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ORIGINAL RESEARCH

Expression of Connective Tissue Growth Factor and Bone Morphogenetic Protein-7 in *Pseudomonas aeruginosa*-induced Chronic Obstructive Pulmonary Disease in Rats

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Abstract

Repeated intratracheal injection of *Pseudomonas aeruginosa* (PA) in male Wistar rats was used to investigate the role of chronic infection in the development of chronic obstructive pulmonary disease (COPD) and the possible involvement of connective tissue growth factor (CTGF) and bone morphogenetic protein-7 (BMP-7) in this process. Injections of PA or normal saline solution were given for 8 weeks and the rats observed for a further 8 weeks. In addition to arterial blood gas, lung function and lung pathology measurement during this time period, protein and mRNA expression of CTGF and BMP-7 were measured, and the correlation of expression of CTGF and BMP-7 with pathological changes in the lung was evaluated. Repeated intratracheal PA infection in rats caused reduction in body weight, hypoxia, carbon dioxide retention, compromised lung function, chronic inflammation, thickening of the tracheal and arterial walls, and emphysema, changes consistent with those of COPD. Rats with PA infection also had increased CTGF and decreased BMP-7 expression, suggesting that both CTGF and BMP-7 are involved in the occurrence and development of airway remodeling. Our findings suggest that repeated airway infection is not only a factor resulting in deterioration of COPD, but is also a risk factor for its development, and that CTGF and BMP-7 are involved in the pathogenesis of this condition.

Introduction

Questions about whether bacterial infection is able to initiate or only to cause exacerbation of chronic obstructive pulmonary disease (COPD) have been present for several decades. Previous studies have shown that bacterial infection is a major cause of acute deteriorations in COPD, and that smoking and inhalation of harmful dust are the main causes of COPD. But in the 1950s, English investigators proposed that although smoking was a major cause of COPD, repeated airway infection and excessive secretion of mucus were also causative factors for COPD (1).

In the 1990s, Sethi et al. (2, 3) proposed a vicious circle hypothesis: smoking, inhalation of harmful dust, respiratory diseases and other events are initiators that may impair mucociliary clearance. Then, after pathogens colonize the airway, the products of these pathogens induce a series of inflammatory reactions that result in an increase in mucus secretion, compromise in mucociliary clearance, degradation of elastic tissue and further

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damage to the respiratory mucosa. The sequential positive feedback of this cycle finally results in the development of COPD.

In clinical practice, COPD patients often have a history of chronic bronchitis or repeated lung infections that cause deterioration in the pathophysiology of COPD. However, in a fraction of COPD patients, known causes of COPD such as smoking and inhalation of harmful dust are not identified. We have previously shown that cigarette smoke and bacterial infection can be used together to establish a stable COPD model in rats (4). But currently, there is no experimental and theoretical evidence to determine whether repeated pulmonary bacterial infection by itself can induce airway remodeling and subsequent progression into COPD.

Pseudomonas aeruginosa (PA) is an opportunistic pathogen that is also a major pathogen causing acute deterioration in COPD (5). PA infection may also result in long-term colonization in a bacterial biofilm (6, 7). Currently, whether PA can cause COPD is still unknown.

Connective tissue growth factor (CTGF) is a fibrogenic growth factor that belongs to the immediate early response gene family. The fibrogenic effect of transforming growth factor- β 1 (TGF- β 1) is mainly mediated by CTGF.

Bone morphogenetic protein-7 (BMP-7) is a member of the TGF superfamily. Studies have confirmed that BMP-7 can inhibit glomerular interstitial fibrosis and can reverse renal glomerular injury in mice (8, 9). Izumi *et al.* (10) showed that BMP-7 could protect rats against TGF- β 1 induced pulmonary fibrosis by inducing the expression of Id2 and Id3.

In the present study, repeated intratracheal injection of PA was used to induce chronic lung infection in rats. Basic pathophysiological characteristics of COPD were examined and, at the same time, pulmonary expression of CTGF and BMP-7 was measured. In this way, the role of bacterial infection in producing the pathophysiological changes of COPD and the potential molecular mechanisms involved were investigated.

Materials and Methods

Animal model and grouping

A total of 96 male Wistar rats (specific pathogen free) weighing 250 ± 10 g were purchased from Slac Experimental Animal Center in Shanghai and fed with basic food and water for an accommodation period of 1 week. The protocol has the approval of the institutional animal committee on animal use. Animals were randomly assigned to a normal saline (NS) group ($n = 48$) and a PA group ($n = 48$). Six rats were sacrificed at each of the following time points: 0 days, 1 day, 1 week, 2 weeks, 4 weeks, 8 weeks, 12 weeks and 16 weeks for pathological and biochemical studies. In the NS group, rats received repeated intratracheal injection of normal saline (0.1 ml) twice weekly (on day 1 and day 4 of each week) for

4 weeks and then once weekly (on day 1 of the week) for the next 4 weeks.

In the PA group, rats received intratracheal injection of PA (ATCC27853; 0.1 ml; Clinical Laboratory Center of the Ministry of Health) at 0.7×10^8 CFU/ml in the same sequence. At the end of the 8 week PA or saline treatment period, the injections were stopped and the rats observed for a further 8 weeks. At each of the 8 time points described above, 6 rats from each group were selected for measurement of body weight and lung function, followed by sacrifice for the detection of blood gases, evaluation of pulmonary pathophysiology, and measurement of CTGF and BMP-7.

Body weight and arterial blood gas

After anesthesia, rats were placed on an electronic balance for the measurement of body weight and were then sacrificed. The abdominal aorta was exposed and arterial blood obtained (0.5 ml) for blood gas analysis (ABL800; Radiometer, Finland) to measure partial pressure of oxygen (PaO₂) and partial pressure of carbon dioxide (PaCO₂).

Measurement of lung function

The AniRes2005 animal lung function analysis system (Beijing Bestlab Biotech Co., Ltd) was used to measure lung function. Animals were intraperitoneally anesthetized with 3% pentobarbital sodium at 0.75 mg/kg, followed by tracheal intubation and connection to a plethysmograph. Expiratory resistance (Re), forced expiratory peak flow (PEF), and ratio of forced expiratory volume in 0.3 second to forced vital capacity (FEV_{0.3}/FVC%) were measured. FEV_{0.3}/FVC% is a measurement reflecting airflow obstruction and is similar to FEV₁/FVC%, a measurement that cannot be used in rats due to their rapid breathing rate.

Pathological examination

At the pre-designated time points, rats were sacrificed and the trachea, left lung, and middle and posterior lobes of right lung were collected and fixed in 10% neutral formalin followed by processing for haematoxylin/eosin staining.

Immunohistochemistry for CTGF and BMP-7

Immunohistochemistry was performed with a two-step method according to the manufacturer's instructions (Envision™ plus; Beijing Zhongshan Golden Bridge Co., Ltd), using HRP conjugated anti-rabbit (PV-6001) and anti-goat (PV-6003) secondary antibodies. After deparaffinization and dehydration, lung sections were treated with citrate buffer at high temperature and high pressure for antigen retrieval. Goat anti-rat CTGF (1:100) polyclonal antibody (Santa Cruz, CA, USA) and rabbit anti-rat BMP-7 (1:250) polyclonal antibody (Abcam, UK) were used as the primary antibodies. Visualization was done with diaminobenzidine staining and counterstaining with haematoxylin. In the

negative control, the primary antibody was replaced with IgG.

On each slide ($\times 200$), 20 fields were selected and positive cell numbers were counted by 2 blinded observers. According to the percentages of positive cells that were recorded, the ICH results were graded from 0–4 using the following scoring scheme: 0: no positive cells; 1: $<25\%$ positive; 2: $25\%–50\%$ positive; 3: $50\%–75\%$ positive; 4: $>75\%$ positive.

Quantitative analysis of pathological findings

Sections (~ 3 mm in thickness) of the middle lobe of right lung were collected in the plane vertical to the bronchi and bronchioles and with maximal perimeter at the sagittal plane, followed by fixation and sectioning. Sections were observed under a microscopic micrometer (107JC, Shangguang Instrument Co., Ltd.) at $\times 400$. The thickness of the tracheal wall and vessel wall was measured at the cross-section of bronchi and bronchioles. A total of 5 bronchial rings were measured and the data were averaged to calculate the thickness of the tracheal and vessel walls.

For goblet cell analysis, the lungs were fixed by inflation with 4% paraformaldehyde and embedded in paraffin. Sections were cut 5 mm thick and stained with periodic acid-Schiff/Alcian-blue. Assessments were performed on stratified random fields (n55) in each animal, as described by Nagai et al. (11). Goblet cell metaplasia was assessed in bronchi with internal diameter measuring 200 mm in cross section, using a mucus grading system (scores 0–3), based on the ratio of goblet cell area to whole cross-sectional epithelial area in each round bronchus. In this grading system, a score of 0 indicates no goblet cells; a score of 1 indicates occupation of up to $1/3$ of the epithelial area; a score of 2 indicates occupation of $1/3$ to $2/3$ of the epithelial area; and a score of 3 indicates occupation of $2/3$ or more of the epithelial area. The mucus score was obtained by averaging the scores of the measured bronchi.

Quantitative analysis of neutrophil count

Sections (~ 3 mm in thickness) of 3 regions of lung were fixed and haematoxylin/eosin stained. Sections were observed under a microscopic micrometer (107JC, Shangguang Instrument Co., Ltd.) at $\times 400$, and the neutrophils were counted. A total of 5 fields were measured and the data were averaged to calculate the neutrophil count.

Real-time RT-PCR

Total RNA was extracted with Trizol (Invitrogen) and used to reverse transcribe into cDNA according to the manufacturer's instructions (Reverse Transcription System; Promega). The gene sequences of GAPDH, CTGF and BMP-7 were obtained from the NCBI Genbank and the primers were designed with Primer 3 and synthesized by Shanghai Sangon Biotech Co., Ltd (Table 1). Real-time PCR was done in a Bio-Rad iCycler thermal

Table 1. Primer sequences

Gene name	5'–3'	Product length (bp)
CTGF	CCAATGACAATACCTTCTGC	148
	GAAAGCTCAAACCTTGACAGG	
BMP-7	GATACCACCATCGAGAGTTC	148
	TGGAGCACCTGATAGACTGT	
GAPDH	GTATGACTCTACCCACGGCAAG	101
	GATCTCGCTCCTGGAAGATG	

cycler. In brief, 1 μ l of cDNA was mixed with 6.25 μ l of SYBR Premix ExTaqTM, each primer (0.25 μ l), and sterilized ultrapure water (final volume: 12.5 μ l).

The PCR conditions were as follows: predenaturation at 94°C for 3 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 55° for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 7 min. The mixture without cDNA served as a negative control. Experiments were done twice and in triplicate. The gene expression in the normal control group was used as a standard and was compared with that in the NS and PA groups. In addition, gene expression was compared between the NS and PA groups.

Statistical analysis

Data were presented as mean with standard deviation, which were compared between NS and PA groups by the Student's *t*-test. Comparisons were performed with repeated ANOVA with post-hoc comparison adjusted by the Bonferroni method. Data were analyzed with SPSS 15.0 statistics software (SPSS Inc, Chicago, IL, US), and a *p*-value < 0.05 was considered statistically significant.

Results

Body weight

Four weeks after PA infection, body weight in the PA group was significantly lower than that in the NS group ($p = 0.0076$). Twelve weeks later, at the end of study, the body weight in the PA group was even more profoundly reduced compared to the NS group ($p < 0.0001$) (Table 2).

Table 2. Body weight at different time points

	NS group	PA group	<i>p</i>
0 days	290.83 \pm 5.85	293.33 \pm 7.53	0.5350
1 day	282.5 \pm 6.89	279.17 \pm 5.85	0.3875
1 week	300 \pm 6.32	299.17 \pm 4.92	0.8040
2 weeks	300 \pm 11.83	297.5 \pm 5.24	0.6463
4 weeks	310.83 \pm 9.7	292.5 \pm 9.35	0.0076
8 weeks	335.83 \pm 5.85	309.17 \pm 8.61	<0.0001
12 weeks	347.5 \pm 9.35	317.5 \pm 6.89	<0.0001
16 weeks	365.83 \pm 7.36	324.17 \pm 7.36	<0.0001

Bold: $p < 0.05$ between two groups.

Table 3. PaO₂ and PaCO₂ at different time points

	PaO ₂			PaCO ₂		
	NS group	PA group	<i>p</i>	NS group	PA group	<i>p</i>
0 days	113.72 ± 6.65	111.28 ± 8.46	0.5918	69.77 ± 2.84	70.88 ± 2.98	0.5215
1 day	109.23 ± 10.67	105.2 ± 8.99	0.4950	66.63 ± 8.12	66.72 ± 5.68	0.9840
1 week	107.95 ± 8.09	110.45 ± 9.26	0.6291	63.12 ± 6.04	63 ± 5.27	0.9723
2 weeks	108.25 ± 5.66	104.72 ± 9.28	0.4443	66.3 ± 5.48	68.32 ± 3.76	0.4742
4 weeks	99.12 ± 7.65	98.95 ± 7.31	0.9700	65.85 ± 5.49	63.2 ± 4.53	0.3833
8 weeks	102.48 ± 4.16	100.45 ± 4.35	0.4274	71.83 ± 2.25	70.82 ± 4.78	0.6474
12 weeks	100.42 ± 5.34	78.35 ± 5.3	<0.0001	64.53 ± 4.13	79.68 ± 5.49	0.0003*
16 weeks	101.22 ± 3.12	71.42 ± 4.11	<0.0001	69.1 ± 3.37	81.53 ± 4.7	0.0004*

Bold: *p* < 0.05 between two groups.

Arterial blood gas analysis

Changes in PaO₂ and PaCO₂ in the two groups are shown in Table 3. By 12 weeks after PA infection, PaO₂ was significantly reduced (*p* < 0.0001), and PaCO₂ significantly increased (*p* = 0.0003) compared to the NS group. And at the end of study, the PaO₂ in the PA group was also significantly lower and the PaCO₂ significantly higher than that in the NS group (*p* < 0.0001 and *p* = 0.0004, respectively).

Lung function

Eight weeks after PA infection, the FEV_{0.3}/FVC had a decreasing tendency, and by 16 weeks after PA infection, it was significantly reduced compared to both baseline (*p* < 0.0001) and NS group (*p* = 0.0017) levels. PEF started to become reduced 1 week after PA infection, and by 8 weeks after PA infection was significantly reduced compared to the NS group (*p* < 0.0001). Re began to increase 1 week after PA infection and was significantly different from that in the NS group at this time (*p* = 0.0007). The changes seen in lung function in the PA rats supported a diagnosis of COPD (Table 4).

Pathological examination of lung

In the normal control group, the mucosal cilia in the trachea and bronchus were intact (not shedding), the ciliated columnar epithelial cells were regularly arranged, and no congestion or infiltration of inflammatory cells were seen in the tracheal wall and surrounding tissues. Bronchioles and alveoli had complete structures, and the alveolar septum was intact.

In the PA group at 4 weeks after PA infection, many glands were observed in the tracheal submucosa, goblet cell numbers were increased, and mucous plugs were present in the glandular ducts. Infiltration of lymphocytes and neutrophils was found in the submucosa of bronchioles and surrounding tissues, the alveolar septum was slightly thickened, congestion and edema were seen in the interstitium, and proliferation of alveolar epithelial cells was noted.

In the PA group at 12 weeks after PA infection, the tracheal epithelial cells were irregularly arranged, hyperplasia of glands and rupture of cartilages were present, the lumen of the bronchioles was irregular, hyperplasia of surrounding smooth muscle was seen, and shedding epithelial cells were found in the lumen. Tracheal

Table 4. Lung function at different time points

	FEV _{0.3} /FVC			PEF			Re		
	NS group	PA group	<i>p</i>	NS group	PA group	<i>p</i>	NS group	PA group	<i>p</i>
0 day	88.65 ± 1.13	89.53 ± 1.15	0.2123	31.2 ± 0.68	31.31 ± 0.6	0.7763	0.42 ± 0.05	0.43 ± 0.07	0.7262
1 days	83.96 ± 1.92	84.47 ± 0.82	0.5639	30.25 ± 1.83	36.07 ± 4.19	0.0109	0.49 ± 0.07	0.5 ± 0.07	0.8415
1 week	88.47 ± 0.78	85.84 ± 0.2	<0.0001	30.4 ± 0.14	28.24 ± 2.01	0.0255	0.4 ± 0.05	0.51 ± 0.02	0.0007
2 weeks	82.3 ± 1.15	81.46 ± 3.14*	0.5497	28.8 ± 0.83	28.39 ± 1.05	0.4717	0.42 ± 0.06	0.44 ± 0.06	0.5528
4 weeks	83.91 ± 13.07	83.04 ± 3.42	0.8779	28.01 ± 3.85	27.9 ± 1.48	0.9487	0.55 ± 0.03	0.6 ± 0.12	0.3209
8 weeks	85.46 ± 2.13	80.6 ± 2.82*	0.0072	28.51 ± 0.67	24.96 ± 0.62*	<0.0001	0.7 ± 0.1*	0.54 ± 0.11	0.0299
12 weeks	85.05 ± 4.2	80.36 ± 2.72*	0.0446	28.74 ± 0.58	26.55 ± 2.83*	0.0928	0.52 ± 0.14 [†]	0.61 ± 0.1*	0.2254
16 weeks	79.24 ± 9.22	56.68 ± 9.17* [†]	0.0017	27.43 ± 1.62*	19.24 ± 2.69* [†]	<0.0001	0.56 ± 0.09	0.64 ± 0.05*	0.0857

**p* < 0.05 vs day 0.

[†]*p* < 0.05 vs last time point.

FEV_{0.3}/FVC, forced expiratory volume in 0.3 sec/forced vital capacity; PEF, forced peak expiratory flow; Re, expiratory resistance.

Bold: *p* < 0.05 between two groups.

remodeling began to occur, the alveolar structure was irregular, and the alveolar wall was thinned, its volume increased, and in some places it had begun to rupture.

At 16 weeks after PA infection, the tracheal epithelial cells were irregularly arranged and shedding, hyperplasia of submucosal smooth muscle was evident, vascular congestion was found, proliferative goblet cells were observed in the bronchi, fibrosis of the submucosa was present, and smooth muscle had begun to thicken. Deposition of extracellular matrix was noted accompanied by airway remodeling. Bronchioles and alveoli showed tube-like or cyst-like expansion, and emphysematous changes were seen. The alveolar space was irregularly enlarged, the alveolar septum ruptured, and some alveoli had merged to form bullae (Figure 1). Goblet cell scoring in the 2 groups is shown in Table 5. From 4 weeks onward,

Table 5. Goblet cell scoring of PA and NS groups (n = 6)

	NS group	PA group	p-value
0 day	0 ± 0	0 ± 0	1.0000
1 day	0.17 ± 0.41	0.33 ± 0.52	0.5670
1 week	0.33 ± 0.52	1 ± 0	0.0102
2 weeks	0.5 ± 0.55	1 ± 0	0.0502
4 weeks	0.5 ± 0.55	1.5 ± 0.55	0.0104
8 weeks	0.67 ± 0.52	1.67 ± 0.52	0.0076
12 weeks	0.83 ± 0.41	2.5 ± 0.55	0.0002
16 weeks	0.67 ± 0.52	2.83 ± 0.41	<0.0001

PA, *Pseudomonas aeruginosa*; NS, normal saline.

Bold: $p < 0.05$ between two groups.

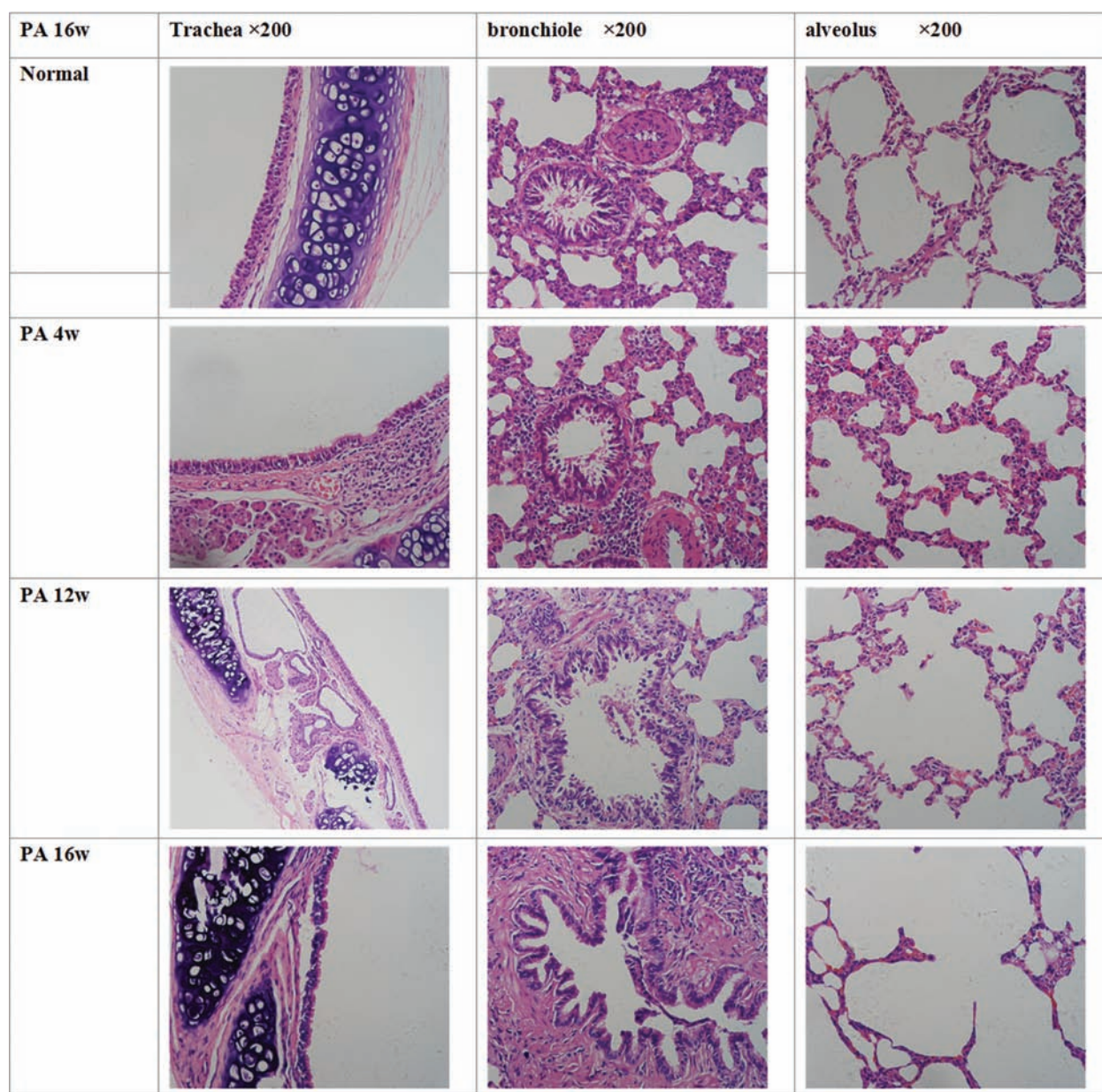


Figure 1. Pathological examination of the lung.

Table 6. Thickness of tracheal wall and vessel wall at different time points

	Thickness of tracheal wall (μm)			Thickness of vessel wall (μm)		
	NS group	PA group	<i>p</i>	NS group	PA group	<i>p</i>
0 day	311 \pm 8.25	309.17 \pm 9.7	0.7317	100 \pm 8.94	108.33 \pm 8.16	0.1228
1 day	310.5 \pm 11.29	306.5 \pm 10.19	0.5340	102.5 \pm 9.35	107.5 \pm 10.37	0.4010
1 week	314 \pm 5.55	314.83 \pm 11.67	0.8776	113.33 \pm 6.06	113.33 \pm 10.33	1.0000
2 weeks	313.67 \pm 8.16	323.67 \pm 4.13	0.0232	115 \pm 10.95	120.83 \pm 13.2	0.4242
4 weeks	320 \pm 10.41	344.33 \pm 10.93	0.0027	112.5 \pm 15.08	134.17 \pm 11.14	0.0178
8 weeks	333 \pm 7.16	384 \pm 9.06	<0.0001	110.83 \pm 13.2	157.5 \pm 15.08	0.0002
12 weeks	344.5 \pm 4.72	494.67 \pm 9.58	<0.0001	116.67 \pm 14.72	155.83 \pm 15.94	0.0013
16 weeks	344.5 \pm 11.88	524.5 \pm 18.59	<0.0001	120 \pm 15.81	163.33 \pm 12.52	0.0004

Bold: *p* < 0.05 between two groups.

significantly more goblet cells were seen in the PA group than in the NS group (all *p* \leq 0.0104).

Quantitative analysis of pathological findings

As seen in the pathological examination, the tracheal wall began to thicken in the PA group as the duration of the PA infection lengthened, and this change was accompanied by hyperplasia of glands. Two weeks after PA infection, the tracheal wall was significantly thicker than that in the NS group (*p* = 0.0232). The walls of small blood vessels also thickened, and were significantly thicker than in the NS group at 4 weeks after PA infection (*p* = 0.0178). At the end of study, both the tracheal wall and the blood vessel wall in the PA group were significantly thicker than those in the NS group (*p* < 0.0001 and *p* = 0.0004, respectively) (Table 6).

Neutrophil counts in trachea, bronchiole and alveolus

Changes in neutrophil count in the two groups are shown in Table 7. From 4 weeks after the beginning of PA inhalation until the end of the study, the neutrophil count in the PA group was significantly higher than that in the NS group in the trachea (all *p* \leq 0.0002), the bronchiole (all *p* \leq 0.0007), and the alveolus (all *p* \leq 0.0008).

Protein expression of CTGF and BMP-7 in the lung

CTGF-positive staining was mainly found in the airway in ciliated epithelial cells, blood vessels and alveolar inter-

stitial cells. In the NS group, only a few airway ciliated epithelial cells and alveolar interstitial cells were positive for CTGF and the staining was light. In the PA group, CTGF expression in the airway ciliated epithelial cells and alveolar interstitial cells began to increase at 2 weeks after PA infection, especially in the airway ciliated epithelial cells. The CTGF expression in the PA group was higher than that in the NS group. At the end of study, the CTGF expression in the PA group was significantly higher than that in the NS group. BMP-7 positive staining was mainly found in the airway ciliated epithelial cells and a few smooth muscle cells surrounding the airway.

In the NS group, the majority of airway ciliated epithelial cells were positive for BMP-7. In the PA group, BMP-7 expression decreased gradually with the prolongation of PA infection. At the end of study, CTGF protein expression in the PA was significantly higher than that in the NS group at 2, 4, 12 and 16 weeks (all *p* < 0.0493, Figure 2A). In the PA group, from the first day after PA infection until the end of the study, BMP-7 protein expression was significantly lower than that in the NS group (all *p* < 0.0493, Figure 2B). Representative images are shown in Figure 2C.

mRNA expression of CTGF and BMP-7 in the lung

Findings in real-time RT-PCR

CTGF mRNA expression increased progressively with the prolongation of PA infection. In the PA

Table 7. Neutrophil counts in trachea, bronchiole and alveolus

	Trachea			Bronchiole			Alveolus		
	NS group	PA group	<i>p</i>	NS group	PA group	<i>p</i>	NS group	PA group	<i>p</i>
0 day	2.0 \pm 0.71	1.8 \pm 0.45	0.5729	1.8 \pm 0.45	2.2 \pm 0.45	0.1547	2.2 \pm 0.84	2.4 \pm 1.14	0.7365
4 weeks	4.6 \pm 1.14	20.6 \pm 6.69	0.0002	4.4 \pm 1.95	14.4 \pm 3.65	0.0001	3.4 \pm 1.14	19.0 \pm 7.94	0.0008
12 weeks	6.0 \pm 1.58	14.4 \pm 3.05	0.0001	5.2 \pm 1.48	11.6 \pm 2.07	0.0001	4.8 \pm 1.79	16.8 \pm 4.81	0.0002
16 weeks	4.8 \pm 1.64	11.8 \pm 2.59	0.0002	4.2 \pm 1.48	10.6 \pm 2.88	0.0007	3.0 \pm 1.58	13.8 \pm 2.86	0.0001

Bold: *p* < 0.05 between two groups.

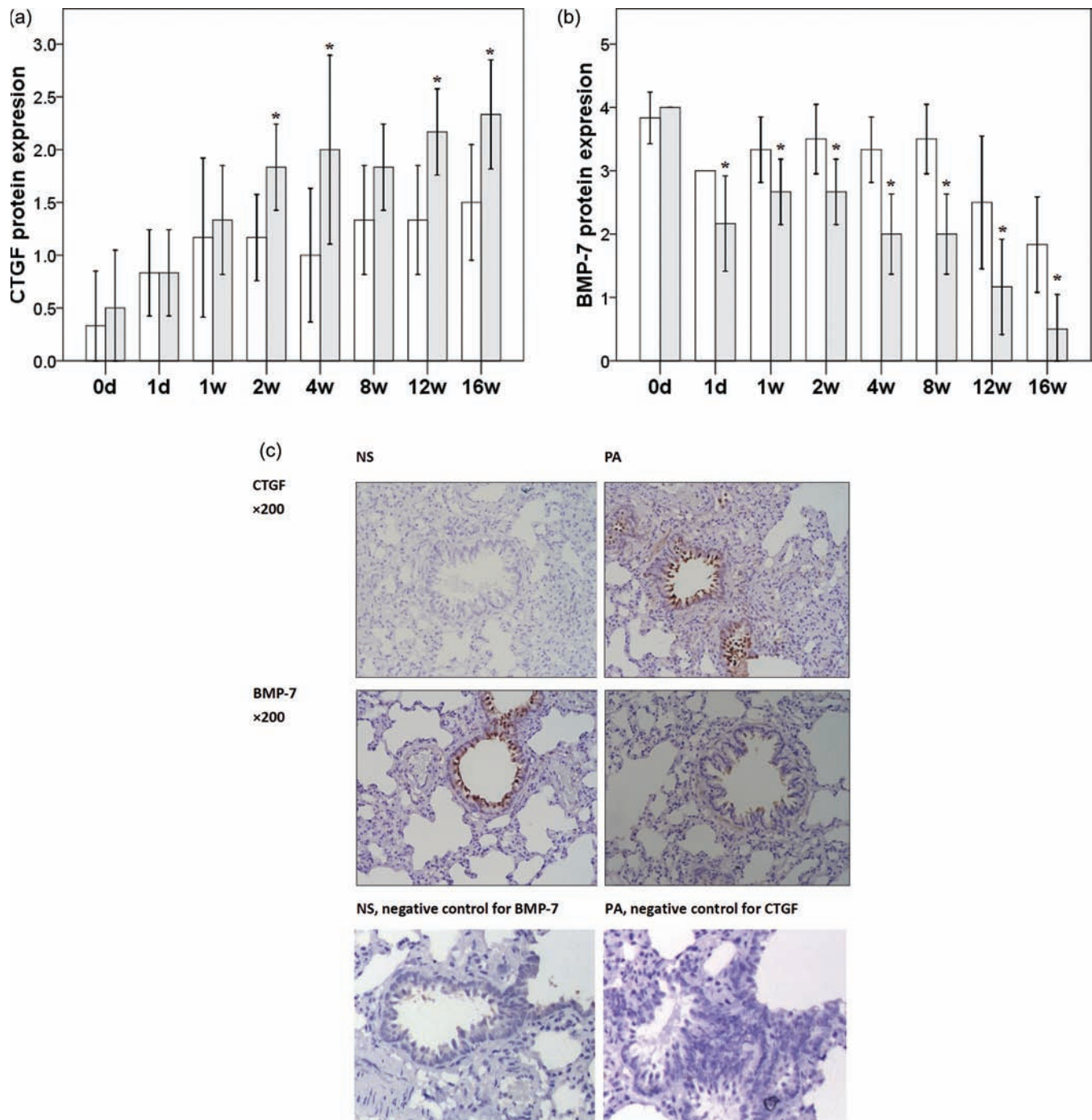


Figure 2. Protein expression of CTGF and BMP-7. A. CTGF protein expression B. BMP-7 protein expression. C. representative images (×200) of CTGF and BMP-7 staining in PA and NS groups. * $p < 0.05$ vs control group at same time point.

group, from 1 day after PA infection onward, CTGF expression was significantly increased compared to the NS group ($p < 0.0001$). By the end of study, CTGF expression in the PA group had increased to approximately 5 times that in the NS group ($p < 0.0001$) (Figure 3). BMP-7 mRNA expression decreased gradually in the PA group with the prolongation of infection, and was significantly lower than that in the NS group from 1 day after PA infection ($p = 0.0219$) until the end of the study ($p = 0.0056$, Figure 4).

Association of thickness of tracheal wall and vessel wall with expression of CTGF and BMP-7

CTGF was positively and BMP-7 negatively correlated with tracheal and vessel wall thickness (Figure 5, all $p < 0.0001$). In addition, CTGF expression was significantly negatively correlated to BMP-7 expression ($r = -0.8911$, $p < 0.0001$).

Discussion

In earlier studies, the reported evidence was not considered sufficient to support an important role of

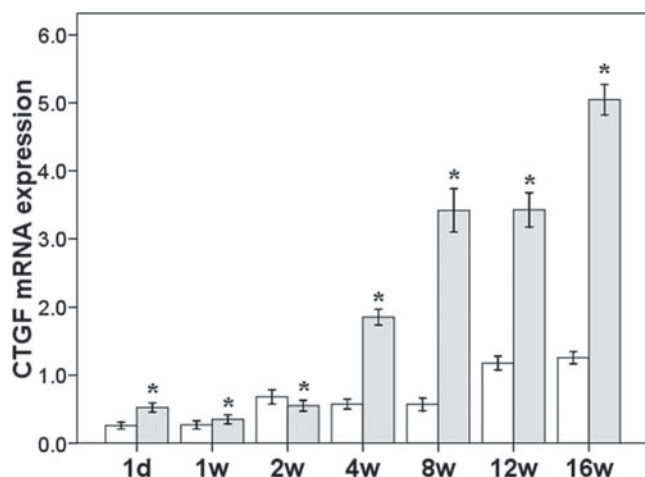


Figure 3. mRNA expression of CTGF in the lung. * $p < 0.05$ vs control group at same time point.

bacterial infection in chronic bronchitis. And in COPD, infection (particularly bacterial infection) is usually regarded as a concomitant phenomenon that has no role in the pathogenesis of this condition. In the last 20 years, however, molecular, cell and immunological techniques have been used to investigate the role of bacterial infection in COPD. Sethi et al. (2, 3) found that infection, especially bacterial infection, was not only a major cause of acute deterioration of COPD, but an important factor in the pathogenesis of COPD. In the current study, PA, a pathogen known to be involved in the pathogenesis of COPD (5, 6) and to be susceptible to colonization in the lung (7), was used to induce chronic lung infection.

In previous studies, sulfur dioxide or smoking in combination with lipopolysaccharide and elastase were usually used to establish a COPD animal model. In the present study, these classical methods were not employed, but instead bacterial infection alone was used to establish the COPD model using the method described by Xu et al. (12) with slight modifications. This method, in which

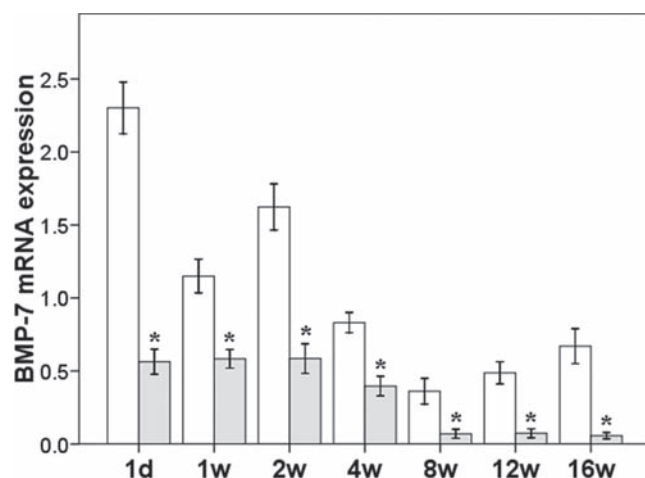


Figure 4. mRNA expression of BMP-7 in the lung. * $p < 0.05$ vs control group at same time point.

intratracheal injection of a PA solution is performed under anesthesia to induce COPD, has the advantages that the amount of bacteria given is controlled and possible infection of researchers is avoided. In a pilot study, we determined that 0.7×10^8 CFU/ml PA was a dose at which the bacteria could induce chronic lung infection but at which an acceptable survival rate of the infected rats was ensured.

Currently, pathological examination and lung function measurements are used to confirm whether or not a COPD animal model is successful. In the present study, our results showed that repeated PA infection at the dose selected caused reduction in body weight, hypoxia, carbon dioxide retention and compromised lung function, accompanied by chronic airway inflammation and lung and airway remodeling. These findings are consistent with the pathophysiology seen in COPD.

The CTGF gene was first cloned from the cDNA library of human umbilical vein endothelial cells. CTGF is a polypeptide rich in cysteine containing 349 amino acids (12, 13). It is known to be increased in COPD (14) and causes cells to shift from an epithelial to a mesenchymal phenotype (15) and in this way to promote remodeling. If CTGF is blocked, the vascular remodeling seen in cigarette smoke-induced pulmonary hypertension is reduced (16).

Our results showed that in the absence of bacterial infection, CTGF expression was not found in the lung. With the prolongation of PA infection, pulmonary CTGF expression increased gradually. This expression was found mainly in the airway ciliated epithelial cells, trachea and surrounding interstitium, findings consistent with those previously reported. When the correlation between CTGF expression and tracheal and vessel wall thickness was evaluated, the results showed CTGF expression to be closely correlated with changes in both thicknesses, a finding that suggests that CTGF is involved in PA-induced airway remodeling.

We speculated that PA infection might increase expression of TGF- β which then induced CTGF expression. CTGF would then act on downstream nuclear factors that promote the proliferation of smooth muscle cells in the airway and blood vessels and the transformation of smooth muscle cells into fibroblasts, increase the synthesis of extracellular matrix, mediate the infiltration of leukocytes, thicken the tracheal wall and vessel wall, and, in this way, finally induce airway remodeling. This hypothesis should be confirmed in future studies.

BMP-7, also known as osteopontin-1, is a member of the TGF- β superfamily. In the kidney, it reverses TGF- β 1 induced epithelial to mesenchymal cell transformation and reverses renal injury (8, 9, 17). In the liver, it inhibits hepatic fibrosis and promotes liver regeneration (18).

Pulmonary fibrosis is similar in pathology to fibrosis of other organs. Thus, we speculate that BMP-7 might exert a protective effect on the lung. There is evidence

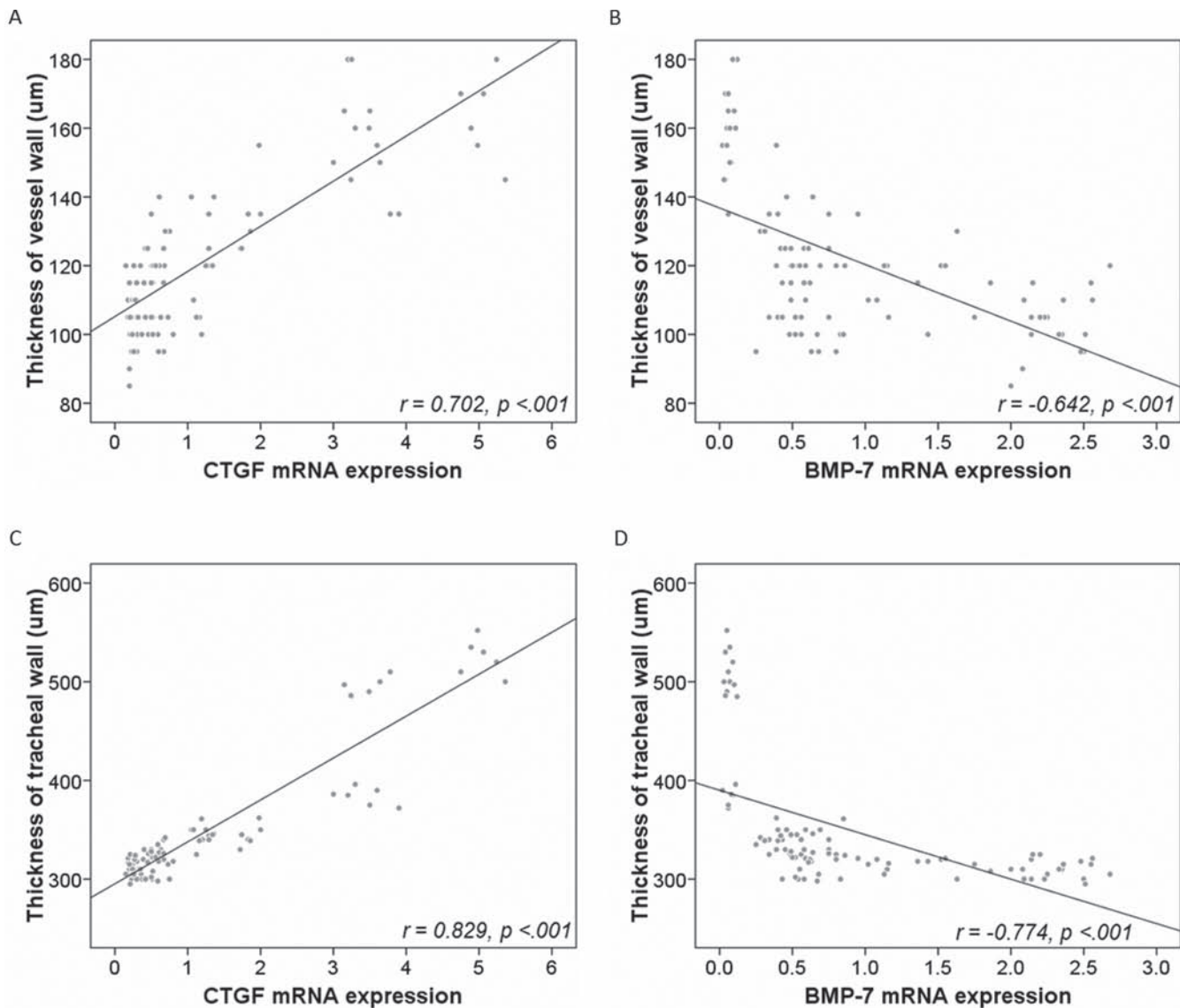


Figure 5. Correlation of lung pathology with expression of CTGF and BMP-7 (r).

showing that the balance between TGF- β and BMP signaling pathways is crucial for the repair, regeneration and fibrosis of the lung (19, 20).

In the present study, with prolongation of PA infection, the BMP-7 expression in the airway mucosal epithelial cells and a few peri-tracheal smooth muscle cells became gradually reduced. In addition, correlation analysis showed BMP-7 expression to be negatively associated with the thickness of tracheal wall and vessel and negatively related to CTGF expression.

These findings suggest that the role of BMP-7 in the lung might be similar to that in the liver and kidney, and that BMP-7 can inhibit the development of pulmonary fibrosis and protect the lung.

A curious finding (Table 4) was that lung function in PA rats was significantly worse than NS rats at 1 week after initiation of PA inhalation, but did not become significantly worse again until the 8- to 16-week period. This finding might be due to the small sample size. Another explanation might be that 1 week is the period of acute

inflammation and the edema present at that time might be greater than at 2 or 4 weeks and be responsible for the significant difference seen at 1 week.

Taken together, repeated intratracheal injection of PA may cause reduction in body weight, decrease in arterial PaO₂, increase in PaCO₂, a compromise in lung function, chronic inflammation in the bronchi at different levels, thickening of tracheal wall, stenosis of trachea, airway remodeling and emphysema. These characteristics are consistent with the pathology of COPD. In addition, BMP-7 expression is reduced and CTGF expression increased in the airway epithelial cells and interstitial cells in the alveoli and surrounding the blood vessels, changes that may contribute to the above pathological changes. Thus, in addition to quitting smoking, measures such as timely and effective control of infection, strengthening of immune function, and prevention of chronic lung inflammation and airway remodeling are crucial for the prevention of COPD.

Declaration of Interest Statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This study was supported by the Natural Science Foundation of Fujian province, China (Grant No. Z0516077).

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