Cigarette smoke upregulates pulmonary vascular matrix metalloproteinases via TNF-α signaling

J. L. Wright, H. Tai, R. Wang, X. Wang, and A. Churg

Department of Pathology, University of British Columbia, Vancouver, British Columbia, Canada

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Wright, J. L., H. Tai, R. Wang, X. Wang, and A. Churg. Cigarette smoke upregulates pulmonary vascular matrix metalloproteinases via TNF-α signaling. Am J Physiol Lung Cell Mol Physiol 292: L125–L133, 2007. First published August 11, 2006; doi:10.1152/ajplung.00539.2005.—Cigarette smoke exposure causes vascular remodeling and pulmonary hypertension by poorly understood mechanisms. To ascertain whether cigarette smoke exposure affects production of matrix metalloproteinases (MMPs) in the pulmonary vessels, we exposed C57Bl/6 (C57) mice or mice lacking TNF-α receptors (TNFRKO) to smoke daily for 2 wk or 6 mo. Using laser capture microdissection and RT-PCR analysis, we examined gene expression of MMP-2, MMP-9, MMP-12, MMP-13, and tissue inhibitor of metalloproteinase (TIMP-1) and examined protein production by immunohistochemistry for MMP-2, MMP-9, and MMP-12 in small intrapulmonary arteries. At 2 wk, mRNA levels of TIMP-1 and all MMPs were increased in the C57, but not TNFRKO, mice, and immunoreactive protein for MMP-2, MMP-9, and MMP-12 was also increased in the C57 mice. Increased gelatinase activity was identified by in situ and bulk tissue zymography. At 6 mo, only MMP-12 mRNA levels remained increased in the C57 mice, but at a much lower level; however, MMP-2 mRNA levels increased in the TNFRKO mice. We conclude that smoke exposure increases MMP production in the small intrapulmonary arteries but that, with the exception of MMP-12, increased MMP production is transient. MMPs probably play a role in smoke-induced vascular remodeling, as they do in other forms of pulmonary hypertension, implying that MMP inhibitors might be beneficial. MMP production is largely TNF-α dependent, further supporting the importance of TNF-α in the pathogenesis of cigarette smoke-induced lung disease.

chronic obstructive pulmonary disease; vascular remodeling; pulmonary hypertension

PULMONARY HYPERTENSION is a serious complication of cigarette smoke-induced chronic obstructive pulmonary disease (COPD), a complication associated with markedly increased mortality, as well as increased morbidity (reviewed in Ref. 34). Pulmonary hypertension in COPD has been viewed as a response to hypoxia and loss of vascular bed secondary to emphysema; however, more recent data suggest that these factors are actually not the cause of pulmonary hypertension in most patients (34). We have been investigating the pathogenesis of pulmonary hypertension in animal models of cigarette smoke exposure and have suggested that pulmonary hypertension is associated with vascular remodeling and that the latter phenomenon is caused by direct, smoke-mediated upregulation of vasoproliferative and vasoconstrictive mediators within the vascular walls (35, 36).

Vascular remodeling in pulmonary hypertension of various causes is associated with an increase in smooth muscle and an increase in extracellular matrix (29). Although there are no data relating to cigarette smoke exposure, in other models of experimental pulmonary hypertension, matrix metalloproteinases (MMPs) appear to have an important role in vascular remodeling, probably through smooth muscle cell and endothelial cell migration and proliferation and through MMP-mediated liberation of smooth muscle cell mitogens and growth factors (9, 18). MMPs are also important in regulating the balance between deposition and resorption of matrix (28).

MMP and tissue inhibitor of metalloproteinase (TIMP) levels are increased in the vessels in human idiopathic pulmonary arterial hypertension (2, 25). In animals, inhibition of MMP activity decreases vascular remodeling in the monocrotaline (9)- and hypoxia (16)-induced models of pulmonary hypertension. MMPs are also known to be important during the recovery phase of hypoxia-induced pulmonary hypertension, where they appear to remove newly deposited matrix proteins (30, 32). Thus MMPs function in progression and reversal of pulmonary hypertension (see DISCUSSION).

We previously showed in a mouse model that TNF-α appears to be central to cigarette smoke-induced inflammation and the development of emphysema and that TNF-α release is closely related to MMP-12 (macrophage metalloelastase) activity (6). TNF-α is also known to upregulate MMP activity in some cell types, at least in vitro (4, 17). Here, we have used laser capture microdissection (LCM) and real-time RT-PCR to investigate whether cigarette smoke exposure is associated with abnormal MMP production in the small intrapulmonary arteries and also whether changes in MMP levels in these vessels are driven by TNF-α.

METHODS

The research protocol was approved by the University of British Columbia.

Materials

C57Bl/6 (C57) mice were purchased from Charles River Laboratories (Montreal, PQ, Canada). Mice lacking the p55 and p75 TNF-α receptors (TNFRKO mice) were obtained through the courtesy of Dr. Jacques Peschon (Amgen, Seattle, WA). The original mice were created in strain 129 stock and then backcrossed five generations into C57Bl/6 stock. 2R1 research cigarettes were purchased from the University of Kentucky.

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**Smoke Exposure**

Experimental groups consisted of three mice. Control animals were exposed to room air. Smoke exposure consisted of the whole smoke from four 2R1 cigarettes and a nose-only apparatus, as described previously by us (8), 5 days/wk for 2 wk or 6 mo. At 24 h after the last smoke exposure, mice were killed by CO₂ inhalation.

**Tissue Preparation for LCM**

The trachea was cannulated, and the lungs were inflated with 100% alcohol. The trachea was then clamped, and the lungs were removed from the chest cavity, sectioned, and embedded in paraffin. With use of RNase protection techniques, 5-μm sections were cut and stained with the Arcturus (Arcturus, Mountain View, CA) stain for subsequent LCM.

**LCM, mRNA Extraction, and RT Reaction**

Utilizing the Arcturus Pixcell II LCM apparatus, we collected the muscularized vessels adjacent to the membranous or respiratory bronchioles onto LCM caps (Fig. 1). The caps were placed on 500-μl Eppendorf (Mississauga, ON, Canada) tubes and stored at −80°C until the RNA extraction and isolation procedure. We used the PicoPure RNase protection kit (Arcturus, Mountain View, CA) for RNA extraction and isolation, as described by us previously (35), to combine all samples from an individual animal as one data point. First-strand cDNA was synthesized using Superscript II RNase H reverse transcriptase (Invitrogen, Burlington, ON, Canada), and real-time RT-PCR were carried out in LightCycler glass capillaries using a reaction mixture with LightCycler-FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany). Each set of PCR included water as a negative control and five dilutions of standards, which were created by cloning part of the transcript of interest into a cloning vector (Invitrogen, Carlsbad, CA). GAPDH was used as a housekeeping gene.

The primer sequences were as follows: CGG ATT TGG CCG TAT TGG GC (forward) and TGA TGG CAT GCA CTG TGG TC (reverse) for GAPDH (518 bp) (10), CGG TTT ATT TGG CGG AC (forward) and TGC GAT GAG CTT AGG G (reverse) for MMP-2 (GenBank accession no. NM008610; 351 bp), TGT ACG GAC CGG AAC C (forward) and CGG TCC TTA TCG TAC TAC G (reverse) for MMP-9 (GenBank accession no. NM013599; 322 bp), TTT GAC CCA CTG CCG C (forward) and GTG ACA CGA CCG AAC AG (reverse) for MMP-12 (GenBank accession no. AK089523; 221 bp), GTA ATC GCA TTG TGA GAG T (forward) and ATC AGG TGA TCC TTG GG (reverse) for MMP-13 (GenBank accession no. NM008607; 270 bp), and CGC CGG TCA TGA GAA AC (reverse) for TIMP-1 (GenBank accession no. NM011593; 187 bp).

**Immunohistochemistry and Morphometric Analysis**

Immunohistochemical evaluation of MMP-2, MMP-9, and MMP-12 was carried out using standard techniques, with negative controls obtained by omission of the primary antibody. Antibodies reactive to murine MMP-2 and MMP-12 (catalog nos. sc-8835 and sc-8839, respectively, Santa Cruz Biotechnology, Santa Cruz, CA) were used in dilutions of 1:200 and 1:100, respectively. The antibody for MMP-9 (a generous gift from Dr. Robert Senior, Washington University, St. Louis, MO) was used in a dilution of 1:500. We were unable to find a suitable antibody for MMP-13 immunochemistry. After the primary antibodies were added, the slides were incubated overnight at 4°C. Avidin-biotin block (Dako, Mississauga, ON, Can-

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**Fig. 1.** Laser capture microdissection (LCM) technique: bronchovascular bundle before dissection, with the arterial branch to be captured indicated by an arrow (A), and after dissection, with the remnant of the same branch indicated by an arrow (B). C: LCM cap, with the dissected vessel.
Zymography

A separate set of C57 and TNFRKO mice were utilized for zymography, with three animals in each control or 2-wk smoke-exposed group.

**In situ gel zymography.** The left lung was inflated with buffer (50 nM Tris·HCl, pH 7.4, 1 mM CaCl$_2$), sectioned in a sagittal plane, placed in a frozen section mold, covered with Tissue-Tek (Sakura Finetek, Torrance, CA), and frozen at −80°C until it was cut into 20-μm-thick sections, which were analyzed in duplicate. The frozen sections were thawed, covered by buffer, and incubated at 37°C for 30 min. The sections were then covered with 30 μl of a 1:1 mixture of 1 mg/ml Oregon green 488-conjugated gelatin (Molecular Probes, Eugene, OR) and 1% low-melting-temperature agarose (Sigma, St. Louis, MO) and placed in a dark box. After 1 h of incubation at room temperature, the sections were incubated for 48 h in a 37°C humidified chamber. Gelatinolytic activity was determined as black holes in the background green fluorescent substrate.

**Whole lung zymography.** The right lung and pulmonary artery were individually flash frozen in liquid nitrogen and stored at −80°C until analysis. To obtain a signal for the pulmonary artery, we utilized three pulmonary arteries for each data point. Bulk lung tissue was analyzed for each individual animal. The tissue was homogenized and then incubated with 1 mM p-aminophenylmercuric acetate for 30 min. The samples were loaded into a type I collagen-10% SDS polyacrylamide gel. After the gel was washed twice with 2.5% Triton X-100 for 10 min, it was incubated in assay buffer for 3 h. The reaction was stopped by a 15% acetic acid wash, and the gel was stained with Coomassie Brilliant Blue G-250. The bands were analyzed by densitometry using Multi-Analysis software (Bio-Rad, Hercules, CA).

Data Analysis and Statistics

The crossing-point curves values generated by the LightCycler and the standard curves were used to determine relative concentrations of each mRNA of interest and to correct them for loading with the corresponding GAPDH value. We compared the mRNA, immunohistochemical, and zymographic data from control and smoke-exposed animals by analysis of variance. For ease of viewing the gene expression and immunohistochemical data, the corresponding values are expressed normalized to the mean control value for each MMP and TIMP-1, but all statistical analyses were performed using the raw data. GAPDH was used as the housekeeping gene; examination of the crossing points showed no differences between control and smoke-exposed animals (data not shown).
RESULTS

At 2 wk, the smoke-exposed C57 mice showed an ~4-fold increase in MMP-2 (P = 0.002) and MMP-9 (P = 0.002), a 12-fold increase in MMP-12 (P = 0.017), and a 15-fold increase in MMP-13 (P = 0.005) gene expression levels compared with controls (Fig. 2). At 2 wk, TIMP-1 mRNA expression was also increased in the smoke-exposed C57 mice compared with control animals (P = 0.05; Fig. 3). These values for MMP gene expression corresponded to an increased staining intensity on immunohistochemical analysis (P = 0.026 for MMP-2, P = 0.002 for MMP-9, and P = 0.006 for MMP-12; Figs. 4 and 5). By contrast, there was no increase in mRNA levels or in immunohistochemical staining for any of these genes or proteins in the TNFRKO mice.

At 2 wk, in situ zymographs of the smoke-exposed C57, but not TNFRKO, mice showed evidence of gelatinase activity, which appeared as darkly staining vessels, in the arteries adjacent to the bronchioles (Fig. 6). Tissue zymography is illustrated in Fig. 7. The bulk lung tissue gelatinase zymographs of the C57 animals showed significant increases in MMP-9 (P = 0.02) and MMP-2 (P = 0.01) in the smoke-exposed animals but no significant differences in the TNFRKO mice. The pulmonary artery zymographs of the C57 mice showed significant increases in MMP-9 (P = 0.001) and MMP-2 (P = 0.02) in the smoke-exposed animals but no significant differences in the TNFRKO mice.

At 6 mo, the smoke-exposed C57 mice continued to have an approximately doubled level of mRNA for MMP-12 (P = 0.034; Fig. 8). The immunohistochemical analysis showed an increase in MMP-12 staining compared with control (Fig. 9) that did not achieve statistical significance. Gene expression and immunohistochemical protein levels of MMP-2, MMP-9, and MMP-13 in the C57 mice were not different from control, and the same was true for TIMP-1 (Figs. 8 and 10). The

Fig. 4. Immunohistochemical staining for MMP-2 (A and B), MMP-9 (C and D), and MMP-12 (E and F) in smokers (B, D, and F, respectively) and controls (A, C, and E, respectively) at 2 wk. Scale bars, ~50 μm.
TNFRKO mice showed an increased (~3.5 times) mRNA level of MMP-2 compared with control ($P = 0.001$); other values were not different from control (Fig. 8). Immunohistochemical analysis showed a slight, but not significant, increase in staining intensity for MMP-2 (Fig. 9).

**DISCUSSION**

In this study, we have shown for the first time that cigarette smoke exposure induces increases in gene expression, protein levels, and activity in the small pulmonary arteries of several different MMPs that are potentially important in vascular remodeling. By contrast, TNFRKO mice did not show increases in MMP levels, except for a small increase in MMP-2 at 6 mo.

In general, pulmonary hypertension is associated with neointimal formation and increased muscularization of the muscular vessels, with extension of smooth muscle into the usually poorly muscularized distal arteries, along with increased production of extracellular matrix (9, 18). This type of vascular remodeling requires cell proliferation and cell migration, as well as deposition of new matrix. The exact mechanism by which cells proliferate and migrate is not known, but bioactive mediators, such as endothelin, vascular endothelial growth factor (VEGF), nitric oxide, and prostacyclin, appear to play a role (18).

Until recently, vascular remodeling and pulmonary hypertension in chronic obstructive pulmonary disease have been largely ignored, although their effect on prognosis (22) is significant. In previous studies, we found that acute exposure to cigarette smoke is associated with cellular proliferation (31) and upregulation of vasoactive mediators that control cell proliferation and vasoconstriction, notably endothelin and VEGF, in the small pulmonary arterial branches (37). Increased production of these mediators is persistent over many months of smoke exposure (35, 36), and mediator levels correlate with the extent of vascular remodeling and levels of pulmonary arterial pressure (36).

The MMPs are a diverse group of Zn-containing endopeptidases that are able to degrade most extracellular matrix components. Depending on their structures, they can be divided into several different groups, and we have chosen examples of collagenase (MMP-13), gelatinase (MMP-2 and MMP-9), and metalloelastase (MMP-12) for the present study. All these MMPs, with the exception of MMP-13, have been implicated in the genesis of pulmonary hypertension and/or are believed to be increased in the lungs of patients with COPD. MMP-13 was examined because it is probably the closest murine MMP in terms of function to human MMP-1 (mice lack MMP-1), which has been found to be increased in COPD (19) and has been implicated in the hypoxia-induced pulmonary

![Fig. 5. Mean intensity of immunohistochemical staining of MMP-2, MMP-9, and MMP-12 in muscularized small intrapulmonary arteries in C57 or TNFRKO mice after 2 wk of cigarette smoke exposure. Smoke exposure significantly increased staining intensity in MMP-2, MMP-9, and MMP-12 in C57, but not TNFRKO, mice. Values are means ± SD. *$P < 0.05$ vs. control.](image-url)
hypertension model (28). There is also considerable interaction between the MMPs, in that MMP-2 activates pro-MMP-13 to MMP-13, which in turn activates pro-MMP-9 (reviewed in Ref. 28), and these interactions may occur in the vessels as well.

Although MMP-12 was originally described in alveolar macrophages, it is not limited to these cells. MMP-12 has recently been identified in normal human bronchial epithelial cells (24), where it has been shown to be upregulated by cigarette smoke (23). MMP-12 has also been detected in a glioma cell line, in a variety of primary brain neoplasms (21), and in cultured smooth muscle cells derived from human aorta atherosclerotic plaques (38). We believe that this is the first description of MMP-12 production in pulmonary arterial branches.

There is evidence in the hypoxia- or monocrotaline-induced models of pulmonary hypertension that administration of a broad-spectrum MMP inhibitor in vivo (16) reduces vascular remodeling, and inhibition of MMPs by GM-6001 in a cultured pulmonary artery system caused suppression of monocrotaline-induced cell proliferation and matrix production followed by regression of vascular wall thickening (9). The inhibitor appeared to have its greatest effect on MMP-2, although activity of other MMPs was also decreased.

The present study extends these concepts to cigarette smoke, demonstrating that a few weeks of smoke exposure upregulates a wide variety of MMPs in the small pulmonary arteries. Other vascular mediators upregulated by smoke exposure may additionally play a role in MMP activity. Endothelin-1 is a possible paracrine mediator here, inasmuch as it is known to be a potent mitogen for smooth muscle cells, and we have shown its upregulation in the vessels acutely and after chronic smoke exposure (35, 36). Endothelin-1 is known to activate MMPs in the heart (1) and in the systemic vasculature (11). There may also be a feedback loop between ET-1 and MMPs, since MMP-2 has been found to cleave big ET-1 to active ET-1 and to increase vasoconstriction in the systemic vasculature (13).

Increased MMP production appears to occur very early after experimental insults such exposure to hypoxia and monocrotaline; the present study shows that this is also true in the vascular reaction to tobacco smoke. However, with smoke exposure, upregulation of production of most of the MMPs is not persistent: there is a marked increase in production after 2 wk of smoke exposure, but at 6 mo only a small increase in MMP-12 is observed in the C57 mice. TIMPs inhibit MMP activities and, thus, may effect matrix protein turnover (32). The present study indicates that TIMP-1 and MMP are upregulated simultaneously in the C57 mice; our findings are similar to those reported for lungs in idiopathic pulmonary hypertension (32) and for a rat model of hypoxia-induced pulmonary hypertension (25). Although we cannot make definitive comments on the balance between MMP and its TIMP, gelatinolytic activity in the vessels of the smoke-exposed animals demonstrated by in situ zymography indicates that there is still an excess of MMP activity.

The exact effects of increased MMP production in this setting are unclear. One possibility is that early remodeling events set the stage for eventual functional abnormalities in the vessels. In particular, MMP activity allows myofibroblasts to migrate (28), but in addition, cleavage of matrix components by the MMP can release fibroblast growth factor and transforming growth factor-β, as well as other growth factors, such as epidermal growth factor (reviewed in Ref. 27), thus stimulating cell growth and activity. MMPs also degrade matrix, and collagen degradation products themselves appear to be a stimulus for fibroblast and vascular smooth muscle cell proliferation (33). This continued cycle eventually may lead to endothelial dysfunction and decreased vascular compliance.

An alternate possibility is that early upregulation of MMPs in the smoke model prevents matrix deposition in the vessels in the hypoxia- or monocrotaline-induced models of pulmonary hypertension. This would result in decreased MMP activity and a reduced propensity for vascular remodeling.
the face of increased levels of the mediators that drive remodeling (e.g., endothelin and VEGF) but that with time these latter mediators predominate, since, in contrast to the MMPs, they remain continuously upregulated (36). This upregulation leads to a net deposition of matrix and greatly increased muscle in the vessels, with subsequent vascular rigidity and, eventually, pulmonary hypertension. In this regard, it is known that provisional matrix components, such as tenascin-C and osteopontin, are important in vascular remodeling and are directly related to metalloproteinase activity. For example, tenascin-C is increased in monocrotaline-induced (20) or hemodynamically mediated (26) pulmonary hypertension and can be increased by MMP activity (9). Osteopontin, which is synthesized by smooth muscle cells, is a survival factor that is able to rescue MMP inhibitor-induced smooth muscle cell apoptosis (9), and osteopontin appears to be increased during

Fig. 7. Gelatin zymographs of bulk lung tissue (A and B) and isolated main pulmonary arteries (C and D) after 2 wk of smoke exposure in C57 and TNFRKO mice. Smoke increased MMP-2 and MMP-9 in C57, but not TNFRKO mice. Values are means ± SD. ∗P < 0.05 vs. control. Control, open bars; smoke exposure, cross-hatched bars.

Fig. 8. Gene expression levels of MMP-2, MMP-9, MMP-12, and MMP-13 in muscularized small intrapulmonary arteries isolated by LCM after 6 mo of cigarette smoke exposure in C57 and TNFRKO mice. In C57 mice, only MMP-12 remained elevated. Smoke significantly upregulated MMP-2 compared with control in TNFRKO mice. Values are means ± SD. ∗P < 0.05 vs. control.
vascular remodeling, where its effects are mediated by transforming growth factor-β (3).

The second conclusion from our study is that TNF-α is crucial to the increased production of MMPs in the pulmonary arteries after smoke exposure, since TNFRKO mice failed to show an upregulation of MMP production, except for MMP-2 at 6 mo. This idea would be consistent with observations of other systems where proinflammatory cytokines, including TNF-α, have been shown to increase MMP-12 production in human peripheral blood-derived macrophages (12) and human bronchial epithelial cells (23) and to increase MMP-9 expression in vascular smooth muscle cells (4) and endothelial cells (14). However, it is important to note that our data do not distinguish between a direct effect of TNF-α and indirect effects related to the inflammatory cascade.

The relation between TNF-α and MMP-12 production in the lung is particularly interesting. Mice lacking MMP-12 do not develop emphysema after chronic smoke exposure (15), and we have shown that the role of MMP-12 appears to that of liberating active TNF-α from pro-TNF-α, with subsequent neutrophil influx and matrix attack by neutrophil-derived proteases (5). This situation may set up a positive-feedback loop, in which TNF-α causes production of MMP-12, which in turn releases more TNF-α.

TNF-α appears to play a central role in the development of emphysema. TNFRKO mice are ~70% protected against cigarette smoke-induced air space enlargement (7). Our present data indicate that TNF-α is likely to be important in smoke-induced vascular remodeling as well, and it is possible that positive-feedback loops similar to those described above would also apply to the vasculature.

**GRANTS**

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