Complete reversal of fatal pulmonary hypertension in rats by a serine elastase inhibitor

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Progression of pulmonary hypertension is associated with increased serine elastase activity and the proteinase-dependent deposition of the extracellular matrix smooth muscle cell survival factor tenascin-C (refs. 1,2). Tenascin-C amplifies the response of smooth muscle cells to growth factors[3], which are also liberated through matrix proteolysis[4]. Recent organ culture studies using hypertrophied rat pulmonary arteries have shown that elastase inhibitors suppress tenascin-C and induce smooth muscle cell apoptosis[5,6]. This initiates complete regression of the hypertrophied vessel wall by a coordinated loss of cellularity and extracellular matrix. We now report that elastase inhibitors can reverse advanced pulmonary vascular disease produced in rats by injecting monocrotaline, an endothelial toxin. We began oral administration of the peptidyl trifluoromethylketone serine elastase inhibitors M249314 or ZD0892 21 days after injection of monocrotaline. A 1-week treatment resulted in 92% survival, compared with 39% survival in untreated or vehicle-treated rats. Pulmonary artery pressure and muscularization were reduced by myocyte apoptosis and loss of extracellular matrix, specifically elastin and tenascin-C. After 2 weeks, pulmonary artery pressure and structure normalized, and survival was 86%, compared with 0% in untreated or vehicle-treated rats. Although concomitant treatment with various agents can reduce pulmonary hypertension[7], we have documented complete regression after establishment of malignant monocrotaline-induced disease.

We injected rats with the alkaloid toxin monocrotaline to induce an endothelial injury leading to severe pulmonary hypertension 21 days later. Then we gavage-fed the rats one of two peptidyl trifluoromethylketone serine elastase inhibitors, ZD0892 or M249314, or their respective administration vehicles. There was an increase in elastase inhibitory activity after M249314 over the first 6 hours, which decreased over the next 6 hours but remained elevated before the next dose (Fig. 1*a*). The monocrotaline-induced elastolytic activity in the lungs of vehicle or untreated rats, which was increased 900% above that in normal lung tissue at 21 days and a further 800% at 28 days, was decreased to levels obtained with normal saline controls after administration of ZD0892 or M249314 (Fig. 1*b*).

Inhibition of serine elastase activity reversed the progressive mortality of untreated or vehicle-treated rats, which started at 23 days after injection (Fig. 1*c*). Only 39% of untreated or vehicle-treated rats survived to 28 days after injection of monocrotaline, whereas 92% of rats treated for 1 week with either elastase inhibitor were alive at 28 days (only one died in each group). When treated with the elastase inhibitor M249314 for 2 weeks (ending on day 35), the survival was still 86% (only one additional animal died), but by 30 days all the untreated rats had died.

That the cause of death in the vehicle-treated or untreated group was related to progressive pulmonary hypertension and that survival represented reversal of the disease were both reflected in hemodynamic assessments. In untreated or vehicle-treated rats, there was an increase in mean pulmonary artery pressure of 200%, from about 19 mm Hg to about 38 mm Hg by day 21 after treatment with monocrotaline, and a further increase to about 44 mm Hg over the next week (Fig. 1*d*). In contrast, there was a progressive reduction in pulmonary artery pressure in conjunction with treatment with elastase inhibitor, such that by day 35, values were not significantly different from normal. This represented a selective reduction in pulmonary artery pressure, as systemic arterial pressures were unchanged (data not shown). Reduced pulmonary artery pressure was associated with arrest in the progression of right ventricular hypertrophy (Fig. 1*e*). In patients, after correction of congenital heart disease, reversal of right ventricular hypertrophy lags behind a reduction in right ventricular pressure[8], and future long-term studies will be necessary to confirm that this also occurs in our model.

We next confirmed that the mechanism causing reversal of progressive pulmonary hypertension and arrest of right ventricular hypertrophy was related to the regression of severe structural changes in peripheral pulmonary arteries. We quantified muscularization of distal pulmonary arteries by barium–gelatin perfusion, a technique that distends vessels evenly and results in reproducible assessments. Most pulmonary arteries 15–50 μm in diameter were normally non-muscular, as in saline-injected rats, with only a small proportion (about 17%) being muscularized (Fig. 2*a*). This muscularized subpopulation increased to about 65% by 21 days and to about 84% by day 28 in rats treated with monocrotaline. Treatment with elastase inhibitors induced progressive regression of this abnormal peripheral vascular muscularization, reducing the muscularized population to within the normal range (about 21%) by day 35. In addition, normally muscularized pulmonary arteries showed regression of medial hypertrophy (Fig. 2*b* and *c*). We quantified these changes in wall thickness for arteries in the ranges of 50–100 μm and 101–200 μm in diameter (Fig. 2*d* and *e*). Both populations of muscular pulmonary arteries showed an increase in thickness of about 800% after injection of monocro-

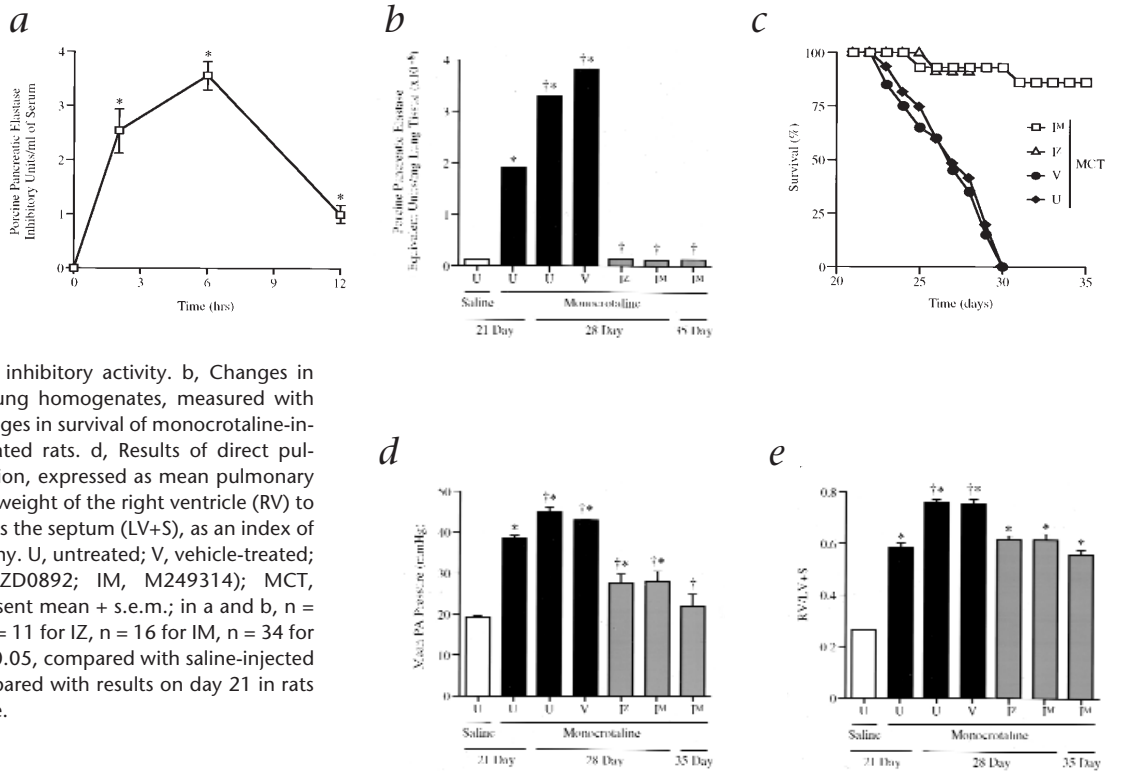

Fig. 1 Serine elastase inhibition induces survival by reversal of pulmonary hypertension. Inhibition of monocrotaline-induced elastolytic activity results in survival, and is related to a reversal of pulmonary hypertension and arrest in progression of right ventricular hypertrophy. a, Elastase inhibitory activity assays of circulating plasma inhibitory activity. b, Changes in elastolytic activity in rat lung homogenates, measured with fluorogenic elastin. c, Changes in survival of monocrotaline-injected, treated and untreated rats. d, Results of direct pulmonary artery catheterization, expressed as mean pulmonary artery pressure. e, Ratio of weight of the right ventricle (RV) to that of the left ventricle plus the septum (LV+S), as an index of right ventricular hypertrophy. U, untreated; V, vehicle-treated; I, inhibitor-treated (IZ, ZD0892; IM, M249314); MCT, monocrotaline. Data represent mean + s.e.m.; in a and b, n = 3; in d and e, n = 6; in c, n = 11 for IZ, n = 16 for IM, n = 34 for V, and n = 27 for U. *, $P < 0.05$, compared with saline-injected controls; †, $P < 0.05$, compared with results on day 21 in rats treated with monocrotaline.

taline, which was progressively reversed by the elastase inhibitors, reaching normal levels by day 35. During progressive monocrotaline-induced pulmonary hypertension, there was a continuous reduction in the number of peripheral pulmonary arteries, assessed as an increasing ratio of alveoli to arteries[1] (Fig. 2*f*). The increase in the ratio of alveoli to arteries (reduction in vessels) of more than 200% after injection of monocrotaline has been attributed to closing-off and resorption of peripheral vessels, which could result from monocrotaline-induced endothelial injury and proteolytic degradation of basement membranes. After 1 week of treatment with elastase inhibitors (day 28), this ratio was substantially lower than that of time-matched untreated or vehicle-treated rats. As there was no substantial decrease below the values obtained on day 21, it is possible, however, that new vessel growth is impeded by elastase inhibitor treatment if endothelial cell migration is suppressed[9]. However, the progressive derangement of endothelial cells, including loss, seen by ultrastructural analysis in rats injected with monocrotaline was arrested by treatment with elastase inhibitor, and endothelial structure began returning to normal (data not shown, but consistent with published reports[10]). That the persistent reduction of small vessels is consistent with a return to normal pulmonary artery pressure is in keeping with a published study[11].

We investigated the cellular mechanism involved in the reversal of the monocrotaline-induced peripheral arterial muscularity with elastase inhibitors on non-distended vessels. Consistent with published reports[5,6], deposition of the matrix component tenascin-C, associated with medial hypertrophy at 21 days after injection of monocrotaline, correlated directly with evidence of vascular cell proliferation, that is, cells positive for the proliferating cell nuclear antigen (PCNA), and inversely with apoptosis, which remained relatively absent (Fig. 3*a*, *e*, *i*, *m*, *q* and *r*). Expression of tenascin-C and PCNA was increased both in untreated and vehicle-treated rats at 28 days (about 180%, relative to day 21), whereas apoptosis was absent or minimal (Fig. 3*b*, *c*, *f*, *g*, *j*, *k*, *n*, *o*, *q* and *r*). Regression of medial hypertrophy after 1 week, at the 28-day time point, induced by elastase inhibitor was attributed to suppression of tenascin-C and a concomitant induction of apoptosis of about 1,000% and a suppression of PCNA of about 500% (relative to values on day 21) (Fig. 3*d*, *h*, *l*, *p*, *q* and *r*). In addition, there was a decrease in other $\alpha_v\beta_3$ ligands, such as osteopontin (data not shown, but consistent with published organ culture studies[6]). The mechanism of elastase inhibitors induced apoptosis can be related to loss of tenascin-C mediated $\alpha_v\beta_3$ ligation, as blockade of $\alpha_v\beta_3$ recapitulates the response to elastase inhibition in organ culture. In addition to loss of cellularity by apoptosis and despite elastase inhibition, reversal of hypertrophy of the vessel wall was associated with resorption of the pathogenic increase in extracellular matrix, as reflected by measurements of elastin content (Fig. 3*s*).

Endothelial injury and loss of barrier function may induce elastase activity during the initiation and progression of pulmonary hypertension in the monocrotaline model, as well as perhaps other clinical and experimental forms of this disease[1]. In cell culture, serum factors can increase activity of an endogenous vascular elastase in smooth muscle cells (SMCs) by a mitogen-activated protein kinase signaling mechanism involving increased nuclear expression of the transcription factor acute myelogenous leukemia, (AML)-1 (ref. 12). In cultured pulmonary artery SMCs, increased elastase activity can release growth factors stored in the extracellular matrix, such as fibroblast growth factor 2 (ref. 4). The proliferative response of SMCs to growth factors can be amplified by the glycoprotein tenascin-C, which is induced by a β_3 integrin-dependent intracellular signaling mechanism resulting from ligation of RGD (Arg-Gly-Asp) binding sites exposed by proteolysis of collagen[13]. Tenascin-C

clusters β_3 integrins and rearranges the actin cytoskeleton, causing co-clustering of growth factor receptors and facilitating their phosphorylation after ligation[3].

In contrast, inhibition of elastase, by arresting ongoing collagen degradation, may prevent the β_3 integrin-dependent signal, which induces tenascin-C, and loss of SMC interaction with tenascin-C could initiate the apoptotic cascade. Indeed, apoptotic SMCs in culture are rescued by the addition of exogenous tenascin-C and subsequent β_3 integrin ligation[3]. The possibility of an $\alpha_v\beta_3$ integrin survival signal is further supported by studies in which blocking endothelial cell $\alpha_v\beta_3$ integrin interactions with either cyclic peptides or functional blocking antibodies induces apoptosis and vessel loss[14,15]. The $\alpha_v\beta_3$ integrin pathway in angiogenic endothelial cells seems to suppress p53 and the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, increase the ratio of B-cell leukemia/lymphoma 2 (Bcl2) to Bcl2-associated X protein (bax) (ref. 16) and sustain extracellular-signal-regulated kinase activity[17]. Thus, α_v knockout mice have an embryonic lethal phenotype[18]. The idea that the $\alpha_v\beta_3$ integrin survival pathway is essential in remodeling vessels[15] is further supported by our observation that SMC apoptosis was induced by elastase inhibitors in hypertensive and not normal vessels[5]. Another observation in our study was the loss of the excess extracellular matrix which accompanies apoptosis. This matrix may be degraded by enzymes produced by apoptotic or viable SMCs which are unaffected by the elastase inhibitors used[6].

These findings indicate that increased serine elastase activity is essential to the progressive proliferative response resulting in excessive muscularity of distal vessels and is also necessary for survival of these SMCs. Therefore, selective elastase inhibitors can induce apoptosis, which, in association with resorption of the extracellular matrix, leads to regression of the hypertrophied vessel wall. This is directly related to normalization of pulmonary artery pressure, arrested right ventricular hypertrophy, and improved survival, thus offering a new and potentially clinically applicable therapeutic approach to treating severe pulmonary hypertension.

Methods

Experimental design. Adult male Sprague-Dawley rats (250–300 g in body weight; Charles River Laboratories, Wilmington, Massachusetts) were assigned to different experimental groups 21 days after a subcutaneous injection of saline or 60 mg/kg monocrotaline (Sigma) to induce pulmonary hypertension. In addition to a group of untreated rats, the experimental groups included rats that received twice-daily gavage tube feedings of either one of two selective serine elastase inhibitors, ZD0892 or M249314, at a dose of 240 mg/kg per day (gifts from C. Veale, Zeneca Pharmaceuticals, Wilmington, Delaware) or their administration vehicle (5% polyethylene glycol or dimethyl sulfoxide; Sigma). Rats were examined after 7 days of treatment (on day 28). Based on initial findings, an additional treatment group was given M249314 for 14 days (ending on day 35). ZD0892 and M249314 are peptidly trifluoromethylketones with an N-terminal 4-$(CH_3O)C_6H_4CO$ group, making them orally bioavailable serine elastase inhibitors[19]. They are very selective for neutrophil elastase (inhibition constant, about 6.7 nM), but also inhibit pancreatic elastase (inhibition constant, about 200 nM), and endogenous vascular elastase[6], but not metalloproteinases, cysteine proteinases or other serine proteinases. Inhibitors were chosen to target the increased elastase activity in monocrotaline-induced vascular disease[5], and doses were chosen based on published studies[20]. In experimental animals, ZD0892 has no detectable untoward systemic effects and indeed suppresses inflammation and its adverse sequelae[20]. All protocols were approved by the Animal Care Committee of The Hospital for Sick Children.

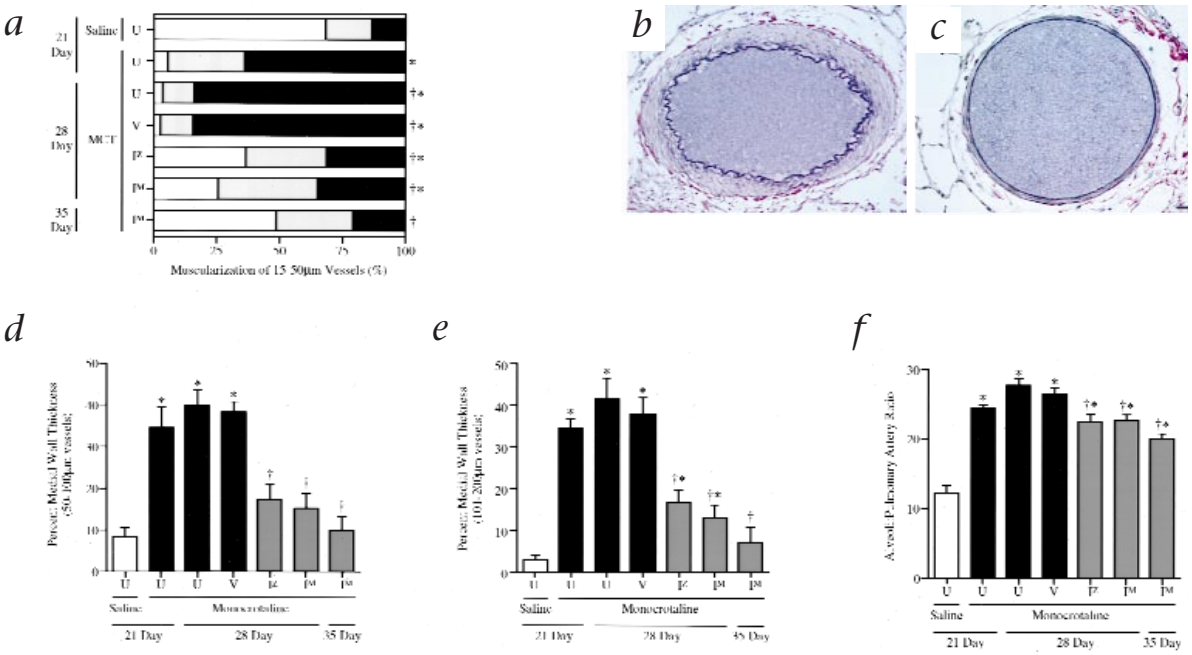

Fig. 2 Reversal of pulmonary arterial muscular changes by elastase inhibition. Elastase inhibition reverses pulmonary artery muscular changes and improves the ratio of artery to alveoli, which indicates no further loss of small arteries. Morphological assessments were made on barium-perfused arteries. ***a***, The presence and degree of muscularization in normally non-muscular pulmonary arteries (15–50 μm; □), as a percent of total pulmonary arteries (muscularized arteries (■) are compared by statistical analysis). ■, partially muscular. ***b–e***, Changes in medial thickness in pulmonary arteries that are normally muscular for a vehicle-treated rat (*b*) and an inhibitor-treated rat (*c*). *d* and *e*, Quantification of these changes, as a percent of the total diameter, for vessels 50–100 μm (*d*) and 101–200 μm (*e*) in diameter. ***f***, Ratio of alveoli to pulmonary arteries. U, untreated; V, vehicle-treated; I, inhibitor-treated (I^Z, ZD0892; I^M, M249314). Graphed data represent mean + s.e.m. of $n = 4–7$. Scale bar represents 10 μm. *, $P < 0.05$, compared with saline-injected controls; †, $P < 0.05$, compared with results on day 21 in rats treated with monocrotaline (*f*, compared with monocrotaline, day 28).

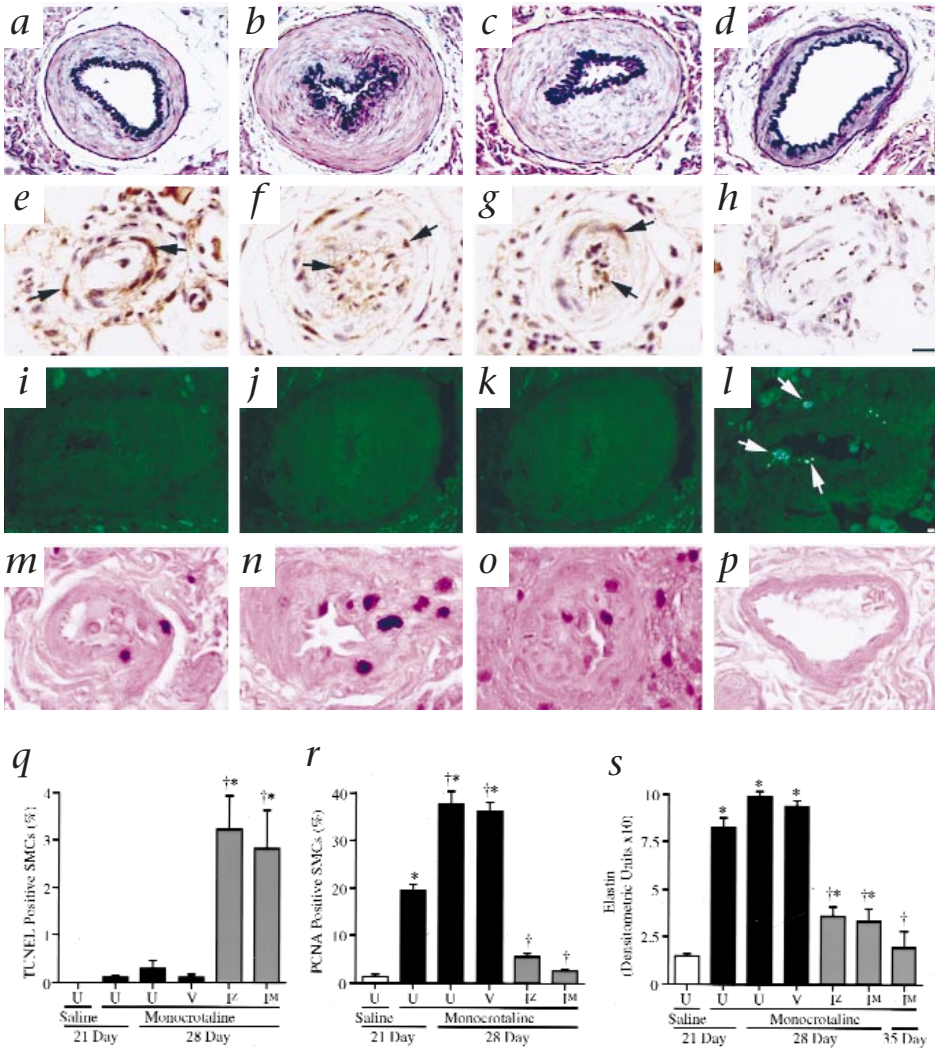

Fig. 3 Cellular mechanism responsible for reversal of pulmonary artery muscularity. Elastase inhibition arrests tenascin-C accumulation and proliferation and induces apoptosis and loss of extracellular matrix (such as elastin). For *a–p* (days refer to time after injection of monocrotaline): First column, day 21; second column, day 28; third column, day 28; last column, day 28. ***a–d***, Saline-perfused pulmonary arteries stained with Movat pentachrome stain. ***e–h***, Pulmonary arteries after tenascin-C immunohistochemistry. Arrows, positive brown peroxidase staining. ***i–l***, *In situ* TUNEL assays identifying apoptosis. White arrows, TUNEL-positive vascular cells. ***m–p***, Proliferating vascular cells, shown by immunohistochemistry for PCNA; dark nuclei are PCNA-positive cells. ***q*** and ***r***, Percent of SMCs that are TUNEL-positive (*q*) or PCNA-positive (*r*). ***s***, Densitometric quantification of elastin. U, untreated; V, vehicle-treated; I, inhibitor-treated (I^Z, ZD0892; I^M, M249314). Graphed data represent mean + s.e.m. of $n = 4$. Scale bars represent 5 μm. *, $P < 0.05$, compared with †, $P < 0.05$, compared with results on day 21 in rats treated with monocrotaline.

Elastase assays. Elastases were extracted from lung tissue as described,[20] and 10-μg protein samples were analyzed for their ability to degrade fluorescein-conjugated bovine neck ligament elastin (EnzChek Elastase Assay Kit; Molecular Probes, Eugene, Oregon). Analyses used the assay kit protocol, with extraction buffer as a negative control and porcine pancreatic elastase as a positive control. For the inhibitory assay, 3 μl rat serum from an intracardiac bleed was challenged with 1 unit porcine pancreatic elastase.

Survival endpoints. Criteria determined and set forth by the Animal Care Committee at the Hospital for Sick Children as 'humane endpoints' were monitored, and rats meeting these criteria were required to be killed and counted as a 'loss' in survival measurements. These endpoints included a sustained bradycardia of less than 100 beats/min, arterial oxygen tension of less than 80%, and abrupt weight loss with a reduction in body weight of more than 10% per day for 2 d. Rats were evaluated for these criteria by the veterinary staff of our Animal Facility, who recommended killing without knowledge of the treatment group.

Hemodynamic measurements. Direct pulmonary artery catheterization was done and pressures were measured (ES 1000 electrostatic recorder; Gould, Cleveland, Ohio), in rats anesthetized by isofluorane (Zeneca, Mississauga, Canada) general anesthesia, using a closed-chest technique as described[10]. Systemic blood pressure was determined by direct left carotid catheterization and was measured by a blood pressure analyzer (Statham DC pressure transducer, Chato Rey, Puerto Rico, and MacLab/8 data acquisition system; Lab Instruments, Castle Hill, Australia).

Right ventricular hypertrophy. The right ventricle was dissected from the left ventricle plus the septum, and these were weighed separately. The weight ratio was then calculated as the ratio of weight of the right ventricle to that of the left ventricle plus the septum.

Morphometric assessments. After the hemodynamic measurements were completed, lung tissue was prepared for morphometric analysis of the vasculature, using the barium injection method that has been reported in detail[10]. Light-microscopic slides were analyzed by investigators 'blinded' to sample identity, as described[10]. All barium-filled arteries more than 15 μm in external diameter were assessed for the presence of muscularity, and the wall thicknesses of normally muscular arteries with external diameters in the ranges of 50–100 μm and 101–200 μm were measured as a percent of the lesser curvature (magnification, ×400). Morphologies of endothelial cells were compared by transmission electron microscopy, as described[10].

Immunohistochemistry and detection of apoptosis. Immuno-histochemistry, using techniques that have been described[5], was done on lung sections perfused with saline and fixed in 4% paraformaldahyde. For detection of PCNA, a monoclonal antibody against PCNA (1:100 dilution; Dako, Carpinteria, California) was used. To quantify apoptosis, TUNEL assays (terminal deoxinucleotidyl transferase-mediated dUTP-biotin nick end-labeling) were done using the Apoptag *in situ* detection system (Oncor, Gaithersberg, Maryland). Nuclear morphology was examined by labeling with 2 μg/ml propidium iodide. The relative number of proliferating and apoptotic cells was quantitatively assessed as a percent of total

propidium-iodide-stained cells. Tenascin-C was identified with a polyclonal antibody against rat formaldehyde-fixed TN (1:100 dilution; a gift from H. Erickson, Duke University Medical Center, Durham, North Carolina), and osteopontin was detected with a monoclonal antibody raised against rat bone osteopontin (clone MPIIIB10_1; 11 ng/ml; Department of Biological Sciences, University of Iowa, Iowa City, Iowa). Estimates of total elastin were made using the Movat pentachrome stain, with which elastin is identified with iron hematoxylin. The relative abundances of the extracellular matrix components examined were graded quantitatively using the Image-Pro Plus program for Macintosh (Media Cybernetics, Silver Spring, Maryland) as described[5]. The program does planimetry and densitometry on positive staining above a uniform, 'background' cut-off. Subsequent multiplication of both the total area positively stained and the average density of staining provides a relative densitometric unit of the extracellular matrix protein being examined.

Statistical analysis. Data from multiple experiments are expressed as mean ± standard error, and statistical significance was determined using one-way analysis of variance followed by Fisher's least-significant-difference test of multiple comparisons to establish individual groups differences.

Acknowledgments

We thank J. Klein and L. Morikawa for technical assistance, D. Osmond of the Department of Physiology, University of Toronto for systemic pressure measurements, as well as the Laboratory Animal Services staff at the Hospital for Sick Children. We also thank J. Jowlabar, J. Matthews and J. Edwards for secretarial assistance. This work was supported by grants from the Heart and Stroke Foundation of Canada grant #3170, the Medical Research Council of Canada grant #PG13920 and the Primary Pulmonary Hypertension Cure Foundation. M.R. is a research endowed chair of the Heart and Stroke Foundation. K.N.C. holds a K.M. Hunter/Medical Research Council of Canada doctoral fellowship.

RECEIVED 9 FEBRUARY; ACCEPTED 13 APRIL 2000

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