S100A4/Mts1 Produces Murine Pulmonary Artery Changes Resembling Plexogenic Arteriopathy and Is Increased in Human Plexogenic Arteriopathy

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S100A4/Mts1 confers a metastatic phenotype in tumor cells and may also be related to resistance to apoptosis and angiogenesis. Approximately 5% of transgenic mice overexpressing S100A4/Mts1 develop pulmonary arterial changes resembling human plexogenic arteriopathy with intimal hyperplasia leading to occlusion of the arterial lumen. To assess the pathophysiological significance of this observation, immunohistochemistry was applied to quantitatively analyze S100A4/Mts1 expression in pulmonary arteries in surgical lung biopsies from children with pulmonary hypertension secondary to congenital heart disease. S100A4/Mts1 was not detected in pulmonary arteries with low-grade hypertensive lesions but was expressed in smooth muscle cells of lesions showing neointimal formation and with increased intensity in vessels with an occlusive neointima and plexiform lesions. Putative downstream targets of S100A4/Mts1 include Bax, which is pro-apoptotic, and the pro-angiogenic vascular endothelial growth factor (VEGF). The increase in S100A4/Mts1 expression precedes heightened expression of Bax in progressively severe neointimal lesions but in non-S100A4/Mts1-expressing cells. VEGF immunoreactivity did not correlate with severity of disease. The relationship of increased S100A4/Mts1 to pathologically similar lesions in the transgenic mice and patients occurs despite differences in localization (endothelial versus smooth muscle cells). (*Am J Pathol* 2004, 164:253–262)

In children with congenital heart disease and a left-to-right shunt, longstanding pulmonary hypertension is associated with arterial remodeling in a process described as plexogenic arteriopathy (reviewed in¹). The earliest changes involve muscularization of peripheral arteries

followed by medial thickening of muscular arteries. Subsequently, there is a decrease in arterial concentration and neointimal formation initially characterized by migration of smooth muscle cells and later associated with fibroelastosis, dilatation complexes, plexiform lesions, and angiomatoid formation in the adventita.^{2–4} Insight into the cellular mechanisms that are associated with the evolution of these changes remains unclear in part due to the lack of a suitable animal model in which the full spectrum of the pathology is replicated. While pulmonary hypertension can be reproduced experimentally in rodents^{5–10} by exposure to hypobaric hypoxia or by injection of the toxin monocrotaline, structural remodeling of pulmonary arteries is limited to abnormal muscularization of distal vessels, medial hypertrophy of muscular arteries, and loss of arteries. Features related to neointimal thickening and the evolution of the plexogenic lesion are lacking.

It is therefore of interest that when Ambartsumian et al¹¹ created a transgenic mouse that overexpressed S100A4/Mts1 in all tissues, a subset (approximately 5%) developed pulmonary arterial changes resembling plexogenic lesions. S100A4/Mts1 is a member of the S100 family of calcium-binding proteins whose biological role is poorly understood. The S100 family members as a group are involved in numerous physiological functions including cell proliferation and differentiation, extracellular signal transduction, intercellular adhesion, and cell motility.¹² The S100A4/Mts1 gene was initially isolated as a gene differentially expressed in highly metastatic mouse mammary adenocarcinoma cells.¹³ Its expression confers a metastatic phenotype^{14,15} and correlates with an advanced stage of human tumors.¹⁶ It has been hypothesized that the S100A4/Mts1 protein participates in tumor progression and metastasis by affecting the motility of tumor cells possibly by controlling phosphorylation

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of the heavy chain of non-muscle myosin II.^{17,18} The association of S100A4/Mts1 with cellular stress fibers, F-actin, and tropomyosin may also be important in regulating cytoskeletal dynamics and cell motility.¹⁹

S100A4/Mts1 also interacts with p53 and modulates its phosphorylation, DNA binding capacity, and transcriptional activity.²⁰ The binding of S100A4/Mts1 to p53 may indicate a role for S100A4/Mts1 in the process of apoptosis. Tumor suppressors, such as p53, exert an important effect in preventing the excess proliferation of cells and act through downstream cell cycle proteins such as Bax, which has a pro-apoptotic function, and Bcl-2 which inhibits apoptosis.²¹

In addition to interacting with intracellular proteins S100A4/Mts1 may also exert an effect extracellularly as a secreted protein.^{22,23} One of its extracellular effects appears to be related to angiogenesis involved in tumor progression.²³ Increased vascular endothelial growth factor (VEGF) expression has also been observed in the lungs of rats who develop pulmonary hypertension secondary to hypoxia²⁴ and in the endothelial proliferative response associated with the plexiform lesions of advanced pulmonary hypertension in humans.^{25,26}

The presence of plexiform lesions in the pulmonary arteries of a mouse overexpressing S100A4/Mts1 suggests that this protein may be important in the pathogenesis of clinical plexogenic arteriopathy and pulmonary hypertension. We therefore characterized the plexogenic lesions in the murine pulmonary arteries immunohistochemically and contrasted them with human disease. Specifically, we examined the pulmonary arteries from children with congenital heart defects who had undergone surgical lung biopsy for staging of their pulmonary hypertension. Quantitative immunohistochemistry for S100A4/Mts1 was carried out and, to explore possible mechanisms of action for S100A4/Mts1, the expression of Bax, Bcl-2, and VEGF was also examined. We detected S100A4/Mts1 in the endothelial cells of plexogenic lesions of mice and in the smooth muscle cells of the neointima in human pulmonary arteries. The expression of this protein was most intense in obstructive lesions and preceded detection of Bax, which was limited to endothelial and non-S100A4/Mts1-expressing neointimal cells. S100A4/Mts1 immunoreactivity did not correlate with that of VEGF, which we found unchanged in lesions of increasing severity in these patients with congenital heart defects.

Materials and Methods

Construction of S100A4/Mts1 Transgenic Mice

Transgenic mice were generated as previously described on a CBA $\times$ C57/Black-6 background.¹¹ Briefly, strains of transgenic mice were created with the 450-bp cDNA sequence of the murine *s100A4/mts1* gene under the control of the 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase gene promoter.

Patients Studied and Histological Assessments

Lung biopsy tissue was obtained from patients (age range, 10 months to 13 years) in whom, on the basis of age and hemodynamic assessment, the severity of pulmonary vascular changes was of potential prognostic concern with regard to surgical correction. The majority of patients in whom lung biopsy material was analyzed had an isolated atrial septal defect (ASD), ventricular septal defect (VSD), or a complete atrioventricular septal defect (AVSD). One patient had a double-outlet right ventricle (DORV), another had tetralogy of Fallot (TOF), and one other had a univentricular heart. The lung biopsy grade assigned to each patient and their clinical information are shown in Table 1.

Consecutive specimens were selected to include the range of vascular pathology. Use of archived patient tissue samples for immunohistochemical study was approved by the Research Ethics Board at The Hospital for Sick Children, Toronto, Canada. The protocol used in obtaining the lung biopsy tissue has been previously described^{2,27} and involved clamping the lung tissue in the inflated state in the right upper lobe to include a section of approximately $1 \times 1 \times 2$ cm. The tissue was then fixed in the inflated state in 1% glutaraldehyde 4% formaldehyde for 10 minutes, sections were cut perpendicular to the clamp marks and were then fixed in 10% neutral-buffered formalin and subsequently embedded in paraffin.

All lung biopsy sections were graded after Movat pentachrome staining using morphometric² and Heath-Edwards criteria³ as follows: morphometric grade A, extension of muscle into small peripheral arteries that are normally non-muscular or, in addition, there is a mild increase in wall thickness of the normally muscular arteries (less than 1.5 times normal); grade B, as in grade A, there is increased extension of muscle but, in addition, there is more severe medial hypertrophy of normally muscular arteries; mild grade B, medial thickness is greater than 1.5 but less than 2 times normal; severe grade B, medial thickness is more than twice normal; grade C, in addition to the findings of severe grade B, arterial concentration and usually artery size are reduced; Heath-Edwards grade I, medial hypertrophy; grade II, cellular intimal proliferation; grade III, occlusive neointimal fibrosis; grade IV, dilatation complexes; grade V, angioma formation.

Immunohistochemistry Assessment

Immunohistochemistry studies were carried out on both mouse and human lung tissue using S100A4/Mts1,²⁸ Bax (rabbit polyclonal antibody raised against amino acids 1–171 of mouse Bax protein; Santa Cruz Biotechnology, Santa Cruz, CA), This antibody does not cross-react with other S-100 proteins as shown in sections taken from Mts1 knock-out mice which are completely negative (unpublished observations). Bcl-2 (mouse monoclonal antibody corresponding to amino acids 1–205 of human Bcl-2; Santa Cruz Biotechnology) and VEGF (rabbit poly-

Table 1. Clinical Data and Lung Biopsy Findings of Human Patients

Number	Age	Sex	Diagnosis	Lung biopsy grade
1	2 y 2 m	M	ASD	Heath-Edwards 0-I Morphometric A
2	13 y 4 m	F	ASD, cardiomyopathy	Heath-Edwards I Morphometric B
3	1 y 7 m	M	DORV, VSD	Heath-Edwards 0-I Morphometric B
4	1 y 5 m	M	ASD	Heath-Edwards I Morphometric B
5	1 y 1 m	M	AVSD	Heath-Edwards I-II Morphometric B-C
6	1 y 4 m	M	Single LV, absent mitral valve, s/p PA band	Heath-Edwards II Morphometric B
7	7 m	M	VSD × 2, ASD, PDA	Heath-Edwards II Morphometric B-C
8	1 y 7 m	M	VSD	Heath-Edwards II Morphometric C
9	10 m	M	AVSD	Heath-Edwards III Morphometric C
10	2 y 3 m	F	VSD	Heath-Edwards III Morphometric ND
11	1 y 7 m	M	AVSD	Heath-Edwards III-IV Morphometric C
12	3 y 3 m	F	AVSD	Heath-Edwards IV Morphometric C
13	3 y 3 m	M	TOF, absent pulmonary valve, hemitruncus	Heath-Edwards IV Morphometric C
14	5 y 3 m	F	AVSD	Heath-Edwards IV Morphometric ND

Number refers to the arbitrary designation of each patient; y, year; m, month; ASD, atrial septal defect; DORV, double-outlet right ventricle; VSD, ventricular septal defect; AVSD, atrioventricular septal defect; LV, left ventricle; s/p, status post; PDA, patent ductus arteriosus; TOF, tetralogy of Fallot; ND, not determined. Grading of biopsies is described in Material and Methods.

clonal recognizing amino acids 1–140 of human VEGF; Santa Cruz Biotechnology, Santa Cruz, CA). Tissue sections (7 μ m) were deparaffinized in xylene and sequentially rehydrated in graded alcohols (100 to 50%) and water. Slides were then microwaved for 10 minutes in 10 mmol/L citrate (pH 6.0) and after cooling equilibrated in phosphate-buffered saline (PBS, pH 7.6) before endogenous peroxidase was blocked by treating sections with 0.3% (v/v) hydrogen peroxide at room temperature for 30 minutes. Sections were then rinsed in PBS containing 1% (v/v) fetal bovine serum (FBS) before the primary antibody was added. The primary antibody was diluted in PBS containing 10% (v/v) FBS to a final concentration of 1:400 for S100A4/Mts1, 1:10 for Bax and Bcl-2, and 1:25 for VEGF. Negative controls substituted PBS and 10% FBS or irrelevant antibody for the primary antibody. The specificity of the S100A4/Mts1 antibody has been demonstrated previously.²⁸ Sections were incubated in the presence of the primary antibody overnight at 4°C. Thereafter, sections were washed in PBS and immunohistochemical detection of S100A4/Mts1, Bax, Bcl-2, and VEGF was carried out using species-specific reagents based on the avidin-biotin peroxidase method (Vector Laboratories, Burlingame, CA).

Immunohistochemistry for α -smooth muscle actin (horseradish peroxidase-conjugated mouse monoclonal IgG2, clone IA4, undiluted; Dako, Glostrup, Denmark) and von Willebrand factor (horseradish peroxidase-coupled rabbit monoclonal, undiluted; Dako) were carried out following the protocol described by the manufacturer.

For detection of proliferating cell nuclear antigen (PCNA), samples were incubated with a peroxidase-conjugated anti-mouse IgG prepared according to the manufacturer's instructions (Amersham International). All immunostained sections were then exposed to 0.5 mg/ml diaminobenzadine (DAB) diluted in 50 mmol/L Tris buffer (pH 7.6), containing 0.02% hydrogen peroxide and lightly counterstained with hematoxylin, or eosin for PCNA.

For S100A4/Mts1, arteries were examined from 14 individual patients and then pooled into four groups based on the pathological classification: group 1, patients 1 to 5 (Heath Edwards grade 0-I, I, and I-II, morphometric grades A, B, and B-C); group 2, patients 6 to 8 (Heath Edwards grade II, morphometric grades B and C); group 3, patients 9 to 11 (Heath Edwards grade III and III-IV, morphometric grade C); and group 4, patients 12 to 14 (Heath Edwards grade IV, morphometric grade C). For analysis of Bax and VEGF, expression arteries were examined from eight individual patients: group 1, patients 1 and 3; group 2, patients 6 and 7; group 3, patients 9 and 10; and group 4, patients 12 and 13. Photomicrographs of all arteries accompanying terminal and respiratory bronchioli in each section (4 to 36 arteries per section) were saved in Photoshop (Version 5.5, Adobe Systems Incorporated) then analyzed for intensity of DAB staining using Image-ProPlus (Version 3.0.1, Media Cybernetics). Measurement of DAB staining was carefully isolated to pulmonary arteries. The intensity of staining was measured for each individual artery and then data were

Figure 1. Representative photomicrographs of mouse lung tissue. **A:** Movat pentachrome staining of lung tissue from a transgenic mouse engineered to overexpress S100A4/Mts1 showing an occlusive neointimal lesion consistent with plexogenic arteriopathy. **B:** A representative Movat pentachrome-stained pulmonary artery from an age-matched, non-transgenic mouse shows a normal thin walled vessel with a patent lumen. **C:** Lung tissue section stained with hematoxylin and eosin from a transgenic mouse engineered to overexpress S100A4/Mts1, demonstrates increased perivascular inflammatory cells. **D:** Immunoperoxidase staining with an antibody to von Willebrand factor in a vessel with extensive neointimal formation from a mouse engineered to overexpress S100A4/Mts1 shows that the cells lining the lumen are endothelial. **E:** Plexogenic-like lesion in a S100A4/Mts1 transgenic mouse stained with α -actin shows numerous neointimal smooth muscle cells. Original magnification, $\times 40$. Bar, 50 μ m.

pooled into the four groups based on histological classification as described above. For S100A4/Mts1, 35 to 52 arteries per group were analyzed and for Bax and VEGF, 24 to 44 arteries per group were analyzed. The mean staining intensity was determined for each group and all data are presented as mean $\pm$ SEM. Statistical significance was evaluated by analysis of variance, and post hoc comparisons were made with the Fisher's protected least significant difference test.

Results

Histology of Murine Plexiform Lesions

In approximately 5% of transgenic S100A4/Mts1-overexpressing mice the pulmonary arteries demonstrate plexiform-like lesions (Figure 1A). There is intimal thickening with virtual occlusion of the vessel lumen, and thinning of the muscular media. These changes are not seen in

Figure 2. Representative photomicrographs of lung tissue from diseased, transgenic mice engineered to overexpress S100A4/Mts1. **A:** Immunoperoxidase staining for S100A4/Mts1 in pulmonary arteries with occlusive neointimal lesions shows expression in endothelial cells and in some periadventitial cells which appear by morphology to be inflammatory. **B:** Immunoperoxidase staining for Bax is present in some of the periadventitial cells surrounding an artery with an occlusive neointimal lesion as indicated by the **arrows**. **C:** Pulmonary artery stained for PCNA. **Arrows** indicate an occasional positive cell within the neointima of the lesion but most are present in the periadventitia. **D:** Staining for VEGF reveals the absence of positive cells within the artery. Original magnification, $\times 40$.

vessels from age-matched, non-transgenic control mice (Figure 1B) or non-diseased transgenic mice. There also appears to be extensive inflammation surrounding the vessels affected with pulmonary vascular disease in transgenic mice as evidenced by the presence of numerous perivascular inflammatory cells (Figure 1C). These cells were not noted in the lungs of non-diseased transgenic mice or non-transgenic control mice. Immunohistochemical staining for von Willebrand factor and smooth muscle α -actin was performed to characterize the cellular composition of the mouse lesions. Von Willebrand factor was localized to endothelial cells lining the obstructed lumen of the pulmonary artery (Figure 1D) and the majority of the cells of the neointima of the plexiform lesion were positive for smooth muscle α -actin (Figure 1E).

S100A4/Mts1, Bax, PCNA, and VEGF in Mouse Lung

Immunohistochemistry was used to examine the expression of S100A4/Mts1 in the pulmonary arteries of dis-

eased transgenic S100A4/Mts1-overexpressing mice. S100A4/Mts1 protein was present in the endothelial cells of the plexiform lesion as well as in a few surrounding cells which appeared to be inflammatory in nature (Figure 2A). In an attempt to ascertain whether expression of S100A4/Mts1 could be related to its ability to induce Bax or VEGF, further immunohistochemical determinations were made. As shown in Figure 2B, Bax was evident in a few cells surrounding the artery but not in the cells of the plexigenic lesion. To determine whether there was clonal expansion of a cell population resistant to Bax expression, staining for PCNA was carried out. However, there appeared to be only a few PCNA-positive cells, mostly within the murine adventitia (Figure 2C). Immunodetection of VEGF, an indicator of angiogenesis, was relatively weak, with staining of the some cells in the lung but no positive cells within the artery (Figure 2D). The relative absence of VEGF in mouse lung and pulmonary vasculature contrasts sharply with the intense immunoperoxidase staining for VEGF observed in mouse skin that was used as a positive control (data not shown).

Figure 3. Representative photomicrographs of human lung biopsy tissue after immunoperoxidase staining for S100A4/Mts1. **A:** Vessel from patient graded 0-IB showing normal pulmonary artery with no immunodetectable S100A4/Mts1. **B:** Vessel showing a typical grade IB lesion with severe medial hypertrophy but without immunoreactivity for S100A4/Mts1. **C:** An artery from a patient with grade IVC disease with occlusive neointimal proliferation and strong positive staining for S100A4/Mts1 particularly in the intima compared to the media of the vessel. S100A4/Mts1 was not detected in all cells and appears to be localized in a subpopulation of intimal cells. **D:** A plexogenic lesion from a patient with grade IVC disease with staining of the smooth muscle cells and sparing of the endothelial cells (arrows). Immunoreactivity for S100A4/Mts1 was present in the lung parenchyma at a similar level in all grades of pulmonary vascular disease. Original magnification, $\times 40$.

Expression and Localization of S100A4/Mts1 in Patient Samples

When we examined the expression of S100A4/Mts1 in lung biopsies taken from children with pulmonary hypertension secondary to congenital heart disease we found that expression of S100A4/Mts1 increases with the severity of pulmonary vascular lesions (Figures 3 and 4). In the first group of patients (Heath Edwards grades 0-I, morphometric grades A and B), S100A4/Mts1 immunoreactivity is minimal in the pulmonary arteries (Figure 3, A and B) but with intimal proliferation and obliterative disease there is increased intensity of immunostaining for S100A4/Mts1, predominantly in the neointima (Figure 3C). When there are plexiform lesions (Heath Edwards grade IV) there is very intense immunostaining for S100A4/Mts1 in the intima (Figure 3D). Immunoreactivity for S100A4/Mts1 is present in the lung parenchyma at a similar level in all grades of pulmonary vascular disease. In contrast to the mouse, S100A4/Mts1 appears to be

expressed in the smooth muscle cells with sparing of the endothelial cells (Figure 3D). Figure 4 shows quantitative analysis of immunoreactivity for S100A4/Mts1 in the arteries of various groups of patients with significant increases noted in groups II and III *versus* group I and a further increase in intensity of immunoreactivity in group IV patients.

We also investigated the relationship between S100A4/Mts1 and Bax.²⁰ As shown in Figures 5 and 6 there is a significant increase in the level of Bax immunoreactivity in the groups with more severe grades of pulmonary hypertension. In these human plexogenic lesions, Bax is localized to endothelial cells and non-S100A4/Mts1 expressing medial smooth muscle cells (Figure 5). Note a vessel with medial hypertrophy (Figure 5A) and no immunoreactivity compared with a vessel from a group IV patient (Figure 5C) where the Bax immunoreactivity co-distributes with cells expressing von Willebrand factor but is not present in the smooth muscle cells that are positive for S100A4/Mts1. Immunoreactivity for Bax is significantly

Figure 4. The expression of S100A4/Mts1 in pulmonary arteries from children with congenital heart disease increases as the severity of pulmonary hypertension increases. The intensity of immunohistochemical staining for S100A4/Mts1 in lung biopsy tissue was quantified as described in Materials and Methods. Groups II, III, and IV are significantly different from group I (P value <0.05 compared to group I). Graphs represent mean values $\pm$ SEM.

greater in groups III and IV compared with patients from groups I and II (Figure 6). Whereas Bax is pro-apoptotic, Bcl-2 is thought to be anti-apoptotic. Immunohistochemistry was used to assess Bcl-2 expression in both human and mouse lung tissue but expression of Bcl-2 was not detectable in these tissues although it was seen in human tonsillar tissue which was used as a positive control.

VEGF expression was weak and relatively constant across all grades (I-IV) of pulmonary hypertension in all groups of patients and localized predominantly to isolated cells within the arterial media (data not shown). There were no significant quantitative differences when comparing vessels in the different groups (Figure 7).

Discussion

The presence of plexiform-like lesions in the pulmonary arteries of mice engineered to overexpress S100A4/Mts1 is intriguing and is the only murine model of this severe phenotype. The lesions observed in the mouse lungs resemble those seen in the end stages of pulmonary hypertension. Earlier stages (eg, medial thickening) were not observed consistent with our previous studies of advanced vascular disease² and the mice examined either had lesion-free lungs or demonstrated this plexiform arteriopathy. The similar minimal immunodetection of S100A4/Mts1 in human lungs with no pulmonary hypertension or with only the early stages of disease, suggests

that S100A4/Mts1 is not involved in the initial response to elevated pulmonary pressures, such as medial hypertrophy, but may be of functional significance in the development of more severe arterial lesions seen in end-stage disease.

The pulmonary arterial lesions noted in the mice are composed of the same cells observed in the human lesions, mainly smooth muscle and endothelial cells. Therefore these cells are not tumor cells that have metastasized to the lung. However, in mice we noted that S100A4/Mts1 is expressed in the endothelial cells whereas in human vessels S100A4/Mts1 is expressed in the intimal smooth muscle cells. Immunoreactivity for S100A4/Mts1 protein was localized to the pulmonary artery endothelial cells of mice that developed abnormal plexiform-like lesions in association with an increased number of inflammatory cells. This peri-arterial inflammation was not present in normal control mice nor was it detected in transgenic, non-diseased mice. The presence of numerous inflammatory cells surrounding affected vessels in diseased, transgenic mice suggests that an inflammatory or infectious insult triggers cellular events that lead to the development of plexogenic arteriopathy in these mice. We do not, however, have further analyses in these mice to indicate the severity of the pulmonary hypertension or whether there was evidence of systemic vascular disease, except that analyses of the liver revealed no vascular abnormalities.

The expression of S100A4/Mts1 in mice and humans may be related to translation or stability of the protein.^{11,29} Alternatively, the immunoreactivity observed was related to the product of transcription of the endogenous and heterologous promoter in an endothelial cell-specific manner. For example, it has been previously observed that lipopolysaccharide can induce *s100A4/mts1* gene expression in murine macrophages³⁰ and that phorbol 12-myristate 13-acetate or serum can induce S100A4/Mts1 in mouse fibroblasts.³¹

The similar histological phenotype in both mouse and human tissues despite differences in localization of S100A4/Mts1, leads us to speculate that the development of disease may be related to the secreted protein. S100A4/Mts1 has been shown to be secreted^{22,23} and could act, in either a paracrine or autocrine fashion, on smooth muscle cells to increase their proliferation and migration or transdifferentiation³² in association with development of the plexogenic arteriopathy.

A recent report suggests that S100 proteins, released from inflammatory cells at sites of inflammation, could act as ligands for the receptor for advanced glycation end products (RAGE) which when activated, induce pro-inflammatory adhesion molecules, cytokines, tissue-destroying elastases, and matrix metalloproteinases (MMPs).³³ This mechanism is consistent with results from our laboratory showing the importance of induction of serine elastase and MMPs and subsequent alterations in the extracellular matrix in the pathogenesis of pulmonary hypertension.^{4,34–37}

Other sequelae of increased S100A4/Mts1 may be relevant to the pathophysiology of pulmonary hypertension. *In vitro* studies suggested that an association between S100A4/Mts1 with p53 and Bax²⁰ may lead to

Figure 5. Representative photomicrographs of human lung biopsy tissue. **A:** Note the relative absence of Bax in a pulmonary artery graded 0–1B. **B:** A grade IVC vessel stained with von Willebrand factor to demonstrate endothelial cells within the plexogenic lesion. **C** and **D:** Serial sections comparing the distribution of Bax (**C**) and S100A4/Mts1 (**D**) expression in grade IVC disease. Bax expression is noted in the endothelial cells and non-S100A4/Mts1-expressing medial smooth muscle cells (**arrows**). S100A4/Mts1 expression occurs primarily in the intima. Original magnification, $\times 40$.

growth of apoptosis-resistant cells.⁹ In our studies we observed that increased S100A4/Mts1 expression preceded an increase in Bax immunoreactivity. However, expression of S100A4/Mts1 and Bax occurred in distinct cell populations. Therefore, if S100A4/Mts1 is influencing Bax expression, we can only speculate that this would only be through a paracrine effect. Since previous work in our laboratory²⁷ indicated that apoptosis is not a prominent feature of vascular lesions, increased intensity of immunoreactivity for the pro-apoptotic factor Bax could reflect increased levels of the protein and resistance to apoptosis.³⁸

The observation that S100A4/Mts1 has a significant stimulatory effect on angiogenesis²³ and reports that VEGF is up-regulated in pulmonary hypertension^{24–26} prompted our investigation into the expression of VEGF both in diseased transgenic mouse lung and in lung biopsy tissue from children with pulmonary hypertension secondary to congenital heart disease. In the mouse no significant enhanced immunostaining was seen for VEGF within the plexiform-like lesions. In human tissue, VEGF was detected at similar levels of intensity despite increas-

ing severity of disease and was found primarily in smooth muscle cells. This is at variance with studies by Hirose et al²⁵ in which increased VEGF immunoreactivity was observed within plexiform lesions but in keeping with the report by Geiger et al²⁶ The exact role and significance of VEGF in pulmonary hypertension therefore remains unclear at this time.

In summary, we have demonstrated that a mouse engineered to overexpress the calcium-binding protein S100A4/Mts1 develops severe obliterative and plexiform-like pulmonary vascular disease when there is evidence of inflammation. Patients with severe neointimal formation progressing to plexiform lesions also show marked immunoreactivity for S100A4/Mts1 in smooth muscle cells. The development of a similar phenotype with differential cellular expression is consistent with the speculation that S100A4/Mts1 exerts its effect as a secreted protein. Understanding the mechanism of action of S100A4/Mts1 could help explain why some children with pulmonary hypertension progress onto end-stage disease whereas others do not.

Figure 6. There is increased expression of Bax in the pulmonary arteries from children with pulmonary hypertension secondary to congenital heart disease in severe pulmonary hypertension (grades III and IV). The intensity of immunohistochemical staining for Bax in lung biopsy tissue was quantified as described in Materials and Methods. Groups III and IV are significantly different from group I (P value <0.05). Graphs represent mean values $\pm$ SEM.

Figure 7. Expression of VEGF in the pulmonary arteries from children with pulmonary hypertension secondary to congenital heart disease remains relatively constant despite increasing severity of disease. The intensity of immunohistochemical staining for VEGF in lung biopsy tissue was quantified as described in Materials and Methods. Graphs represent mean values $\pm$ SEM.

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