs [16] and present squamous cell metaplasia with a relative drop in the amount of mucous and ciliated cells in the tracheobronchial tree [17]. Moreover, surfactant synthesis and ornithine decarboxylase activity, the rate-limiting enzyme in polyamine synthesis, are significantly lower in type II pneumocytes isolated from VAD rats, indicating impaired functional and proliferative capacity. These alterations are associated with changes in ECM and BM protein content and distribution. It has been reported that VAD lungs contain less elastin, but more collagen, than controls. Collagen content is especially high in areas of interstitial pneumonitis but is low in small arteries and arterioles and remains at a normal level in peribronchial ECM [16]. In a recent work, we showed that collagens I and IV, the main interstitial and BM collagens, respectively, display an increased presence in the lungs of VAD rats and, in parallel, the alveolar BM doubles in thickness and appears to have an ectopic deposition of collagen I fibrils inside [18]. Treatment with RA restores, at least partially, both the functional lung properties and the ECM structure and composition, suggesting the implication of the ECM in lung function alterations [18,19].

Although some work has been done on the ECM composition and structure in VAD lung [15,16,18,19], very little is known about BMs' molecular composition. Therefore, by considering that BM molecules influence cell behaviour [9,20], its molecular composition is modified in many diseases [21–24] and its structure is altered in VAD [18], we analyzed the chain composition of collagen IV and laminin as well as the ECM degradation capacity in the lungs of VAD rats before and after RA treatment.

2. Methods and materials

2.1. Animal treatment

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

2.2. Tissue sample preparation

The blood and lung samples for biochemical studies were processed as previously described [25] and stored frozen until they were used.

2.3. Solubilization of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), noncollagenous domains of collagen IV [NC1(IV)] and laminin chains

Pieces of frozen lung (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 supernatant was stored at −80°C until use for MMP and TIMP determination. The processing of the pellets differed depending on whether NC1(IV) or laminin chains were solubilized.

To solubilize the NC1(IV) fragments, the pellet was digested extensively with 0.3 mg/ml of bacterial collagenase (high-purity collagenase, type VII, Sigma) at 37°C for 48 h in a digestion buffer consisting of 50 mM Hepes (pH 7.5), 10 mM $CaCl_2$, 0.05% NaN_3 and 5 μl/ml of the above-mentioned protease inhibitor cocktail. The suspension was centrifuged, and EDTA, at a final concentration of 25 mM, was added to the supernatant and stored at −80°C until used. Pellet digestion was repeated to confirm that no more NC1 collagen IV fragments were solubilized.

To solubilize laminin chains, the pellet was resuspended and sonicated in Tris–HCl 25 mM (pH 7.5) containing 4% sodium dodecyl sulfate (SDS) and 4% β-mercaptoethanol. It was then boiled for 10 min. The suspension was centrifuged, and the supernatant was stored at −80°C until use.

2.4. Protein quantification by Western blotting

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were carried out as previously described [25]. The proteins solubilized from equal lung weights were separated in either 4%–10% polyacrylamide gradient gels under reducing conditions (laminins) or 4%–20% polyacrylamide gradient gels under nonreducing conditions (collagen IV α-chains and TIMPs) and were transferred electrophoretically to polyvinylidene fluoride paper at 150 mA constant current for 18 h at 4°C. Specific rabbit polyclonal antibodies against laminin 1 (Sigma; diluted 1/4000), laminin α5 (Santa Cruz; diluted 1/200), TIMP1 and TIMP2 (Santa Cruz; diluted 1/100) and mouse monoclonal antibodies (H11, H22, H31, H44, H52, H65; diluted 1/100) against all the six human (IV) chains, prepared as described elsewhere [26], were used for immunodetection purposes. Anti-rabbit or anti-mouse IgG conjugated to alkaline phosphatase (Promega; diluted 1/7500) was used as the secondary antibody. After colour development, blots were scanned with an HP Scanjet 5400c, and protein bands were quantified using the ImageJ Programme. Linearity ranges were established by blotting different amounts of each sample. Control samples were included in each blot as a reference for the relative quantification of protein bands.

2.5. Gelatin zymography

The MMPs (MMP2 and 9) in the samples were analyzed by gelatin zymography. Samples were diluted with SDS-PAGE sample buffer containing 1% SDS and separated in 10% polyacrylamide gels with 1 mg/ml gelatin (type A 300 bloom, Sigma) at 130 V for 30 min followed by 170 V for 1.5 h at 4°C. After electrophoresis, gels were washed with Milli-Q water and incubated in 2.5% Triton X-100 for 15 min to remove SDS. Then, gels were equilibrated in 50 mM Tris–HCl (pH 7.5) and 2.5% Triton X-100 twice for 30 min with agitation. Finally, gels were incubated in 50 mM Tris–HCl (pH 7.5), 5 mM $CaCl_2$, 2 μM $ZnCl_2$ and 0.05% NaN_3 for 16 h at 37°C with agitation. They were stained for 2 h with 0.05% Coomassie Brilliant Blue R250, 10% acetic acid and 50% methanol in water and thereafter destained with 10% acetic acid and 25% methanol in water to reveal clear bands, which are indicative of gelatin digestion. Gels were scanned with an AGFA Snapscan 1236u, and bands were analyzed using the ImageJ Programme. Linearity ranges were established by loading different amounts of each sample. Control samples were included in each zymogram as a reference for the relative quantification of protein bands.

2.6. Total RNA extraction and quantitative polymerase chain reaction (PCR)

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 in Ref. [18]. Total RNA was isolated from lung samples by 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) and random primers. The primer sets from each candidate gene were designed by the Primer3 software and synthesized by Sigma (Table 1). Gene expression measurement was performed using the AB 7900HT Fast Real-Time PCR System and the SYBR Green Master Mix (Applied Biosystems) following the manufacturer's instructions. Standard curves were generated for each gene to determine PCR efficiency and the quantification of the copies of each mRNA in the samples. The linear regression analyses of the standard curves documented an R^2 value of 0.99 in all cases. Normalization of samples was performed by the reference gene, β_2-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.

2.7. Statistical analysis

Statistical analyses were performed using the 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 obtained from the three groups were analyzed by one-way analysis of variance followed by the Bonferroni test. Differences were considered significant when the P value was less than .05.

3. Results

3.1. Plasma and lung retinoids

As already reported [18,25], the retinol concentration in VAD rats plasma was lowered 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 both VAD and RR lungs were lowered by more than 95%.

3.2. Chain composition of collagen IV

The relative changes in chain composition of type IV collagen in the lungs of the control, VAD-treated and RA-treated animals were analyzed by Western blotting and are shown in Fig. 1. VAD increased the amount of chains α1, α3 and α4 in relation to controls (30%, 43% and 56%, respectively). No difference between the control and VAD groups was observed for chains α2 and α5. RA treatment returned the amounts of chains α1, α3 and α4 to values that were not significantly different from controls. The content of chains α2 and α5 was not modified by RA.

3.3. mRNA for collagen IV chains

The quantitative mRNA analysis of the different collagen IV chains was carried out by real-time reverse transcriptase (RT)-PCR and is shown in Fig. 2. As reported at the protein level, VAD increased the *COL4A1*, *COL4A3* and *COL4A4* mRNAs when compared to controls (70%, 70% and 100%, respectively). RA treatment reduced these levels to the control values. Neither VAD nor RA modified the amount of *COL4A2* and *COL4A5* mRNAs. The correspondence between the changes in protein and in their respective mRNA indicates that VAD and retinoic acid act on the collagen IV chain expression mainly at the mRNA level.

3.4. Laminin chain content

The amount of laminin α5, β1 and γ1 chains in the lungs of the control, VAD-treated and RA-treated animals was determined by Western blotting and is shown in Fig. 3. In contrast to the effect on collagen IV, VAD reduced chains α5 and γ1 and chain β1 by about 30% and 50%, respectively. The RA treatment on VAD animals returned the amounts of chains β1 and γ1 to the control values but did not affect chain α5.

3.5. mRNA for laminin chains

The mRNA for several laminin chains was quantified by real-time RT-PCR as shown in Fig. 4. As with collagen IV, the changes in laminin mRNA levels paralleled those in the protein content. VAD lowered the mRNAs from *LAMA5* and *LAMC1* genes by approximately 30% and the mRNA from *LAMB1* by around 50%. The mRNAs for the two other major laminin chains in the lung, α2 and α4, were also measured. The mRNA from *LAMA4* gene was reduced by 50% and that from *LAMA2* was reduced by 25% in VAD lungs. RA returned the mRNA for β1 and γ1 to values not significantly different from the controls, but did not modify the mRNA values for any of the α chains. As with the collagen IV chains, these results indicate that the effects of VAD and RA on laminin expression are mainly noted at the mRNA level.

3.6. MMP activity

Retinoids influence the expression of MMPs, which are active players in ECM degradation and remodelling. Therefore, the activities of MMP2 and MMP9 in lung extracts were measured by gelatin zymography. These results are shown in Fig. 5A and B. Compared to the control value, a significant drop in both MMP2 and MMP9 (30% and 40%, respectively) was observed in VAD lungs. Treatment of VAD animals with RA recovered the control values of MMP9, but not those of MMP2 (Fig. 5B). The activity of MMP2 in all the lung samples was greater than that of MMP9 (Fig. 5A).

3.7. Expression of TIMPs

The collagenolytic activity of an organ depends on the balance between its proteolytic activity and its concentration of protease inhibitors. Therefore, to further characterize the lung's collagenolytic

Table 1
Primer sequences used in quantitative RT-PCR

Gene name	Forward (5' to 3')	Reverse (5' to 3')	Construct length (bp)
COL4A1	CTCTGCTCTATGTCCAAGGAAACG	CAGACGTTGTTGATGTTGCAGAAG	118
COL4A2	CCGGATATGTACAAGGGTGAAAAG	GCTTCCTTTCTGTCCTGAGACTCC	153
COL4A3	GGTTTTCCCTTCTTTTCGTACAAGG	TGAATAATCATTTCGTGATGCAAAGTTAC	152
COL4A4	CCCCTGGTCACTGATATTTATCCTC	GTGACCCCGAGATCCCTTTTC	133
COL4A5	GTTTGCCTGGTGATCCTGGTTAC	GTTGCCCAATATTTCCTTTTGCTC	153
LAMA2	TCCAGACTGTCAACCCTGTAACTG	ACAGCCTTTCTGATTTTCCTCTTG	157
LAMA4	CAGAGCCATCACCTCGAAGC	TGCATTCTTTATTATCTCATCAAGTTCC	157
LAMA5	GACCTCTACTGCAAGCTGGTTGG	ATCGATGGCGTTGCTCACAG	132
LAMB1	CAGGAGCTCTTCTGGGGAGAC	AACACCCTCCACACAGACACAC	120
LAMG1	GAGAAGACAAGGGAGGCACAG	TGGTACTGGTGGCATTCTTCTG	124
B2MG	CAGTTCCACCCACCTCAGATAGA	ATTCAGTGTGAGCCAGGATGTAG	118

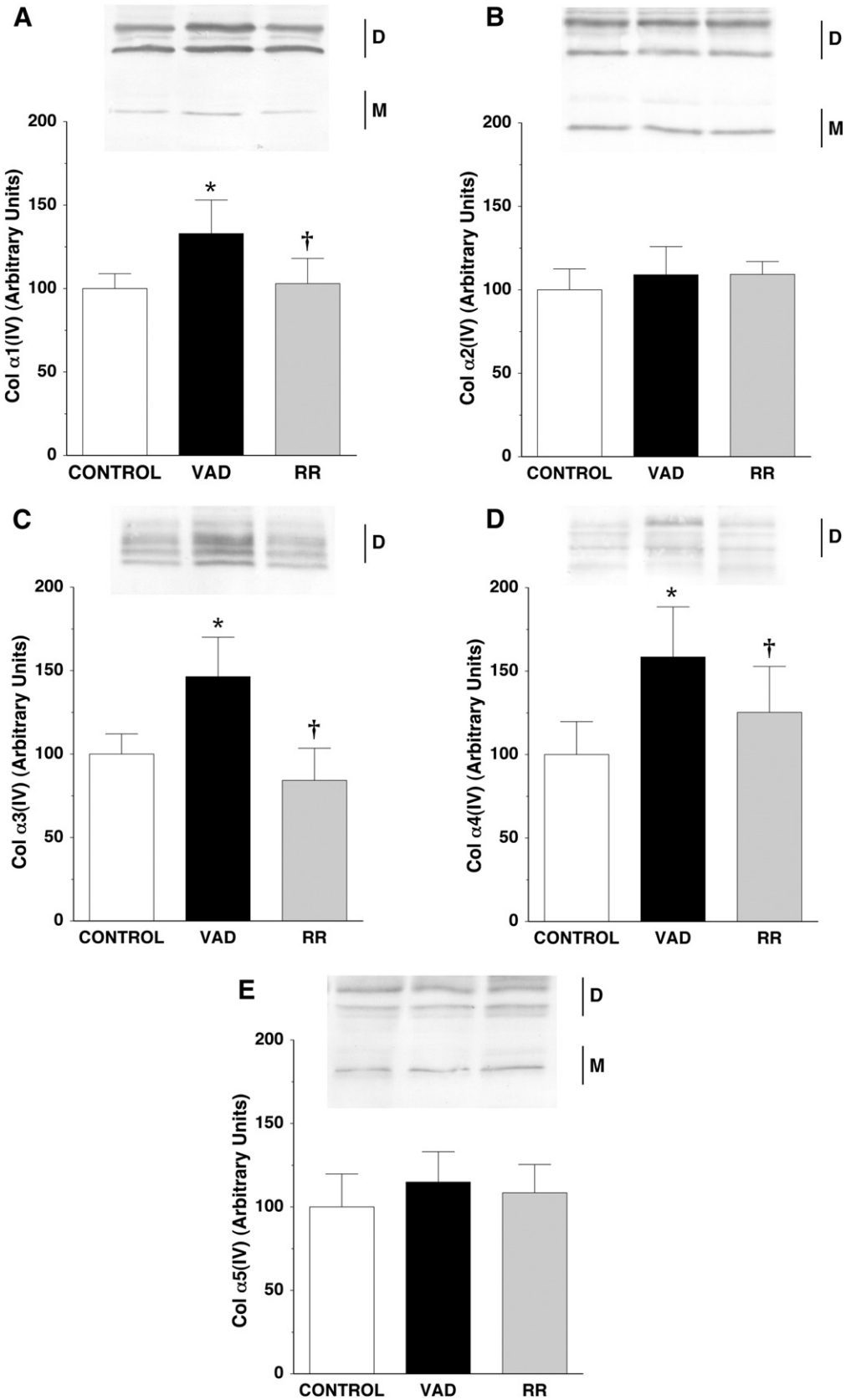

Fig. 1. Quantification of the collagen IV α chains in the lungs of control, VAD and RR rats. Collagenase-solubilized material from control, VAD and RR rat lungs was separated by SDS-PAGE and immunoblotted with specific monoclonal antibodies for each α(IV) chain. The monomeric and dimeric subunits of the NC1(IV) domains (M and D, respectively, in the corresponding insert) were scanned to evaluate the relative content of each α chain in the different lungs. Histograms represent the densitometric values as a percentage of the control group. Data are mean±S.D. *Significantly different from the control group; †significantly different from the VAD group; $P<.001$. Inserts show a representative Western blot of the quantified bands.

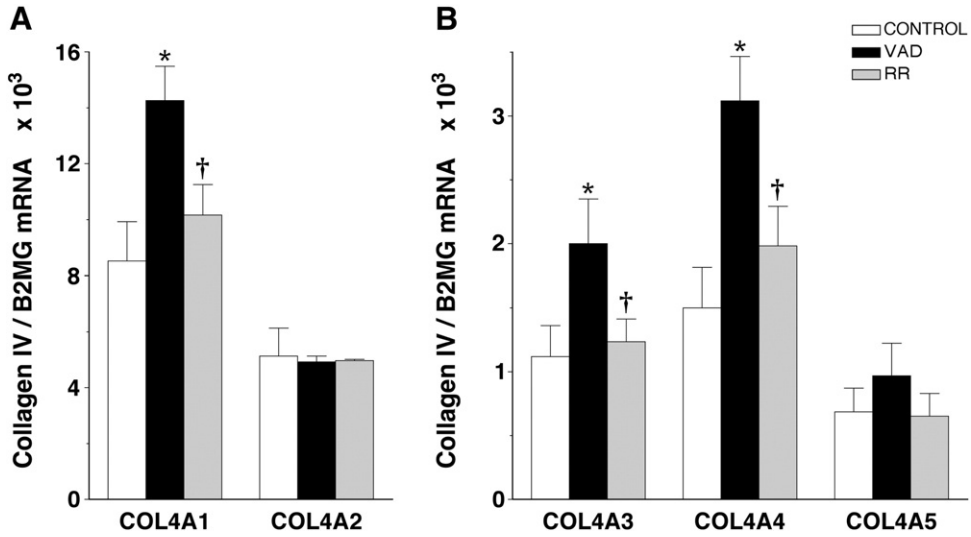

Fig. 2. Quantification of mRNA for each α(IV) chain in the lungs of control, VAD and RR rats. Total RNA was extracted from the lungs, and the mRNA for the α chains of collagen IV was quantified by real-time RT-PCR. (A) Relative mRNA content for chains α1(IV) and α2(IV). (B) Relative mRNA content for chains α3(IV), α4(IV) and α5(IV). The histograms show the number of mRNA molecules for each α chain normalized to the mRNA for β_2-microglobulin, determined identically in the same sample. Values are mean±S.D. *Significantly different from the control group; †significantly different from the VAD group; $P<.005$.

capacity, the expressions of TIMP1 and TIMP2, natural inhibitors of MMPs, were determined by Western blot (Fig. 6). Neither VAD nor RA treatment produced any significant change in the expression of these inhibitors.

4. Discussion

We have recently shown that retinoid deficiency results in the thickening of the alveolar BM and increases the lung content of type IV collagen [18]. These alterations are reverted by RA, except for BM thickness which does not recover completely. In the present work, we have studied whether the changes in collagen IV induced by VAD and RA treatment are chain specific and if laminins, another major BM component, are also modified. The ECM degrading capacity of the lung has been also analyzed.

In agreement with increased collagen IV content, its α chains also increased in VAD compared to the control lungs; however, this change is chain specific. An increase in chains α1, α3 and α4 was noted without the corresponding increment in chains α2 and α5. Chain-specific variations in the collagen IV expression have been also observed in the kidneys of animals with either VAD or experimentally induced renal diseases, such as chronic serum sickness nephritis [25,27]. However, the variations in the collagen IV expression differed between these three situations. The renal expression of all the collagen IV chains increased during the course of experimental nephritis, and the most significant was that of chains α1, α2 and α4 [27]. Conversely, in VAD kidneys, the expression of chains α1, α4 and α5 increased, whereas that of α2 and α3 decreased [25]. All these results suggest that variations in the collagen IV chain expression are both disease and tissue specific. From the studies conducted on the hexameric noncollagenous domains obtained by collagenase digestion of the BMs from normal tissues, only three collagen IV protomers with a different chain composition and tissue distribution have been identified: the ubiquitous α1α2α1 (IV) trimer and the more tissue-restricted α5α6α5(IV)and α3α4α5(IV) trimers [9]. Therefore, the uncoordinated change of the α(IV) chains in VAD BMs raises the following question: which partners in the assembly of the extra-chains form the triple-helical collagen IV protomer? In the case of the VAD lung, one possibility would be the formation of $[\alpha1(IV)]_3$ homotrimers and/or of unusual heterotrimers such as α3α4α1(IV). In the latter case, α5 was replaced with the α1 chain. These two chains are those that show the highest degree of identity in their noncollagenous domains which contain the recognition sequences for chain assembly specificity [28–31]. In fact, α1(IV) and α5(IV) chains have been suggested to associate in order to form collagen IV networks independently of the other four chains [31]. The existence of collagen IV protomers composed solely of α1(IV) chains has been reported in the culture medium of embryo-derived parietal yolk sac carcinoma cells [32]. However, other authors have not found any formation of α1(IV) homotrimers using Chinese hamster ovary cells transfected with full-length cDNA for the mouse α1(IV) chain [33]. Alternatively, or additionally, synthesized extra-chains could be secreted as monomers. The secretion of monomeric α1(IV) chains by cultured cells and their presence in the tissues *in vivo* have already been described [33,34]. Regardless of unusual protomers being formed or monomeric chains being secreted, the structure and composition of BMs could be disturbed, resulting in alterations to cell viability and behaviour, and finally in organ malfunction and damage.

Laminins form networks which, together with those of collagen IV, constitute the basic scaffold of BMs. Therefore, the alveolar BM thickening which we previously observed in VAD lungs [18] could also result from an increment in laminin content. However, unlike that which occurs with collagen IV, laminins decrease in VAD lungs. The protein levels of laminin α5, β1 and γ1 chains are significantly lower in VAD than in the control lungs. Additionally, the mRNA levels for chains α2 and α4 are also lower in VAD lungs, indicating that the corresponding proteins may decrease. As chains β1 or γ1 are shared by most laminin molecules, and α2, α4 and α5 are the major α chains in the adult rat lung, measured laminin chains may be considered representative of total laminin content. Therefore, our results indicate that the laminin network in the lung BMs of VAD animals is diminished. The fact that collagen IV increases but laminin decreases suggests that the previously observed thickening of the alveolar BM may be due, in part, to the disorganisation and loosening of collagen and laminin networks.

RA reverts all the modifications of the collagen IV chains induced by VAD in the lung to the control values. This result is in agreement with our previous finding of the reduction to control values of the elevated total collagen IV of VAD lungs by RA [18]. With laminins,

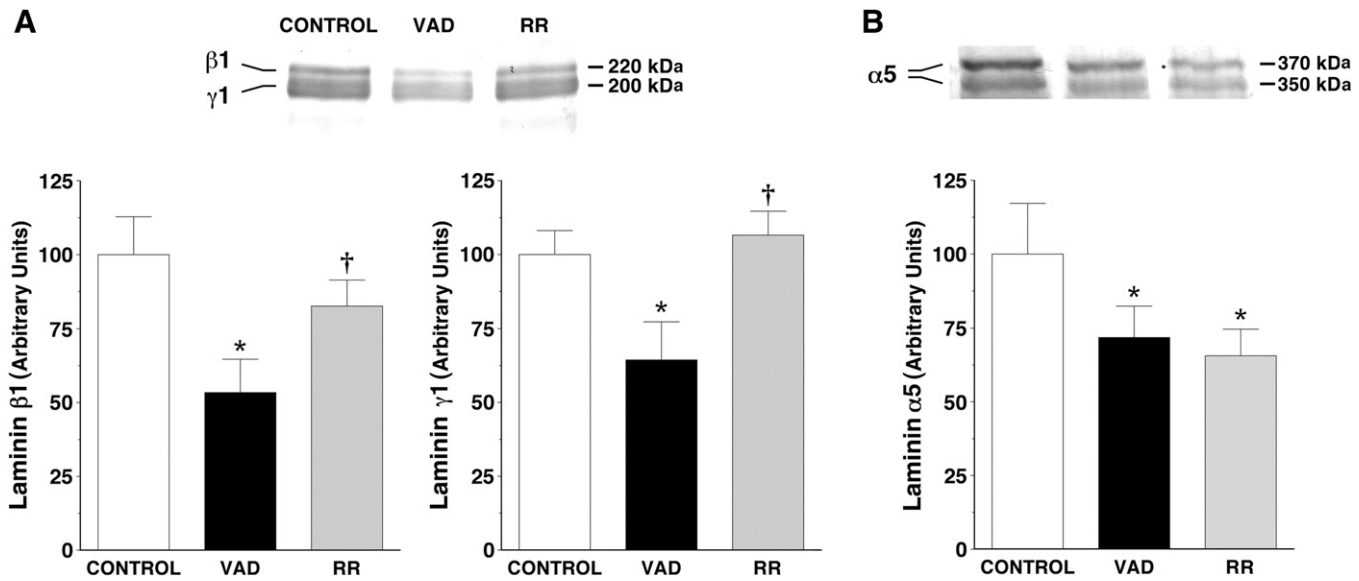

Fig. 3. Quantification of the laminin chains in the lungs of control, VAD and RR rats. Solubilized laminin chains from control, VAD and RR rat lungs were separated by SDS-PAGE and immunoblotted with specific polyclonal antibodies against (A) laminin-1 and (B) laminin α5 chain. The polyclonal antibodies against laminin-1 were used for the quantitation of the laminin β1 and γ1 chains. Two bands of 370 and 350 kDa, which both had the same distribution percentage between experimental groups, were observed with the anti-laminin α5 antibodies. The specific bands were scanned to evaluate the relative content of each chain in the different lungs. Histograms represent the densitometric values as a percentage of the control group. Data are mean±S.D. *Significantly different from the control group; †significantly different from the VAD group; $P<.001$. Insets show a representative Western blot of the quantified bands.

however, RA restores only the control values of chains β1 and γ1 but has no effect on either the protein or mRNA content of the α chains analyzed. This fact poses a question as to which α chain will be overexpressed to substitute the defective ones in RR lungs. Although our study does not provide the answer, there are two main possibilities. It is known that laminin α1 is expressed in murine lung in the early stages of organogenesis but is drastically down-regulated in later embryonic stages [35]. However, the resumed synthesis of chain α1 in mature organs has been described in the glomeruli of mice deficient in the α3(IV) collagen chain [36]. The renewed expression of laminin α1 associated with the decline in α3(IV) and α4(IV) collagen does not seem to occur in the RR lungs since we did not observe any laminin α1-reactive band by Western blot with anti-laminin1 antibodies (data not shown). The other possibility is the up-regulation of the laminin α3 chain expression. Laminin α3 mRNA has been described to be highly expressed in adult mouse lungs but is present at low levels in rat lungs [13]. However, freshly isolated, primary type I alveolar cells are able to assemble *in vitro* laminin-311-rich matrix fibres, which contain the laminin α3 chain [37]. An abnormal, ectopic deposition of this chain associated with the decrease in other ECM proteins has also been reported. For instance, the laminin α2 chain is normally expressed in the crypts of the small intestinal mucosa in control specimens but is absent in inflamed Crohn's disease specimens. This loss of the α2 expression is associated with an up-regulation of both the laminin α1 and α3 expression [21]. There is also a third possibility: that of the surplus β and γ chains forming dimers which either remain inside the cells or are secreted into the BM. Secretion of chains β3 and γ2, presumably as dimers, has already been reported in a human fibrosarcoma cell line which expressed these chains, but not the α3 chain [38]. Conversely, other authors using transfected embryonic kidney cells have found that the laminin α1 chain can be secreted as intact protein

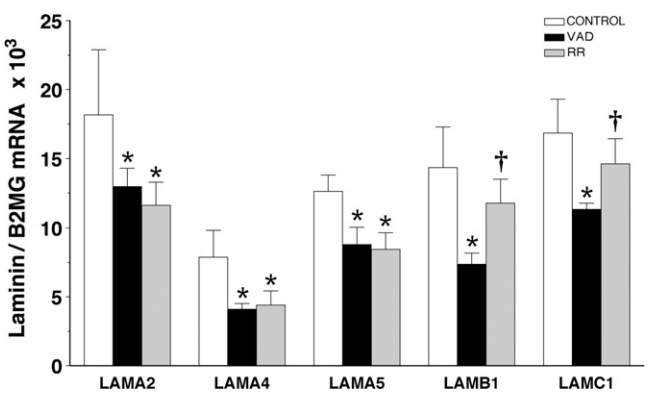

Fig. 4. Quantification of mRNA for the laminin chains in the lungs of control, VAD and RR rats. Total RNA was extracted from the lungs, and the mRNA for the laminin chains was quantified by real-time RT-PCR. The histograms show the number of mRNA molecules for each chain normalized to the mRNA for β_2-microglobulin, determined identically in the same sample. Values are mean±S.D. *Significantly different from the control group; †significantly different from the VAD group; $P<.005$.

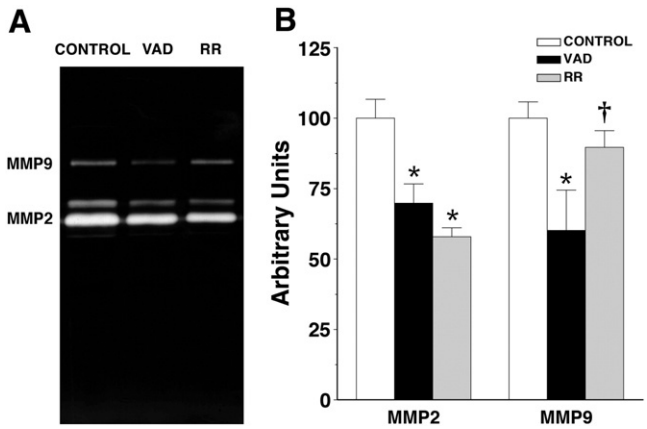

Fig. 5. Quantification of the MMPs in the lungs of control, VAD and RR rats. (A) MMP2 and MMP9 in lung extracts were analyzed by gelatin zymography. (B) The zymogram was negatively scanned, and the densitometric values were presented as a percentage of the control group. Data are mean±S.D. *Significantly different from the control group; †significantly different from the VAD group; $P<.005$.

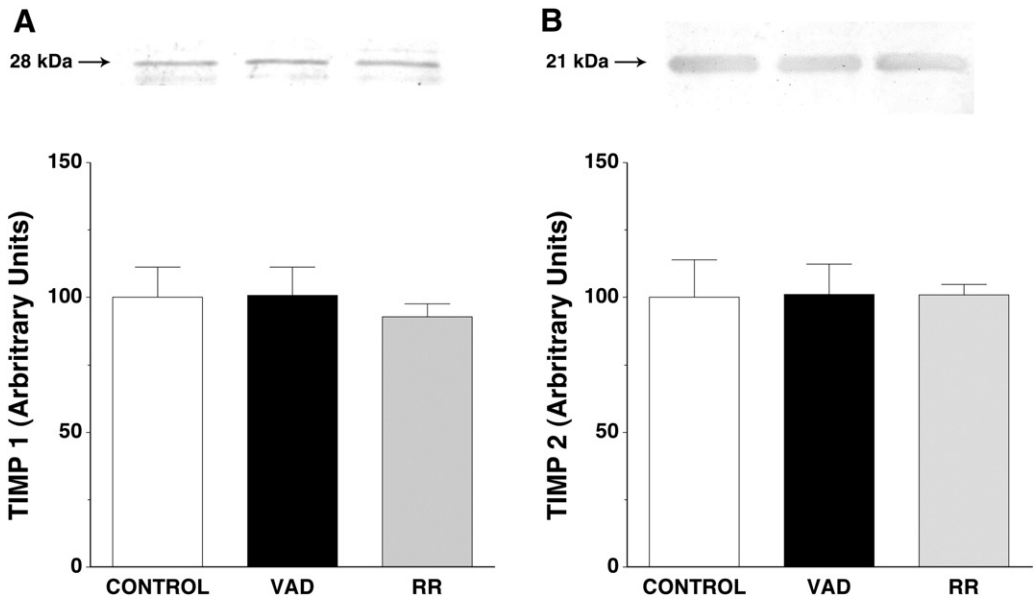

Fig. 6. Quantification of the TIMP1 and TIMP2 in the lungs of control, VAD and RR rats. Solubilized material from the control, VAD and RR rat lungs was separated by SDS-PAGE and immunoblotted with specific polyclonal antibodies for TIMPs. The bands corresponding to TIMP1 (A) and TIMP2 (B) were scanned, and the densitometric values were presented as a percentage of the control group. Data are mean±S.D. There were no statistically significant differences between groups. Insets show a representative Western blot of the quantified bands.

in the absence of its β and γ partners, whereas the β1 and γ1 chains remain as intracellular dimers in the absence of the α1 chain expression [39]. These results are indicative of a chain- and/or cell-type-specific laminin chain assembly and secretion. Whatever the possibility, it may contribute to BM alteration and contacting cell dysfunction.

All the modifications in the chains of both collagen IV and laminin correlated with those in their corresponding mRNA ($r=0.878$, $P=.001$ for collagen IV chains and $r=0.931$, $P=.007$ for laminin chains). This parallelism indicates that the expressions of collagen IV and laminin in both VAD and RR lungs are mainly controlled at the mRNA level. Moreover, we have already shown a similar correlation for collagens I and IV in the lung and kidney, respectively [18,25].

Proteolytic degradation of BM macromolecules may be a contributing factor to the modifications in its structure and composition. Therefore, we analyzed two MMPs that can degrade type IV collagen and laminin, MMP2 and MMP9, and two of their physiological inhibitors, TIMP1 and TIMP2. The absence of retinoids results in a decrease in the amount of both MMPs which, in the case of MMP9 but not in that of MMP2, returns to control values by RA administration. On the contrary, neither the absence of retinoids nor RA treatment produces any change in TIMP1 or TIMP2. A decrease in MMPs without a parallel reduction in TIMPs shifts VAD lungs towards a lower ECM proteolytic capacity, which may contribute to the observed accumulation of collagen IV. In an earlier study, we also showed a reduction in both MMP2 and MMP9 with no modification of TIMP1 or TIMP2 in VAD kidneys [25]. Therefore, lack of retinoids produces the same effect on MMPs and TIMPs in both kidneys and lungs. However, other authors in a recent report found no change in MMP2 and MMP9 activity in rat hearts with reduced vitamin A and retinoic acid concentrations [40]. Although their protocol to induce the vitamin A deficiency is very similar to ours, there are some differences which might account for discrepancy. On the one hand, these authors use female rats; on the other hand, their postweaning rats are fed a diet with a low vitamin A content which results in decreased retinoid concentration in the heart but the same serum retinol levels as controls. Alternatively, the response of the proteolytic status to VAD might be tissue specific. One fact favours this possibility: opposing results have been reported on the effect of RA in the expressions of MMP2 and 9 by different cell types in culture or tissues *in vivo* [41–44].

The mechanism by which retinoids produce changes in BM composition and ECM proteolytic capacity is complex and poorly understood. RA exerts its effects on the protein expression by binding to nuclear receptors, RARs and RXRs, which, as homo- or heterodimers, bind to specific DNA sequences (RARE) in gene promoters or interact with a plethora of transcription factors and other coactivator or corepressor proteins. A RARE has been identified in the promoter region of the murine *LAMB1* gene, and this response element is recognised by RARs [45]. In our study, the observation that the mRNA of the laminin γ1 chain decreases simultaneously with that of laminin β1 and MMP9 in VAD and increases in response to RA suggests a coordinated expression of the three proteins. No RARE has been reported in the promoter of the laminin γ1 gene. However, two enhancer elements, one of which has a number of near matches to RARE, have been found in the first intron of the gene in F9 embryonal carcinoma cells induced to differentiate and to synthesize laminin γ1 by exposure to RA and dibutyryl cAMP [46]. Binding of RARα together with the transcription coactivator P300 to the MMP9 gene promoter, despite the absence of a consensus RARE, has been also described in rat mammary glands after weaning and in murine dendritic myeloid cells after RA treatment [44,47]. Moreover, the rat laminin γ1 gene promoter contains motifs that recognise Sp1, a transcription factor which can interact physically with RARs and recruit them to the promoter [48,49]. Recruitment of RAR/RXR by GABPα and p300 at the CD 18 proximal promoter from a distal RARE to form an enhanceosome has been also shown in myeloid cells in the presence of RA [50]. Thus, regulation of the expressions of laminin chains β1 and γ1 and of MMP9 by either RA, via the direct RAR binding to the gene promoter RARE or the recruitment of RAR either by P300/CBP or another transcriptional coactivator, is consistent with our finding of their decline in VAD and their increase after RA administration. As regards collagen IV chains, there are few studies on their gene

promoters, and most of those available address the common bidirectional promoter for chains α1–α2. To our knowledge, no RARE has been described in this proximal bidirectional promoter, but it contains two GC box binding sites for Sp1 [51]. Sp1 has been reported to mediate the stimulatory effect of TGF-β1 on type IV collagen gene transcription and protein synthesis [52]. Furthermore, we have demonstrated that TGF-β1 is increased in VAD and returns to the control values with RA treatment [18]. Therefore, TGF-β1 might be a mediator of the effects of the absence or presence of RA on the α1(IV) chain expression, but this does not explain the uncoordinated expression of chain α2(IV). Nevertheless, other sequence elements have been described in the common promoter to have differential effects on the transcription of genes *COL4A1* and *COL4A2*, as well as additional regulatory elements that are located downstream of the transcriptional start sites of both genes and may be involved in their differential expression [53,54]. Whether the same or similar transcriptional regulatory elements are present in the other α(IV) genes remains to be analyzed. Despite the aforementioned studies, the mechanism by which RA exerts its effects on the expression of collagen IV and laminin chains remains largely unknown, and more studies are needed to clarify it satisfactorily.

In conclusion, our studies show that retinoid deficiency modifies the expression and chain composition of collagen IV and laminin, as well as the amount of MMPs. Most of these effects are due to deficiency of RA because treating VAD rats with RA restores the control values of collagen IV chains, laminin β–γ chains and MMP9. Other retinoid deficiency or signalling factors, such as oxidative stress which continues to increase after RA administration, may be responsible for the alterations to laminin α chains and MMP2. Bearing in mind that collagen IV and laminin are able to specifically modulate the gene expression and to induce cell differentiation; that they mediate their effects through membrane receptors, mainly integrins; and that these receptors can bind differentially to distinct collagen IV and laminin chains, we suggest that changes in collagen IV and laminin composition may be mediators of tissue malfunction and damage in VAD.

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