SC-39026, a Serine Elastase Inhibitor, Prevents Muscularization of Peripheral Arteries, Suggesting a Mechanism of Monocrotaline-Induced Pulmonary Hypertension in Rats

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In rats injected with the toxin monocrotaline, altered synthesis and distribution of pulmonary artery elastin suggest that increased elastase activity may be important in the development of vascular changes and progressive pulmonary hypertension. To test this hypothesis, male Sprague-Dawley rats (250–300 g) were given 40 mg/kg of the elastase inhibitor SC-39026 in a carboxymethylcellulose vehicle or vehicle only by gavage, 12 hours before and twice daily for 8 days after a single subcutaneous injection of either monocrotaline (60 mg/kg) or saline. Thirteen days after injection, indwelling cardiovascular catheters were inserted under pentobarbital anesthesia, and at 15 days after injection, pulmonary and systemic hemodynamic measurements were recorded with the animals awake. At post-mortem examination, the lungs were perfused and morphometric techniques applied for light and electron microscopic evaluation. Saline-injected rats given either SC-39026 or vehicle were similar in all features assessed. In contrast, monocrotaline-injected rats given SC-39026 had significantly lower mean pulmonary artery pressure than those given vehicle (21.0±1.6 vs. 27.5±0.8 mm Hg, p<0.05), and this correlated with a significant reduction in the number of abnormally muscularized arteries at alveolar wall level (r²=0.89, p<0.001). SC-39026 did not significantly reduce monocrotaline-induced medial hypertrophy of muscular arteries, endothelial injury, and associated subendothelial edema; nor was there a significant increase in the proportion of the medial elastin, although a trend was apparent. Additional groups of monocrotaline injected rats were followed 3 weeks after injection, but both SC-39026 and vehicle-treated rats were similar at this point. Our data suggest that increased serine elastase activity associated with endothelial injury may mediate early abnormal pulmonary vascular smooth muscle differentiation resulting in muscularization of normally nonmuscular peripheral arteries and pulmonary hypertension induced in rats by injection of the toxin monocrotaline. Lack of persistence of this protective effect suggests that there may be continued elastase activity in this model. Failure to inhibit medial hypertrophy with SC-39026 suggests that a different mechanism or a different elastase may be involved in this structural change. (Circulation Research 1989;64:814–825)
hyperplasia, \textsuperscript{1,4} and progressive increases in collagen and elastin synthesis and accumulation in the media. \textsuperscript{3,5-7} Structural and biochemical studies in our laboratory in which the toxin monocrotaline was used to induce pulmonary hypertension in rats, suggest that increased elastase activity may initiate and contribute to the progression of these vascular changes. \textsuperscript{6,7} Fragmentation of the internal elastic lamina is noted as early as 4 days after injection of monocrotaline\textsuperscript{6} and precedes the muscularization of peripheral arteries observed at day 8 and the rise in pulmonary artery pressure and resistance observed at day 12. \textsuperscript{8} Continued elastase activity is suggested both by an increase in elastin synthesis out of proportion to elastin accumulation and by abnormal distribution of the medial elastin as interlamellar islands rather than as thicker elastic laminae, features observed at day 28 after monocrotaline injection. \textsuperscript{6}

While previous investigators have shown that inhibitors of collagen synthesis are effective in reducing right ventricular and medial hypertrophy, \textsuperscript{9,10} the intention of the present study was to explore the hypothesis that proteolytic, in particular elastolytic activity, induced by the toxin monocrotaline, may be initiating the neomuscularization of peripheral arteries, the medial thickening of the more proximal muscular arteries, and the associated increased connective tissue protein synthesis. We administered an elastase inhibitor, SC-39026 (Searle Pharmaceuticals, Skokie, Illinois) \textsuperscript{11} and assessed whether this decreased or prevented the development of the above vascular changes. SC-39026 is a new agent, a 2-chloro-4-(1-hydroxyoctadecyl) benzoi acid, which inhibits neutrophil serine elastase. \textsuperscript{11} The latter is a protease that degrades type IV collagen as well as elastin. \textsuperscript{12} We hypothesized that since alterations in type IV collagen and other basement membrane proteins may be associated with changes in cell phenotype, \textsuperscript{13,14} increased elastase activity in normally nonmuscular peripheral arteries might lead to differentiation of precursor cells (pericytes and intermediate cells) to mature smooth muscle. \textsuperscript{2} Moreover, increased elastase activity in the vessel wall could also stimulate elastin synthesis and smooth muscle hypertrophy as has been suggested in vitro. \textsuperscript{15} Neutrophil sequestration in the lung occurs within the first few hours after monocrotaline injection, \textsuperscript{16} and while its role in the pathogenesis of vascular disease is questionable, it may be the source of increased elastase activity in rats.

We assessed the development of pulmonary hypertension in the rats by implanting indwelling cardiovascular catheters. Structural changes in the peripheral and hilar arteries, previously observed following monocrotaline injection, were analyzed by morphometric techniques. Muscularization of normally nonmuscular peripheral arteries (i.e. "extension of muscle") and medial hypertrophy of normally muscular arteries were assessed by light microscopy. Increased medial thickness of the hilar pulmonary artery as well as endothelial injury, subendothelial edema and the proportion of elastin in the media were evaluated by transmission electron microscopy.

**Materials and Methods**

**Study Design**

Pathogen-free male Sprague-Dawley rats (250-300 g) were used. Half were assigned at random to be given a single subcutaneous injection of monocrotaline (60 mg/kg) and the other half received an equal volume of 0.9% saline. Monocrotaline solutions were prepared from the crystalline compound (Transworld Chemicals, Rockville, Maryland) as previously described. \textsuperscript{6} Half of the rats in each group were further assigned at random to receive by gavage either the elastase inhibitor SC-39026 (40 mg/kg/dose) suspended in carboxymethylcellulose vehicle or an equal volume of vehicle only. Carboxymethylcellulose vehicle was prepared by dissolving 1 g of the compound (Sigma Chemical Co, St. Louis, Missouri) in 100 ml of warmed distilled water. The rats were gavaged twice daily starting 12 hours before and continuing for 8 days after the monocrotaline or saline injection to provide a "window" around day 4. This was the time monocrotaline-induced increased elastase activity was first suspected on the basis of what appeared to be increased fragmentation of the internal elastic lamina of the hilar pulmonary artery. \textsuperscript{7}

On day 13, after the monocrotaline or saline injection, the rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (33 mg/kg), and indwelling catheters were inserted into the abdominal aorta under direct vision \textsuperscript{17} and into the pulmonary artery by a modification \textsuperscript{18} of a closed-chest technique previously described. \textsuperscript{19} Pressure measurements and cardiac output were recorded 48 hours later to allow sufficient time for recovery from the effects of anesthesia. The heart and lungs were then prepared for morphological assessments. \textsuperscript{18,20,21} Day 15 was chosen for physiological and morphological studies of the pulmonary vasculature to assure that satisfactory end-points would be obtained. This allowed for a few days beyond the time after monocrotaline injection, when a significant increase in pulmonary artery pressure and muscularization of peripheral arteries was previously observed. \textsuperscript{8} Throughout the experimental period, food and water were supplied ad libitum. The number of rats with complete hemodynamic and morphologic data in each of the groups is indicated in the tables and figures.

To assess whether the results observed 2 weeks after monocrotaline injection in rats treated with SC-39026 were persistent at 3 weeks, a limited study was carried out. We assessed two groups of rats injected with monocrotaline, one treated with SC-39026, the other treated with vehicle as in the previously described protocol. Assessment of hemodynamic and morphological features of the heart...
and lungs were carried out as in the previous groups with the exception that ultrastructural analyses were not performed.

**Hemodynamic Measurements**

Pressures were recorded from the pulmonary artery and aorta using physiological transducers (MS20, Electromedics, Englewood, Colorado) and an electrostatic recorder (ES1000, Gould, Cleveland, Ohio). Blood samples, 0.5 ml each, were obtained from the pulmonary and aortic catheters and blood gases were analyzed (Corning Glass Works, Medfield, Massachusetts); the hematocrit was determined from an additional 0.1-ml sample drawn from the aortic catheter. Oxygen consumption was measured as previously described,\(^{18,20}\) with adjustments made for temperature and pressure and the cardiac output was calculated by the Fick principle using an oxygen carrying capacity derived from the measured hematocrit.

**Tissue Preparation for Morphological Assessment**

After the hemodynamic measurements were obtained, the rats were weighed and then anesthetized with an intraperitoneal injection of sodium pentobarbital (33 mg/kg). The animals were ventilated (Rodent Ventilator Model 683, Harvard Apparatus, South Natick, Massachusetts) through a tracheostomy at a rate of 75 breaths per minute and a stroke volume of 3.5 ml. The heart and lungs were then exposed via a median sternotomy. Heparin (500 units) was injected into the right ventricle and allowed to circulate for 2 minutes. The central pulmonary artery was cannulated with a 5-cm length of polyethylene tubing (PE 90, i.d. 0.86x.o.d. 1.27 mm) through a right ventriculotomy incision and then perfused with preheated (37°C), heparinized (0.5% vol/vol) phosphate-buffered saline at 20 cm water pressure for 5 minutes to clear the lungs of blood. The left pulmonary artery was then temporarily occluded, and the left lung was covered in a small plastic bag to avoid fixation while the right lung was perfused at the pressure measured in vivo, for 10 minutes with a cold (4°C), 1% glutaraldehyde in 4% formaldehyde solution. The right pulmonary artery was then occluded, the lung was removed, and a block of tissue, approximately 5x5x2 mm, containing the hilar pulmonary artery was then taken. The tissue was diced into small blocks approximately 1x1x2 mm, fixed for 1 more hour, and then placed in 0.1 M phosphate buffer and processed for transmission electron microscopy.

The left pulmonary artery was then unclamped and injected with a hot (60°C), radiopaque barium-gelatin mixture at 100 cm water pressure for 5 minutes, and then clamped. The lung was distended through the trachea with 10% formaldehyde at 36 cm water pressure and perfused continuously for 3 days. A block of tissue 10x10x5 mm was obtained from the midsection of the lung for light microscopic analysis.\(^{18,20}\) The right ventricle (RV) of the heart was dissected from the left ventricle plus septum (LV+S) and weighed separately.\(^{21}\) Ventricular weights were expressed as the ratio RV/(LV+S) and also as the ratio of the weight of the RV to final body weight (FBW).

**Light Microscopic Analysis**

Lung tissue for histological evaluation was embedded in paraﬁn and stained by the elastic Van Gieson method. Slides were analyzed without knowledge of treatment group. Using previously described morphometric techniques,\(^{18,20}\) all barium-filled arteries in each tissue section were analyzed at \(\times400\) magnification, for an average of 97 arteries per section (range, 81–120). Each artery was first "landmarked" according to its accompanying airway, as being either preacinar or related to a terminal bronchiolus, respiratory bronchiolus, alveolar duct, or alveolar wall, and its external diameter was measured. Each artery was further described as being either muscular (with a complete medial muscular coat), partially muscular (with an incomplete coat), or nonmuscular (no muscle apparent). Extension of muscle into peripheral, normally nonmuscular arteries was evaluated by the percentage of muscular and partially muscular arteries at each airway level. Medial wall thickness, an index of medial hypertrophy, was calculated for each muscular and partially muscular artery as a percent wall thickness (%WT).\(^{18,20}\) For each rat, an average wall thickness for arteries of the various sizes (50–99 and 100–149 \(\mu m\)) was calculated. In each of 10 consecutive fields at \(\times250\) magnification, all alveoli and arteries were counted. Arterial density was expressed as the number of arteries per 100 alveoli.

**Transmission Electron Microscopic Analysis**

Tissue blocks were dehydrated and embedded in Epon, from which thin sections (1 \(\mu m\)) were cut and stained with toluidine blue. Blocks containing the hilar pulmonary artery were cut into ultrathin sections (600–900 \(\AA\)), prepared on copper grids, stained with 5% uranyl acetate and 0.4% lead citrate, and viewed in a transmission electron microscope (model 201, Philips Electronic Instruments, Mount Vernon, New York). For each animal, at least three consecutive photomicrographs that included the full thickness of the hilar artery were taken at \(\times3,600\) magnification for measurement of wall thickness as well as assessment of the thickness of elastic laminae of the media. Similarly, for each animal, at least eight consecutive photomicrographs that included the intima were taken at \(\times17,500\) magnification for assessment of endothelial cell morphology, width of subendothelial space, and thickness of internal elastic lamina. Photomicrographs were printed in 20x20 cm format and analyzed with a semiautomatic computerized system (Interactive Image Analysis System IBAS-1, Zeiss, Thornwood, New York).

The intima of the hilar pulmonary artery was examined for evidence of monocrotaline-induced
changes. Ten consecutive endothelial cells per artery were assessed, and the number showing evidence of injury (i.e., vacuolation, pallor, and foamy cytoplasm) was recorded and expressed as a percent of injured endothelial cells per artery. The subendothelium, defined as the region between the abluminal edge of the endothelium and the abluminal border of the internal elastic lamina, was examined for evidence of edema by measuring its thickness at three equidistant points on each print. Thus, at least 24 measurements were recorded per vessel and averaged. The thickness of the internal elastic lamina in each photomicrograph was derived from the digitized surface area, divided by the measured length; from eight assessments, an average thickness per artery was calculated.

The media was defined as the region between the abluminal edge of the internal elastic lamina and the abluminal border of external elastic lamina. The thickness of the media of the hilar artery was measured at three equidistant points on each of the photomicrographs, and an average thickness was calculated for each vessel. The sum of the digitized surface areas of all the elastic laminae in the media was calculated and related to the digitized surface area of the media. Assessments from each of the prints taken from a given artery were averaged.

**SC-39026 Plasma Levels**

After completion of these studies, six additional weight-matched male Sprague-Dawley rats were gavaged with SC-39026 for 6 days. Just before the first dose and 9 hours after the last dose, 1 ml of tail vein blood was obtained, and plasma levels were determined by the Department of Drug Metabolism at Searle Research Laboratories, Skokie, Illinois, using a high-performance liquid chromatography assay. Values before gavage with SC-39026 ranged from 0.10 to 0.24 μg/ml (background activity), whereas levels 9 hours after the last dose were 0.30–0.38 μg/ml in two rats and 1.30–4.17 μg/ml in the remaining four. Since only values greater than 0.6 μg/ml are considered to represent a therapeutic level (Dr. G. Fuller, Searle, personal communication), it appears that there is considerable variation in the degree of absorption of the compound after oral administration in rats.

**Analysis of Data**

Two-way analysis of variance was used to compare the effects of monocrotaline and SC-39026 in the four groups: saline/vehicle, saline/SC-39026, monocrotaline/vehicle, and monocrotaline/SC-39026. When significant variance was found, Duncan's test of multiple comparisons was used to determine which groups were different. Linear regression analysis was used to detect whether, in individual monocrotaline injected rats, there was a relation between the mean pulmonary artery pressure and structural and ultrastructural differences in the pulmonary arteries. This analysis was also applied to determine the relation between the amount of elastin in the vessel wall, the wall thickness of the pulmonary artery and the degree of muscularization of peripheral arteries. In the limited study carried out to assess differences in the SC-39026 treated and untreated monocrotaline injected rats at 3 weeks after injection, comparisons are based on a Student's 𝑡 test. Differences were considered statistically significant at 𝑝<0.05. Mean values±SEM are given in the figures and tables, and the number of rats assessed in each group is noted.

**Results**

**Growth, Hemodynamic Measurements and Ventricular Weights at 2 Weeks**

Complete data were obtained in 33 rats: seven saline/vehicle, seven saline/SC-39026, 10 monocrotaline/vehicle, and nine monocrotaline/SC-39026. Both saline/vehicle and saline/SC-39026 groups had similar final body weights. The monocrotaline groups had lower final weights than the saline rats (𝑝<0.001, ANOVA), a feature not significantly altered by SC-39026. There were no significant differences between the four groups in hematocrit, cardiac output, arterial blood gas values, and systemic arterial pressure (Table 1).

The mean pulmonary artery pressures of the saline/vehicle and saline/SC-39026 groups were similar, 16.4±1.1 and 17.4±0.9 mm Hg, respectively (Figure 1). The monocrotaline groups had signifi-
There was considerable overlap in RV/FBW as well as in the RV/(LV+S) values among the four groups. The saline/vehicle and saline/SC-39026 groups had similar values for RV/(LV+S) and RV/FBW. Monocrotaline injection resulted in higher values for RV/(LV+S) (p < 0.05) and for RV/FBW (p < 0.001); ANOVA). Values for RV/FBW but not for RV/(LV+S) were, however, significantly lower in the monocrotaline/SC-39026 group than in the monocrotaline/vehicle group (p < 0.05, Duncan's Multiple Range Test). In five of nine rats in the monocrotaline/SC-39026 group, pulmonary artery pressure values were within the range of saline controls (Figure 1).

There was considerable overlap in RV/FBW as well as in the RV/(LV+S) values among the four groups. The saline/vehicle and saline/SC-39026 groups had similar values for RV/(LV+S) and RV/FBW. Monocrotaline injection resulted in higher values for RV/(LV+S) (p < 0.05) and for RV/FBW (p < 0.001); ANOVA). Values for RV/FBW but not for RV/(LV+S) were, however, significantly lower in the monocrotaline/SC-39026 group than in the monocrotaline/vehicle group (p < 0.05, Duncan's Multiple Range Test) (Table 2).

**Table 2. Assessments of Right Ventricular Hypertrophy and Light Microscopic Measurements of Medial Hypertrophy and Arterial Density 2 Weeks After Injection**

<table>
<thead>
<tr>
<th>GP</th>
<th>RV/FBW (g/kg)</th>
<th>RV/LV</th>
<th>% Wall thickness 50–99 µm</th>
<th>% Wall thickness 100–149 µm</th>
<th>Arterial density (arteries/100 alveoli)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/V</td>
<td>0.61±0.03</td>
<td>0.26±0.01</td>
<td>3.85±0.21</td>
<td>4.12±0.32</td>
<td>4.62±0.28</td>
</tr>
<tr>
<td>M/SC-I</td>
<td>0.54±0.03*</td>
<td>0.24±0.01</td>
<td>3.71±0.13</td>
<td>3.73±0.16</td>
<td>4.73±0.17</td>
</tr>
<tr>
<td>S/V</td>
<td>0.50±0.02</td>
<td>0.23±0.01</td>
<td>3.49±0.17</td>
<td>2.73±0.22</td>
<td>4.33±0.20</td>
</tr>
<tr>
<td>S/SC-I</td>
<td>0.32±0.02</td>
<td>0.23±0.01</td>
<td>3.45±0.07</td>
<td>2.49±0.06</td>
<td>4.68±0.22</td>
</tr>
</tbody>
</table>

GP, group; n, number; RV/FBW, ratio of right ventricular to final body weight; RV/LV, ratio of right ventricular weight to left ventricular + septal weight; M/V and M/SC-I, monocrotaline-injected rats treated with vehicle and SC-39026 inhibitor, respectively; S/V and S/SC-I, saline-injected rats treated with vehicle and SC-39026 inhibitor, respectively.

* p < 0.05, M/V compared with M/SC-I.
† p < 0.05, saline compared with monocrotaline.
‡ p < 0.001, saline compared with monocrotaline.

**Light Microscopic Analysis at 2 Weeks**

Light microscopic structural analysis of the peripheral pulmonary vasculature was performed on 25 of the 33 unselected rats: six saline/vehicle, six saline/SC-39026, six monocrotaline/vehicle, and seven monocrotaline/SC-39026.

**Extension of muscle.** Saline/vehicle and saline/SC-39026 rats had only a small percentage of arteries muscularized at the alveolar wall level (1.9±1.4 and 0.4±0.4%, respectively) (Figure 2). Monocrotaline-injected animals had significantly greater muscularization of alveolar wall and duct arteries, compared with saline rats (p < 0.001 for both, ANOVA). The monocrotaline/vehicle group had 24.1±2.6% alveolar wall arteries muscularized. Treatment of monocrotaline-injected rats with SC-39026 resulted in a decreased percentage of alveolar wall arteries muscularized (10.0±3.6%, p < 0.05, Duncan's Multiple Range Test). A trend toward a decreased percent of alveolar duct arteries muscularized was apparent, although the difference was not statistically significant. In the monocrotaline/vehicle rats, there was no significant correlation between the percent alveolar wall arteries muscularized and the level of mean pulmonary artery pressure. Values were high for both parameters. In the monocrotaline/SC-39026 group, however, there was a significant correlation between the level of pulmonary artery pressure and the percent of alveolar wall arteries muscularized (r² = 0.89, intercept = 15.245, slope = 0.599, 95% confidence limits of the slope = 0.599±1.96, p < 0.001) (Figure 3).

**Medial wall thickness of muscular arteries.** The saline/vehicle and saline/SC-39026 groups had similar values for medial wall thickness (%WT) of muscular arteries of 50–99 and 100–149 µm external diameter (Table 2). The monocrotaline-injected rats had significantly increased %WT compared with the saline-injected animals in arteries of 100–149 µm external diameter only (p < 0.001, ANOVA). Treatment of the monocrotaline injected rats with SC-39026 did not result in a significantly decreased...
%WT, although there was a trend in that direction (Table 2).

Arterial density. There was no difference among the four groups in the number of arteries per 100 alveoli (Table 2). Lung volumes and absolute number of alveoli per mm² were also similar.

Ultrastructural Analysis at 2 Weeks

Ultrastructural analysis of the hilar pulmonary artery was performed on 18 of 33 unselected rats: three saline/vehicle, three saline/SC-39026, six monocrotaline/vehicle, and six monocrotaline/SC-39026.

Intima. Both saline/vehicle and saline/SC-39026 groups had few injured endothelial cells per artery and a barely measurable subendothelial space (Figures 4 and 5). In contrast, monocrotaline-injected animals had a high proportion of injured endothelial cells per artery and evidence of subendothelial edema judged by a wide subendothelial space (p<0.001, ANOVA for both comparisons). Treatment of monocrotaline rats with SC-39026 did not

FIGURE 2. Extension of Muscle: Percent of peripheral arteries muscularized at the alveolar wall and alveolar duct level. M, monocrotaline; V, vehicle; SC-I, elastase inhibitor SC-39026; S, saline. M/V (n=6), M/SC-I (n=7), S/V (n=6), S/SC-I (n=6). Values are mean±SEM for each group. M groups had abnormally increased muscularization of arteries at alveolar wall and alveolar duct level, compared with S groups, ***p<0.001 (ANOVA). The M/SC-I group compared with the M/V group had decreased muscularization at alveolar wall level, *p<0.05 (Duncan's Multiple Range Test) with a similar trend at alveolar duct level, although not statistically significant.

FIGURE 3. Correlation of mean pulmonary artery pressure with extension of muscle into alveolar wall arteries. M, monocrotaline; V, vehicle; SC-I, elastase inhibitor SC-39026. M/V (n=6), M/SC-I (n=7). There is no significant correlation between the two measurements in M/V rats whereas in M/SC-I rats r²=0.89, slope=15.245, intercept=0.599 (95% confidence limits=0.599±1.96), p<0.001.

FIGURE 4. Ultrastructural features of the intima of hilar pulmonary arteries (endothelium and subendothelial space). M, monocrotaline; V, vehicle; SC-I, elastase inhibitor SC-39026; S, saline. M/V (n=6), M/SC-I (n=6), S/V (n=3), S/SC-I (n=3). Values are mean±SEM for each group. The number of "injured" endothelial cells per artery as defined in the text and the width of the subendothelial space is increased in M groups, ***p<0.001 for both (ANOVA). No significant differences exist between M/V and M/SC-I groups.
FIGURE 5. Electron photomicrographs of the hilar pulmonary arteries. A saline/vehicle rat (top left) and a saline/SC-39026 rat (bottom left) show normal endothelial (E) cells and virtually no subendothelial (S) space. In contrast, endothelial cells in the hilar pulmonary artery of a monocrotaline/vehicle (top right) and a monocrotaline/SC-39026 (bottom right) rat show considerable swelling and pallor, with well-defined subendothelial space. SM, smooth muscle cell. Thickness of internal elastic lamina (iel) is similar in all photomicrographs, but the proportion of media occupied by elastic laminae (mel) is reduced in the photomicrographs from the monocrotaline rats (top and bottom right). Magnification, ×6,138; bar, 1.5 μm.
alter the severity of these abnormalities. The width of the internal elastic lamina was similar in saline and monocrotaline-injected rats and was unaffected by SC-39026 (Figure 6).

**Media.** The thickness of the media of the hilar pulmonary artery was similar in both saline groups (Figure 7), but in monocrotaline injected rats, it was increased \( (p<0.01, \text{ANOVA}) \). Values in monocrotaline/SC-39026 and monocrotaline/vehicle groups were similar. The ratio of the surface area of the elastic laminae of the media to the surface area of the media was similar for saline/vehicle and saline/SC-39026 rats but was decreased in the monocrotaline groups \( (p<0.001, \text{ANOVA}) \). Moreover, qualitatively, the elastic laminae appeared fragmented (Figure 4). Treatment of monocrotaline-injected rats with SC-39026 did not result in a significantly increased proportion of media occupied by elastic laminae, although a trend was apparent (Figures 4 and 6) and the laminae appeared less fragmented (Figure 4). In individual monocrotaline-injected animals, the proportion of elastic laminae in the media did not correlate significantly with the thickness of the media or the mean pulmonary artery pressure.

**Hemodynamic and Structural Changes at 3 Weeks.**

At 3 weeks after monocrotaline injection, maintaining catheters in the pulmonary artery of the rats was particularly difficult because they tended to flip out, probably because of the very high levels of pulmonary artery pressure. This phenomenon has been previously described.\(^2\) We were therefore able to compare only four SC-39026 and four vehicle-treated monocrotaline-injected rats with respect to pressures, but eight and 12 rats, respectively, with regard to right ventricular hypertrophy. Light microscopic morphometric assessment was carried out on the eight rats in which pressures were obtained. At the 3-week time point, there were no significant differences in the two groups with respect to pulmonary artery pressure, cardiac output, and right ventricular hypertrophy expressed either as the ratio of right to left ventricular weight or as the ratio of right ventricular to body weight, nor was there a significant difference in the degree of muscularization of peripheral arteries or in the medial wall thickness of normally muscular arteries (Table 3).

**Discussion**

We have previously reported that in rats, injury to the pulmonary vascular endothelium by the pyrrolizidine alkaloid metabolite of monocrotaline precedes muscularization of the normally nonmuscular distal pulmonary vascular bed and the rise in pulmonary artery pressure and increase in pulmonary vascular reactivity.\(^8\) That muscularization of distal vessels precedes pulmonary hypertension was also evident in studies by Meyrick et al, in which rats were fed *Crotalaria spectabilis* seeds.\(^20\) Since administration of the elastase inhibitor SC-39026 for 7 days after injection of monocrotaline reduced extension of muscle into normally nonmuscular peripheral arteries correlating with a reduction in the severity of pulmonary hypertension at 2 weeks, our study supports the hypothesis that elastase activity may initiate this vascular change. Muscularization of peripheral arteries is thought to result from the differentiation of precursor cells (pericytes) to mature smooth muscle cells.\(^2\) Cellular differentiation may be regulated by changes in basement membrane and other extracellular matrix proteins.\(^13,14\) Thus, SC-39026 may have prevented muscularization of peripheral arteries by inhibiting degradation by elastase of elastin or type IV collagen, a basement membrane component, or by some other as yet undescribed antiproteolytic effect.

The reduction in pulmonary artery pressure was not related to a change in cardiac output, mean systemic arterial pressure, hematocrit or arterial oxygen tension. We report pulmonary artery pressure and not pulmonary vascular resistance as we did not record left ventricular end-diastolic pressure. However, we established that there was no significant change in cardiac output, and it has been observed previously by our group\(^8\) and others\(^20\) that there is no significant change in left ventricular end diastolic pressure after monocrotaline injection. For these reasons, the pulmonary artery pressure changes likely reflect similar alterations in pulmonary vascular resistance. In six of nine rats, the values of pulmonary artery pressure in the monocrotaline-injected rats treated with the inhibitor SC-39026 were similar to those in the saline injected controls, whereas in the other three rats, only a partial reduction in pulmonary hypertension may have been achieved. The variability in response may be due to the variation from rat to rat in the severity of monocrotaline-induced vascular damage or in the absorption characteristics of the orally administered SC-39026.\(^11\) While the two may certainly interrelate, the relatively uniform response in the untreated monocrotaline injected rats and the variability in SC-39026 absorption demonstrated in a similarly treated group suggest that the latter may be a more likely explanation.

Associated with the decreased pulmonary artery pressure at 2 weeks in monocrotaline injected rats treated with SC-39026, right ventricular hypertrophy was less as judged by the ratio of right ventricle to final body weight. That there was only a trend toward a decrease in the ratio of right to left ventricular weights is due, most likely, to the considerable overlap in values even with saline injected rats.

In previous studies, we observed increased fragmentation of the internal elastic lamina of more proximal and larger muscular pulmonary arteries, at 4 days after injection of the toxin monocrotaline in association with endothelial damage.\(^7\) This preceded the development of medial hypertrophy and the increase in elastin synthesis\(^8\) observed 16 days after injection. We therefore hypothesized that increased elastase activity was also important in the
pathogenesis of the increased medial wall thickness of more proximal normally muscular arteries. In the present study, however, we were unable to show a significant reduction in the severity of monocrotaline-induced medial hypertrophy with administration of the elastase inhibitor. We had anticipated that an increase in the proportion of elastic laminae in the media of the hilar pulmonary artery associated with a decrease in fragmentation might more sensitively reflect some degree of elastase inhibition. However, the difference comparing SC-39026 treated and non-treated monocrotaline-injected rats was small and statistically insignificant. Thus, it seemed that a different mechanism or elastase may be responsible for medial hypertrophy.

We wondered whether observation of the rats for 3 weeks after injection of the toxin monocrotaline might reveal a more pronounced effect of the inhibitor on medial hypertrophy and medial elastin. We did not, however, observe any significant difference in SC-39026 treated or untreated monocrotaline-injected rats. Moreover, the decrease in muscularization of peripheral arteries, pulmonary artery pressure, and right ventricular hypertrophy previously observed in SC-39026-treated rats at 2 weeks did not persist. It is possible that a higher or more constant level of inhibitor may be necessary to achieve a sustained response. This is currently being explored with the Searle company. We have not had success with an intravenous preparation because the compound crystallized (authors' unpublished observations), so a food-admix is now being prepared.

The source of the elastase, which may be involved in the muscularization of distal vessels, is not known. Since the inhibitor is specific for neutrophil elastase one might speculate that the neutrophils observed early in the lung are the source, but their appearance is transient and it is questionable whether they are related to the pathogenesis of the vascular changes particularly since animals such as mice, that have the greatest inflammatory response to monocrotaline do not develop vascular changes. The spectrum of elastase activity of vascular endothelial cells is not known, and it is conceivable that, in response to the endothelial injury induced by monocrotaline, an elastase is released that would be inhibited by SC-39026. It has been shown that smooth muscle cells produce a serine elastase, but it is not known whether it is inhibited by SC-39026.

While neutrophils are observed early and transiently in association with monocrotaline injection, the presence of platelets and microthrombi are even more evident, so platelet production or interaction with elastase may be important. An increased number of alveolar macrophages are associated with the development and progression of monocrotaline-induced pulmonary hypertension, but they produce primarily metalloelastases.

**Figure 6.** Mean width of internal elastic lamina (iel) of hilar pulmonary arteries and proportion of medial elastic laminae. M, monocrotaline; V, vehicle; SC-I, elastase inhibitor SC-39026; S, saline. Values are mean±SEM for each group. There were no differences between the four groups in the mean width of the iel. Quantification of elastic laminae of the media of the four groups. M rats have a decreased proportion of medial elastin relative to S rats ***p<0.001, (ANOVA) and in M/SC-I (SC-39026), rats, there is a trend apparent toward a proportion similar to that in S rats. The numbers (n) in each group are given in Figure 4.

**Figure 7.** Thickness of the media of the hilar pulmonary artery. M, monocrotaline; V, vehicle; SC-I, elastase inhibitor SC-39026; S, saline. Values are mean±SEM for each group. There is a significant difference between monocrotaline and saline groups, **p<0.01 (ANOVA). The numbers (n) in each group are given in Figure 4.
While our study suggests an antielastase effect of SC-39026, it is possible that this compound alters the metabolism of monocrotaline in such a way as to make it less toxic. This seems unlikely since the degree of endothelial injury and subendothelial swelling we observed was similar in the treated and untreated monocrotaline-injected rats. An increase in the activity of angiostatin converting enzyme (a serine protease) has been observed in association with an early phase of monocrotaline pulmonary toxicity,31,32 but SC-39026 has been shown to not inhibit this enzyme.11

We conclude that we have evidence in the monocrotaline-injected rat model indicating that serum elastase may be associated with muscularization of peripheral arteries leading to the development of pulmonary hypertension. Further in vitro and immunocytochemical studies will be necessary to establish the mechanism whereby elastase activity in the small peripheral vessels leads to the differentiation of smooth muscle cells from precursor cells and especially whether this is related to degradation of the component of the basement membrane that is type IV collagen. Also, further studies will be necessary to show that the mechanism of induction of abnormal muscularization of peripheral arteries after monocrotaline is similar when chronic hypoxia is the stimulus or when this feature results from the high pulmonary blood flow and pressure produced by a congenital cardiac defect with a left-to-right shunt.1

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References

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TABLE 3. Hemodynamic and Structural Measurements 3 Weeks after Injection of Monocrotaline

| GP (n) | Pressures (mm Hg) | Arterial blood gases | Hematocrit (%) | Cardiac output (ml/kg/min) | Right ventricular hypertrophy | Extension of muscle (% | Wall thickness (µm)
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<tbody>
<tr>
<td></td>
<td>PA</td>
<td>Ao</td>
<td>pH</td>
<td>Po2</td>
<td>RV/LV FBW</td>
<td>RV/Alveolar wall</td>
<td>Alveolar duct</td>
</tr>
<tr>
<td>M/V</td>
<td>38±3</td>
<td>104±3</td>
<td>7.41±0.2</td>
<td>81±3</td>
<td>33±2</td>
<td>34±3</td>
<td>325±7</td>
</tr>
<tr>
<td>M/SC-1</td>
<td>53±8</td>
<td>98±4</td>
<td>7.44±0.2</td>
<td>75±9</td>
<td>33±1</td>
<td>39±4</td>
<td>405±45</td>
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KEY WORDS: pulmonary hypertension • elastase • elastin • vascular smooth muscle • cell differentiation
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