Morphology of the Internal Elastic Lamina in Arteries from Pulmonary Hypertensive Patients: a Confocal Laser Microscopy Study

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The development and progression of pulmonary hypertension lesions involve continuous remodeling of the arterial wall, including the extracellular matrix components. The integrity of the internal elastic lamina may represent a barrier to cell migration and formation of intimal proliferative lesions. Some patients with congenital cardiac shunts develop precocious intimal occlusive lesions, whereas others evolve with isolated medial hypertrophy. We studied the 2-D and 3-D morphology of the internal elastic lamina of peripheral pulmonary arteries to search for any difference regarding the type of histological lesion. Fifteen lung biopsies collected for diagnostic purposes from patients with congenital shunts and 6 control lungs (mean ages, 15.8 and 14.7 mo) were studied using the confocal laser scanning microscope, under predetermined conditions of laser intensity, brightness and contrast. We measured the thickness of the internal elastic lamina and determined the number of gaps and projections of elastic tissue towards the medial and intimal layers. The mean internal elastic lamina thickness was significantly higher in arteries from cases with isolated medial hypertrophy when compared with controls and to those with proliferative lesions ($P < .05$). The number of gaps of the internal elastic lamina was higher in arteries $>100 \mu\text{m}$ in diameter from the group with intimal lesions when compared to the cases presenting with isolated hypertrophy, but did not differ from the controls. There was a

positive linear correlation between the external arterial diameter and the thickness of the internal elastic lamina ($r = .74$, $P < .001$) in cases presenting isolated medial hypertrophy. The increased thickness and smaller number of gaps of the internal elastic lamina may act as a barrier that prevents smooth muscle cell migration in patients with pulmonary hypertension without intimal proliferative lesions. On the other hand, a greater number of gaps does not represent, by itself, unrestrained migration, because controls also showed fenestrated laminae.

KEY WORDS: Arteries, Congenital cardiac defects, Elastic tissue, Extracellular matrix, Pulmonary circulation, Pulmonary hypertension.

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The severity of pulmonary hypertension occurring in the evolution of patients with increased pulmonary flow associated with congenital cardiac shunts varies among cases, and early correction of the defect usually arrests the development of the vascular lesions. From the morphological point of view, the peripheral pulmonary arteries undergo modifications that comprise early muscularization of intra-acinar branches, medial hypertrophy, and intimal proliferative lesions, besides a decrease in their number relative to the number of alveoli (1). Adventitial alterations that parallel the modifications in the medial layer have also been documented (2). The mechanism of continuous arterial remodeling includes signaling by the endothelial cells to the medial smooth muscle cells (3, 4) that dedifferentiate (5, 6) and migrate to the intimal layer, turning to the secretory phenotype and synthesizing extracellular matrix components (7), following a course that also takes place in other types of vascular processes (8, 9). Degradation of extracellular matrix components by enzymes such as elastases leads to the release of growth factors (10), promoting vascular cell proliferation and enabling migration of the

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smooth muscle cells to the intima, through openings of the internal elastic lamina. Moreover, a continuous process of synthesis/deposition of these matrix proteins occurs in the arterial wall (11), leading finally to the neointimal formation and increased medial fibrosis.

Although some pulmonary hypertensive patients may present precociously with severe intimal occlusive lesions, others show isolated medial hypertrophy of arteries for long periods (12), allowing reversibility of the pulmonary hypertension after restoration of normal pulmonary flow. Experimental studies have shown that inhibitors of elastase and matrix metalloproteinases stop the evolution of vascular lesions and even promote regression of the thickened vessel wall (13).

We studied the two and three-dimensional morphology of the internal elastic lamina of peripheral muscular pulmonary arteries from patients with congenital shunts and pulmonary hypertension, searching for differences related to the severity and type of the vascular lesions.

MATERIAL AND METHODS

This study was approved by the Scientific Committee of the Heart Institute (InCor), University of São Paulo Medical School, Brazil.

Fourteen lung biopsies from patients with congenital cardiac shunts collected for diagnostic purposes were included in the study, as well as 6 control lung fragments obtained from necropsies of children without cardiopulmonary disease.

The ages ranged from 3 to 60 months (mean = 15.8 mo, median = 10.5 mo) and from 3 to 72 months (mean = 14.7, median = 10 mo) in the hypertensive and control groups, respectively. All patients presented mean pulmonary arterial pressure of >30 mmHg. The cardiovascular defects are shown in Table 1. All individuals from the control group had causes of death not related to the cardiovascular system, and the necropsies were performed within the first 7 hours after death.

A qualitative analysis of the vascular disease was performed in the biopsies, according to the presence of hypertrophy of the medial layer and of intimal proliferative lesions. Occasionally, rare arteries presented inflammatory cells permeating their walls, but this was not a usual feature.

The lung biopsies were collected with the airways distended, according to methods described elsewhere (1), and the necropsy lungs were injected with 10% formalin through the trachea. After routine histological processing, we obtained 30- μ m-thick sections from paraffin-embedded tissue. Sections were stained with Evans' blue for 10 minutes in order to enhance the natural fluorescence of

TABLE 1. Age, Diagnosis, and Type of Arterial Lesion of the Pulmonary Hypertensive Patients

Age (mo)	Gender	Heart Defect	Histological Findings
3	F	TGA, VSD	IPL
7	M	DORV	IMH
7	F	AVSD	IMH
8	F	AVSD	IMH
8	M	AVSD	IPL
9	M	VSD, ASD	IMH
10	F	DORV	IPL
11	M	VSD	IMH
12	M	AVSD	IMH
14	F	DILV, Discordant VA	IPL
22	M	DILV, DORV	IPL
27	M	VSD, PDA	IPL
36	M	VSD	IPL
60	F	VSD, PDA	IPL

TGA = transposition of the great arteries; VSD = ventricular septal defect; DORV = double outlet right ventricle; AVSD = atrioventricular septal defect; ASD = atrial septal defect; DILV = double inlet left ventricle; VA = ventriculo-arterial connection; PDA = patent *ductus arteriosus*; IPL = intimal proliferative lesions; IMH = isolated medial hypertrophy.

elastin and were examined with the confocal laser microscope Odyssey XL (Noran Instruments, Middleton, WI) equipped with an argon krypton laser.

Laser intensity was set at the limits between 30 and 50%, the wavelength was 568 nm, and optimal conditions of brightness and contrast were used to examine pre and intra-acinar arteries. The samples were imaged with a 40 $\times$ dry objective to measure the external diameter through the short axis of each vessel and a 100 $\times$ oil immersion objective to analyze the segment of elastic lamina. In each case, at least three transversally oriented arteries were scanned (Z series), pre and intra-acinar in location. Twenty-four to 32 optical sections of a segment of internal elastic lamina from each artery were obtained, at scanning intervals of 0.5 μ m. The linear extension of the elastic segments analyzed (transversal sections in 2-D imaging) varied from case to case, ranging from 41.3 μ m to 141 μ m (mean, 76.1 μ m).

In the median optical section, we determined the following:

The mean thickness of the internal elastic lamina (measurements made at intervals of 10 μ m along the linear extension of the lamina)—Figure 1

The number of gaps along the linear extension of the internal elastic lamina (expressed as number of gaps per 100 μ m)

The number of segments of elastic tissue projecting from the elastic lamina towards the medial and intimal layers (expressed as number of projections/100 μ m), and taken as an index of the intensity of elastic tissue deposition in the arterial wall.

The group of pulmonary hypertensive patients was split into one presenting isolated medial hypertrophy of arteries (6 cases, median age = 8.5 mo) and another showing any kind of intimal proliferative arterial lesion (8 cases, median age = 18 months; Table 1).

FIGURE 1. A, cross-section of a segment of the internal elastic lamina (2-D image) during the linear measurements of the thickness (transversal lines). There are projections of the elastic tissue (Proj.) and one gap.

We also searched for differences between arteries larger (mostly the preacinar ones) and smaller than $100\ \mu\text{m}$ in diameter.

Group comparisons were done using a one-way analysis of variance test and Tukey's test for pairwise comparison procedures. Statistical significance was set at the 5% alpha error level ($P < .05$).

After doing the quantifications above, we obtained a three-dimensional image of each analyzed elastic lamina segment, using the resources of the software Intervision and the Work Station *Indy* from Silicon Graphics Computer Systems, Inc.

RESULTS

Mean Thickness of the Internal Elastic Lamina

The mean thickness was $1.94 \pm 0.37\ \mu\text{m}$ in the group showing isolated medial hypertrophy, $1.63 \pm 0.40\ \mu\text{m}$ in the cases presenting intimal proliferative arterial lesions, and $1.38 \pm 0.22\ \mu\text{m}$ in the controls ($P = .041$, ANOVA; Fig. 2). A significant difference was detected between the controls and the group with isolated medial hypertrophy (Tukey test, $P = .033$).

There was a positive and significant linear correlation ($P < .001$) between the mean thickness of the elastic lamina and the external diameter of the artery in the three groups. Linear regression analysis showed that the greater slope was found for the group with isolated medial hypertrophy (Fig. 3).

Number of Gaps

The mean number of gaps of the internal elastic lamina was not statistically different among the three groups: 3.05 ± 0.01 gaps/ $100\ \mu\text{m}$ in the group with isolated medial hypertrophy, 4.84 ± 0.04 gaps/ $100\ \mu\text{m}$ in cases presenting intimal proliferative lesions, and 5.89 ± 0.02 gaps/ $100\ \mu\text{m}$ in the control group ($P = .237$, ANOVA, Fig. 4). However, a significant differ-

FIGURE 2. Mean thickness (SD) of the internal elastic lamina in the three groups, given in micrometers. Med. Hypertr., cases showing isolated medial hypertrophy of the pulmonary arteries; Intimal prolif., cases showing intimal proliferative lesions of the pulmonary arteries.

FIGURE 3. Linear correlation between the IEL thickness and the external diameter of peripheral pulmonary arteries from the three groups. IMH, cases showing isolated medial hypertrophy of the pulmonary arteries ($r = .74$); PL, cases showing intimal proliferative lesions of the pulmonary arteries ($r = .70$); C, controls ($r = .73$).

ence was detected when considering only preacinar arteries (greater than $100\ \mu\text{m}$ in diameter— $P = .046$, ANOVA). The group with isolated medial hypertrophy had fewer gaps than the controls ($P = .037$, Tukey test), but did not differ significantly in this regard from the group with intimal proliferative lesions.

Comparing arteries smaller and larger than $100\ \mu\text{m}$ in diameter, the number of gaps was greater in the smaller ones, in the three groups (Table 2).

Number of Projections

Cases presenting intimal proliferative lesions showed a greater number of projections of elastic tissue when compared to those with isolated medial

FIGURE 4. Mean number of gaps present along 100 μm of length of the internal elastic lamina in preacinar arteries. Med. Hypertr., cases showing isolated medial hypertrophy of the pulmonary arteries; Intimal prolif., cases showing intimal proliferative lesions of the pulmonary arteries; C, controls.

hypertrophy and to controls (Fig. 5). However, no statistically significant difference was detected among the groups ($P = .14$, ANOVA).

3-D Images

The resultant 3-D images obtained from the Z series study demonstrated in the cases with isolated medial hypertrophy a thick and even internal elastic lamina, with very few and small or no gaps and projections at all (Fig. 6A), except in small intra-acinar branches, which showed a greater number of fenestrations (Fig. 7). On the other hand, pre and intra-acinar arteries from cases presenting intimal proliferative lesions showed a thinner and perforated elastic lamina, with abundant projections (Fig. 6B). Arteries from the control group presented a thin and perforated lamina, with very few projections (Fig. 6C).

DISCUSSION

During the course of pulmonary hypertension, either in its primary form or due to congenital cardiac shunts, the remodeling of peripheral pulmonary arteries depends on several stimuli that cause smooth

TABLE 2. Mean Numbers (SD) of Gaps of the IEL in Arteries Smaller and Larger than 100 μm from the Three Groups

External Diameter	Number of Gaps/100 μm		
	C	IMH	IPL
<100 μm	5.6 (3.9)	4.1 (2.4)	5.4 (5.5)
>100 μm	3.2 (2.0)	1.0 (1.4)	1.7 (1.5)

IPL = proliferative lesions; IMH = isolated medial hypertrophy; C = controls.

FIGURE 5. Mean number of projections of elastic tissue present along 100 μm of extension of the internal elastic lamina. Med. Hypertr., cases showing isolated medial hypertrophy of the pulmonary arteries; Intimal prolif., cases showing intimal proliferative lesions of the pulmonary arteries; C, controls.

muscle cell proliferation and synthesis/degradation of the extracellular matrix components (3, 7). Endothelial cells are known to play a major role in the maintenance of this process, as they present abnormal function and morphology (14), releasing growth factors besides vasoactive substances and factors that induce endovascular elastase (15).

Increased synthesis of elastin and collagen is a feature not only in pulmonary but also in systemic arteries in response to hypertension (16). If initially this production may be considered as protective to the vessel wall, it may in the long term result in increased stiffness and contribute to the persistence of hypertension (17). Moreover, these extracellular matrix components are also deposited in the neointima, occluding the arterial lumen.

It has been experimentally demonstrated that increased elastolytic activity in the arterial wall is related to progressive pulmonary hypertension (11), and that such activity can mediate mitogenic activity in pulmonary artery smooth muscle cells by the release of fibroblast growth factor (10).

In the context of neointimal formation, the internal elastic lamina acts as a barrier to the migration of transformed smooth muscle cells. On the other hand, the presence of “defects” or holes in its structure would facilitate such process. So, it could be assumed that the high turnover of elastin in peripheral pulmonary arteries would lead to an increased number of fenestrations of the internal elastic lamina and be associated to severe occlusive morphological lesions. Our study demonstrated fewer gaps in preacinar arteries from the group showing isolated medial hypertrophy, although only statistically significant in comparison with the controls. In one hand, this finding could corroborate the “protective effect” of a continuous elastic lamina against cell migration. On the other hand, however, the controls also showed a great number of gaps, and this led us to the inference that the presence of gaps in the elastic lamina probably is not, by itself, the determinant factor of intimal

FIGURE 6. **A**, 3-D reconstruction of a segment of the IEL from a case with isolated medial hypertrophy (IMH). The structure of the IEL is homogeneous, with no gaps and very few projections. **B**, 3-D image of a segment of the IEL from a patient presenting intimal proliferative (IP) lesions. Notice that the lamina is fenestrated and that its thickness varies. Several projections are directed towards the medial layer (behind). **C**, 3-D reconstruction of a segment of the IEL from a control case, showing a thin lamina with fenestrated appearance. Depths (Z-axis) of imaging are respectively 12 μm (**A**); 15 μm (**B**), and 12.5 μm (**C**). In the three images, the frontal surface represents the intimal aspect of the elastic layer.

smooth muscle cell migration. Although we cannot decide, on morphological basis, which is the definitive feature, elastin degradation and its resultant products seem to be essential in promoting migration and proliferation of smooth muscle cells. If we assume that medial hypertrophy represents the initial phase of arterial wall response to the injury triggered by the increased blood flow, we can appreciate that the initial mechanism of elastic deposition is followed

FIGURE 7. Three-dimensional reconstruction of the elastic laminae of a small intra-acinar artery (38 μm in diameter) from the group with isolated medial hypertrophy. Both the internal and the external laminae are visible. There are focal and few fenestrations in the internal lamina, and more extensive ones in the external lamina. Depth (Z-axis) = 12 μm .

by an elastolytic phase and a return to the fenestrated aspect of the lamina.

The fact that arteries smaller than 100 μm in diameter present a greater number of gaps than the larger ones in all groups also militates against the idea that these discontinuities determine migration, because intimal proliferative lesions usually occur in preacinar arteries. Moreover, some authors have demonstrated in animals that the number of fenestrae of the internal elastic lamina increases during normal postnatal development, and that they are larger when the blood flow is artificially augmented (18) in systemic arteries. In the present work we have not evaluated the size of the gaps of the elastic lamina, only their number. That information could help to determine whether the gap dimension is related to the process of migration/proliferation of smooth muscle cells to the intimal layer and if there are differences between small and large arteries regarding this aspect.

Our results also demonstrated that an increased deposition of elastin on the elastic lamina occurred in both groups presenting pulmonary hypertension, because they showed a greater thickness of the lamina when compared with that of controls. Such thickness correlated positively to the external diameter of the vessel, indicating a higher elastin deposition in preacinar arteries, the resistance vessels of the lungs. The finding of a greater regression coefficient (slope) in patients with isolated medial hypertrophy leads us to assume that in this group elastin deposition is greater than in the others, exceeding degradation, and that elastolytic activity must be low.

We interpreted the projections towards the intimal and medial layers as elastic fibers or fibrils not fully incorporated to the internal elastic lamina in the context of increased elastin synthesis. Alternatively, they could be the consequence of continuous elastin degradation. Although not statistically significant, our finding of a greater number of projections in the group with intimal occlusive lesions suggests a faster turnover of elastin in these arteries, as a probable consequence of increased activity of local enzymes.

Another point that deserves discussion is the age of patients in the two subgroups of pulmonary hypertension. In the present study, the age range was broader and the median greater in those showing intimal proliferative lesions. This could suggest that the differences in the structure of the internal elastic lamina detected between the two groups could be ascribed not to a different "pattern" of response to increased flow but rather to the age. If so, one could interpret the finding of a thicker elastic lamina, with few fenestrations, to be a stage of development of the arterial lesions in pulmonary hypertension. Only an age-controlled study would provide an answer to this hypothesis.

As far as we are aware, this work represents the first approach to the 3-D structure of the arterial elastic laminae in patients with pulmonary hypertension. Although confocal laser microscopy is not presently widely available for routine analysis, it must be considered a very powerful tool in the investigation of the arterial wall biology in both health and disease, because it allows the 3-D analysis of the morphology and has added the advantage of employing fluorescent-labeled antibodies.

Summing up, our findings reveal that patients presenting isolated medial hypertrophy as the main morphological lesion of pulmonary hypertensive vessels show a thick, continuous, and apparently stable elastic lamina that can act as a real barrier to the migration of smooth muscle cells to the intima and enhance the response to the elevated tensile stress in the arterial wall. On the other hand, when intimal proliferative lesions occur, the elastin deposition, although increased, is accompanied by high turnover, what gives to the internal elastic lamina the aspect of a fenestrated and irregular structure.

The underlying enzymatic mechanisms of such morphological findings of the internal elastic lamina remain to be fully elucidated. The different individual responses that lead to these distinct morphological patterns may be present at birth or may appear in the course of the disease. Further studies involving patients from a larger age range may contribute to elucidate this point.

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