Pulmonary hypertension in TNF-α-overexpressing mice is associated with decreased VEGF gene expression

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Fujita, Masaki, Robert J. Mason, Carleyne Cool, John M. Shannon, Nobuyuki Harа, and Karen A. Fagan. Pulmonary hypertension in TNF-α-overexpressing mice is associated with decreased VEGF gene expression. J Appl Physiol 93: 2162–2170, 2002. First published August 9, 2002; 10.1152/japplphysiol.00083.2002.—Tumor necrosis factor-α (TNF-α) transgenic mice have previously been found to have characteristics consistent with emphysema and severe pulmonary hypertension. Lungs demonstrated alveolar enlargement as well as interstitial thickening due to chronic inflammation and perivascular fibrosis. In the present report, we sought to determine potential mechanisms leading to development of pulmonary hypertension in TNF-α transgenic mice. To determine whether sustained vasoconstriction was an important component of this pulmonary hypertension, nitric oxide was administered and hemodynamics were measured. Nitric oxide (25 ppm) failed to normalize right ventricular pressure in transgene-positive mice, suggesting that the pulmonary hypertension was not due to sustained vasoconstriction. Structural analysis of the pulmonary arteries found adventitial thickening and a trend toward medial hypertrophy in pulmonary arteries of transgene-positive mice, suggesting that vascular remodeling had occurred. Echocardiographic measurement of the percent fractional shortening of the left ventricle as a measurement of ventricular function in vivo revealed that left ventricular dysfunction was not contributing to pulmonary hypertension. We examined expression of genes known to be important in regulation of vascular tone and structure. Messenger RNA expression of vascular endothelial growth factor and its receptor flk-1 was reduced compared with transgene-negative littermates at all ages. Endothelial and inducible nitric oxide synthase mRNA levels were similar in both groups. Endothelin-1 mRNA was also decreased in TNF-α transgenic mice. Interestingly, female transgenic mice had decreased survival rate compared with male transgenic mice. We conclude that chronic overexpression of TNF-α is associated with decreased vascular endothelial growth factor and flk-1 gene expression, pulmonary vascular remodeling, and severe pulmonary hypertension, although the precise mechanism is unknown.

Although the association between emphysema and pulmonary hypertension is well recognized, the precise mechanism of this association remains unclear (63). Our laboratory recently reported (16) that tumor necrosis factor-α (TNF-α)-overexpressing transgenic mice have age-dependent chronic inflammation, severe alveolar enlargement, loss of elastic recoil, and physiological changes consistent with emphysema. These mice also develop severe pulmonary hypertension and right ventricular hypertrophy similar to that observed in tight-skinned mice with emphysema and cor pulmonale (36). TNF-α has been reported as important in both the systemic (38) and pulmonary circulations (16, 27). Subtle, pulmonary vascular remodeling has been previously reported in mice with hypoxia-induced pulmonary hypertension with thickening and neomuscularization of small resistance pulmonary arteries (11, 23, 57).

Several regulators of pulmonary vascular tone and structure have been implicated in the development of pulmonary arterial hypertension. Vascular endothelial growth factor (VEGF) and its receptors flk-1 and flt-1 (8, 50, 59), endothelin-1 (ET-1) (19), and endothelial cell nitric oxide (NO) synthase (ecNOS) (11, 14, 18, 56, 57) have all been reported as important in modulating the development of pulmonary hypertension.

The importance of VEGF in the development of pulmonary hypertension is not clear. VEGF and its receptor flk-1 are expressed in the characteristic plexiform lesion of human primary pulmonary hypertension (24, 61) and may play a role in the disordered angiogenesis found in the plexiform lesion of primary pulmonary hypertension (61). However, others have reported se-
were hypoxia-induced pulmonary hypertension with inhibition of VEGF receptor flk-1 (59), whereas overexpression of VEGF protected against the development of pulmonary hypertension in two different models (4, 46). A decrease in VEGF and flk-1 may also be important in the development of emphysema (28, 29). TNF-α has been reported to induce expression of VEGF in human neutrophils (62) with reports of both increased and decreased expression of flk-1 in human endothelial cells (20, 48).

ET-1 is increased in human pulmonary hypertension (58), and an ET-1 antagonist has recently been approved for the treatment of pulmonary hypertension (54). However, modest overexpression of human ET-1 in mice was not associated with the development of pulmonary hypertension (25).

Inhaled NO has been used to attenuate pulmonary hypertension in humans with primary pulmonary hypertension (49), persistent pulmonary hypertension of the neonate (26), and acute respiratory distress syndrome (52) and in association with chronic obstructive pulmonary disease (41, 66). In animal studies, NO attenuates the development of hypoxic pulmonary hypertension (30). In mice, loss of ecNOS leads to enhanced acute hypoxic vasoconstriction and susceptibility to chronic pulmonary hypertension after modest hypoxia and is associated with vascular remodeling (11, 56, 57), whereas overexpression of eNOS prevents hypoxia-induced pulmonary hypertension (5).

Thus we hypothesized that chronic overexpression of TNF-α in mice leads to changes in expression of important modulators of pulmonary vascular tone and structure, leading to pulmonary hypertension. To address the contributions of known modulators of pulmonary vascular tone and structure to the development of pulmonary hypertension in TNF-α transgenic mice, we measured expression of genes known to be important in the development of pulmonary hypertension in transgenic-positive mice and -negative littermate controls. Using morphometric analysis, we determined the extent of remodeling of the media and adventitia in transgenic mice. To determine whether the pulmonary hypertension in transgenic mice was due to sustained vasoconstriction, we tested the ability of inhaled NO to decrease pulmonary pressures. Additionally, to determine whether left ventricular dysfunction was causing increased pulmonary pressures, we evaluated left ventricular function by echocardiography.

MATERIALS AND METHODS

Animals. Surfactant protein (SP)-C/TNF-α transgenic mice overexpressing TNF-α were a kind gift of Y. Miyazaki (Department of Clinical Immunology, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan). The transgenic mice were crossed with C57BL/6 mice and bred in an animal facility documented to be free of murine-specific pathogens. All transgenic mice were identified by PCR analysis of genomic DNA by use of primers reported previously (40). Transgene-negative littermates were used as controls. Transgenic mice have severalfold increased TNF-α protein and message levels (16). All animals were studied after having been bred and raised at an altitude of 5,280 ft. (Denver, CO).

Histological analysis. Mice aged 6–8 mo were killed by intraperitoneal injection of pentobarbital sodium. Lungs were inflated at 25 cmH2O static pressure by intratracheal instillation of 4% paraformaldehyde in PBS. After ~5 min, lungs were taken and immersed in same buffer overnight at 4°C, followed by immersion in 70% ethanol, and were paraffin embedded. Tissue sections were stained with either hematoxylin and eosin, Sirius red (total collagen), and/or pentachrome (immature collagen, blue; mature collagen, yellow) (16).

Morphometry. Morphometry of the pulmonary artery was performed according to established methods previously described with minor modifications (2, 10, 22). Ten pulmonary arteries from mice age 6–8 mo ranging 30–100 μm in size were digitally captured by use of Scion image software. The radius of pulmonary artery (in μm), thickness of smooth muscle layer, and thickness of perivascular fibrosis were measured by NIH Image software version 1.6. The area of the lumen of the vessel was determined, followed by capturing of the total area encompassing the media and lumen and finally the area encompassing perivascular area, media, and lumen. The radius was then calculated for each measured area and used for analysis by using the equation area = πr². Medial thickness was determined by measuring the radius of the external medial area and dividing by the internal radius of the artery. Adventitial fibrosis was determined by measuring the radius of perivascular fibrosis (collagen staining) and dividing by the internal radius of the artery plus media. Only round-shaped pulmonary arteries were chosen for morphometric analysis. Eight transgene-positive and six transgene-negative mice were used in these studies.

Cardiovascular physiology. In a separate group of animals age 6–8 mo, after induction of anesthesia (ketamine-xylazine, 15 mg/kg), a 26-gauge needle connected with a pressure transducer (Gulton-Statham, Costa Mesa, CA) was inserted percutaneously into the left ventricular and right ventricular chambers via a subxyphoid approach (11). The pressure waveform was monitored during the procedure to ensure accurate measures, and right and left ventricular pressures were recorded. Right ventricular pressure was measured from seven transgene-positive and 11 transgene-negative mice. Left ventricular pressure was measured in three transgene-positive and nine transgene-negative mice.

NO inhalation. NO was administered to mice age 6–8 mo by placing the head of the animal in a hood flushed with a mixture of room air plus NO to a final concentration of 25 ppm NO (Scott Medical Products, Plumsteadville, PA) for 5 min. NO concentration was monitored by continuous electrochemical gas analysis (Pulmonox II; Pulmonox Medical) (11). Right and left ventricular pressures were measured by the method described above. For measurement of right ventricular pressure, five transgene-positive and six transgene-negative mice were used. For left ventricular measurements, four transgene-positive and five transgene-negative mice were used.

Echocardiography. To determine whether left ventricular dysfunction contributes to pulmonary hypertension, M-mode and Doppler echocardiography were performed in a blinded fashion as previously described (44). Mice were anesthetized by intraperitoneal injection of triethanolamine. Triethanolamine, a sedative that does not achieve a surgical level of anesthesia, was used because of the less invasive nature of echocardiographic measurements compared with the measurement of right and left ventricular pressures. Body fur was shaved off the chest, and electrodes were attached to...
limbs for electrocardiograph monitoring. M-mode echocardiography was recorded by use of a 5-MHz transducer. Anterior wall thickness, posterior wall thickness, left ventricular end-systolic diameter (ESD), left ventricular end-diastolic diameter, and posterior wall retarded slope were measured. Percent fraction shortening of the left ventricle as a measurement of ventricular function in vivo was calculated as (EDD – ESD)/EDD, where EDD is end-diastolic diameter. Mitral inflow, aortic outflow, tricuspid inflow, and pulmonic outflow velocity were obtained by use of a 10-MHz pulsed Doppler probe. The highest velocity with an adequate waveform was used for measurements. Eight transgene-positive and eight transgene-negative mice were used in these studies.

RNA extraction and ET-1 Northern hybridization. Lung RNA was prepared by a guanidine isothiocyanate-cesium chloride gradient procedure as previously described (65). RNA samples were electrophoresed, transferred to a nylon membrane, and hybridized with 32P-labeled murine probes specific for murine ET-1 (6). Numbers of animals at age 1 and 6 mo were three transgene-positive and three transgene-negative and at age 2.5 mo three transgene-positive and two transgene-negative.

RNase protection assay for VEGF, flk-1, and flt-1. RNase protection assay was performed according to the method previously described (65). Briefly, the probes of VEGF (9), flk-1 (39), and flt-1 (15) were designed as 244, 303, and 356 bp, respectively, on the basis of their DNA sequence data.

RT-PCR was carried out, and cDNA was attained. The cDNA was then cloned into a plasmid (pGEM4Z, Promega, Madison, WI) containing a DNA polymerase promoter and DNA sequences confirmed by sequence analyzer (ABI Prism 377 automated sequencer, Applied Biosystems, Foster City, CA). Linearized plasmid was used as a template for a riboprobe system (Promega) and labeled with 32P-labeled murine probes for ecNOS and iNOS.

RESULTS

Measurements of pulmonary hypertension and pulmonary vasoreactivity. Previously, our laboratory reported right ventricular hypertrophy and increased right ventricular pressure in TNF-α transgenic mice (16). In the present report, we expand on the previous data (16), demonstrating that right ventricular pressure was increased in transgenic mice compared with negative littermates at age 6–8 mo (Fig. 1A). There was no difference in left ventricular pressures (Fig. 1B). To test whether the pulmonary hypertension in TNF-α mice was due to sustained vasoconstriction of the pulmonary circulation, we tested the ability of NO inhalation to vasodilate the pulmonary circulation and reduce systolic right ventricular pressure in transgene-
positive and transgene-negative mice. Inhalation of NO decreased right ventricular pressure in both transgenic and nontransgenic mice to the same extent but failed to normalize right ventricular pressure in transgene-positive mice (Fig. 1A). We found no effect on systemic pressure (systolic left ventricular pressure) after inhaled NO in either transgene-negative or transgene-positive mice (Fig. 1B).

Lung histology and morphometry. To determine whether the pulmonary hypertension in TNF-α transgenic mice was associated with pulmonary vascular remodeling, we examined pulmonary arteries in fixed lungs by using hematoxylin and eosin and connective tissue stains (Sirius red and pentachrome for collagen). As shown in Fig. 2, TNF-α transgenic mice age 6–8 mo had perivascular fibrosis and adventitial thickening compared with transgene-negative littermates. This was characterized by increased collagen deposition (Fig. 2). Digital morphometric analysis confirmed the adventitial thickening in transgenic mice (Table 1). Additionally, there was a trend toward increased pulmonary arterial medial hypertrophy in transgenic compared with nontransgenic mice in hematoxylin and eosin staining (Table 1).

Echocardiography. To determine whether the pulmonary hypertension in TNF-α transgenic mice was due to left ventricular dysfunction, echocardiography was performed on mice at age 6 mo. The percent fraction shortening of the left ventricle as a measurement of ventricular function in vivo was calculated. Echocardiography demonstrated no differences of fractional shortening or left ventricular wall thickness between transgene- and nontransgenic mice (Table 2). In these studies, right ventricular size, function, or estimated pulmonary arterial pressure could not be determined.

Messenger RNA profile. To determine the expression of important modulators of vascular growth and tone, mRNA levels of specific genes were determined. Lung ET-1 expression, as measured by Northern hybridization, decreased with increasing age in transgenic mice compared with nontransgenic mice (Fig. 3A) With the use of RT-PCR, neither ecNOS nor iNOS was different in transgenic vs. nontransgenic mice at age 6 mo (Fig. 3B). With the use of RNAase protection assay, VEGF and its receptor flk-1 were decreased in transgene-positive mice compared with transgene-negative mice at all ages. Expression of flt-1 was not different in transgenic vs. nontransgenic mice at any age (Fig. 3C).

Survival. Cumulative survival of mice of either gender was determined. Although there were fewer male than female mice, at 10 mo >90% of male TNF-α transgenic mice were alive, whereas ~70% of female mice were alive (Fig. 4).

DISCUSSION

Overexpression of TNF-α in mice leads to pathological and physiological findings consistent with emphysema and severe pulmonary hypertension. In the present report, we further characterized the pulmonary hypertension and evaluated several mechanisms that may lead to pulmonary vascular disease in TNF-α mice. Remodeling of pulmonary arteries, manifest by perivascular fibrosis, increased adventitial area, and a trend toward increasing medial thickness, was present in TNF-α transgenic mice with severe pulmonary hypertension and emphysema. The right ventricular pressure in transgene-positive, hypertensive animals also failed to normalize in response to NO, suggesting that the pulmonary hypertension was not primarily due to sustained vasoconstriction but due to vascular remodeling. Neither NO synthase was decreased nor

Table 1. Morphometric results of pulmonary vasculature in SP-C/TNF-α transgenic mice and transgene-negative littermates

<table>
<thead>
<tr>
<th>Tg(−)</th>
<th>Tg(+)</th>
</tr>
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<tbody>
<tr>
<td>Radius, μm</td>
<td>29.61 ± 4.05</td>
</tr>
<tr>
<td>SMCMedia</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td>Adventitial Thickening, SMC/Radius</td>
<td>0.267 ± 0.033</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals studied with 10 arteries per animal evaluated. Tg(+), transgenic mice over expressing tumor necrosis factor-α (TNF-α); surfactant protein (SP)-C/TNF-α; Tg(−), transgene-negative littermates; SMC, smooth muscle layer. *P = 0.055; †P < 0.05.

Fig. 2. Pulmonary artery from representative SP-C/TNF-α transgenic mice (Tg(+)) and control mice (Tg(−)). Slides were stained with hematoxylin and eosin (A: Tg(+), B: Tg(−)), pentachrome (fibrosis with mature collagen, yellow) (C: Tg(+), D: Tg(−)), and Sirius red (collagen, red) (E: Tg(+), F: Tg(−)), demonstrating a perivascular fibrosis and adventitial thickening in Tg(+) but not Tg(−) lungs. Magnification: ×200.
was ET-1 increased as potential contributors to the development of pulmonary hypertension. However, there was a decrease in the expression of VEGF and its receptor, flk-1, in TNF-α/H9251 transgenic mice. Because VEGF acting through its receptor flk-1 may have a protective role in the pulmonary circulation, we conclude that chronic overexpression of TNF-α leads to pulmonary vascular remodeling and pulmonary hypertension, possibly by decreasing expression of VEGF and its receptor flk-1.

Several recent reports suggest that VEGF likely plays an important role in maintenance of normal pulmonary vascular structure. In patients with primary pulmonary hypertension, VEGF and flk-1 are found in plexiform lesions (24, 61), suggesting that VEGF may play a role in the pathogenesis of pulmonary hypertension by stimulating dysregulated angiogenesis (61). VEGF is also increased in rats with hypoxia- and monocrotaline-induced pulmonary hypertension and vascular remodeling (8, 47). However, in-

Table 2. UCG B-mode data of LV wall thickness and motion in Tg(+) and Tg(−) at age 6 mo

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>AW-s, mm</th>
<th>AW-d, mm</th>
<th>LVID-s, mm</th>
<th>LVID-d, mm</th>
<th>PW-s, mm</th>
<th>PW-d, mm</th>
<th>%FS</th>
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<tr>
<td>Tg(−)</td>
<td>8</td>
<td>1.022 ± 0.058</td>
<td>0.519 ± 0.039</td>
<td>2.311 ± 0.144</td>
<td>3.601 ± 0.146</td>
<td>1.040 ± 0.081</td>
<td>0.698 ± 0.053</td>
<td>36.08 ± 1.83</td>
</tr>
<tr>
<td>Tg(+)</td>
<td>8</td>
<td>1.074 ± 0.054</td>
<td>0.534 ± 0.041</td>
<td>2.199 ± 0.115</td>
<td>3.495 ± 0.093</td>
<td>1.161 ± 0.071</td>
<td>0.681 ± 0.058</td>
<td>37.10 ± 2.37</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. s, Systolic; D, diastolic; AW, anterior wall; LVID, left ventricular intercept diameter; PW, posterior wall; FS, fraction shortening.
hinition of the VEGF receptor flk-1 caused pulmonary hypertension characterized by thickening of the medial layer of pulmonary arteries in normoxic rats (59). Additionally, in severely hypoxic rats treated with a flk-1 inhibitor, more marked pulmonary hypertension developed with a marked increase in endothelial cell proliferation in the pulmonary artery. The authors suggest that VEGF, acting through flk-1, inhibits endothelial cell death (59). Thus, when flk-1 is blocked, endothelial cell death is enhanced, and proliferation of apoptosis-resistant endothelial cells and smooth muscle cells leads to vascular remodeling and pulmonary hypertension (59). In other studies, adenovirus-mediated gene transfer and cell-based gene transfer of VEGF both attenuated the development of hypoxia- and monocrotaline-induced pulmonary hypertension, respectively (4, 46). Although reports have suggested a role for VEGF in the pathogenesis of pulmonary hypertension, these recent reports suggest that VEGF is important in attenuating the development of pulmonary hypertension, possibly by protecting endothelial cells from injury and apoptosis (3, 17). In the present study, decreased expression of VEGF and flk-1 message was associated with severe pulmonary hypertension in TNF-α-overexpressing mice. Although we report hemodynamics in older transgenic animals, derangements in VEGF and flk-1 message were present in the youngest animals, suggesting that the decrease in VEGF and flk-1 expression may have had a permissive role in the development of pulmonary hypertension. This is in agreement with the hypothesis that VEGF, acting through flk-1, has a protective effect on the integrity of the pulmonary circulation.

Decreased expression of VEGF and flk-1 may also play a role in the development of emphysema in TNF-α transgenic mice (28, 29, 59). Decreased VEGF and flk-1 were found in the lungs of patients with emphysema and was associated with an increase in number of apoptotic pulmonary endothelial and epithelial cells (28). This suggests that a primarily vascular process might contribute to the development of severe emphysema (37). Because of the development of emphysema, we cannot exclude an absolute decrease in vessel number contributing to the development of pulmonary hypertension and vascular remodeling in TNF-α transgenic mice. However, in our laboratory’s previous report, it was found that there was evidence of right ventricular hypertrophy before the development of severe emphysema (16), suggesting that vascular disease may precede the onset of severe airway disease in TNF-α transgenic mice.

Altered regulation of endogenous pulmonary vasodilators or vasoconstrictors may play a role in the development of pulmonary hypertension. Expression of the endogenous vasodilator NO and the enzyme responsible for its production play an important role in experimental pulmonary hypertension (12, 31). Previously, ecNOS was reported as reduced in pulmonary hypertension patients and correlated with severity of morphological change (18). Congenital deficiency of ecNOS in mice leads to sustained pulmonary hypertension and right ventricular hypertrophy after chronic hypoxia (57), and ecNOS-null mice were also reported to be hyperresponsive to mild hypoxia (11). Overexpression of ecNOS attenuates pulmonary hypertension (5). Previous reports have suggested that TNF-α decreased ecNOS message in pulmonary artery endothelial cells (67). However, decreased ecNOS expression was not present in hypertensive TNF-α transgenic mice, suggesting that this likely did not play a role in the development of pulmonary hypertension. Additionally, in both rats and mice, iNOS is increased in the lung with hypoxia-induced pulmonary hypertension (13, 33, 51), and decreased iNOS may be important in the regulation of pulmonary vascular tone (14). Although TNF-α has been reported to induce iNOS expression (60) and iNOS expression may increase with inflammation, we did not observe changes in iNOS message with TNF-α overexpression. Lastly, TNF-α itself may also act to enhance vasoconstriction in the pulmonary circulation (7).

ET-1, a potent endogenous vasoconstrictor, is localized in smooth muscle cells of arteries and bronchioles, inflammatory cells, epithelium of bronchioles and pleura, and endothelium in the lung. ET-1 may contribute to collagen deposition in the lung (42) and contribute to perivascular fibrosis in pulmonary hypertension (25, 35). ET-1 is also increased in the lungs of rats with spontaneous pulmonary fibrosis at Denver altitude (55) and after chronic hypoxia (32, 43). Mice overexpressing human prepro-ET-1 have modest increases in lung ET-1 peptide and age-dependent pulmonary inflammation and perivascular fibrosis similar to that seen in this report (25). However, the mice did not have evidence of pulmonary hypertension at any age studied (25). In the present report, ET-1 message did not increase with age in TNF-α overexpressors as in negative littermates, suggesting that ET-1 likely did not have a causative or sustaining role in the development of pulmonary hypertension.

Inhaled NO has been used in several clinical situations, including primary pulmonary hypertension, acute respiratory distress syndrome (52, 53), and persistent pulmonary hypertension of the newborn (26). NO also attenuates the pulmonary hypertension associated with pulmonary hypertension and vascular remodeling in TNF-α transgenic mice. However, in our laboratory’s previous report, it was found that there was evidence of right ventricular hypertrophy before the development of severe emphysema (16), suggesting that vascular disease may precede the onset of severe airway disease in TNF-α transgenic mice.

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associated with chronic obstructive pulmonary disease (41, 66). To determine whether pulmonary hypertension in TNF-α mice could be reversed by administration of a vasodilator, inhaled NO was administered to transgenic mice with pulmonary hypertension. Although right ventricular pressure decreased slightly in both TNF-α transgenic and nontransgenic mice, NO did not normalize right ventricular pressure in transgenic mice, suggesting that the pulmonary circulation was remodeled, consistent with the histological results. Although this suggests that sustained vasoconstriction was not the primary mechanism of pulmonary hypertension in TNF-α transgenic mice, it does not rule out that dysregulation of pulmonary vascular tone did not play a role in the early development of pulmonary hypertension.

Consistent with our previous findings of normal left ventricular weights in TNF-α-overexpressing mice, left ventricular wall thickness by echocardiography was also similar between transgene-positive and transgene-negative mice (16). Although TNF-α is known to increase systemic resistance (38), the fractional shortening was similar between the two groups confirming that left ventricular function was not suppressed in TNF-α transgenic mice and was not likely the cause of pulmonary hypertension.

Human primary pulmonary hypertension is more common in young women but is not associated with earlier death compared with afflicted men (34). In the present study, although survival was decreased in all TNF-α transgenic compared with wild-type mice, female mice had a significantly higher mortality rate starting at 6 mo of age. Elevated pulmonary artery pressure is associated with higher mortality in patients with emphysema (45), but, in the present study, female mice did not have more severe pulmonary hypertension compared with male mice. Interestingly, there were fewer male transgenic mice born, consistent with observations in human familial pulmonary hypertension.

A significant limitation of the present study is the small number of animals in some groups in which we measured gene expression for ET-1, VEGF, flk-1, and flt-1. Although we did observe differences in VEGF and flk-1 but not flt-1, using increased numbers of animals might lead to a stronger conclusion regarding lung RNA levels in TNF-α transgenic mice. We are confident that the numbers of animals used to assess hemodynamics, vasoreactivity, and vascular remodeling were sufficient to support our conclusions.

In conclusion, overexpression of TNF-α resulted in severe pulmonary hypertension and emphysema. Remodeling of the pulmonary circulation likely represents the major reason for pulmonary hypertension and may be related to derangements in expression of VEGF and its receptor flk-1. Although the mechanism leading to derangements in VEGF expression in TNF-α transgenic mice is not known, it may be related to overexpression of TNF-α or as a result of the presence of emphysema. Given recent studies demonstrating the development of pulmonary hypertension and emphysema with the VEGF receptor flk-1 antagonist (29, 59), we speculate that decreased VEGF and flk-1 expression due to TNF-α overexpression may place into motion events leading to severe lung disease.

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REFERENCES


Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J* 15: 427–438, 2001.


