Cardiovascular, Pulmonary and Renal Pathology

Carbon Monoxide Suppresses Bleomycin-Induced Lung Fibrosis

Zhihong Zhou,* Ruiping Song,*
Cheryl L. Fattman,[‡] Sara Greenhill,* Sean Alber,[†]
Tim D. Oury,[‡] Augustine M.K. Choi,* and
Danielle Morse*

From the Division of Pulmonary, Allergy, and Critical Care Medicine,* and the Departments of Cell Biology and Physiology[†] and Pathology,[‡] University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

Idiopathic pulmonary fibrosis is an incurable fibrosing disorder that progresses relentlessly to respiratory failure. We hypothesized that a product of heme oxygenase activity, carbon monoxide (CO), may have anti-fibrotic effects. To test this hypothesis, mice treated with intratracheal bleomycin were exposed to low-concentration inhaled CO or ambient air. Lungs of mice treated with CO had significantly lower hydroxyproline accumulation than controls. Fibroblast proliferation, thought to play a central role in the progression of fibrosis, was suppressed by *in vitro* exposure to CO. CO caused increased cellular levels of p21^{Cip1} and decreased levels of cyclins A and D. This effect was independent of the observed suppression of MAPK's phosphorylation by CO but was dependent on increased cGMP levels. Further, CO-exposed cells elaborated significantly less fibronectin and collagen-1 than control cells. This same effect was seen *in vivo*. Suppression of collagen-1 production did not depend on MAPK or guanylate cyclase signaling pathways but did depend on the transcriptional regulator Id1. Taken together, these data suggest that CO exerts an anti-fibrotic effect in the lung, and this effect may be due to suppression of fibroblast proliferation and/or suppression of matrix deposition by fibroblasts. (Am J Pathol 2005, 166:27–37)

Fibrosis of the lung interstitium represents the ultimate pathological response to injury. Disease states as diverse as systemic collagen-vascular diseases, infection, acute lung injury, and occupational exposure to inhalants can result in lung fibrosis. Although the pathophysiological changes associated with different types of lung injury

vary, all involve some degree of accumulation and activation of chronic inflammatory cells, fibroblast proliferation, and deposition of extracellular matrix proteins such as collagen.

Carbon monoxide (CO) is a biologically active molecule produced by the human body in states of health and disease; the enzyme heme oxygenase is responsible for the elaboration of CO by catabolism of heme. The inducible form of heme oxygenase, HO-1, is a stress-responsive molecule that is critical to the defense of cellular homeostasis. There is evidence that CO exerts protective effects in the setting of lung injury.¹ Inhaled CO has also been shown to be protective in animal models of lung transplantation, asthma, and ischemia-reperfusion.^{2–4} The mechanism by which CO might provide pulmonary cytoprotection has not been entirely elucidated, but down-regulation of proinflammatory cytokines such as tumor necrosis factor- α along with augmentation of the anti-inflammatory cytokine interleukin-10 appear to play a role.⁵ CO also has well-described anti-proliferative properties, and has been shown to inhibit smooth muscle cell proliferation and limit vascular intimal hyperplasia in response to injury.^{6–10}

An important feature in idiopathic pulmonary fibrosis/usual interstitial pneumonia (UIP) is the presence of fibroblast foci, microscopic aggregates of fibroblasts that proliferate and contribute to the accumulation of extracellular matrix molecules. Current evidence suggests that the presence and extent of fibroblastic foci in the affected lung represent the morphological change most closely associated with subsequent progression to dense fibrosis.¹¹ Given the known anti-proliferative properties of CO, we chose first to test whether CO could protect against lung fibrosis *in vivo* and could inhibit fibroblast proliferation *in vitro*. We then investigated whether CO was capable of inhibiting matrix production by fibroblasts.

Supported by the Veteran's Administration (Research Career Development Award to D.M.), the GEMI Fund (grant to D.M.), and the National Institutes of Health (grants HL60234, HL55330, and AI42365 to A.M.K.C.).

Accepted for publication September 15, 2004.

Address reprint requests to Danielle Morse, M.D., Division of Pulmonary, Allergy, and Critical Care Medicine, NW 628 UPMC Montefiore, 3459 Fifth Ave., Pittsburgh, PA 15213. E-mail: morseed@msx.upmc.edu.

Materials and Methods

Animals

Male CBA/J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were allowed to acclimate for 1 week with rodent chow and water *ad libitum*. All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and were approved by the Animal Care and Use Committee of University of Pittsburgh School of Medicine.

Bleomycin Administration

Bleomycin (Bristol-Myers Squibb, Princeton, NJ) was administered intratracheally at a dose of 0.075 U/mouse in 50 μ l of sterile saline. All intratracheal instillations were performed by cannulation of the trachea via the mouth with a 20-gauge feeding needle. The mice were lightly sedated with isofluorane for this procedure.

Carbon Monoxide Exposures

Mice or macrophages were exposed to compressed air with varying concentrations of CO (0 to 500 ppm). For cell culture experiments, the atmosphere included 5% CO₂ for adjustment of pH. Details of CO administration have been previously described.¹

Hydroxyproline Assay

Fourteen days after bleomycin administration, mouse lungs were harvested for determination of hydroxyproline content. The left lungs of all animals were used for this assay. Details of this technique have been previously described.^{12,13}

Histopathological Scoring System

Lung sections stained with hematoxylin and eosin and trichrome stains were scored for fibrosis by a lung pathologist (T.O.) in a blinded manner. Lungs were examined under high power and scored for a total of 10 random high-power fields per specimen starting at the center of the area with the most severe fibrosis and then scoring every other high-power field in a radial manner from this starting point (the original starting point is not scored). Any high-power view containing less than 50% alveolar parenchyma was not scored. Fibrotic/reparative changes in the interstitium and alveolar walls were assessed according to the percentage of tissue involved using a 0 to 4 grading system. A score of 0 corresponded with absence of fibrosis, a score of 1 indicated the involvement of 1 to 25% of the tissue in the field, a score of 2 corresponded with 25 to 50%, 3 with 50 to 75%, and 4 with 75 to 100% involved tissue. Scores were then averaged per treatment group (bleomycin alone or bleomycin with CO) and compared to control using a Student's

t-test. Results were considered significant if *P* = 0.05 or less.

Cell Treatment and Reagents

Human fetal lung fibroblasts (MRC-5) (purchased from the American Type Culture Collection, Rockville, MD) and mouse lung fibroblasts were maintained and grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.1% gentamicin. The guanylate cyclase inhibitor 1H(1,2,4)oxadiazolo(4,3-a)quinoxalin-1 (ODQ) (10 μ mol/L; Calbiochem-Novabiochem, San Diego, CA) was dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO). SB203580 (20 μ mol/L), a selective inhibitor of p38 MAPK; UO126 (20 μ mol/L), a selective inhibitor of mitogen-activated protein kinase kinase 1/2 (MEK1/2); and SP600125 (20 μ mol/L), a selective c-jun n-terminal kinase (JNK) inhibitor (Calbiochem, Darmstadt, Germany), were dissolved in dimethyl sulfoxide. The inhibitor of protein kinase A, adenosine 3',5'-cyclic monophosphorothioate, Rp-isomer, triethylammonium salt (Rp-cAMPS) (20 μ mol/L; Calbiochem-Novabiochem), and the adenylate cyclase inhibitor 2',5'-dideoxyadenosine (10 μ mol/L; Biomol Research Laboratories, Plymouth Meeting, PA) were dissolved in dH₂O. Transforming growth factor (TGF)- β (R&D Systems, Minneapolis, MN) was reconstituted in sterile 4 mmol/L HCl containing 0.1% bovine serum albumin.

Cell Counts and [³H]Thymidine Incorporation

Proliferation assays were performed in either CO-containing atmosphere or control atmosphere as outlined above. Cells were seeded at 5×10^3 cells/well in a 12-well plate and cultured overnight in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were serum-starved for 24 hours (0% serum) before induction of cell proliferation (10% fetal bovine serum; Hyclone, Logan, UT). Cell counting and [³H]thymidine incorporation studies were performed as previously described.⁸ Data are presented as the mean counts/minute/well. Experiments were performed in quadruplicate.

Cell Cycle Analysis by Flow Cytometry

After serum starvation for 48 hours, fibroblasts were stimulated with 10% fetal bovine serum for 24 hours. Cells were harvested and analyzed by flow cytometry as previously described.⁸ Experiments were performed in triplicate.

Cell Extracts and Western Blot Analysis

Cellular protein extracts were electrophoresed under denaturing conditions (10 to 12.5% polyacrylamide gels) and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Total and activated/phosphorylated forms of mitogen-activated protein kinase (MAPK) were detected using rabbit polyclonal antibody

ies directed against the total and phosphorylated forms of these MAPKs, according to the manufacturer's suggestions (New England Biolabs Inc., Beverly, MA). Phosphorylated p38, JNK, and extracellular signal-regulated kinase (ERK) were normalized to the total amount of p38, JNK, and ERK, respectively, detected in the same membrane. p21^{Cip1} was detected using a rabbit anti-human polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Cyclin A and cyclin D were detected using a rabbit anti-human polyclonal antibody; fibronectin was detected using mouse monoclonal antibody (Santa Cruz Biotechnology); collagen-1 was detected using rabbit polyclonal antibody (Abcam Inc., Cambridge, MA); and β -actin was detected using anti-human β -actin monoclonal antibody (Sigma Chemical Co., St. Louis, MO). Primary antibodies were detected using horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibodies (Pierce, Rockford, IL). Peroxidase was visualized using the Enhanced chemiluminescence assay (Amersham Life Science Inc., Arlington Heights, IL) according to manufacturer's instructions and stored in the form of autoradiograph (Biomax MS; Eastman Kodak, Rochester, NY). Digital images were obtained using an image scanner equipped with FotoLook and Photoshop software. When indicated, membranes were stripped (62.5 mmol/L Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, and 100 mmol/L β -mercaptoethanol, 30 minutes, 50°C).

cGMP and cAMP Immunoassays

Cellular levels of cyclic guanosine 3',5'-monophosphate (cGMP) and cyclic adenosine 3',5'-monophosphate (cAMP) were quantified using a commercially available immunoassay (Biomol). Fibroblasts were incubated in the presence or absence of CO (250 ppm) and cell lysates were analyzed for cGMP and cAMP content, as suggested by the vendor.

Statistical Analysis

Data are expressed as the mean $\pm$ SE. Differences in measured variables between experimental and control group were assessed using Student's *t*-test. Groups containing multiple comparisons were analyzed by analysis of variance, and Bonferroni's correction was used in determining *P* values. Statistical difference was accepted at *P* < 0.05.

Results

Inhaled CO Inhibits Hydroxyproline Deposition in the Lungs of Bleomycin-Treated Mice

We hypothesized that inhaled CO could exert an anti-fibrotic effect on the lung. To assess the effect of inhaled low-concentration (250 ppm) CO on fibrosis, mice were treated with intratracheal bleomycin and then exposed to

Figure 1. Low concentration of inhaled CO inhibits hydroxyproline deposition in the lungs of bleomycin-treated mice. **A:** Mice were treated with bleomycin and subsequently exposed to either room air (*n* = 8) or continuous inhaled CO at a concentration of 250 ppm (*n* = 6) for a period of 14 days. Lungs were subsequently harvested and analyzed for hydroxyproline content. Mice exposed to continuous CO had significantly lower lung hydroxyproline content than controls (*P* = 0.001 by analysis of variance; *, *P* = 0.001 versus saline using Bonferroni's correction; #, *P* = 0.025 versus control using Bonferroni's correction). Lungs of mice exposed to intratracheal saline and CO (*n* = 3) had equivalent hydroxyproline content to intratracheal saline controls (*n* = 6). **B:** Mice were treated with bleomycin and subsequently exposed to either room air (*n* = 8) or 3 hours per day inhaled CO at a concentration of 250 ppm (*n* = 6) for a period of 14 days. Lungs were subsequently harvested and analyzed for hydroxyproline content. Mice exposed to 3 hours per day CO had significantly lower lung hydroxyproline content than controls (*P* < 0.0001 by analysis of variance; *, *P* < 0.0001 versus saline using Bonferroni's correction; #, *P* = 0.001 versus control using Bonferroni's correction).

either room air or continuous inhaled CO for a period of 14 days. Lungs were subsequently harvested and analyzed for hydroxyproline content. Mice exposed to CO had significantly less accumulation of lung hydroxyproline than controls, indicating a lesser degree of fibrosis (Figure 1A). To determine whether transient exposure to CO could effect the same change, we treated mice in an identical manner with bleomycin and subsequently exposed the experimental group to only 3 hours of inhaled CO (250 ppm) daily. This shorter exposure to CO inhibited hydroxyproline deposition to a similar degree (Figure 1B).

In a separate experiment, histopathological changes were evaluated 14 days after bleomycin exposure. Variable lung injury was observed in both bleomycin and bleomycin + CO treatment groups, but the CO-treated animals had less injury on average than bleomycin alone as assessed by an independent blinded observer (*n* = 6 in each group). Representative sections are illustrated in Figure 2A. Figure 2B summarizes the results from the scoring system outlined in the Materials and Methods section. Lungs from CO-treated animals had statistically

A

Figure 2. Histology of bleomycin-treated mouse lungs and Western blot for matrix components. Lungs were harvested from mice treated with bleomycin alone (**A: A and B**) or bleomycin with CO 250 ppm (**A: C to F**); H&E stainings of representative sections are shown. Although variability in degree of injury was observed in both groups, animals treated with CO had less injury on average than animals treated with bleomycin alone as assessed by an independent blinded observer. **B:** A summary of histology scores assigned to experimental groups is represented. Details of the scoring method are provided in the Materials and Methods section. Animals receiving bleomycin and CO had significantly lower fibrotic/repairative scores than animals receiving bleomycin alone. **C:** A summary of the distribution of histological scores [from 0 = no fibrosis to 4 = interstitial fibrosis affecting 76 to 100% of the high-power field (HPF)] is shown. **Black bars** represent bleomycin alone and **gray bars** represent bleomycin with CO treatment. There is a range of injury and repair in both experimental groups but the CO-treated animals are less severely affected overall. **D:** Western blot analysis of fibronectin and collagen-1 content of whole mouse lung exposed to bleomycin alone (Bleo) or bleomycin with CO 250 ppm (Bleo/CO). Each lane represents protein from the lung of a single animal. This blot is representative of $n = 6$ animals analyzed in each group. The densitometry shown in **E** and **F** quantitates the blot shown (*, $P < 0.05$ versus saline; #, $P < 0.05$ versus Bleo). Original magnifications: $\times 4$ (**A, left column**); $\times 20$ (**A, right column**).

lower fibrosis scores than lungs from animals treated with bleomycin alone ($P = 0.006$). Figure 2C summarizes in bar graph form the distribution of severity of fibrosis in both experimental groups. The scores range from 0 (no

fibrosis) to 4 (greatest involvement with interstitial fibrosis) as outlined in the Materials and Methods section. The y axis gives the number of high-power field assigned a given score. As can be seen from the graph there was a

A

B

Figure 3. CO suppresses fibroblast proliferation *in vitro*. Cultured human fetal lung fibroblasts (MRC-5) were exposed to CO (250 ppm) or control incubator air for 1 week and cells were counted daily. **A:** Cells exposed to CO exhibited a significantly slower rate of growth than their control counterparts (*, $P < 0.005$ compared with nonexposed control). **B:** Flow cytometry of serum-stimulated fibroblasts. Cells exposed to CO-containing atmosphere are less likely to proceed to S and G₂/M phase after serum stimulation than are control cells.

range of severity of fibrosis in both the bleomycin group and the bleomycin + CO group but the former group was more severely affected overall.

Fibrosis was further assessed by Western blotting of whole lung protein extract from bleomycin-exposed and bleomycin + CO-exposed mice 14 days after exposure. Collagen-1 and fibronectin levels were assessed for six mice in each group; a representative blot is shown in Figure 2D. When measured by densitometry the difference in fibronectin and collagen-1 content were significantly lower in the CO-treated group (2E).

CO Suppresses Fibroblast Proliferation in Vitro

Given the known anti-proliferative effect of CO on cultured smooth muscle cells,^{6–9} we postulated that inhibition of fibroblast proliferation might contribute to the suppression of hydroxyproline accumulation by CO. To test the effect of CO on fibroblast growth, cultured human fetal lung fibroblasts (MRC-5) were exposed to CO (250 ppm) or control incubator air for 1 week and cells were counted daily. As illustrated by the growth curve in Figure 3A, exposure to CO significantly inhibited fibroblast proliferation.

To assess the effect of CO exposure on cell cycle, fibroblasts were analyzed by flow cytometry. After syn-

chronization of MRC-5 fibroblasts by serum starvation for 48 hours, cells were stimulated to proliferate with 10% fetal bovine serum. Fibroblasts were stained with propidium iodide and cell cycle was analyzed at 24 hours after stimulation by flow cytometry. In the absence of CO, there was an increase in the percentage of cells in S and G₂/M phase. This increase was blocked by exposing cells to 250 ppm CO (Figure 3B). These data suggest that the effect of CO is associated with arrest of fibroblast cycle progression at the G₀/G₁ phase of the cell cycle.

CO Exposure Augments p21^{Cip1} Expression and Suppresses Cyclins A and D1 in Vitro

Given the findings above indicating that CO promotes cell-cycle arrest in the G₀/G₁ phase, we sought to gain a more detailed molecular understanding of this arrest point. We examined expression of cell-cycle regulatory proteins that are known to function as control elements for transit through G₁ and into S phase. Using Western blot analysis, we tested the amounts of cyclins D and A, and the cyclin-dependent kinase inhibitor p21^{Cip1} in serum-stimulated MRC-5 fibroblasts after exposure to CO (250 ppm) or control incubator air (time 0). Cells were harvested at 8 and 24 hours after serum stimulation. Figure 4 demonstrates that expression of cyclins A and D decreased after exposure to CO, whereas expression of p21^{Cip1} increased.

CO Exposure Affects Phosphorylation in all Three Branches of the MAPK Cascade in Serum-Stimulated Fibroblasts

It has been demonstrated in various cell types that all three MAPK pathways (JNK, p38, and ERK) may be involved in proliferation and cell-cycle progression.^{14–16} CO has previously been shown to exert some of its actions via the MAPK pathways.^{5,8} To explore the role of the MAPK pathways in the CO effect on fibroblast proliferation, we analyzed fibroblast lysates by Western blotting for phosphorylation of JNK, p38, and ERK after serum stimulation and exposure to CO. Serum-starved MRC-5 fibroblasts were exposed to CO (250 ppm) or control incubator air for 2 hours before serum stimulation. Cells were replaced in either CO-containing atmosphere or control atmosphere and harvested at time points ranging from 5 minutes to 1 hour for analysis by Western blotting. As shown in Figure 5A, exposure to CO decreased phosphorylation of ERK, JNK, and p38 at 30 minutes. The decrease in JNK phosphorylation was sustained at 1 hour, whereas the CO effect on p38 was not sustained and ERK phosphorylation was if anything increased by CO treatment at the 15-minute and 1-hour time points.

To test whether deletion of select genes contributing to the MAPK pathways would diminish the effect of CO on fibroblast proliferation, lung fibroblasts were extracted from *Jnk1*^{−/−}, *p38β*^{−/−}, and *MKK3*^{−/−} mice. Early passage serum-starved fibroblasts were stimulated with serum in the presence or absence of CO. Deletion of these

Figure 4. CO exposure augments p21^{Cip1} expression and suppresses cyclins A and D *in vitro*. Serum-starved MRC-5 fibroblasts were exposed to CO (250 ppm) or control incubator air (time 0) and subsequently serum stimulated. **A:** Cells were replaced in either CO-containing atmosphere or control atmosphere and harvested at 8 and 24 hours for analysis by Western blotting. Expression of cyclins A and D was decreased after exposure to CO, whereas expression of p21^{Cip1} was increased. **B:** Densitometry for the same experiment and represents an average of two to three blots for each time point. Values were normalized to the RA control for each blot to adjust for overall film lightness (*, $P < 0.05$ compared with RA control for same time point).

select MAPK genes did not affect the anti-proliferative effect of CO on these fibroblasts (Figure 5B). This finding was confirmed by use of chemical inhibitors of MAPKs. Selective inhibitors of p38 MAPK (SB203580), MEK1/2 (UO126), and JNK (SP600125) were added to fibroblasts in culture before treatment with CO and serum stimulation. Proliferation was assessed by [³H]thymidine uptake. The results are illustrated in Figure 5C. The white bars represent CO-treated cells, and the black bars represent room air controls. Although chemical inhibition of both JNK and ERK did decrease the baseline proliferative response of the fibroblasts to serum stimulation, it did not abrogate the effect of CO on [³H]thymidine uptake. Even when all three pathways were inhibited concurrently (mix), CO still exerted an additional anti-proliferative effect. These data confirm that CO capable of inhibiting fibroblast proliferation through a mechanism that does not depend on its dampening effect on the MAPK pathways.

CO Treatment of Fibroblasts Increases the Levels of Intracellular cGMP, and Inhibition of cGMP Abolishes the Anti-Proliferative Effect of CO

Given that the MAPK pathways did not appear to be crucial to the effect of CO on proliferation, we sought the involvement of an alternative signaling pathway. Elevated intracellular cAMP levels have been shown to inhibit progression of fibroblasts through the mid to late G₁ phase of the cell cycle,¹⁷ making this a reasonable target for investigation. Stimulation of fibroblasts with CO did not

result in a significant increase in cAMP as measured by enzyme-linked immunosorbent assay, however, and neither inhibition of cAMP production or of protein kinase A activity [using chemical inhibitors of protein kinase A, Rp-cAMPS (Rp), and adenylyl cyclase, 2',5'-dideoxyadenosine] could diminish the anti-proliferative effect of CO (data not shown).

Although the role for cGMP in fibroblast proliferation is less well defined than is the role of cAMP, elevated cGMP levels in smooth muscle cells have been shown to inhibit proliferation and decrease expression of cyclin D1 and A.^{18,19} To determine whether cGMP stimulation by CO is important in our system, we exposed MRC-5 fibroblasts to CO (250 ppm) and assessed for changes in intracellular cGMP by enzyme-linked immunosorbent assay at times ranging from 15 minutes to 24 hours. There was a statistically significant increase in cGMP peaking at 1 hour and returning to baseline by 4 hours (Figure 6A). To test whether the inhibitor of guanylate cyclase, ODQ, can prevent the increase in intracellular cGMP stimulated by CO, we repeated this experiment in the presence or absence of ODQ. As expected, ODQ abolished the CO-stimulated rise in cGMP (Figure 6B). Fibroblasts were again exposed to CO after serum stimulation in the presence of ODQ and [³H]thymidine uptake was measured. The anti-proliferative effect of CO was completely abolished by treatment with ODQ, whereas treatment with vehicle alone (dimethyl sulfoxide) had no effect on the fibroblast response to CO (Figure 6C).

To test whether inhibition of guanylate cyclase could also reverse the effect of CO on the expression of cell-cycle regulatory proteins, we repeated the measurement of p21^{Cip1} and cyclin D in serum-stimulated fibroblasts,

Figure 5. The role of MAPK signaling in the anti-proliferative effects of CO. Fibroblasts were stimulated with serum and exposed to CO (250 ppm) or control incubator air. **A:** Cells were subsequently analyzed for MAPK phosphorylation by Western blotting at time points ranging from 5 minutes to 1 hour. **B:** Densitometry for the same experiment is shown. Bar graphs represent an average of two to three blots for each time point and all values were normalized to RA control for each blot (*, $P < 0.05$ compared with RA control for same time point). To test whether deletion of select genes contributing to the MAPK pathways would affect fibroblast proliferation, lung fibroblasts were extracted from lungs of *Jnk1*^{-/-}, *p38β*^{-/-}, and *MKK3*^{-/-} mice. Early passage serum-starved fibroblasts were stimulated with serum in the presence or absence of CO. **C:** Deletion of these select MAPK genes did not affect the anti-proliferative effect of CO on these fibroblasts (*, $P < 0.005$ compared with corresponding room air control). **D:** To confirm this finding, the experiment was repeated using chemical inhibitors of the MAPK pathways. The **black bars** represent ambient air controls and **white bars** represent CO-exposed fibroblasts. Dimethyl sulfoxide, vehicle control; SB, p38 MAPK inhibitor SB203580; SP, JNK inhibitor SP600125; UO126, MEK1/2 inhibitor UO126; mix, all three inhibitors combined. JNK and ERK inhibition slowed fibroblast proliferation at baseline, but none of the inhibitors abrogated the effect of CO (*, $P < 0.05$ compared with corresponding room air control).

this time treating the cells with ODQ before CO exposure. Cells were harvested at 8 and 24 hours after serum stimulation. Figure 6D demonstrates that treatment with ODQ reversed the previously observed stimulatory effect of CO on p21^{Cip1} and the inhibitory effect of CO on cyclins A and D.

CO Suppresses TGF- β -Induced Fibronectin and Type I Collagen Production by Fibroblasts Independently of the MAPK Pathways or Guanylate Cyclase

Although fibroblast proliferation is likely to play a role in the pathogenesis of fibrosis, proliferative potential does not necessarily correlate with matrix deposition.²⁰ To test whether CO could have an effect on the elaboration of matrix molecules, MRC-5 fibroblasts were stimulated with TGF- β in the presence or absence of CO (250 ppm).

Protein from cell lysate and supernatant was collected at 48, 96, and 144 hours for analysis of matrix components by Western blotting. There was a time-dependent increase in extracellular collagen type I and intracellular fibronectin produced by control cells, but this was inhibited at all time points by exposure to CO (Figure 7, A and B). This same experiment was repeated in the presence or absence of chemical inhibitors of the MAPK pathways and guanylate cyclase. As shown in Figure 8A, fibronectin production was suppressed by CO exposure even in the presence of the p38 MAPK inhibitor SB203580, the JNK inhibitor SP600125, the MEK1/2 inhibitor UO126, or the guanylate cyclase inhibitor ODQ, indicating that the effect of CO is independent of these pathways. To confirm this finding, we tested whether CO is capable of inhibiting the production of fibronectin in fibroblasts lacking the MKK3, p38 β , or Jnk1 genes. As shown in Figure 8B, the deletion of these MAPK genes did not reduce the impact of CO on the production of fibronectin.

Figure 6. CO treatment of fibroblasts increases the levels of intracellular cGMP, and inhibition of cGMP abolishes the anti-proliferative effect of CO. **A:** MRC-5 fibroblasts were exposed to CO (250 ppm) and assessed for changes in intracellular cGMP by enzyme-linked immunosorbent assay at times ranging from 15 minutes to 24 hours. There was a statistically significant increase in cGMP peaking at 1 hour and returning to baseline by 4 hours (*, $P < 0.05$ compared with room air control). **B:** Fibroblasts were exposed to CO (250 ppm) for 1 hour in the presence or absence of an inhibitor of guanylate cyclase, ODQ. The rise in intracellular cGMP stimulated by CO was inhibited by ODQ (*, $P < 0.05$ compared with room air control; #, $P < 0.05$ compared with CO 1 hour). **C:** Fibroblasts were again exposed to CO after serum stimulation in the presence of ODQ. The anti-proliferative effect of CO was completely abolished by treatment with ODQ, whereas treatment with vehicle alone (dimethyl sulfoxide) had no effect on the fibroblast response to CO (*, $P < 0.05$ compared with room air control; #, $P < 0.005$ compared with room air control). **D:** Western blotting of fibroblasts similarly exposed to CO (250 ppm) with or without ODQ for a period of 8 hours. The effects of CO on cyclins A and D and p21^{Cip1} were completely abolished by treatment with ODQ.

The transcriptional regulator Id1 is known to play a role in cellular growth and differentiation and was recently shown to be highly expressed by myofibroblasts within fibrotic foci in experimentally induced pulmonary fibrosis.²¹ When fibroblasts lacking the gene for Id1 are exposed to TGF- β in the presence of CO, the effect of CO on the production of type I collagen is completely lost (Figure 9A). Of note, deletion of the Id1 gene does not

prevent suppression of fibroblast proliferation by CO (Figure 9B).

Discussion

Fibrosis represents an important response to tissue injury. In the lung, however, excessive fibrosis can lead to impairment of gas exchange and ultimately respiratory failure. No intervention has been demonstrated to reverse fibrosis in the human lung, and there are few therapies that show promise for slowing or halting the progression of fibrosis. The roles of heme oxygenase-1 and its enzymatic product, carbon monoxide, have not been extensively examined in the context of pulmonary fibrosis, and this is what we sought to investigate. Using a murine model of bleomycin-induced fibrosis, we have demonstrated that inhaled low-concentration CO, even when dosed intermittently, can inhibit the deposition of tissue hydroxyproline. It was recently reported that adenoviral transfer of HO-1 cDNA protects against bleomycin-induced lung fibrosis in C57BL/6 mice,²² and we have similar findings from our own laboratory using CBA/J mice (data not shown). This indicates that increasing endogenous production of CO may have the same effect as exogenous application of CO.

Given the known anti-proliferative effect of CO,^{6–9} we postulated that at least part of the mechanism for this decrement in lung fibrosis might be through inhibition of fibroblast proliferation. The effect of CO on fibroblast growth has not, to our knowledge, previously been examined. We have confirmed in this study that CO is capable of inhibiting proliferation in fibroblasts. We have further shown that this inhibition involves changes in the expression of cell-cycle regulatory proteins (including cyclins D and A, and the cyclin-dependent kinase inhibitor p21^{Cip1}) via a cGMP-dependent mechanism. Our finding that CO inhibits phosphorylation of all three MAPK pathways in the same fibroblasts is interesting, but we have shown using both chemical MAPK inhibitors and genetic inhibition that this effect is not critical for the anti-proliferative action of CO. The inhibition of the MAPK pathways by CO may be a mechanism for other cellular effects of CO, but this remains a question for future investigations.

Although it appears intuitive that fibroblast proliferation should play a pivotal role in the initiation and progression of fibrosis, studies dealing with the proliferative characteristics of fibroblasts obtained from human idiopathic pulmonary fibrosis lungs have yielded conflicting results. Some investigators have shown that fibroblasts derived from fibrotic tissues proliferate significantly faster than do fibroblasts from normal lungs,²³ whereas others have shown that these same fibroblasts grow more slowly than normals.²⁰ Raghu and associates²⁴ found that fibroblast proliferation was higher in cells derived from areas of early fibrosis compared with normal areas, but slower in cells obtained from densely fibrotic tissue. This finding is consistent with a growing body of evidence that there is phenotypic and behavioral heterogeneity among tissue fibroblasts. It may well be that inhibiting fibroblast prolif-

A

B

Figure 7. CO suppresses TGF- β -induced fibronectin and type I collagen production by fibroblasts. MRC-5 fibroblasts were treated with TGF- β (2 ng/ml) in the presence or absence of CO (250 ppm); protein from cell lysate and supernatant was collected at 2 days, 4 days, and 6 days for analysis of matrix components by Western blotting. **A:** Intracellular fibronectin was dramatically decreased by exposure to CO at all time points. **B:** Similarly, there was a time-dependent increase in extracellular collagen type I produced by control cells, but this was inhibited at all time points by exposure to CO. Densitometry for both experiments is shown in **C**; each graph represents an average of three blots (*, $P < 0.05$ relative to RA value for same time point). Values were normalized to the RA control for each blot to adjust for overall differences in intensity among films.

C

eration will have an impact on fibrosis at one stage of the process, or one region of the lung, and have no impact in other stages or regions. It is also possible that the effect of CO on fibroblast proliferation is not the sole mecha-

nism for its action. Carbon monoxide is known to have anti-apoptotic effects in a number of cell types,^{25,26} and if CO prevents apoptosis of epithelial cells, this could theoretically offer protection against bleomycin-induced injury. Additionally, CO has been shown to inhibit plasminogen activator inhibitor-1 (PAI-1) production by macrophages under hypoxic conditions,⁴ and increased fibroblast elaboration of PAI-1 was recently linked to increased matrix production.²⁷ We have not seen CO-related decreases in PAI-1 production by fibroblasts *in vitro* or in bleomycin-exposed lung however (unpublished data), so this does not appear to be an important effect of CO in our models. We have shown that CO does markedly decrease the expression of fibronectin and collagen-1 both *in vivo* and *in vitro*. Unlike the effect of CO on fibroblast proliferation, the suppression of matrix production is independent of guanylate cyclase as well as of the MAPK pathways but depends at least in part on the transcriptional regulator Id1. Inhibition of matrix production by CO through a direct action on fibroblasts represents a novel and surprising finding. This effect of CO is unique among the many previously described functions of this gaseous molecule in biological systems.

Interestingly, cigarette smoking has been associated with prolonged survival in idiopathic pulmonary fibrosis.²⁸ Smokers also have less overall interstitial cellularity and significantly lower granulation/connective tissue

A

B

Figure 8. Suppression of fibronectin production by CO does not depend on the MAPK pathways or cGMP. **A:** MRC-5 fibroblasts were treated with chemical inhibitors of the MAPK pathways or cGMP in the presence or absence of CO (250 ppm); protein from cell lysate was collected at 48 hours for analysis of fibronectin production by Western blotting. Intracellular fibronectin was decreased by exposure to CO even in the presence of signaling pathway inhibitors. C, control; SB, p38 MAPK inhibitor SB203580; SP, JNK inhibitor SP600125; UO, MEK1/2 inhibitor UO126; ODQ, guanylate cyclase inhibitor ODQ. **B:** Mkk3^{-/-}, p38^{-/-}, and Jnk1^{-/-} mouse lung fibroblasts along with their wild-type controls were treated with TGF- β (2 ng/ml) in the presence or absence of CO (250 ppm); protein from cell lysates was collected at 0 and 4 days for Western blot analysis of fibronectin production. Intracellular fibronectin was decreased by exposure to CO in all fibroblast lines.

Figure 9. Suppression of type I collagen production by CO depends on the presence of Id1, but suppression of proliferation by CO is independent of Id1. **A:** Both *Id1*^{-/-} and wild-type mouse lung fibroblasts were treated with TGF-β (2 ng/ml) in the presence or absence of CO (250 ppm); supernatant protein was collected at 0 and 4 days for analysis of collagen-1 production by Western blotting. Collagen-1 was significantly decreased by exposure to CO for 4 days in wild-type fibroblasts; but in *Id1*^{-/-} fibroblasts, the effect of CO on collagen-1 production was reversed. Bar graph shows densitometry for the same experiment and represents an average of three blots. Values were normalized to RA control (*, *P* < 0.05 compared with RA value for same time point). **B:** Growth curve for *Id1*^{-/-} fibroblasts without CO exposure (filled triangles) and exposed to CO (open triangles) and wild-type fibroblasts without CO exposure (filled diamonds) and exposed to CO (open diamonds). The absence of *Id1* did not prevent the growth-inhibitory effect of CO. *, *P* < 0.005 compared with non-CO treated control.

scores than nonsmokers when lung biopsies are pathologically graded.²⁸ These unexpected findings remain unexplained, but the results of our studies raise the possibility that CO in cigarette smoke may be involved. Inhibition of fibroblast proliferation and matrix deposition by CO could lead to pathological findings similar to those described by Dr. King and colleagues²⁸ in smokers.

In summary, we have demonstrated that low-concentration inhaled CO can inhibit the pulmonary fibrotic response in a bleomycin rodent model. We postulate that one of the mechanisms for this action is through inhibition of fibroblast proliferation. We have shown that CO inhibits *in vitro* fibroblast proliferation via a cGMP-dependent modulation of cell-cycle regulatory proteins, independent of MAPK pathway. We have also shown that CO is capable of inhibiting the elaboration of matrix molecules by fibroblasts *in vivo* and *in vitro* independently of both guanylate cyclase and the MAPK pathways. The mechanism by which CO exerts this effect remains to be further elucidated, but appears to involve the transcriptional regulator Id1. Further investigation of this mechanism will provide a starting point for future studies of the effect of CO on fibrosis and fibroblast behavior.

Acknowledgments

We thank Dr. Robert Benezra (Department of Immunology, Memorial Sloan-Kettering Cancer Center, New York, NY) for generously donating the *Id1*-null mouse fibroblasts; Dr. Richard A. Flavell (Howard Hughes Medical Institute and Section of Immunobiology, Yale University School of Medicine, New Haven, CT) for his kind gift of the MAPK-deficient mice from which lung fibroblasts were derived for this work; and Dr. Rachel Givelber (Division of PACCM, University of Pittsburgh School of Medicine, Pittsburgh, PA) for her help with statistical analysis.

References

1. Otterbein LE, Mantell LL, Choi AM: Carbon monoxide provides protection against hyperoxic lung injury. *Am J Physiol* 1999, 276:L688–L694
2. Song R, Kubo M, Morse D, Zhou Z, Zhang X, Dauber JH, Fabisiak J, Alber SM, Watkins SC, Zuckerbraun BS, Otterbein LE, Ning W, Oury TD, Lee PJ, McCurry KR, Choi AM: Carbon monoxide induces cytoprotection in rat orthotopic lung transplantation via anti-inflammatory and anti-apoptotic effects. *Am J Pathol* 2003, 163:231–242
3. Chapman JT, Otterbein LE, Elias JA, Choi AM: Carbon monoxide attenuates aeroallergen-induced inflammation in mice. *Am J Physiol* 2001, 281:L209–L216
4. Fujita T, Toda K, Karimova A, Yan SF, Naka Y, Yet SF, Pinsky DJ: Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis. *Nat Med* 2001, 7:598–604
5. Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk W, Davis RJ, Flavell RA, Choi AM: Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 2000, 6:422–428
6. Peyton KJ, Reyna SV, Chapman GB, Ensenat D, Liu XM, Wang H, Schafer AI, Durante W: Heme oxygenase-1-derived carbon monoxide is an autocrine inhibitor of vascular smooth muscle cell growth. *Blood* 2002, 99:4443–4448
7. Morita T, Mitsialis SA, Koike H, Liu Y, Kourembanas S: Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells. *J Biol Chem* 1997, 272:32804–32809
8. Song R, Mahidhara RS, Liu F, Ning W, Otterbein LE, Choi AM: Carbon monoxide inhibits human airway smooth muscle cell proliferation via mitogen-activated protein kinase pathway. *Am J Respir Cell Mol Biol* 2002, 27:603–610
9. Otterbein LE, Zuckerbraun BS, Haga M, Liu F, Song R, Ushva A, Stachulak C, Bodyak N, Smith RN, Csizmadia E, Tyagi S, Akamatsu Y, Flavell RJ, Billiar TR, Tzeng E, Bach FH, Choi AM, Soares MP: Carbon

- monoxide suppresses arteriosclerotic lesions associated with chronic graft rejection and with balloon injury. *Nat Med* 2003, 9:183–190
10. Morse D: The role of heme oxygenase-1 in pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2003, 29(Suppl 3):S82–S86
11. Kuhn III C, McDonald JA: The roles of the myofibroblast in idiopathic pulmonary fibrosis: ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. *Am J Pathol* 1991, 138:1257–1265
12. Woessner JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 1961, 93:440–447
13. Oury TD, Thakker K, Crapo JD, Chang L-Y, Day BJ: Attenuation of bleomycin-induced pulmonary fibrosis by a catalytic antioxidant metalloporphyrin. *Am J Respir Cell Mol Biol* 2001, 25:164–169
14. Campos CB, Bedard PA, Linden R: Activation of p38 mitogen-activated protein kinase during normal mitosis in the developing retina. *Neuroscience* 2002, 112:583–591
15. Kapur R, Chandra S, Cooper R, McCarthy J, Williams DA: Role of p38 and ERK MAP kinase in proliferation of erythroid progenitors in response to stimulation by soluble and membrane isoforms of stem cell factor. *Blood* 2002, 100:1287–1293
16. Lin SY, Liang YC, Ho YS, Tsai SH, Pan S, Lee WS: Involvement of both extracellular signal-regulated kinase and c-jun N-terminal kinase pathways in the 12-O-tetradecanoylphorbol-13-acetate-induced up-regulation of p21(Cip1) in colon cancer cells. *Mol Carcinog* 2002, 35:21–28
17. Kothapalli D, Hayashi N, Grotendorst GR: Inhibition of TGF- β stimulated CTGF gene expression and anchorage independent growth by cAMP identifies a CTGF-dependent restriction point in the cell cycle. *FASEB J* 1998, 12:1151–1161
18. Fukumoto S, Koyama H, Hosoi M, Yamakawa K, Tanaka S, Morii H, Nishizawa Y: Distinct role of cAMP and cGMP in the cell cycle control of vascular smooth muscle cells: cGMP delays cell cycle transition through suppression of cyclin D1 and cyclin-dependent kinase 4 activation. *Circ Res* 1999, 85:985–991
19. Kronemann N, Nockher WA, Busse R, Schini-Kerth VB: Growth-inhibitory effect of cyclic GMP- and cyclic AMP-dependent vasodilators on rat vascular smooth muscle cells: effect on cell cycle and cyclin expression. *Br J Pharmacol* 1999, 126:349–357
20. Ramos C, Montano M, Garcia-Alvarez J, Ruiz V, Uhal BD, Selman M, Pardo A: Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis and tissue inhibitor of metalloproteinases expression. *Am J Respir Cell Mol Biol* 2001, 24:591–598
21. Chambers RC, Leoni P, Kaminski N, Laurent GJ, Heller RA: Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. *Am J Pathol* 2003, 162:533–546
22. Tsuburai T, Suzuki M, Nagashima Y, Suzuki S, Inoue S, Hasiba T, Ueda A, Ikehara K, Matsuse T, Ishigatsubo Y: Adenovirus-mediated transfer and overexpression of heme oxygenase 1 cDNA in lung prevents bleomycin-induced pulmonary fibrosis via a Fas-Fas ligand-independent pathway. *Hum Gene Ther* 2002, 13:1945–1960
23. Jordana M, Schulman J, McSharry C, Irving LB, Newhouse MT, Jordana G, Gauldie J: Heterogeneous proliferative characteristics of human adult lung fibroblast lines and clonally derived fibroblasts from control and fibrotic tissue. *Am Rev Respir Dis* 1988, 137:579–584
24. Raghu G, Chen YY, Rusch V, Rabinovitch PS: Differential proliferation of fibroblasts cultured from normal and fibrotic human lung. *Am Rev Respir Dis* 1988, 138:703–708
25. Liu XM, Chapman GB, Peyton KJ, Schafer AI, Durante W: Carbon monoxide inhibits apoptosis in vascular smooth muscle cells. *Cardiovasc Res* 2002, 55:396–405
26. Zhang X, Shan P, Otterbein LE, Alam J, Flavell RA, Davis RJ, Choi AM, Lee PJ: Carbon monoxide inhibition of apoptosis during ischemia-reperfusion lung injury is dependent on the p38 mitogen-activated protein kinase pathway and involves caspase 3. *J Biol Chem* 2003, 278:1248–1258
27. Tuan TL, Wu H, Huang EY, Chong SS, Laug W, Messadi D, Kelly P, Le A: Increased plasminogen activator inhibitor-1 in keloid fibroblasts may account for their elevated collagen accumulation in fibrin gel cultures. *Am J Pathol* 2003, 162:1579–1589
28. King Jr TE, Schwarz MI, Brown K, Tooze JA, Colby TV, Waldron Jr JA, Flint A, Thurlbeck W, Cherniack RM: Idiopathic pulmonary fibrosis: relationship between histopathologic features and mortality. *Am J Respir Crit Care Med* 2001, 164:1025–1032