Indian Journal of Biochemistry & Biophysics
Vol 46, December 2009, pp 482-490

Pulmonary vascular remodeling in congenital heart disease: Enhanced expression of heat shock proteins

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Received 27 September 2009; revised 7 November 2009

In congenital heart disease (CHD), mechanical wall stress by increased pulmonary artery pressure and pulmonary blood flow is believed to play a pivotal role in the pathogenesis of pulmonary plexogenic arteriopathy (PPA). The pathogenesis of this disease that involves significant pulmonary arterial remodelling, is, however, largely unknown. In the systemic circulation, upregulation of HSP-70 and HSP-27 in the arterial wall occurs in response to acute hypertension, whereas HSP-60 and increased titres of anti-HSP-60 antibodies are associated with atherosclerotic vessel disease. We looked for the involvement of HSPs in the stress response of pulmonary endothelial and vascular smooth muscle cells in different abnormal hemodynamic conditions in patients with CHDs. We analyzed the expression pattern of HSP-27, HSP-70 and HSP-60 in lung biopsies of 38 patients with CHD, using immunohistochemistry. These included 4 individuals with an essentially normal pulmonary circulation, who served as controls. Immunoreactivity against HSP-27 and also against HSP-70 was present in the pulmonary endothelium and vascular smooth muscle cells of patients and controls in a similar pattern. In contrast, expression of HSP-60 was absent in pulmonary arteries of both patients and controls. In patients with advanced PPA, cells within plexiform lesions showed strong staining for HSP-27 and HSP-70, but were again negative for HSP-60. The intensity of immunoreactivity against HSP-70 correlated inversely with medial thickness of pre-acinar arteries ($r = -0.32$; $p = 0.04$). Expression of HSP-27 and HSP-70 did not correlate with hemodynamic parameters, although immunoreactivity against HSP27 tended to be increased in cases with high pulmonary artery pressure ($r = 0.37$; $p = 0.16$) and was highest in patients with flow-associated pulmonary hypertension ($p<0.01$). HSP-27 and HSP-70, but not HSP-60 are engaged in the stress response of cells of small pulmonary arteries in pulmonary plexogenic arteriopathy. HSP-27 and HSP-70 are increasingly expressed in the advanced proliferative lesions of this disease.

Keywords: Histopatholgy, Pulmonary circulation, Remodeling, Congenital heart disease, Heat shock proteins

In patients with congenital heart diseases (CHDs), abnormal pulmonary hemodynamic conditions modify functional and structural properties of the pulmonary vasculature. Elevated pulmonary artery pressure and increased pulmonary blood flow lead to a pattern of progressive pulmonary vascular remodelling, characterized by muscularisation of small pulmonary arteries, intimal proliferation, concentric laminar fibrosis, and ultimately the emergence of focal so-called plexiform lesions within small arterial branches[1,2]. In these patients, pulmonary vascular endothelial and smooth muscle cells are subject to a permanent and increasing mechanical and metabolic stress due to the unfavourable pulmonary hemodynamics. Heat shock proteins (HSPs) are families of proteins that are constitutively expressed in many mammalian cells and have physiological roles in the correct folding and transcellular transport of other proteins[3-5]. Upregulation of some HSP members occurs as a response to various types of stress[4-7]. This upregulation of HSP synthesis is in general considered to represent a powerful physiological, endogenous route for protecting crucial cellular homeostatic mechanisms against disturbing external factors.

Also, in the cardiovascular system, cells respond to environmental stress with the synthesis of HSPs. In the systemic circulation, upregulation of HSPs by cells of the arterial wall can be induced by, amongst others, thermal stress, ischemia, cytokine activation, reactive oxygen species (ROS) and hemodynamic stressors,

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Abbreviations: ABC, avidin-biotin complex; ASD, atrial septal diseases; CHD, congenital heart disease; HSP, heat shock protein; PPA, pulmonary plexogenic arteriopathy; PAP, peroxidase-antiperoxidase; ROS, reactive oxygen species; VSD, ventricular septal diseases; VSMCs, vascular smooth muscle cells.

such as acute hypertension and volume overload[4-8]. These observations indicate that the HSPs probably play an important role in various cardiovascular disease states. Upregulation of HSP-70 and HSP-27 in cardiomyocytes has been reported to protect the heart from ischemic injury[3,4,8]. Several members of the HSP families have been detected in systemic atherosclerotic plaques in human blood vessels. Hypoxia has been shown to increase HSP-27 expression in pulmonary endothelial cells in rats. Furthermore, we have previously demonstrated increased amounts of HSP-70 and HSP-27 in pulmonary vascular cells of neonates with congenital diaphragmatic hernia[9].

On the other hand, it has been speculated that increased expression of HSPs might have detrimental rather than protective effects on cell function and might even propagate disease in specific conditions. The demonstration of HSP-60 expressing cells of arterial atherosclerotic lesions and the circulating antibodies to mycobacterial HSP-65, cross-reacting with the human HSP-60 fits the idea of an immunological contribution to atherogenesis[10]. These observations led us to seek *in vivo* evidence for the engagement of HSP in pulmonary vascular remodelling.

We investigated whether in patients with CHDs, the mechanical and metabolic stress on cells of the pulmonary arterial wall due to unfavourable pulmonary blood flow and pressure conditions induces the expression of HSPs. Expression of members of the HSP families may in congruency with other cardiovascular disease states play a role in the pulmonary vascular remodelling process in these patients. We, therefore, determined and analyzed the *in vivo* micro-anatomical expression pattern of the HSP-70, HSP-27 and HSP-60 in lung biopsies from patients with CHDs associated with abnormal pulmonary hemodynamics and compared these data with those in control subjects. Furthermore, we assessed the intensity of the immunostaining on a semi-quantitative basis and correlated these findings with both hemodynamic and histological data of pulmonary arteriopathy in these patients.

Patients and Methods

Thirty-four patients with CHD, who underwent cardiac catheterization and had increased pulmonary blood flow (pulmonary-to-systemic blood flow ratio >1,2) and/or pulmonary hypertension (mean pulmonary artery pressure >25 mm Hg) and in whom subsequently a lung tissue specimen was obtained, were enrolled in the study after written informed parental consent. Four of the patients had mild left-sided obstructive lesions and an essentially normal pulmonary circulation: these served as control subjects (Group 1). In all patients, complete hemodynamic evaluation was performed during cardiac catheterization, including measurements of pulmonary artery pressure, determination of shunt size by oximetry and calculation of pulmonary and systemic vascular resistance. Blood flow was determined using the dye dilution technique. Ten patients with atrial and/or ventricular septal diseases (ASD and/or VSD) had increased pulmonary blood flow with normal mean pulmonary artery pressure (Group 2).

Eighteen patients had pulmonary hypertension (mean pulmonary artery pressure >25 mm Hg) associated with increased pulmonary blood flow (Group 3). Diagnoses in this group were VSD, with or without patent ductus arteriosus or ASD (n = 13), complete atrioventricular septal disease (n = 4) and one patient with aberrant origin of left pulmonary artery from the aorta. Finally, 6 patients without intracardiac shunts had pulmonary hypertension due to pulmonary venous congestion (Group 4). Diagnoses in this group were (sub)valvular aortic stenosis and/or coarctation of the aorta and/or mitral stenosis. Patient characteristics and hemodynamic findings are presented in Table 1. Open lung biopsy was

Table 1—Patient characteristics, histological and hemodynamical data

	Group 1 Control (n=4)	Group 2 Increased Qp, normal PAP (n=10)	Group 3 Increased Qp increased PAP (n=18)	Group 4 Pulmonary venous congestion (n=6)
Age (yrs) median range	3.3 (1,8-4,8)	6.6 (0,6-32,1)	0.7 (1,1-10,9)	2.4 (0,3-30,7)
mPAP (mm Hg)	13 ± 2	13 ± 3	31 ± 12 *	41 ± 14 *
PWP (mm Hg)	9 ± 2.0	7 ± 2	10 ± 2	16 ± 4 *
Shunt ratio (Qp:Qs)	1.0 ± 0	1.7 ± 0.7 *	3.3 ± 1.7 *	1.0 ± 0
PVRi (WU.m^2)	0.9 ± 0.4	1.4 ±0.8	5.0 ± 4.0 *	5.1 ± 3.8 *

*indicates $p < 0.01$ compared to controls

mPAP indicates mean pulmonary artery pressure; PWP, pulmonary wedge pressure; Qp, pulmonary blood flow; Qs, systemic blood flow; PVRi, indexed pulmonary vascular resistance.

performed during elective cardiac surgery (n = 36) or for diagnostic purpose (n = 2). The study protocol was approved by the Institutional Medical-Ethical Committee.

Tissue specimens

Tissues were formalin fixed under vacuum and subsequently embedded in paraffin. Serial sections were stained with hematoxylin-eosin (HE), Resorcin Fuchsin and Perls' iron stain to allow for proper identification of morphological structures and for correlation with patterns of immuno-reactivity. Pulmonary arteries were categorized as pre-acinar, when accompanied by a bronchus or bronchiolus and as intra-acinar arteries, when no bronchus or bronchiolus was apparent. Vascular changes were described histologically according to standard criteria, including determination of percentage medial thickness, extension of muscularization to intra-acinar arteries, the presence and character of neointimal lesions and the presence of advanced, characteristic vascular lesions, such as concentric laminar intimal fibrosis and plexiform lesions[11,12].

Immunohistochemistry

The peroxidase-antiperoxidase (PAP) technique was used for staining HSP-70 and HSP-60 and the avidin-biotin complex (ABC) method was used for staining HSP-27. The sections (5 µm) of paraffin-embedded lung tissues were deparaffinized in xylene, degraded in ethanol and rinsed in water. For the PAP method, sections were put in methanol/hydrogen peroxide 3% for 20 min to quench off endogenous peroxidase. For antigen-retrieval, (HSP-60, HSP-27) sections were boiled in citric acid buffer for 10 min in a microwave oven. Slides were allowed to return to room temperature over several hours. Thereafter, sections were processed in 5% phosphate-buffered saline (PBS), incubated with 10% normal rabbit serum (PAP) or normal goat serum (ABC) for 15 min to block non-specific binding. The sections were then incubated overnight with either anti-HSP-60 or anti-HSP-27.

After rinsing once with PBS-Tween 0.2% and once with PBS 5%, the sections were incubated for 30 min with rabbit-anti-mouse serum at dilution of 1:25 (DAKO, Glostrup, Denmark), rinsed with PBS 5% and incubated with mouse peroxidase-anti-peroxidase (PAP)-complex 1:300 (Sigma Chemical Co., St. Louis, MO, USA) for 30 min. Chromogen consisted of 3,3′-diaminobenzidine tetrahydrochloride (DAB)/hydrogenperoxide in PBS 5% for 7 min. Sections were rinsed with water, slightly counterstained with Mayer´s hematoxylin and regraded to xylene by increasing alcohol concentrations.

For HSP-27 detection, sections were incubated with biotinylated anti-immunoglobulins (Multilink, Biogenex, San Ramon, CA, USA), dilution 1:50 with 2% normal goat serum and 2% normal human serum for 30 min, followed by incubation with preformed avidin-biotin-alkaline phospatase complex (ABC, Biogenex, San Ramon, CA, USA) 1:50 for 30 min. Chromogen consisted of naphtol AS-MX-phosphate, new fuchsin, sodium nitrite and levamisole in Tris-HCl at pH 8.0 for 30 min. Sections were slightly counterstained with Mayer's hematoxylin and mounted in an aqueous-based mounting medium. Negative controls consisted of replacement of the primary antibody by non-immunogen serum (Dako, Glostrup, Denmark). Specifity on paraffin material was tested by using mamma carcinoma (for HSP-70 and –27) and synovia from patients with juvenile rheumathoid arthritis (for HSP-60) as positive controls.

Tissue samples were analyzed histologically without knowledge of the clinical data. Intensity of the immunohistochemical staining of pulmonary arterial and bronchiolar cells was assessed semi-quantitatively as absent, weak, moderate and strong independently by two investigators who were kept blinded for the tissue identification. The bronchiolar epithelium which stained consistently strong for all three HSP`s was taken as an internal reference for the grading of staining intensity in the vasculature. Inter-observer variability accounted for 10%. Discrepancies in the grading were no more than one grade and occurred only in the weak to moderate range, in these cases the lower grading was chosen.

Statistical analysis

Values are given as mean ± standard deviation, unless indicated otherwise. For comparison of continuous variables between groups analysis of variance (ANOVA) or Kruskal-Wallis tests were used, where appropriate. For comparison of categorical variables between groups, the chi square and Fisher exact tests were used. P-values <0.05 were considered significant.

Results

Histology

The control group revealed normal overall morphology of the pulmonary arteries both at pre-acinar and intra-acinar levels, but medial thickness of pre-acinar arteries was increased, averaging 9.8 ± 2.8% of the outer vessel diameter (mean ± SD). In group 2 (increased pulmonary flow and normotension), a somewhat lesser degree of medial hypertrophy was present (medial thickness being 6.6 ± 3.0% of outer vessel diameter) and mild cellular intimal hyperplasia of some of the pre-acinar arteries was observed. In patients of group 3 (flow-associated pulmonary hypertension), the broad spectrum of lesions of the pulmonary plexogenic arteriopathy was present, consisting of marked medial hypertrophy (medial thickness 15.8 ± 5.0%), extension of muscularization to intra-acinar vessels in 11 out of 18 patients, cellular intimal proliferation, concentric laminar intimal fibrosis, and plexiform lesions. In group 4, all patients showed extension of muscularization together with marked arterialization of pulmonary veins, which is a prominent feature of pulmonary venous congestion. Medial thickness of pre-acinar arteries was 12.8 ± 3.9%. The distribution of vascular lesions is summarized in Fig. 1

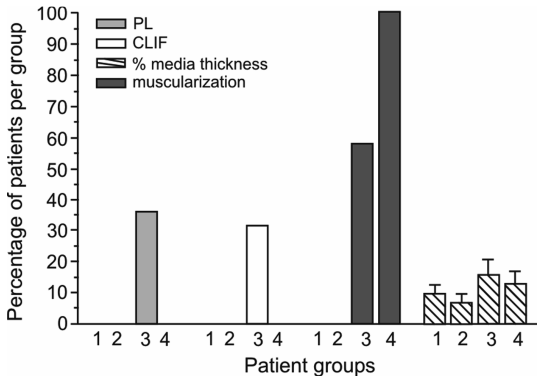

Figure 1—The occurrence of the distinct histological vascular features in the patient groups with different pulmonary hemodynamics.

Cellular localization of HSPs in the pulmonary vasculature

HSP-27

Immunohistochemical analysis revealed evident immunoreactivity to HSP-27 in endothelial and smooth muscle cells of pulmonary arteries at pre-acinar and intra-acinar level in patients and control cases (Fig. 2A). Endothelial and vascular smooth muscle cells of pulmonary arteries with most marked medial hypertrophy and with intimal proliferation consistently showed strong HSP-27 immunoreactivity. When severe concentric intimal hyperplasia was present, pronounced immunostaining of endothelial cells and the media was observed, but only a weak staining of the thick intimal layer (Fig. 2B). In contrast, the cells within plexiform lesions were consistently and strongly HSP-27 positive (Fig. 2C). No differences in staining pattern were observed between the pre-acinar and intra-acinar arteries.

Semi-quantitative assessment of the intensity of immunostaining of endothelial and smooth muscle cells revealed increased immunoreactivity to HSP-27 in patients with abnormal pulmonary hemodynamics, compared to the control subjects (immunohistological grade 2,4 ± 0.6 vs. 1,9 ± 0.3; mean ± SD, p< 0,05). No differences in expression of HSP-27 between patient groups could be demonstrated. When correlated with hemodynamic parameters, the intensity of immunoreactivity to HSP-27 correlated positively with mean pulmonary artery pressure (r = 0.45, p = 0,008) and pulmonary flow (r = 0.41, p = 0.016). However, there was a considerable degree of variability between subjects within the groups. The intensity of immunostaining for HSP-27 did not correlate with histological data, such as medial thickness or the extent of muscularization of intra-acinar arteries.

HSP70

Immunoreactivity to HSP-70 was weak to moderate in endothelial and vascular smooth muscle cells of pre-acinar pulmonary arteries both in patients

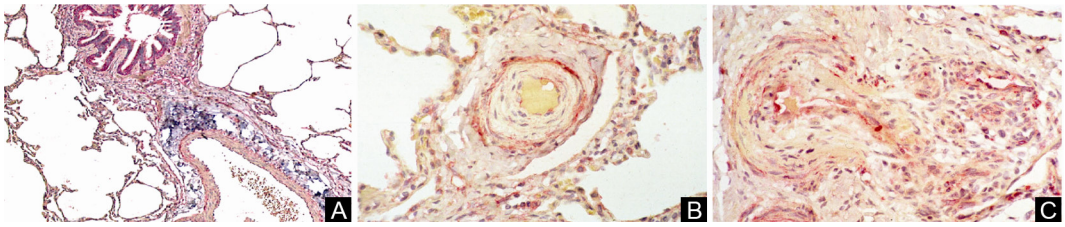

Figure 2—Immunoreactivity to HSP-27 (alkaline phosphatase; red) of a pre-acinar pulmonary artery and the adjacent bronchiolus (**A**, x10). Strong immunoreactivity to HSP-27 of the outer vascular smooth muscle cell layer of a small pulmonary artery with concentric laminar intima fibrosis, whereas almost no staining within the intimal layer in a patient with advanced PPA (**B**, x20). Strong immunoreactivity to HSP-27 in the proliferating cells of a plexiform lesion in a patient with advanced PPA (**C**, x20).

and in control subjects (Fig. 3A). In patients, intra-acinar pulmonary arteries that were most markedly muscularized showed strong immunostaining for HSP-70 (Fig. 3B). In the presence of concentric laminar intimal fibrosis, HSP-70 was barely detectable. In contrast, pronounced immunostaining for HSP-70 was present in the cells of plexiform lesions, comparable to that for HSP-27 (Fig. 3C). The intensity of immunostaining for HSP-70 did not differ significantly between patient groups and did not correlate with any of the hemodynamic parameters of the patients. Intensity of HSP-70 staining correlated negatively with thickness of the media of pulmonary arteries ($r = -0.32$, $p = 0.04$).

HSP60

In striking contrast to the other HSPs that were investigated, expression of HSP-60 was virtually absent in cells of the pulmonary vasculature in controls as well as in patients (Fig. 4A, B). Also, in the advanced lesions of pulmonary plexogenic arteriopathy, such as concentric laminar intimal fibrosis and plexiform lesions, HSP-60 remained below detection level (Fig. 4C).

HSPs localization in cells of the respiratory system

All three HSP subtypes investigated were consistently expressed in bronchial epithelial cells. Intensity of the immunostaining was strong for all three HSPs (Fig. 5A, B, C). In contrast, alveolar epithelium stained negative for HSP-60. Alveolar macrophages and mononuclear cells were occasionally positive for HSP-27 and HSP-70. This was in striking contrast to HSP-60, where staining of alveolar macrophages and mononuclear cells was pronounced in patients as well as controls.

Discussion

In the present immunohistochemical study, we demonstrated immunoreactivity to HSP-27 and HSP-70

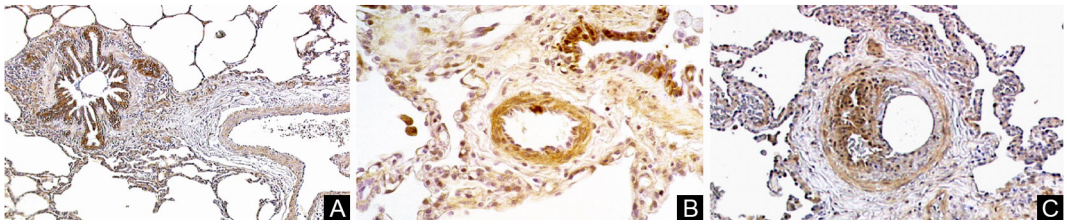

Figure 3—Immunoreactivity to HSP-70 (immunoperoxidase; brown) in pulmonary arteries at pre-acinar (**A**, x10) and intra-acinar level (**B**, x40) in a patient with moderate PPA. Strong immunoreactivity to HSP-70 in a plexiform lesion in a patient with advanced PPA.

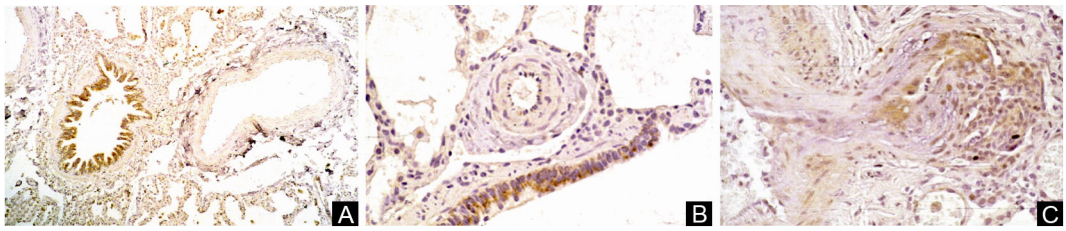

Figure 4—No immunoreactivity to HSP-60 (immunoperoxidase; brown) in cells of the pulmonary vasculature: not in normal pre-acinar pulmonary arterie (**A**, x10), not in pulmonary artery with medial hypertrophy in a patient with early PPA (**B**, x10) and not in a pulmonary artery affected by concentric laminar intimal fibrosis in advanced PPA (**C**, x40).

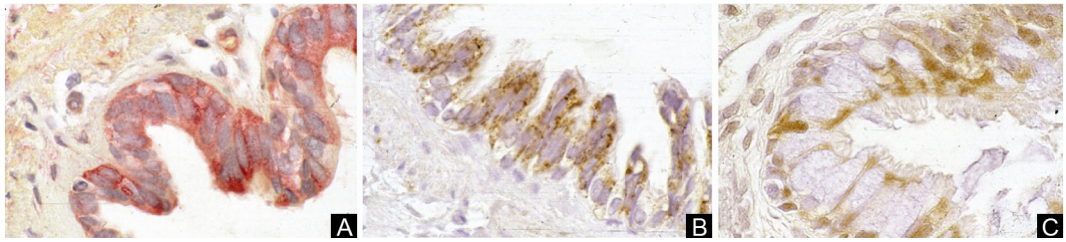

Figure 5—Bronchiolar epithelium: moderate to strong immunoreactivity to HSP-27 (**A**, red, x100), HSP-70 (**B**, brown, x100) and HSP-60 (**C**, brown, x100).

in vascular endothelial and smooth muscle cells of pulmonary arteries. Immunoreactivity to both HSPs was also demonstrated in pulmonary arteries remodelled due to abnormal pulmonary hemodynamics, but in these arteries, the intensity of immunoreactivity to HSP-27 was increased compared to controls and correlated positively with pulmonary artery pressure and magnitude of pulmonary blood flow. The grade of immunoreactivity to HSP-70 did not correlate with pulmonary hemodynamic variables, but correlated negatively with the medial wall thickness of pulmonary arteries. Immunoreactivity to both HSP-27 and HSP-70 was consistently strong in the cells constituting plexiform lesions in patients with pulmonary plexogenic arteriopathy (Fig. 6). In contrast, immunoreactivity to HSP-60 was virtually undetectable in the pulmonary arteries of both control subjects and patients with pulmonary vascular disease in our paediatric patient series.

Although the physiological role of the HSPs and their protective potential under pathophysiological circumstances have been studied in detail in animal models, knowledge of the functions of these proteins in the human pulmonary vasculature is still limited. HSPs are known to be present in the cardiovascular system under normal conditions. HSP-27, a small HSP is normally present in blood vessels, where it is expressed in the cytoplasm of vascular endothelial and smooth muscle cells[13,14]. HSP-27 is involved in regulation of actin microfilament dynamics, where it is supposed to be involved in contractile functions[15]; however, information on its function upon stress in humans is limited. Under physiological conditions, HSP-70 serves the assembly, folding/unfolding and translocation of newly synthesized and the removal of denatured proteins[4].

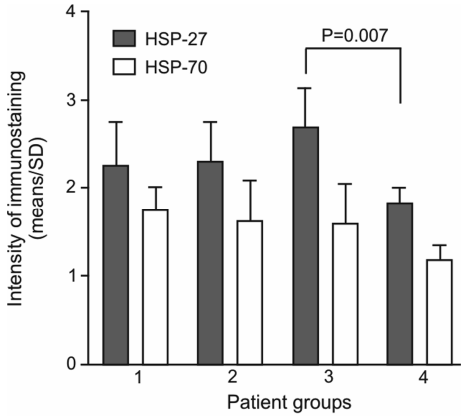

Figure 6—Immunoreactivity to HSP-27 and HSP-70 in media of small pulmonary arteries, as graded semi-quantitatively in the different patient groups. Patients with flow-associated pulmonary hypertension (group 3) revealed significantly higher immunoreactivity to HSP-27 than patients with pulmonary hypertension without increased pulmonary blood flow (group 4)

HSP members are upregulated in various cardiovascular disease states and this induction has been considered to play an important role in maintaining arterial cell homeostasis during vascular remodelling. In the experimental setting, hemodynamic forces, including shear stress, circumferential distension and arterial pressure have been reported to lead to upregulation of members of different HSP families in vascular cells[16-19]. In normal human aorta, intimal and medial cells show a weak homogeneous expression of HSP-70, whereas increased expression has been found in central portions of atheromas around necrotic foci[20].

In rat aorta, both HSP-27 and HSP-70 mRNA and protein synthesis are upregulated in response to acute elevation of arterial pressure[21]. HSP-70 occurs almost exclusively in vascular smooth muscle cells (VSMCs) of the medial layer. Also cyclic strain has been shown to induce HSP-70 expression in arterial VSMCs in the rat and this appears to be upregulated by HSF-1-activation[5,6]. In children with congenital cardiac shunts, vascular endothelial and smooth muscle cells lining the pulmonary arteries are exposed to mechanical stresses due to the abnormal pulmonary circulation[22]. Through only partially unravelled signal transducing pathways, these mechanical forces including increased shear stress and cyclic strain induce the modulation of various vasoactive growth factors, contributing to the remodeling of pulmonary arteries. This remodeling process is characterized by the proliferation of both endothelial and vascular smooth muscle cells, leading to increased vascular muscularization and luminal obstruction by characteristic neointimal and plexiform lesions.

HSP expression and pulmonary hemodynamics

The correlation of HSP-27 expression with both pulmonary artery pressure and increased pulmonary blood flow observed in the present study suggests participation in the adaptation mechanisms of the pulmonary vasculature to unfavourable hemodynamics. Indeed, HSP 27 has been demonstrated experimentally to be involved in morphological changes in vascular endothelial cells, induced by shear stress through activation of the p38/mitogen-activated protein (MAP) kinase activated protein kinase 2 (MAPKAP kinase 2)/HSP25/27 pathway[18]. Both the upregulation

of HSP-27 synthesis and its activation by phosphorylation are considered to play a particular role in the protection of cells against various stresses[18].

In animal models, induction of HSP-70 mRNA, followed by HSP-70 protein production that occurs in systemic vessels due to acute elevation in blood pressure appears to be blunted by chronicity of the hypertension as well as aging[5,6,21]. Long-lasting stressor exposure has been shown to abolish HSP-70 mRNA overexpression in low-flow ischemia experiments *in vitro*[23]. In our patients, the mechanical stresses due to the abnormal pulmonary circulation were obviously not acutely induced. The lack of correlation between HSP70 expression and hemodynamic parameters in the present study might be explained by a blunted response of HSP70 to the relatively longstanding nature of the hemodynamic stressors in these patients.

HSP expression and morphological vascular changes

In our series, the intensity of staining for HSP-70 correlated inversely with the medial thickness of pulmonary arteries. As wall tension and cyclic strain decrease with increasing vessel wall thickness, the diminished HSP-70 expression in thick-walled arteries might reflect reduced mechanical stress inflicted upon the VSMCs in these arteries. In contrast, reduction of the vascular lumen will lead to increased shear stress at the endothelial cells[24]. Indeed, in our study, arteries with severe neointimal lesions showed marked endothelial staining for HSP-27, whereas the neointimal layer of arteries with severe concentric intimal hyperplasia showed reduced expression of HSP27 and HSP70. This was in contrast to the upregulation of HSP70 in coronary intimal hyperplasia after acute balloon-injury in rats and might reflect reduced ability of cells, present in concentric laminar intimal fibrosis, to elicit the stress response[23-25,26]. In patients with PPA, the presence of pronounced concentric laminar intimal fibrosis indicates irreversibility of the vascular disease and poor prognosis[12]. The characteristic plexiform lesions are found mainly at the origin of supernumerary arteries, small thin-walled arteries that originate perpendicular from larger muscular pulmonary arteries. At these specific sites, increased shear stress and mechanical wall stress are present. This increased stress might be associated with the strong immunoreactivity to both HSP-27 and HSP-70 in the cells within the plexiform lesions. On the other hand, plexiform lesions are characterized by abundant, aberrant endothelial cell proliferation and abnormal vasodilatation. Recently, increased expression of VEGF and its receptors has been demonstrated in these vascular lesions[22]. Since HSP-27 and HSP-70 are a prerequisite in proliferation and apoptosis processes and are known to be co-upregulated with VEGF in circumstances of vascular proliferation, the increased expression of HSPs found in plexiform lesions might be associated with the high cellular proliferation rate.

Finally, oxidative injury of the arterial wall has also been proposed as an important mechanism in the development of the characteristic lesions of PPA. We recently demonstrated abundant expression of inducible and endothelial NOS in plexiform lesions in lung biopsies from patients with flow-associated pulmonary hypertension, suggesting local production of large amounts of NO, leading to ROS[27]. Molecular adaptation to oxidative stress includes HSP upregulation[26,27]. HSP upregulation has been demonstrated to inhibit the expression of iNOS in cultured rat PASMC. Since upregulation of HSP-27 and HSP-70 has been shown to increase cellular resistance against oxidative injury, the co-localization of these HSP's and both isoforms of NOS in plexiform lesions found in the present study might represent a protective response to increased oxidative stress.

In the present *in vivo* study, immunoreactivity to HSP-60 could not be demonstrated in the pulmonary vascular cells in normal children, neither in remodeled or diseased pulmonary arteries of children with CHD. This is in contrast to the systemic arterial system in which HSP-60 has been shown to be expressed constitutively and is upregulated in response to hemodynamic stress. HSP-60 has been considered to play an important role in the systemic arterial atherosclosis[20,28]. Although there might be common features in the vascular responses to injury in systemic and pulmonary arteries, our observations indicated that atherosclerosis in systemic arteries and pulmonary obstructive vascular disease are different diseases with a different pathogenesis.

Limitations of the study

The data presented in this study do not allow conclusions regarding causality of the observed relationships between histologic or hemodynamic findings and the presence or absence of the proteins under study. Immunohistochemistry can demonstrate

the presence and, important to this study, the microanatomical localization of a protein, but not its biological activity. Semi-quantitative analysis of immunohistochemical data is an estimation of relative difference in staining intensity and may be hampered by confounding factors, even in the presence of a good internal standard as in our series by consistently uniform staining of the bronchiolar epithelium to all proteins under study. We have taken pains to minimize the possible influence of these factors by rigid standardization of processing of the biopsy material, staining the entire series in one session, and relating the intensity to a standard staining. In this study, the application of Western blots or ELISA to strengthen quantification was not possible, because of the restricted amounts of human lung tissue obtained by lung biopsy.

Conclusion

We conclude that HSP-27 and HSP70 are expressed in remodelling pulmonary arteries of patients with CHD associated with abnormal pulmonary hemodynamics. Furthermore, HSP-27 and HSP-70 are markedly expressed in the cells, forming the advanced complex vascular lesions that ultimately emerge in flow-associated pulmonary hypertension in children with CHDs. Our data indicate that the adaptive cellular mechanisms of the human pulmonary vasculature in response to increased pulmonary flow and pressure include the upregulation of HSP-27 and HSP-70. Their specific roles in the pathogenesis of flow-associated pulmonary hypertension require further investigation. In contrast, HSP-60 could not be detected by immunohistochemistry in pulmonary arteries of control subjects, neither in the course of pulmonary vascular disease in patients with CHD, the latter being in discongruence with systemic arterial disease.

Acknowledgements

R Geiger has been the recipient of a grant (no. J 1282) from the Austrian Science Foundation, Vienna, Austria.

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