Congenital Heart Disease

Impaired Apoptosis of Pulmonary Endothelial Cells Is Associated With Intimal Proliferation and Irreversibility of Pulmonary Hypertension in Congenital Heart Disease

Marilyne Lévy, MD, PhD,*† Christelle Maurey, DVM,* David S. Celermajer, MBBS, DSC, FRACP,‡ Pascal R. Vouhé, MD,† Claire Danel, MD,* Damien Bonnet, MD, PhD,§ Dominique Israël-Biet, MD*||
Paris, France; and Sydney, Australia

Objectives
This study sought to assess the cellular and histologic basis of irreversible pulmonary hypertension (PHT) in the clinical setting of congenital heart disease (CHD).

Background
Although many children with CHD develop pulmonary vascular disease, it is unclear why this complication is reversible after complete repair in some cases but irreversible in others. Because failure of endothelial cell apoptosis might lead to intimal proliferation and lack of reversibility of PHT, we investigated this and other key markers of vasoactivity and angiogenesis in subjects with PHT and CHD.

Methods
We assessed antiapoptotic and proapoptotic markers in vascular and perivascular cells in lung biopsy samples from 18 patients with CHD, 7 with reversible and 11 with irreversible PHT, and 6 control patients. Immunostaining for endothelial nitric oxide synthase, vascular endothelial growth factor, and CD34 (markers of vasoactivity and neoangiogenesis) was also performed.

Results
The antiapoptotic protein Bcl-2 was highly expressed by pulmonary endothelial cells in all cases of irreversible PHT but in no cases of reversible PHT, nor in control patients (p < 0.001). Intimal proliferation was present in 10 of 11 irreversible PHT cases, but never observed in reversible PHT (p < 0.001). Similarly, perivascular inflammatory T-cells expressed more antiapoptotic proteins in irreversible PHT (p < 0.01). Irreversible PHT cases were also more likely to show compensatory upregulation of vascular endothelial growth factor and new small vessel formation at the sites of native vessel stenosis or occlusion (p < 0.001).

Conclusions
Irreversible PHT is strongly associated with impaired endothelial cell apoptosis and antiapoptotic signaling from perivascular inflammatory cells. These changes are associated with intimal proliferation and vessel narrowing, and thereby may contribute to clinical outcomes associated with pulmonary hypertension. (J Am Coll Cardiol 2007;49:803–10) © 2007 by the American College of Cardiology Foundation

Although particular congenital heart diseases (CHD) are well recognized to lead to early and important pulmonary vascular disease (PVD), there is a wide range of pulmonary vascular responses for any given underlying lesion (1,2). For example, truncus arteriosus or atrioventricular septal defect are often but not always associated with PVD in infancy (3). Furthermore, the important clinical parameter of reversibility of PVD is often difficult to predict in patients, even with detailed knowledge of their abnormal intracardiac anatomy and physiology. The pathogenic mechanisms that lead to “irreversible” PVD and thus pulmonary hypertension (PHT) (that which fails to reverse after correction of the underlying anatomical defect) are therefore poorly understood in subjects with CHD.

Apoptosis is the process of normal programmed cell death that allows normal cell turnover and remodeling in the vasculature (4), and recent data have implicated a resistance to apoptosis in the pathogenesis of vascular proliferation in experimental and cell culture studies (5,6). Endothelial dysfunction is also known to complicate PHT in the setting of CHD in vivo in humans (7,8).

We therefore hypothesized that resistance to apoptosis might characterize the pulmonary vessels of subjects with irreversible PHT, with consequent unregulated intimal

From the *UPRES EA4068, UFR Biomédicale des Saints Pères et Faculté de Médecine Paris V, Paris, France; †Service de Chirurgie Cardiaque, Hôpital Necker-Enfants Malades, Paris, France; ‡Faculty of Medicine, University of Sydney, Sydney, Australia; §Service de Cardiologie Pédiatrique, Hôpital Necker-Enfants Malades, Faculté de Médecine Paris V, Paris, France; and the ||Service de Pneumologie, Hôpital Européen Georges Pompidou, Paris, France. This study was supported by Leg Poix, Paris, France.
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proliferation, vessel stenosis, and/or occlusion and compensatory neoangiogenesis with local up-regulation of vascular endothelial growth factor (VEGF). To explore these possibilities, we undertook detailed histology and cellular immunohistochemistry studies of the lungs of subjects with CHD and either reversible or irreversible PHT.

Methods

Study population. Eighteen consecutive patients with CHD and PHT, excluding all patients with trisomy 21, who underwent cardiac surgery at Necker-Enfants Malades Hospital and in whom a lung biopsy specimen was obtained, were enrolled in the study. Table 1 shows their clinical and hemodynamic characteristics and postoperative outcomes. Preoperative hemodynamic evaluation was performed in all patients, including measurements of pulmonary pressure and determination of shunt by oximetry before and after inhaled nitric oxide (NO). In all these patients, preoperative hemodynamic data did not clearly establish whether or not the PHT would be reversible after pulmonary artery banding or lesion repair. Therefore, all of these patients underwent either complete repair or palliative surgery, and lung biopsy was performed for histomorphometric analysis of pulmonary arteries. Patients were separated into 2 groups on an a posteriori evaluation of pulmonary pressure after 1 year. Seven had normal pulmonary arterial pressure (reversible PHT) and 11 still had elevated pulmonary vascular resistance (PVR) and pulmonary arterial pressure (irreversible PHT). These 2 groups were similar in terms of age and preoperative level of PHT. Longer-term postoperative follow-up (median 2 years, range 1 to 10 years) confirmed that pulmonary pressure remained normal in all patients with “reversible” PHT. One of these patients (Patient #1) died with sepsis in the postoperative course of a subsequent operation (total cavopulmonary connection). There were 2 early and 2 late deaths related to PHT in the irreversible PHT group. One patient (Patient #15) who had suprasystemic PHT in the postoperative course is still alive after 10 years, and the pulmonary systolic pressure was estimated as two-thirds of the systemic pressure. Six patients with normal lung histology who died of extracardiac and extrapulmonary causes were studied as control patients.

Tissue specimens. Lung tissue, obtained during cardiac surgery, was formalin-fixed and paraffin-embedded. Serial 5-μm-thick sections were stained with hematoxylin and eosin and modified orcein for elastic fibers to allow identification of morphologic structures and precise correlation with patterns of immunoreactivity. Pulmonary vascular structures were analyzed using quantitative morphometric techniques as previously described (9). Intimal thickening, reduced arterial concentration, occlusive fibrosis, plexiform lesions, and dilatation of distal arteries were looked for and considered as irreversible vascular lesions.

Table 1: Patient Characteristics and Postoperative Outcome

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Gender</th>
<th>Age* (yrs)</th>
<th>Diagnosis</th>
<th>PAP (S/D)</th>
<th>QP/QS</th>
<th>Postoperative PAP (S/D)</th>
<th>Handing</th>
<th>TCPC</th>
<th>Follow-Up (yrs)</th>
</tr>
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<tbody>
<tr>
<td>Reversible PHT</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>M</td>
<td>3</td>
<td>SV</td>
<td>60/25</td>
<td>2</td>
<td>NA</td>
<td>Banding</td>
<td>25/12</td>
<td>TCPC (1), dead</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>4</td>
<td>SV</td>
<td>59/26</td>
<td>2.1</td>
<td>3</td>
<td>Banding</td>
<td>25/10</td>
<td>TCPC (2)</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>5</td>
<td>SV</td>
<td>75/35</td>
<td>1.6</td>
<td>2</td>
<td>Banding</td>
<td>20/10</td>
<td>TCPC (1)</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>6</td>
<td>VSD</td>
<td>55/30</td>
<td>2</td>
<td>3</td>
<td>Closure</td>
<td>30/15</td>
<td>20/10 (7)</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>3</td>
<td>SV</td>
<td>68/28</td>
<td>2.7</td>
<td>2.8</td>
<td>Banding</td>
<td>35/12</td>
<td>20/10 (1)</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>32</td>
<td>ASD</td>
<td>65/35</td>
<td>1.8</td>
<td>2.7</td>
<td>Closure</td>
<td>35/15</td>
<td>30/15 (10)</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>42</td>
<td>ASD</td>
<td>70/33</td>
<td>2.7</td>
<td>2.5</td>
<td>Closure</td>
<td>35/20</td>
<td>22/10 (5)</td>
</tr>
<tr>
<td>Irreversible PHT</td>
<td></td>
<td></td>
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<tr>
<td>8</td>
<td>M</td>
<td>5</td>
<td>TGA/VSD</td>
<td>65/30</td>
<td>1.3</td>
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<td>Senning</td>
<td>80/40</td>
<td>Alive (2)</td>
</tr>
<tr>
<td>9</td>
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<td>2</td>
<td>SV</td>
<td>75/36</td>
<td>1.7</td>
<td>2</td>
<td>Banding</td>
<td>90/38</td>
<td>Alive (1)</td>
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<tr>
<td>10</td>
<td>F</td>
<td>24</td>
<td>SV</td>
<td>67/26</td>
<td>1.6</td>
<td>2</td>
<td>Banding</td>
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<td>Dead</td>
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<tr>
<td>11</td>
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<td>86/36</td>
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<td>Closure</td>
<td>95/50</td>
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<tr>
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<td>VSD</td>
<td>75/35</td>
<td>1.5</td>
<td>2</td>
<td>Closure</td>
<td>90/40</td>
<td>Alive (1)</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>13</td>
<td>SV</td>
<td>62/26</td>
<td>2</td>
<td>NA</td>
<td>Banding</td>
<td>110/55</td>
<td>Dead</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>7</td>
<td>VSD</td>
<td>59/25</td>
<td>2.5</td>
<td>2.5</td>
<td>Closure</td>
<td>85/40</td>
<td>Alive (2)</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>33</td>
<td>ASD</td>
<td>60/25</td>
<td>2.5</td>
<td>2.5</td>
<td>Closure</td>
<td>110/50</td>
<td>70/40 (10)</td>
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<tr>
<td>16</td>
<td>M</td>
<td>3</td>
<td>SV</td>
<td>58/30</td>
<td>1.5</td>
<td>2</td>
<td>Banding</td>
<td>85/40</td>
<td>Dead (2)</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>3</td>
<td>VSD</td>
<td>55/30</td>
<td>1.8</td>
<td>2</td>
<td>Closure</td>
<td>90/40</td>
<td>Alive (1)</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>1</td>
<td>VSD</td>
<td>70/20</td>
<td>1.2</td>
<td>2.3</td>
<td>Closure</td>
<td>60/30</td>
<td>Dead (0.5)</td>
</tr>
</tbody>
</table>

*Age at lung biopsy.

ASD = atrial septal defect; iNO = inhaled nitric oxide; NA = not available; PAP (S/D) = pulmonary arterial pressure (systolic/diastolic) (mm Hg); PHT = pulmonary hypertension; PVR = pulmonary vascular resistance; QP = pulmonary blood flow; QS = systemic blood flow; SV = single ventricle; TCPC = total cavopulmonary connection; TGA = transposition of great arteries; VSD = ventricular septal defect.
Immunohistochemical analysis. Markers of apoptosis were assessed in vascular cells and inflammatory cells using immunohistochemistry with the corresponding primary antibodies, anti–caspase-3 (BD Pharmingen, Le Pont de Claix, France), -p53 (monoclonal anti-human p53 protein, Dako Corp., Carpenteria, California), and -Bcl-2 antibodies (monoclonal anti-human BCL2 oncoprotein, Dako Corp.) (10).

The vascular expression of vasoactive and angiogenic factors, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and VEGF was also assessed with the corresponding primary antibodies anti-eNOS (Transduction Laboratory, Lexington, United Kingdom), anti-iNOS, and anti-VEGF (A-20, Santa Cruz Biotechnology, Santa Cruz, California). In addition, antibodies recognizing endothelial polyclonal anti-CD34 (Dako Corp.) or inflammatory cells (anti-CD3, anti-CD68, anti-CD79, anti-tryptase, and anti-elastase [Dako Corp.]) identifying T lymphocytes, macrophages, B lymphocytes, mast cells, and polymorphonuclear cells, respectively, were used. Briefly, tissue sections were deparaffinized in toluene, rehydrated, and heated for 40 min in buffered citrate at pH 6, or pH 9 for the anti–caspase-3. Slides were incubated in hydrogen peroxide to block endogenous peroxidase activity, washed in tris-buffered saline, and incubated for 1 h with the primary antibodies or with normal serum used as a negative control. Slides were then incubated for 15 min with a biotinylated secondary antibody and stained with streptavidin labeled with peroxidase, according to the manufacturer’s instructions (Dako Corp.). The slides were then counterstained using Harris hematoxylin.

The intensity of the immunostaining in the arteries of external diameter 100 to 200 μm or 50 to 100 μm was graded semi-quantitatively from 0 = no staining to 3 = maximal staining. Inflammatory infiltrates were analyzed in terms of cell type and density, scored as 0 = no labeled cells, to 3 = maximal density. The grading was performed by 2 independent investigators who were blinded to all clinical information.

Statistical analysis. No significant differences were noted between observers, and we have previously documented excellent interobserver correlations for the parameters measured in this study (11). For each marker, because the staining score intensities were similar in arteries of different sizes, we pooled the corresponding results. Data are expressed as a score of immunodetection (mean ± SD of the scores obtained by each investigator). Comparisons between groups were assessed using the nonparametric Mann-Whitney U test analysis. Correlation between vasoactive factor scores was analyzed using the Spearman test correlation. A value of p < 0.05 was considered significant.

Results

Histomorphometric data. Lung tissue specimens showed the presence of intimal proliferation lesions in 10 of 11 cases of irreversible PHT and in no cases of reversible PHT (Fig. 1). Intimal proliferation appeared as more advanced lesions in 8 of these patients, whereas concentric laminar intimal fibrosis and plexiform lesions were observed in the remaining 2. Only 1 patient of this group (Patient #17) did not show intimal proliferation.

Both groups of PHT patients showed a comparably increased mean percentage wall thickness and abnormal extension of muscle into distal pulmonary arteries (Table 2). These values were markedly increased compared with control patients (p < 0.0001). Patients’ values were also markedly increased compared with normal values for age (p < 0.001) (9). Lung biopsies showed normal pulmonary arteries in all subjects in the control group.

![Figure 1](image-url)
Markers of apoptosis. The antiapoptotic marker Bcl-2 was exclusively expressed in irreversible PHT by endothelial cells from arteries with severe intimal fibrosis and never expressed in reversible PHT (Fig. 2A). Very few smooth muscle cells expressed Bcl-2 in irreversible PHT. Markers of apoptosis caspase-3 and p53 were expressed by the endothelial layer in both groups of patients (Figs. 2B and 2C). Immunostaining for caspase-3 and p53 was not observed in smooth muscle cells. Very few cells expressed caspase-3 or p53 in the control group (p < 0.001 compared with CHD-PHT).

Inflammatory infiltrates. In addition to the aforementioned morphologic alterations, patients with PHT differed from control patients in that the majority showed a consistent inflammatory infiltrate in the bronchovascular areas. These infiltrates were present in all patients with irreversible PHT (11 of 11 patients) and in 5 of 7 patients with reversible PHT. Inflammatory cells mainly consisted of CD3+ T lymphocytes (89 ± 23%) with a few macrophages (7 ± 2%) and a few polymorphonuclear cells (3 ± 1%). Mast cells when present were not localized in either inflammatory infiltrate or in the pulmonary arteries. These infiltrates were similar between the 2 PHT groups in terms of cell density and cell type. We then assessed the apoptotic properties of inflammatory cells in the 2 groups. The antiapoptotic protein Bcl-2 was largely expressed in reversible PHT and poorly expressed in reversible PHT (Fig. 3A). Conversely, the proapoptotic proteins caspase-3 and p53 were strongly expressed by inflammatory cells in reversible PHT but not expressed in irreversible PHT (p < 0.001) (Figs. 3B and 3C). Thus, in irreversible CHD-PHT, inflammatory cells showed decreased proapoptotic activity and increased antiapoptotic marker expression, suggesting that the nature of the inflammatory process might play a role in the course of CHD-PHT.

Pulmonary vascular iNOS expression, involved in the inflammatory processes, was similarly increased in the 2 PHT groups compared with control patients (p < 0.001).

Markers of compensatory neoangiogenesis. Endothelial NOS was detected exclusively in vascular endothelial cells of all size arteries. Its expression was higher in irreversible than in reversible PHT patients and particularly intense in severe vascular lesions (eNOS scores 2.4 ± 0.4 and 1.2 ± 0.5, respectively; p < 0.001) (Fig. 4A). In control patients, eNOS expression was lower than in both PHT groups (0.65 ± 0.2, p < 0.05 vs. reversible PHT and p < 0.001 vs. irreversible PHT).

Vascular endothelial growth factor was immunolocalized on vascular endothelial cells of all size arteries (Fig. 4B). Its expression was higher in irreversible than in reversible PHT patients (2.9 ± 0.5 and 0.5 ± 0.1, respectively, p < 0.001). The VEGF expression was comparable in reversible PHT and control patients (0.6 ± 0.1).

The eNOS expression correlated with VEGF expression only in irreversible PHT (r = 0.96; p < 0.001). This, together with CD34 immunostaining in the vicinity of all occluded arteries, suggests neoangiogenesis in irreversible PHT (Fig. 4C).

Discussion

The pathophysiologic mechanisms that determine the reversible or irreversible nature of PHT that commonly complicate certain congenital heart diseases remain unclear. Irreversible PHT is difficult to predict on the basis of preoperative testing and has adverse long-term consequences. In the present study, we document a highly significant association between failure of endothelial cell apoptosis, intimal proliferation, and irreversible PHT in children with high-risk CHD.

Previous studies in idiopathic PHT have recently implicated a failure of vascular smooth muscle apoptosis in the disease pathogenesis (12,13). However, data about endothelial cell apoptosis and proliferation are scarce in idiopathic PHT (5,14) and absent for PHT complicating CHD. Other recent studies have suggested a role for periarterial inflammatory cell mediators in the pathogenesis of PHT (15,16).

For these reasons, we sought to determine whether altered endothelial cell and or periarterial inflammatory cell phenotype might account for the development of irreversible pulmonary vascular changes in CHD. To answer these questions, we analyzed pulmonary biopsies from patients undergoing cardiac surgery for CHD and elevated PVR to analyze vascular expression of apoptotic and angiogenic markers in reversible or irreversible PHT in the setting of CHD (as assessed by the gold standard test of presence or absence of residual PHT judged by hemodynamic status 1 year after surgery).

We first confirmed that all PHT patients showed the expected histomorphologic vascular alterations in terms of arterial wall thickness (1,2), but intimal damage was observed only in irreversible PHT. One patient of the irreversible PHT group did not show any intimal lesion, in keeping with the fact that a few patients can maintain high pulmonary vascular resistances despite mild histologic vascular changes (17). This underscores the fact that histomorphometric parameters are not always sufficient to predict the outcome in PHT.

The role of proapoptotic and antiapoptotic proteins in PHT has been suggested, both as an initiating mechanism in the pathogenesis of PHT and as a potential mechanism...
for the proliferation of apoptosis-resistant vascular cells (5,18). In our study, markers of apoptosis markedly differed between subjects with reversible and irreversible PHT. Indeed, although the proapoptotic markers caspase-3 and p53 were expressed in endothelial cells in patients of both groups, the antiapoptotic protein Bcl-2 was expressed only in the endothelial cells of severely damaged pulmonary arteries of patients with irreversible PHT. The TdT-mediated dUTP-biotin end labeling staining was not performed in these experiments because this stain is not completely specific for apoptosis but also identifies necrotic cells (19), thus suggesting a superior specificity of caspase-3 and p53 protein expression as proapoptotic markers.

This observation is consistent with a recent in vitro study suggesting that pulmonary endothelial cell apoptosis is an early mechanism in the pathogenesis of idiopathic PHT followed by proliferation of apoptotic-resistant cells (5).

In our series, although all PHT patients had proliferation of smooth muscle cells with increased medial wall thickness, the expression of the antiapoptotic protein Bcl-2 was low in smooth muscle cells. Our data may suggest that the remodeling process in the course of CHD-PHT preferentially

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**Figure 2** Vascular Immunostaining for Markers of Apoptosis in Reversible PHT and Irreversible PHT

The antiapoptotic protein Bcl-2 is not expressed in reversible pulmonary hypertension (PHT), but by endothelial cells of severely damaged pulmonary arteries in irreversible PHT in all cases (A). Endothelial cells of both groups expressed markers of apoptosis caspase-3 (B) and p53 (C). The arrow indicates immunostaining in the endothelial layer.
involves an acquired apoptosis-resistant phenotype in endothelial cells rather than a central role of apoptosis-resistant smooth muscle cells as proposed for idiopathic PHT (12,13). Apoptosis-resistant endothelial cells are then more likely to produce intimal proliferation and occlusion of distal intrapulmonary arteries, and then likely to induce compensatory development of neovessels in the vicinity of severely altered native vessels. Consistent with this hypothesis, we found CD34+/H11001 structures surrounding all occluded vessels. Both VEGF and eNOS are involved in neoangiogenesis (20–23). Although eNOS expression was increased in all patients compared with control patients, eNOS expression was significantly higher in irreversible compared with reversible PHT. Increased eNOS expression in advanced pulmonary vascular lesions has already been described in the clinical setting of Eisenmenger syndrome (irreversible PHT) (24,25), but no data are available on reversible PHT in humans.

The current findings are consistent with experimental studies suggesting an adaptive compensatory response to increased pulmonary blood flow and arterial pressure (26,27). In irreversible PHT, however, the overexpression of eNOS is associated with impaired NO-dependent pulmonary vasodilation (7,8). This is perhaps consistent with dysfunction of NO production in favor of superoxide, as documented in advanced systemic arterial disease (28). We also found an overexpression of the angiogenic protein VEGF in association with irreversible PHT, similar to that seen previously in studies performed in Eisenmenger lesions (29,30). Also, VEGF has been implicated in endothelial cell survival by inducing upregulation of the antiapoptotic protein Bcl-2 (31). Therefore, VEGF could induce both
survival of proliferative endothelial cells and compensatory angiogenesis in irreversible PHT. It is of note that severely occluded vessels with strong eNOS and VEGF expression were all surrounded by important CD34+ structures, suggesting a paracrine and autocrine effect on new small vessel formation. This compensatory up-regulation of VEGF in response to occluded vessels has also been found in systemic arteriopathy, such as diabetic retinopathy and age-related macular degeneration (20,32).

Finally, 16 of 18 patients showed inflammatory infiltrates in perivascular areas, mostly consisting of T cells. The contribution of the inflammatory process to the pathogenesis of idiopathic PHT recently has been suggested (15,16), but data on its role in CHD-PHT are scarce (33). Here we have shown the presence of important inflammatory infiltrates located in the bronchopulmonary spaces, but whether these infiltrates are of primary or secondary concern regarding the pathogenesis of pulmonary vascular disease remains unclear.

We now document an additional potential mechanism for an interaction between inflammation and vascular changes associated with CHD: the increased expression of antiapoptotic and reduced expression of proapoptotic factors by infiltrative leucocytes in irreversible PHT. This might suggest that irreversible PHT is associated with an increased capacity of the inflammatory infiltrate to contribute to worsening vascular injury.

In this study, only cross-sectional cellular and histomorphometric data are available, although we had some clinical and hemodynamic information. Thus associations can be
made only between the parameters measured and reversible or irreversible PHT, rather than any cause-and-effect relationship. Our interpretation of the data is based on published information and one logical but hypothetical model of a possible sequence of events involving impaired endothelial apoptosis, intimal proliferation, and PHT (5,18). Further studies would be required to better understand the actual pathophysiological sequence of events. In summary, our current findings highlight the roles of impaired endothelial cell apoptosis and inflammatory cell apoptosis in the pathogenesis of irreversible PHT complicating CHD.

Reprint requests and correspondence: Dr. Marilyne Lévy, Hôpital Necker-Enfants Malades, Service de Chirurgie Cardiaque Pédiatrique, 149 Rue de Sevres, 75015 Paris, France. E-mail: marilyne.levy@nck.aphp.fr.

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