Granulocyte colony-stimulating factor enhances alpha-naphthylthiourea-induced pulmonary hypertension

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Submitted 4 September 2002; accepted in final form 4 January 2003

Azoulay, Élie, Saadia Eddahibi, Élisabeth Marcos, Micheline Levame, Alain Harf, Benoît Schlemmer, Serge Adnot, and Christophe Delclaux. Granulocyte colony-stimulating factor enhances alpha-naphthylthiourea-induced pulmonary hypertension. J Appl Physiol 94: 2027–2033, 2003. First published January 10, 2003; 10.1152/japplphysiol.00807.2002.—Physiopathological discrepancies exist between the most widely used models of pulmonary hypertension (PH), namely monocrotaline- and hypoxia-induced PH. The development of a new model could help in the understanding of underlying mechanisms. Repeated alpha-naphthylthiourea (ANTU) injections (5 mg/kg weekly, 3 wk) induced pulmonary vascular remodeling, which was associated with development of PH and right ventricular hypertrophy. ANTU followed by granulocyte colony-stimulating factor (G-CSF; 25 μg·kg⁻¹·day⁻¹ subcutaneously, 3 days/wk) induced higher pulmonary arterial pressures and right ventricular hypertrophy than ANTU alone. Lidocaine, which inhibits neutrophil functions, inhibited PH exacerbation by G-CSF. Endothelial nitric oxide synthase expression, measured to assess ANTU-related endothelial toxicity, decreased significantly in ANTU-treated rats and fell even more sharply when G-CSF was given. This occurred despite a significant increase in vascular endothelial cell growth factor expression in lung and right ventricle in rats given ANTU alone and even more in rats given ANTU plus G-CSF. Repeated ANTU administration induces PH with vascular remodeling that can be further aggravated by the neutrophil activator G-CSF.

neutrophil; pulmonary hypertension model; lidocaine

PULMONARY HYPERTENSION (PH) occurs in many clinical settings, such as chronic conditions, i.e., primary PH, hypoxic PH after respiratory insufficiency, but also acute diseases like acute respiratory distress syndrome. Therefore, despite different initial injuries, common histopathological features are further evidenced, suggesting common biological pathways (7). Consequently, animal models have been developed to study PH pathophysiology, with the most frequently used being hypoxia- and monocrotaline-induced PH. These two models share common features, such as pulmonary vascular remodeling produced by cell hypertrophy and extracellular matrix protein synthesis in response to endothelial abnormalities (18). Besides these common features, some discrepancies have been evidenced, because monocrotaline-induced PH is characterized by early inflammatory injury, namely vascular platelet sequestration and alveolar neutrophil recruitment in contrast to hypoxia-induced PH (10, 19). Another difference between hypoxic and monocrotaline-induced PH is that lung expression of the angiogenic factor VEGF is markedly decreased after treatment with monocrotaline but unmodified by exposure to hypoxia (16). These discrepancies are of critical importance because they can turn in opposite responses to therapeutic approaches. Indeed, using the tissue inhibitor of matrix metalloproteinase-1 adenosynergic factor VEGF is markedly decreased after treatment with monocrotaline but unmodified by exposure to hypoxia (16). These discrepancies are of critical importance because they can turn in opposite responses to therapeutic approaches. Indeed, using the tissue inhibitor of matrix metalloproteinase and monocrotaline-induced PH. Nevertheless, Hill and colleagues (8) demonstrated that the effect of matrix metalloproteinase inhibition, namely a beneficial effect in monocrotaline-induced PH (unpublished observations) and a detrimental effect in hypoxia-induced PH (23). To further delineate the critical biological mechanisms underlying these functional discrepancies, the development of other models of PH could be a useful approach. In 1984, Hill and colleagues (8) described PH after repeated administration of alpha-naphthylthiourea (ANTU). ANTU induces neutrophil-independent pulmonary toxicity due to acute toxic lung microvascular injury associated with high-permeability edema (5), as evidenced during monocrotaline-induced PH. Nevertheless, Hill and colleagues (8) demonstrated right ventricular (RV) hypertrophy without substantial vascular remodeling 4 wk after repeated ANTU-induced lung injury in rats. The first aim of this study was, consequently, to better characterize histological features of ANTU-induced PH to assess similarities and discrepancies with previously described models.

Neutrophil derivatives, i.e., both leukocyte elastase and reactive oxygen species, seem to be involved in several circumstances associated with acute PH (3, 4, 13, 14, 22). Granulocyte colony-stimulating factor (G-
CSF) is the most important regulatory cytokine capable of stimulating the production of neutrophilic granulocytes from committed hematopoietic progenitor cells, both in vitro and in vivo. G-CSF increases neutrophil counts and primes and enhances many neutrophil functions. Due to these effects, G-CSF has been found to exacerbate experimental acute lung injury, including ANTU-induced acute lung microvascular injury (1, 11). We hypothesized that this last effect may lead to exacerbation of ANTU-related PH and pulmonary artery remodeling. The second aim of this study was, therefore, to assess whether G-CSF worsens ANTU-induced PH in rats.

MATERIALS AND METHODS

Animal Model

One hundred twelve male, specific-pathogen-free Sprague-Dawley rats (Charles River Laboratories, Saint Aubin Les Elbeuf, France), each with a body weight of 225–250 g, were used. The rats were maintained on a standard laboratory diet and housed in a controlled environment with a 12:12-h light-dark cycle. Animals were housed as recommended by the European Convention for Protection of Experimental Animals (decree no. 2001-131 06, February 2001). The local animal subcommittee approved the animal protocols.

Experimental Design

We conducted five sets of experiments. Preliminary experiments done to characterize ANTU-induced PH involved a comprehensive hemodynamic study, measurement of RV hypertrophy, and description of lung morphology. The effects of 5 mg/kg ANTU (Kodak, Rochester, NY) or the vehicle 500-μL DMSO (Sigma Chemical, Sigma Aldrich) given intraperitoneally (ip) once a week for 4 wk were compared in two groups of 12 rats. One week after the fourth injection, the rats were anesthetized with pentobarbital sodium (30 mg/kg body wt ip) and exsanguinated via the abdominal aorta. The following were assessed: body and heart weights; Fulton index; RV, pulmonary arterial, and carotid arterial pressures; lung wet-to-dry weight ratios; and lung morphology.

Measurement of RV hypertrophy using the Fulton index. The heart and lungs were excised and weighed, and the ratio of RV free wall weight over septum plus left ventricular (LV) free wall weight was estimated and used as the index of RV hypertrophy (Fulton index).

Hemodynamic study. Hemodynamic measurements were performed as previously described (16). Rats were anesthetized with ketamine (20 mg/100 g) and xylazine (1 mg/100 g). After exposure of the right jugular vein, a polyvinyl catheter was inserted and manipulated through the RV into the pulmonary artery. A polyethylene catheter was inserted into the right carotid artery. Immediately after insertion of the catheters, pulmonary and systemic arterial pressures were measured by using Gould P23 ID transducers coupled to pressure modules and a Gould TA 550 multichannel recorder. Only pulmonary arterial pressures successfully recorded within 30 min of catheter insertion were used for the study.

Wet-to-dry weight ratios. To determine whether lung edema developed after each ANTU injection, the left lobe of a lung was removed and weighed on a balance before and after drying for 2 days in a vacuum oven at 40°C. The wet-to-dry weight ratio was obtained by dividing wet weight by dry weight. Although this parameter does not detect differences in intravascular blood volume, it seems a reasonable indicator of extravascular pulmonary edema (1).

Lung morphology. The lungs were fixed in the distended state by infusion of 4% aqueous buffered formalin into the trachea at a pressure of 25 cmH2O. The entire specimen was left in a bath of the same fixative for 1 wk. A midsagittal slice of the right lung, including the apical, azygous, and diaphragmatic lobes, was processed for paraffin embedding. Sections 5 μm thick were cut for light microscopy and stained with hematoxylin-phloxin-saffron and orcein-picroindigo-carmine. In each rat, 35–65 intra-acinar vessels were analyzed to assess the distribution of the degree of muscularization according to the accompanying air space, i.e., an alveolar duct or alveolus. Muscularization was defined as the presence of typical smooth muscle cells stained red by phloxin and exhibiting an elongated shape and square-ended nuclei. Intra-acinar vessels were categorized as muscular, partially muscular, or nonmuscular. The external diameter (distance between and including the two external elastic laminae intersected by the diameter) and medial thickness (distance from the luminal surface and the internal elastic laminae to the abluminal surface of the external lamina) were recorded for all muscularized and partially muscularized arteries.

In the second set of experiments, Fulton indexes and lung morphology were assessed after 1, 2, and 3 wk of weekly ANTU or DMSO administration to six groups of eight rats. In six rats, the effect of ANTU was assessed 2 wk after weekly ANTU injections during cyclophosphamide-induced neutropenia (1).

In the third set of experiments, body and heart weights, lung wet-to-dry weight ratios, and lung morphology were assessed after 1, 2, or 3 wk in three groups (one per time point) of eight rats injected weekly with 5 mg/kg ANTU followed by 25 μg/kg G-CSF (Lenograstime, CHUGAI Pharmaceutical) given subcutaneously once a day for 3 days, i.e., 1, 2, and 3 days after ANTU. Human recombinant G-CSF has been found to be effective in rats (1, 11). In a group of 12 rats given four weekly ANTU injections followed by one G-CSF injection on 3 consecutive days, the same parameters were assessed, and a complete hemodynamic study was performed.

In the fourth set of experiments, we assessed whether the exacerbation of PH observed with G-CSF was related to neutrophil-dependent worsening of lung endothelial injury. To this end, we examined the effect of lidocaine (Astra), an inhibitor of neutrophil functions (15), in eight rats after 2 wk of ANTU administration. Lidocaine was given ip in a dose of 50 mg/kg 2 h before and at the time of each of the three G-CSF injections. In preliminary experiments, we evaluated whether lidocaine can modify per se ANTU-induced PH.

Three groups of six rats were treated for 2 wk with DMSO + saline, ANTU + saline, or ANTU + G-CSF, as previously described. One week after the last ANTU injection, the animals were killed. Specimens of the lungs, RV, and LV were harvested, weighed, and homogenized in pH 7.40 lysate buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl2, and 1 mM MgCl2. Homogenates were incubated on ice for 30 min and then centrifuged for 20 min at 10,000 g and 4°C. Supernatants were stored at −80°C for assays of endothelial nitric oxide synthase (eNOS) and VEGF.

eNOS Western blot. The lungs were snap-frozen in liquid nitrogen immediately after removal. After thawing at 0°C, the tissues were sonicated in 0.1 M PBS containing antiproteases (1 μM leupeptin and 1 μM pepstatin A). The homogenates were subjected to SDS-polyacrylamide gel electrophoresis. Proteins in the gel were transferred to a nitro-
cellulose membrane by electroblotting in a transblot Bio-Rad transfer apparatus. Gels, Whatman filter paper, and nitrocellulose membrane were soaked in electroblotting buffer (25 mM Tris-HCl, 193 mM glycine, 20% methanol, pH 8.0) for 15 min before transferring. After protein transfer for 12 h at 4°C, the membrane was blocked with 1× Tris-buffered saline-Tween-20 (0.15 M NaCl, 10 mM Tris-HCl, 0.05% Tween 20, 5% bovine serum albumin, pH 8.0) for 1 h at room temperature. The eNOS protein was detected by incubating the membrane overnight at 4°C with goat polyclonal anti-eNOS (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1,000. Then the membrane was washed three times in 1× Tris-buffered saline-Tween-20. Specific protein was detected by using horseradish peroxidase-conjugated secondary antibody and electrochemiluminescent reagents (Amersham). To quantitate eNOS immunoreactivity, we used a semiautomated image analysis device (NIH image 1.52) that quantifies both the area and the intensity of immunoreactive bands, with the use of a ScanJet II scanner and DeskScan II (Hewlett Packard) software. Results are expressed as arbitrary units.

Lung and heart preparations for VEGF analysis. The lungs and hearts were harvested, weighed, and homogenized in pH lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15

Fig. 1. A: changes in body weight in control rats (solid line), rats given 5 mg/kg alpha-naphthylthiourea (ANTU; dotted line), and rats given both 5 mg/kg ANTU and 25 μg/kg granulocyte colony-stimulating factor (G-CSF; dashed line). B: results of right heart catheterization on day 28 (1 wk after the fourth ANTU injection). Left: the Fulton index is the ratio of right ventricular weight over left ventricular + septal weight. Right: mean pulmonary arterial pressure (MPAP) was obtained as described in MATERIALS AND METHODS. C: changes in the Fulton index during the 4-wk study period in the rats given ANTU alone (dotted line) or ANTU + G-CSF (dashed line). In the control group, the Fulton index was the same on day 0 and at the 4 other time points. Values are means ± SE. *P < 0.05; **controls vs. ANTU; ***ANTU vs. ANTU + G-CSF.
mM Tris, 1 mM CaCl, and 1 mM MgCl (pH 7.40). Homogenates were incubated on ice for 30 min and then centrifuged for 20 min at 12,000 g and 4°C. Supernatants were stored at −80°C for cytokine assays.

VEGF concentrations in lung homogenates and in the RV and LV were quantitated by using a specific ELISA as recommended by the manufacturer. The detection threshold was 5 pg/ml. Results were expressed as picograms per gram.

Statistics

The data are expressed as means ± SE. Data were analyzed by using the nonparametric Kruskal-Wallis test, and, when differences were found among groups, the Mann-Whitney U-test was used to evaluate the significance of differences in continuous variables between control and individual experimental groups. Statistical significance was defined as \( P < 0.05 \). STATVIEW 5.0 software (Abacus Concepts, Berkeley, CA) was used for all statistical tests.

RESULTS

Mortality and Total Body Weight Changes After ANTU Treatment With or Without G-CSF

No deaths occurred among control rats given only 500 µl of DMSO ip. In the 5 mg/kg ANTU group, 10% of the rats died within 24 h after the first injection. No additional mortality was observed after the first or subsequent ANTU injections. In the ANTU + G-CSF group, 15% of the rats died; all excess deaths in this group occurred 1 day after the first G-CSF injection.

As shown in Fig. 1A, control rats gained weight linearly, doubling their weight during the 4-wk study period. During the first 3 wk, no significant differences were observed among the three groups. On day 28, body weight was significantly lower in the ANTU group than in the controls and in the ANTU + G-CSF group than in the ANTU group.

ANTU-related Chronic Lung Vascular Injury

Preliminary experiments. As shown in Fig. 1, rats given ANTU for 4 wk had higher values for the Fulton index and mean pulmonary arterial pressure (MPAP), compared with control rats (0.35 ± 0.003 vs. 0.24 ± 0.003, \( P = 0.0001 \); and 23.5 ± 0.5 vs. 17 ± 0.5, \( P = 0.0004 \); respectively). The ratio of RV weight over total body weight was significantly higher in the rats given 4 wk of ANTU than in the controls. The increase in MPAP was not associated with an elevation in right atrial pressure, and no differences were found for heart rates (ANTU, 295 ± 25 beats/min vs. control, 280 ± 15 beats/min) or carotid arterial pressures (ANTU, 105 ± 10 mmHg vs. control, 112 ± 5 mmHg) compared with the control group. Complete muscularization of the pulmonary artery walls (Fig. 2 shows an example) was seen in more than one-half of the studied arteries. The muscularization was sectorial: some lung sites were spared. The veins were normal. There was mild macrophagic alveolitis but no pulmonary edema. No pulmonary edema was seen in lung sections, which was consistent with the fact that wet-to-dry weight ratios were similar in ANTU-treated rats and in controls (3.98 ± 0.06 vs. 4.11 ± 0.10).

Fig. 2. Lung morphology in rats 1 wk after the fourth injection of 5 mg/kg ANTU. Complete pulmonary arterial muscularization is shown. Sections were stained with hematoxylin-phloxin-saffron. Original magnification, ×400.

Kinetics of ANTU-related RV hypertrophy and pulmonary artery remodeling. As shown in Fig. 1C, weekly ANTU injections caused early RV hypertrophy. Although the Fulton index was slightly higher after one ANTU injection than in the controls, the difference was not significant, and lung morphology was normal except for a few smooth muscle cells (<10%) in a small minority of pulmonary artery walls. After two weekly ANTU injections, the Fulton index was significantly higher than in the controls, and partial muscularization was seen in ~30% of studied pulmonary arteries. The third weekly ANTU injection induced a further significant increase in the Fulton index, with complete muscularization of 30% of studied pulmonary arteries. The Fulton index was slightly lower after the fourth injection. When ANTU was injected into rats with neutropenia (<1,000 leukocytes per liter), a similar pattern of RV hypertrophy and pulmonary artery remodeling occurred (data not shown). Six weeks after the four weekly ANTU injections, the Fulton index and lung morphology were normal.

G-CSF exacerbated ANTU-related chronic lung vascular injury. Figure 1 shows that PH was more severe in the rats given ANTU + G-CSF. At all four time points, the Fulton index was higher in the ANTU + G-CSF group than in the ANTU group, and MPAP was higher in the ANTU + G-CSF group than in the ANTU group on day 28. Complete muscularization was seen in 50, 60, 75, and 90% of studied pulmonary arteries after 1, 2, 3, and 4 wk, respectively (Fig. 3). Adventitial thickening related to connective tissue proliferation in pulmonary artery walls (arrow in Fig. 3, B, C, and D) occurred only in the ANTU + G-CSF group. The orcein-picroindigo-carmine stain showed no elastic fiber changes. Moreover, wet-to-dry weight ratios in the ANTU + G-CSF groups were similar to those in the ANTU and control groups.
Lidocaine reversed the effect of G-CSF. As shown in Fig. 4, RV hypertrophy was not exacerbated when lidocaine was given with ANTU + G-CSF. Preliminary experiments demonstrated that lidocaine did not inhibit ANTU-induced PH (Fulton index at day 14, ANTU + lidocaine: 0.32 ± 0.004 vs. ANTU + vehicle: 0.31 ± 0.007; n = 5 in each group).

Expression of eNOS and VEGF proteins in lungs. Figure 5 shows that eNOS expression in lung and RV tissue was significantly lower in rats given ANTU than in controls and in rats given ANTU + G-CSF than in rats given ANTU. However, eNOS in LV was similar in each of these three groups.

Figure 5B shows that, in contrast to eNOS, VEGF in lung and RV tissue was significantly higher in rats given ANTU than in controls and in rats given ANTU + G-CSF than in rats given ANTU. However, VEGF in LV tissue was similar in the three groups.

**DISCUSSION**

Although the acute pulmonary toxicity of ANTU has been extensively described (5), a single study has reported chronic lung vascular toxicity related to repeated ANTU administration: Hill et al. (8) found PH and RV hypertrophy in rats given ANTU for 4 wk. Although they observed no evidence of pulmonary artery remodeling, this does not rule out focal muscularization as found in the present study. ANTU-related endothelial injury is direct, independent from neutrophils, and related to intrinsic properties of ANTU that induce cell blebbing and signs of necrosis and apoptosis (5). To assess ANTU-related endothelial toxicity, we measured eNOS expression in lung tissues. Repeated ANTU injections were followed by a decrease in lung eNOS expression. This decrease may be involved in the abnormal vascular reactivity shown by Hill and Rounds (9) in the same model. ANTU-induced injury is
known to be confined to the endothelial wall in the alveolocapillary barrier. Endothelial injury occurred despite concomitant increased expression of lung VEGF that is produced mainly by epithelial cells during recovery from lung vascular injury (2). Such an increase in VEGF has been interpreted as limiting endothelial injury. In the present study, it is likely that a regulatory mechanism contributed to enhance lung VEGF that limits endothelial injury, because VEGF has been shown to be an endothelial cell survival factor capable of preventing apoptosis (21), and its overexpression protects against hypoxic PH (17).

Our results show that ANTU-induced PH shares common features with hypoxia-induced PH: a mild level of PH and the absence of alveolar inflammatory cell recruitment due to the absence of alveolar macrophage activation (1). Although the muscularization seen in our animals resembled that evidenced in hypoxia-related PH, our animals were not hypoxic, as recently shown by arterial blood-gas measurements 6 and 24 h after ANTU injection (unpublished observations), the period of most severe lung edema in the study by Hill and colleagues (8). It would be of interest to further assess the role of both endothelin and serotonin systems, which are important mediators involved in the pulmonary vascular remodeling and the development of hypoxic PH (6).

Taken together, these results are consistent with the concept that repeated endothelial injury seems to be the main factor leading to PH. Interestingly, this model of repeated administration of a toxic compound could mimic the effects of the chemotherapy course. Along this line, PH has been reported after mitomycin therapy (12, 20).

To the best of our knowledge, this is the first study designed to measure the additive effect of neutrophils on chronic lung vascular injury. All features characterizing ANTU-related PH were exacerbated by G-CSF. G-CSF is known to prime neutrophils for reactive oxygen species release and upregulation of adhesion molecules. Thus sequestration and activation of neutrophils...
phils toward previously injured endothelial cells may exacerbate ANTU-related toxicity, causing a further decrease in eNOS and a further increase in VEGF. Furthermore, the inhibitory effect of lidocaine, which prevents neutrophil activation and adhesion to endothelial cells (15), suggests that neutrophils are at the epicenter of PH exacerbation by G-CSF. These results emphasize the fact that, despite the absence of a role of neutrophils in initial injury, they can aggravate endothelial lesions, further enhancing vascular remodeling. The relevance of such findings is highlighted by the fact that ANTU-related PH could mimic some chemotherapy regimens, a clinical situation for which G-CSF is often prescribed.

In conclusion, repeated endothelial injury related to ANTU administration caused PH sharing similarities with both hypoxia- and monocrotaline-induced PH. G-CSF enhanced ANTU-related PH via a mechanism involving neutrophils, demonstrating that neutrophils can also exacerbate pulmonary remodeling observed during PH.

This study was supported by a grant from Aventis Pharma France.

REFERENCES


