3. Busse W, Elias JA, Sheppard D, Banks-Schlegel S. Airway remodeling and repair. Am J Respir Crit Care Med 1999;160:1035-42.
4. Auerbach I, Springer C, Godfrey S. Total population survey of the frequency and severity of asthma in 17 year old boys in an urban area in Israel. Thorax 1993;48: 139-41.
5. American Thoracic Society. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. Am J Respir Crit Care Med 2000;162:2341-51.
6. Serra-Batlles J, Plaza V, Morejon E, Comella A, Brugues J. Costs of asthma according to the degree of severity. Eur Respir J 1998;12:1322-6.
7. Barnes PJ. Against the Dutch hypothesis: asthma and chronic obstructive pulmonary disease are distinct diseases. Am J Respir Crit Care Med 2006;174:240-3.
8. Montuschi P, Corradi M, Ciabattoni G, Nightingale J, Kharitonov SA, Barnes PJ. Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. Am J Respir Crit Care Med 1999;160:216-20.
9. MacNee W. Oxidative stress and lung inflammation in airways disease. Eur J Pharmacol 2000;429:195-207.
10. Merker MP, Pitt BR, Choi AM, Hassoun PM, Dawson CA, Fisher AB. Lung redox homeostasis: emerging concepts. Am J Physiol Lung Cell Mol Physiol 2000;279: L413-7.
11. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A 1990;87:1620-4.
12. Sugiura H, Ichinose M, Oyake T, Mashito Y, Obuchi Y, Endoh N, et al. Role of peroxynitrite in airway microvascular hyperpermeability during late allergic phase in guinea pigs. Am J Respir Crit Care Med 1999;160:663-71.
13. Kaminsky DA, Mitchell J, Carroll N, James A, Soultanakis R, Janssen Y. Nitrotyrosine formation in the airways and lung parenchyma of patients with asthma. J Allergy Clin Immunol 1999;104:747-54.
14. Sugiura H, Liu X, Kobayashi T, Togo S, Ertl RF, Kawasaki S, et al. Reactive nitrogen species augment fibroblast-mediated collagen gel contraction, mediator production, and chemotaxis. Am J Respir Cell Mol Biol 2006;34:592-9.
15. The ENFUMOSA Study Group. The ENFUMOSA cross-sectional European multicentre study of the clinical phenotype of chronic refractory asthma. European Network for Understanding Mechanisms of Refractory Asthma. Eur Respir J 2003;22: 470-7.
16. Ichinose M, Sugiura H, Yamagata S, Koarai A, Shirato K. Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. Am J Respir Crit Care Med 2000;162:701-6.
17. Marshall HE, Merchant K, Stamler JS. Nitrosation and oxidation in the regulation of gene expression. FASEB J 2000;14:1889-900.
18. Bellocq A, Azoulay E, Marullo S, Flahault A, Fouqueray B, Philippe C, et al. Reactive oxygen and nitrogen intermediates increase transforming growth factor-beta1 release from human epithelial alveolar cells through two different mechanisms. Am J Respir Cell Mol Biol 1999;21:128-36.
19. Koarai A, Ichinose M, Sugiura H, Yamagata S, Hattori T, Shirato K. Allergic airway hyperresponsiveness and eosinophil infiltration is reduced by a selective iNOS inhibitor, 1400W, in mice. Pulm Pharmacol Ther 2000;13:267-75.
20. Sadeghi-Hashjin G, Folkerts G, Henricks PA, Verheyen AK, van der Linde HJ, van Ark I, et al. Peroxynitrite induces airway hyperresponsiveness in guinea pigs in vitro and in vivo. Am J Respir Crit Care Med 1996;153:1697-701.
21. Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, et al. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature 1998;391:393-7.
22. Ichinose M, Sugiura H, Yamagata S, Koarai A, Tomaki M, Ogawa H, et al. Xanthine oxidase inhibition reduces reactive nitrogen species production in COPD airways. Eur Respir J 2003;22:457-61.
23. Saleh D, Ernst P, Lim S, Barnes PJ, Giaid A. Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. FASEB J 1998;12: 929-37.
24. Radomski MW, Palmer RM, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc Natl Acad Sci U S A 1990;87:10043-7.
25. Ito K, Hanazawa T, Tomita K, Barnes PJ, Adcock IM. Oxidative stress reduces histone deacetylase 2 activity and enhances IL-8 gene expression: role of tyrosine nitration. Biochem Biophys Res Commun 2004;315:240-5.
26. Adcock IM, Ford P, Barnes PJ, Ito K. Epigenetics and airways disease. Respir Res 2006;7:21.
27. Ito K, Barnes PJ, Adcock IM. Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12. Mol Cell Biol 2000;20:6891-903.
28. Filep JG, Beauchamp M, Baron C, Paquette Y. Peroxynitrite mediates IL-8 gene expression and production in lipopolysaccharide-stimulated human whole blood. J Immunol 1998;161:5656-62.
29. Meagher LC, Cousin JM, Seckl JR, Haslett C. Opposing effect of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. J Immunol 1996;156:4422-8.
30. ten Brinke A, Zwinderman AH, Sterk PJ, Rabe KF, Bel EH. "Refractory" eosinophilic airway inflammation in severe asthma: effect of parenteral corticosteroids. Am J Respir Crit Care Med 2004;170:601-5.
31. Kobzik L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D, et al. Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. Am J Respir Cell Mol Biol 1993;9:371-7.
32. Belda J, Leigh R, Parameswaran K, O'Byrne PM, Sears MR, Hargreave FE. Induced sputum cell counts in healthy adults. Am J Respir Crit Care Med 2000;161:475-8.

Am J Physiol Lung Cell Mol Physiol 295: L800–L808, 2008.
First published September 12, 2008; doi:10.1152/ajplung.90264.2008.

Peroxynitrite augments fibroblast-mediated tissue remodeling via myofibroblast differentiation

Tomohiro Ichikawa, Hisatoshi Sugiura, Akira Koarai, Satoru Yanagisawa, Masae Kanda, Atsushi Hayata, Kanako Furukawa, Keiichiro Akamatsu, Tsunahiko Hirano, Masanori Nakanishi, Kazuto Matsunaga, Yoshiaki Minakata, and Masakazu Ichinose

Third Department of Internal Medicine, Wakayama Medical University, School of Medicine, Kimiidera, Wakayama, Japan

Submitted 7 April 2008; accepted in final form 5 September 2008

Ichikawa T, Sugiura H, Koarai A, Yanagisawa S, Kanda M, Hayata A, Furukawa K, Akamatsu K, Hirano T, Nakanishi M, Matsunaga K, Minakata Y, Ichinose M. Peroxynitrite augments fibroblast-mediated tissue remodeling via myofibroblast differentiation. *Am J Physiol Lung Cell Mol Physiol* 295: L800–L808, 2008. First published September 12, 2008; doi:10.1152/ajplung.90264.2008.—Irreversible airflow limitation in asthma is associated with airway remodeling in which the differentiation of fibroblasts to myofibroblasts plays a pivotal role. In asthmatic airways, excessive production of reactive nitrogen species (RNS) has been observed. The aim of this study is to evaluate whether peroxynitrite, one of the RNS, can affect the differentiation of fibroblasts to myofibroblasts. Human fetal lung fibroblasts were treated with various concentrations of authentic peroxynitrite or a peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1), and the expressions of α-smooth muscle actin (α-SMA) and desmin, markers of myofibroblast differentiation, were evaluated. The releases of transforming growth factor-β_1 (TGF-β_1) and ECM proteins including fibronectin and collagen I were assessed. To clarify the mechanism in this differentiation, the effect of anti-TGF-β antibody or NF-κB inhibitors on the α-SMA expression and ECM production was assessed. Peroxynitrite and SIN-1 significantly augmented the α-SMA expression compared with control in a concentration-dependent manner ($P < 0.01$ and $P < 0.05$, respectively). Peroxynitrite significantly increased desmin and TGF-β_1 production ($P < 0.01$). Peroxynitrite enhanced the translocation of NF-κB into the nucleus confirmed by immunocytostaining and immunoblotting. Peroxynitrite-augmented α-SMA expression was blocked by NF-κB inhibitors, MG132 and caffeic acid phenethyl ester (CAPE), and anti-TGF-β antibody. CAPE completely inhibited the peroxynitrite-augmented TGF-β_1 release. The production of fibronectin and collagen I was significantly increased by peroxynitrite ($P < 0.01$) and inhibited by anti-TGF-β antibody. These results suggest that RNS can affect the differentiation to myofibroblasts and excessive ECM production via a NF-κB-TGF-β_1-dependent pathway.

reactive nitrogen species; airway remodeling; asthma; α-smooth muscle actin; nuclear factor-κB

ASTHMA IS A DISORDER CHARACTERIZED by chronic inflammation of the airways, airflow limitation, airway hyperresponsiveness (AHR), and changes in the airway architecture sometimes termed airway remodeling (9). Airway remodeling plays a pivotal role in increasing the severity and irreversible airflow limitation in asthma and leads to the refractoriness of this disease in spite of treatment with high doses of corticosteroids (1a, 8). The parenchymal cells of the airway, including epithelial cells, airway smooth muscles, endothelial cells, and fibroblasts are responsible for the maintenance of the airway structure and are involved in the progression of airway remodeling (27). In airway remodeling, subepithelial fibrosis is one of the pathological features and is characterized by excessive deposition of ECM protein including collagens I, III, and V, fibronectin, and tenascin in the lamina reticularis of the basement membrane (12). In an in vitro model, epithelial injury reportedly differentiated lung fibroblasts into myofibroblasts, which leads to excessive ECM production (24). In a chronic allergen-challenged murine model, the differentiation of fibroblasts to myofibroblasts in the airway was observed (33). In addition, there were more myofibroblasts within the airway walls of asthmatic patients (10, 30), especially refractory asthmatic patients, compared with healthy subjects in pathological examinations suggesting that the differentiation of fibroblasts to myofibroblasts is a key process of airway remodeling in asthma (14).

Excessive production of nitric oxide (NO) during inflammatory and immune processes leads to the formation of reactive nitrogen species (RNS) including peroxynitrite and nitrogen dioxide (4). These RNS are formed from NO and superoxide anion or via the hydrogen peroxide/peroxidase-dependent nitrite oxidation pathway (35). Especially peroxynitrite is an extremely powerful oxidant and is presumed to be largely responsible for many of the adverse effects of excessive NO generation. Excessive RNS production has been reported to cause tissue injury, lipid peroxidation, and nitration of tyrosine residues (35). Furthermore, peroxynitrite caused airway inflammation (31) and AHR (28) in asthmatic animal models. In fact, excessive production of 3-nitrotyrosine, which is a footprint of RNS production, has been observed in the airways of asthmatic patients (15, 29). These data suggest that increased RNS production can be an important contributor to the pathophysiology of asthma.

Transforming growth factor-β_1 (TGF-β_1) is a key mediator in a variety of pathological processes, including fibroblast-mediated repair responses. TGF-β_1 is believed to be a major regulator of tissue remodeling (5) and is reportedly overexpressed in asthmatic airways (23, 36). It has been reported that the expression of TGF-β_1 is modulated by transcriptional factor complexes including NF-κB (7, 18, 19) and activator protein-1 (AP-1) (11, 19). These pathways potentially play a central role in the progression of airway remodeling in asthmatic airways.

Address for reprint requests and other correspondence: M. Ichinose, Third Dept. of Internal Medicine, Wakayama Medical Univ., School of Medicine, 811-1 Kimiidera, Wakayama 641-8509, Japan (e-mail: masakazu@wakayama-med.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The effect of RNS on airway remodeling process has not been fully understood. It has been reported that RNS augment fibroblast-mediated collagen gel contraction and fibroblast chemotaxis toward fibronectin (34), suggesting that RNS can affect the tissue repair process. However, the contribution of RNS to myofibroblast differentiation, which plays a pivotal role in airway remodeling observed in asthma, has not been elucidated yet. In addition, the molecular mechanism involved in the RNS-mediated tissue remodeling process also remains unclear.

The present study was therefore designed first to determine whether RNS could affect the differentiation of lung fibroblasts to myofibroblasts. Next, we assessed the effect of peroxynitrite on the release of TGF-β_1 and ECM proteins including fibronectin and collagen I in lung fibroblasts. Finally, we investigated the mechanism of differentiation of lung fibroblasts to myofibroblasts by peroxynitrite.

MATERIALS AND METHODS

Materials. Commercially available reagents were obtained as follows: anti-TGF-β_1 antibody (clone: 9016.2), TGF-β_1, biotinylated anti-TGF-β_1, neutralizing anti-TGF-β antibody, and anti-IgG were from R&D Systems (Minneapolis, MN); peroxynitrite was from

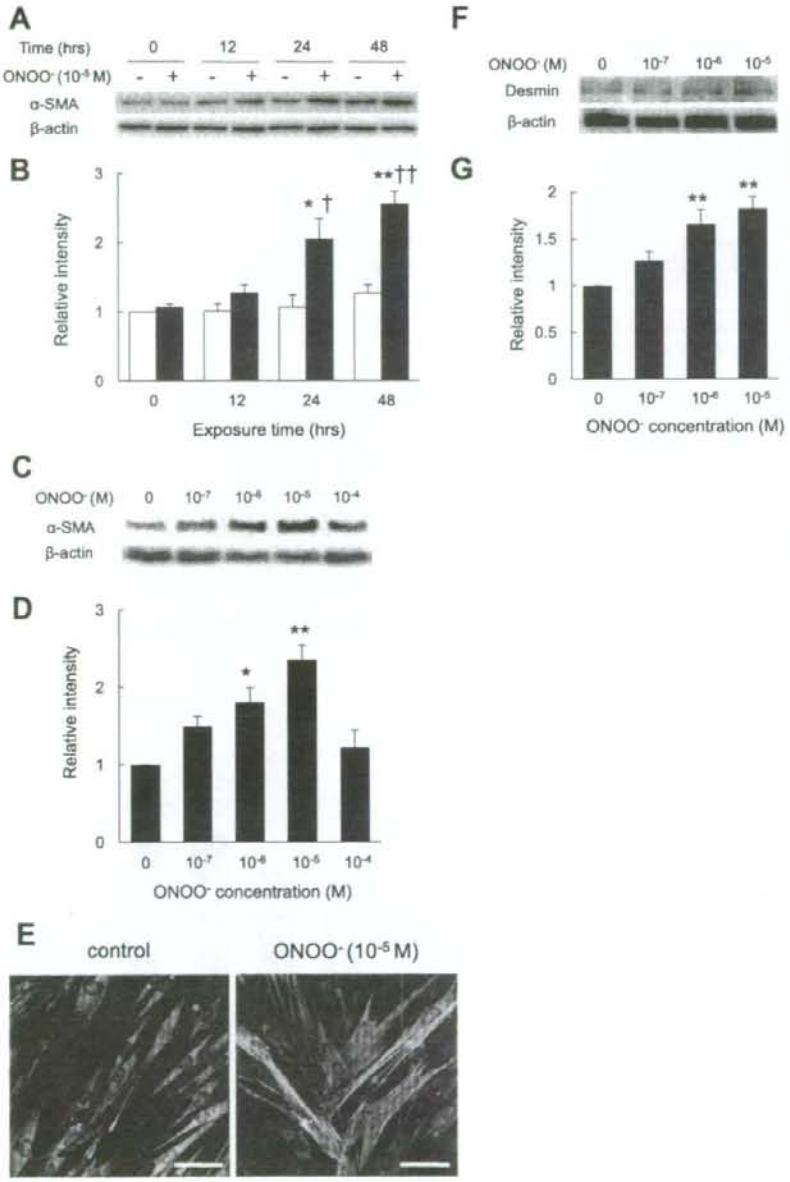


Fig. 1. Effect of authentic peroxynitrite ($ONOO^-$) on α-smooth muscle actin (α-SMA) and desmin expression in human fetal lung fibroblasts (HFL-1). Cultured cells were treated with 10^{-5} M authentic peroxynitrite (filled bars) or vehicle (open bars) and harvested at various time points. α-SMA expression was analyzed by Western blotting (*A*) and quantified by densitometry (*B*). Cultured cells were treated with various concentrations of authentic peroxynitrite for 48 h. α-SMA expression was analyzed by Western blotting (*C*) and quantified by densitometry (*D*). Cells were treated with 10^{-5} M authentic peroxynitrite (*right*) or vehicle (*left*) for 48 h. α-SMA expression was determined by immunocytostaining. Bars = 50 μm (*E*). Cultured cells were treated with various concentrations of authentic peroxynitrite for 48 h. Desmin expression was analyzed by Western blotting (*F*) and quantified by densitometry (*G*). Each band intensity of α-SMA or desmin was normalized with the corresponding β-actin band intensity. All values are expressed as means ± SE for 4 separate experiments. $*P < 0.05$, $**P < 0.01$, compared with the values of control. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, compared with the values of vehicle-treated control.

Upstate Biotechnology (Temecula, CA); 3-morpholinosydnonimine hydrochloride (SIN-1), 3,3',5,5'-tetramethylbenzidine (TMB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), monoclonal anti-human fibronectin antibody, polyclonal anti-human fibronectin antibody, anti-rabbit IgG antibody, and ebselen, a peroxynitrite scavenger, were from Sigma (St. Louis, MO); MG132, a proteasomal inhibitor, and caffeic acid phenethyl ester (CAPE), a specific NF-κB inhibitor (25), were from Calbiochem (La Jolla, CA); DMEM, FCS, and antibiotic-antimycotic were purchased from Invitrogen Life Technologies (Grand Island, NY).

Cell culture. Human fetal lung fibroblasts (HFL-1) were obtained from American Type Culture Collection (Rockville, MD). Normal human adult lung fibroblasts (NHLF) were obtained from Cambrex (Walkersville, MD). The cells were cultured on tissue culture dishes (Falcon; Becton-Dickinson, Lincoln Park, NJ) with DMEM supplemented with 10% FCS, 100 μg/ml penicillin, 250 μg/ml streptomycin, and 2.5 μg/ml fungizone. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. HFL-1 and NHLF cells were passaged every 4–5 days at a 1:4 ratio. HFL-1 cells were used between the 14th and 18th passages and NHLF cells were used between the 4th and 6th passages. To evaluate mediator production in the monolayer culture, cells were seeded in six-well tissue culture plates at a cell density of 1×10^5 per milliliter. At 90% confluence, cells were treated with various concentrations of peroxynitrite in serum-free DMEM (SF-DMEM). For the investigation of the effect of neutralizing anti-TGF-β antibody on peroxynitrite-modulated mediator release, neutralizing anti-TGF-β antibody (10 μg/ml) was also added to the media. The supernatants were harvested after 48-h treatment with peroxynitrite and stored at −80°C until later assay.

Determination of cell viability. For monitoring cell viability, peroxynitrite or vehicle-treated cells were incubated with MTT solution at a final concentration of 1 mg/ml for 4 h at 37°C. After incubation, DMSO was added into each well. The absorbance of each sample at 570 nm was determined by a spectrophotometer using a reference wavelength of 630 nm.

Western blotting. Cells were seeded in 60-mm dishes at a density of 1×10^5 per milliliter. At 90% confluence, cells were starved with SF-DMEM for 24 h. Then, cells were treated with various concentrations of authentic peroxynitrite or SIN-1 in the presence or absence of ebselen, MG132, CAPE, or anti-TGF-β neutralizing antibody for 48 h. Cells were washed with 4°C PBS and homogenized in cell lysis buffer (35 mM Tris·HCl, pH 7.4, 0.4 mM EGTA, 10 mM $MgCl_2$, 1 μM phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, and 1 μg/ml leupeptin). To obtain the nuclear fraction, a Nuclear Extraction Kit (Active Motif, Carlsbad, CA) was used according to the manufacturer's instructions. Samples were solubilized in SDS-PAGE sample buffer. Equal amounts of protein were loaded and separated by electrophoresis on 12.5% SDS-polyacrylamide gels. After electrophoresis, the separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). The following antibodies were used for detection of the target protein: mouse monoclonal anti-α-smooth muscle actin (α-SMA) antibody (1:5,000 dilution; Sigma), mouse monoclonal anti-desmin antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-β-actin antibody (1:10,000 dilution; Sigma), rabbit polyclonal anti-collagen I antibody (1:5,000 dilution; Rockland Immunochemicals, Gilbertsville, PA), mouse monoclonal anti-NF-κB p65 antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), or mouse monoclonal anti-lamin A/C antibody (1:400 dilution; Santa Cruz Biotechnology). Bound antibodies were visualized using peroxidase-conjugated appropriate second antibodies and enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, United Kingdom) with a chemiluminescence imaging system (Luminocapture AE6955; Atto, Tokyo, Japan). For detection of NF-κB p65 and desmin, SuperSignal West Femto (Pierce, Rockford, IL), a higher sensitivity substrate, was used. Each band intensity was quantified by densitometry (ImageJ; National Institutes of Health, Frederick, MD).

Measurement of TGF-β_1 and fibronectin. TGF-β_1 and fibronectin in the media of the monolayer culture were determined by ELISA (34). Quantification of TGF-β_1 was performed as follows: plates were coated with monoclonal anti-TGF-β_1 antibody at 4°C overnight. After being washed three times (5 min each), standards and samples were added and incubated at room temperature for 2 h. To measure TGF-β_1, all samples were assayed both with and without acidification and neutralization to convert the latent form of TGF-β_1 to the active form. To accomplish this, a 500-μl sample was mixed with 100 μl of 1 N HCl and, after 10 min at room temperature, neutralized with 100 μl of 1.2 N NaOH/0.5 M HEPES. Bound antigen was detected after adding biotinylated anti-TGF-β_1 antibody for 1 h at room temperature. Horseradish peroxidase (HRP)-streptavidin (1:20,000 dilution) was added for 1 h. Bound HRP was detected with TMB. The reaction was stopped with 1 M H_2SO_4, and the product was quantified at 450 nm with a microreader. Fibronectin was assayed with an ELISA that specifically detects human but not bovine fibronectin (34). Plates were coated with monoclonal anti-fibronectin antibody at 4°C overnight. After being washed three times, standards and samples were added and incubated at room temperature for 2 h. Bound antigen was detected after adding polyclonal anti-human fibronectin antibody (1:2,000 dilution) at room temperature for 1 h. HRP-conjugated anti-rabbit IgG antibody (1:10,000 dilution) was added at room temperature for 1 h. Bound HRP was detected with TMB. The reaction was stopped with 1 M H_2SO_4, and the product was quantified at 450 nm with a microreader.

Immunochemical localization of α-SMA and NF-κB p65. HFL-1 cells were seeded in an 8-well chamber slide at a density of 1×10^5 per milliliter and cultured for 24 h, and then the medium was replaced with SF-DMEM for 24 h. The cells were incubated with 10^{-5} M peroxynitrite for various time points for NF-κB assessment and for 48 h for α-SMA assessment. After washing, cells were fixed with freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature. The cells were then permeabilized with 0.1% Triton

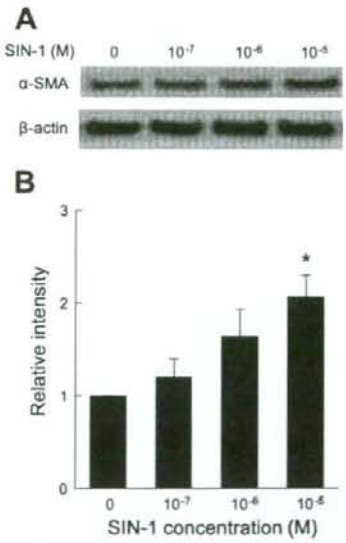


Fig. 2. Effect of the peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1) on α-SMA expression in HFL-1. Cultured cells were treated with various concentrations of SIN-1 for 48 h and harvested. α-SMA expression was analyzed by Western blotting (*A*) and quantified by densitometry (*B*). Each α-SMA band intensity was normalized with the corresponding β-actin band intensity. All values are expressed as means ± SE for 4 separate experiments. *$P < 0.05$, compared with the values of control.

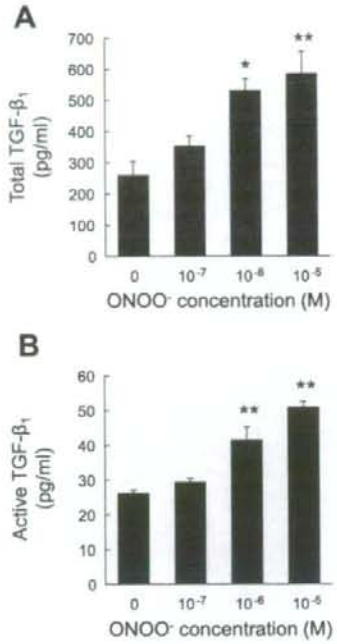


Fig. 3. Effect of peroxynitrite on transforming growth factor-β_1 (TGF-β_1) release by HFL-1. Cultured cells were treated with various concentrations of peroxynitrite. After 48 h, media were harvested and assayed for total or active TGF-β_1 by ELISA. All values are expressed as means ± SE for 4 (*A*; total TGF-β_1) or 3 (*B*; active TGF-β_1) separate experiments. $*P < 0.05$, $**P < 0.01$, compared with the values of control.

X-100 in PBS for 10 min at room temperature and blocked with 1% skim milk in PBS for 1 h at room temperature and rinsed with PBS. Then, they were incubated with mouse monoclonal anti-α-SMA antibody (1:400 dilution; Sigma) or mouse monoclonal anti-NF-κB p65 antibody (1:100 dilution; Santa Cruz Biotechnology) in 1% skim milk at 4°C overnight. After washing, the cells were incubated with FITC-conjugated anti-mouse IgG antibody (1:1,000 dilution; Sigma) in 1% skim milk for 60 min at room temperature and then viewed with an epifluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan) and photographed with a digital camera (DMX1200C; Nikon) under ×400 magnification.

Statistical analysis. Data were expressed as means ± SE. Experiments with multiple comparisons were evaluated by one-way analysis of variance followed by Scheffé test to adjust for multiple comparisons. An unpaired two-tailed Student's *t*-test was used for single comparisons. Probability values of <0.05 were considered significant.

RESULTS

To determine whether peroxynitrite induces the differentiation of fibroblasts to myofibroblasts, we assessed the expression of α-SMA, most commonly used as a molecular marker of myofibroblasts, in HFL-1 cells by Western blotting treated with various concentrations of peroxynitrite. Authentic peroxynitrite significantly augmented α-SMA expression compared with the control in a time-dependent manner (at 48 h, 2.6-fold increase; $P < 0.01$; Fig. 1, *A* and *B*). Peroxynitrite also significantly augmented α-SMA expression at 48 h in a concentration-dependent manner (at 10^{-5} M, 2.4-fold increase; $P < 0.01$; Fig. 1, *C* and *D*). Furthermore, peroxynitrite induced morphological changes in HFL-1 cells. More stress fibers, estimated for α-SMA immunostaining, were observed in the peroxynitrite-treated cells (Fig. 1*E*). We also confirmed the peroxynitrite-mediated myofibroblast differentiation by assessing the expression of desmin, which is reportedly another marker of the differentiation (37). The expression of desmin was significantly upregulated by peroxynitrite in a concentration-dependent manner (at 10^{-5} M, 1.8-fold increase; $P < 0.01$; Fig. 1, *F* and *G*). The peroxynitrite donor SIN-1 also significantly augmented α-SMA expression in a concentration-dependent manner (at 10^{-5} M, 2.1-fold increase; $P < 0.05$; Fig. 2).

To confirm whether the increased α-SMA expression was directly mediated by peroxynitrite, we assessed the effect of ebselen, a peroxynitrite scavenger, on the peroxynitrite-augmented α-SMA expression. Ebselen completely inhibited the augmentation (at 5 μM; $P < 0.01$; Supplemental Fig. 1, *A* and *B*, available in the data supplement online at the *AJP-Lung Cellular and Molecular Physiology* web site). Furthermore, we investigated the effect of peroxynitrite on cell viability and

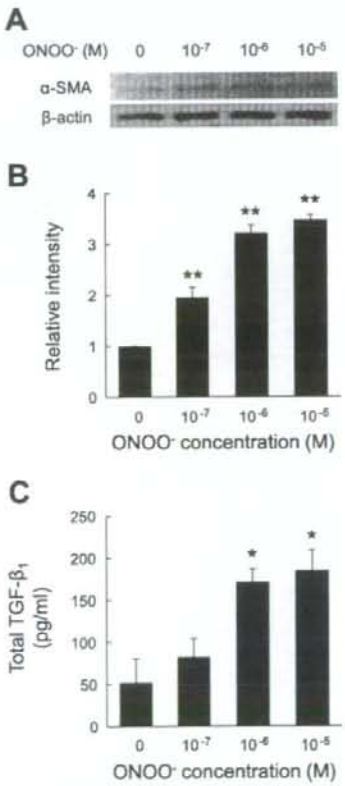


Fig. 4. Effect of peroxynitrite on α-SMA expression and TGF-β_1 release in normal human adult lung fibroblasts (NHLF). NHLF cells were treated with various concentrations of peroxynitrite for 48 h. Cells and media were harvested. α-SMA expression was analyzed by Western blotting (*A*) and quantified by densitometry (*B*). Each α-SMA band intensity was normalized with the corresponding β-actin band intensity. Media were assayed for total TGF-β_1 by ELISA (*C*). All values are expressed as means ± SE for 3 separate experiments. $*P < 0.05$, $**P < 0.01$, compared with the values of control.

proliferation because high doses of peroxynitrite have been reported to have cytotoxic effect. A concentration of 10^{-5} M or less peroxynitrite has no cytotoxic effects, and cell proliferation was not affected by 10^{-5} M peroxynitrite (Supplemental Fig. 1, *C* and *D*).

To determine whether peroxynitrite augments TGF-β_1 release by HFL-1 cells, we measured the TGF-β_1 concentration in the media. Both total and active TGF-β_1 amounts were accumulated in the media in a time-dependent manner (data not shown). Peroxynitrite significantly increased total TGF-β_1 (at 10^{-5} M, 587 ± 69 vs. 251 ± 44 pg/ml; $P < 0.01$; Fig. 3*A*) and active TGF-β_1 release(at 10^{-5} M, 51.0 ± 1.7 vs. 26.3 ± 0.9 pg/ml; $P < 0.01$; Fig. 3*B*) from HFL-1 cells in a concentration-dependent manner.

We assessed the effect of peroxynitrite on α-SMA expression and TGF-β_1 release in NHLF. Peroxynitrite significantly augmented α-SMA expression (at 10^{-5} M, 3.5-fold increase; $P < 0.01$; Fig. 4, *A* and *B*) and total TGF-β_1 release (at 10^{-5} M, 184 ± 25 vs. 52 ± 28 pg/ml; $P < 0.05$; Fig. 4*C*).

To clarify how peroxynitrite augments TGF-β_1 release by HFL-1 cells, we assessed the translocation of NF-κB into the nucleus, which is thought to regulate TGF-β_1 expression. Translocation of NF-κB p65 into the nucleus was assessed by immunocytostaining and Western blotting. After treatment

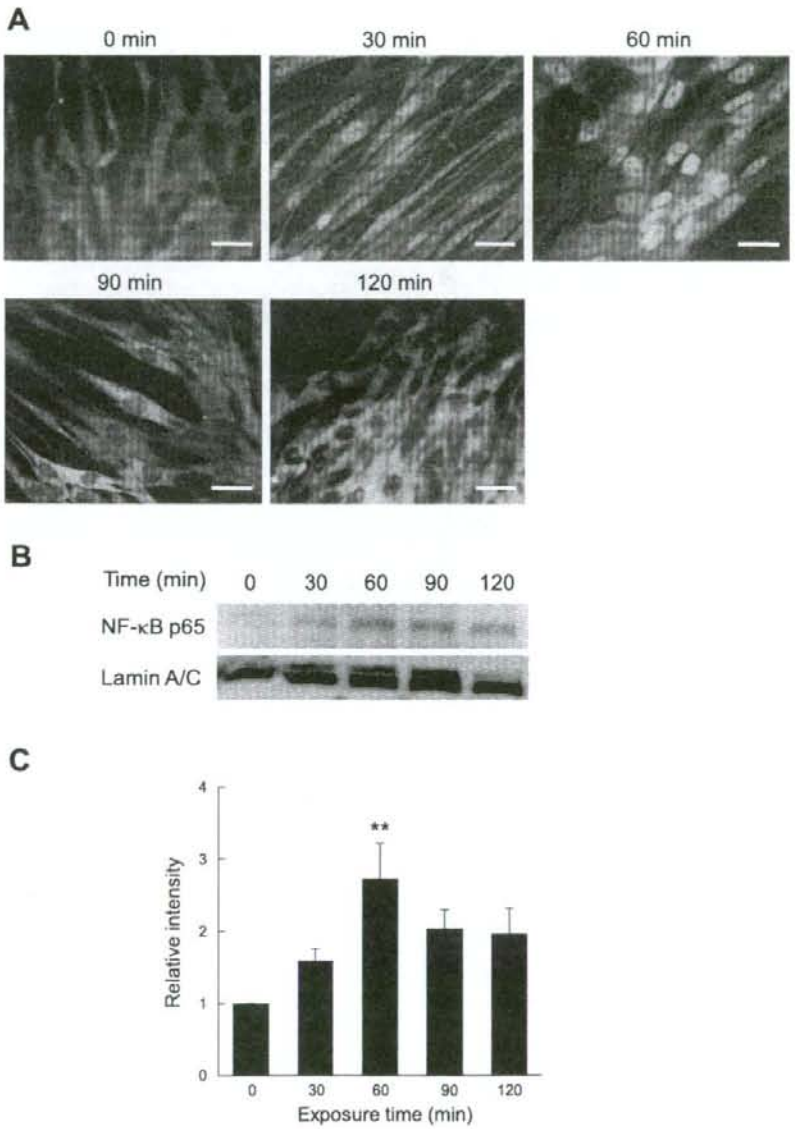


Fig. 5. Effect of peroxynitrite on translocation of NF-κB p65 into nucleus in HFL-1. HFL-1 cells were treated with 10^{-5} M peroxynitrite for 0, 30, 60, 90, and 120 min, and the intracellular localization of NF-κB p65 was determined by immunocytostaining (*A*). The amount of NF-κB p65 in the nuclear fraction was analyzed by Western blotting (*B*) and quantified by densitometry (*C*). Each NF-κB p65 band intensity was normalized with the corresponding lamin A/C band intensity. All values are expressed as means ± SE for 4 separate experiments. $^{**}P < 0.01$, compared with the values of control. Bars = 50 μm.

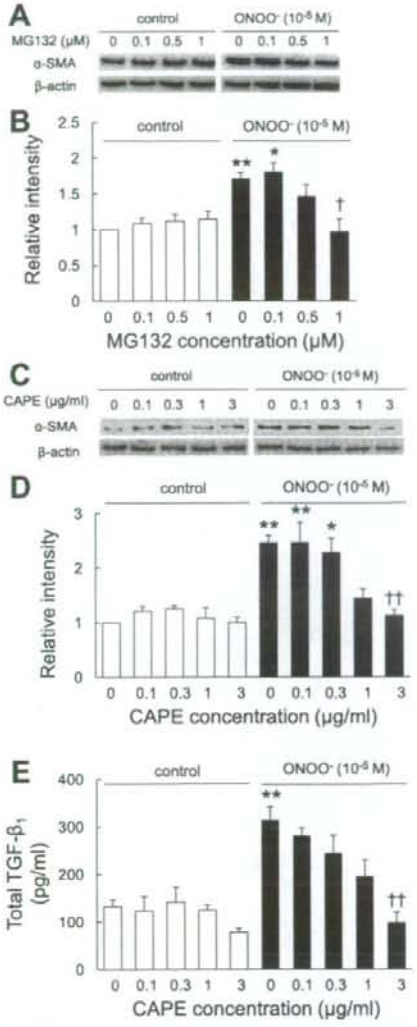


Fig. 6. Effects of proteasomal inhibitor MG132 and a specific NF-κB inhibitor, caffeic acid phenethyl ester (CAPE), on peroxynitrite-augmented α-SMA expression and TGF-β_1 release in HFL-1. Cultured cells were treated with various concentrations of MG132 in the presence (filled bars) or absence (open bars) of 10^{-5} M peroxynitrite for 48 h. α-SMA expression was analyzed by Western blotting (*A*) and quantified by densitometry (*B*). Each α-SMA band intensity was normalized with the corresponding β-actin band intensity. All values are expressed as means ± SE for 4–6 separate experiments. Cultured cells were treated with various concentrations of CAPE in the presence (filled bars) or absence (open bars) of 10^{-5} M peroxynitrite for 48 h. α-SMA expression was analyzed by Western blotting (*C*) and quantified by densitometry (*D*). Each α-SMA band intensity was normalized with the corresponding β-actin band intensity. All values are expressed as means ± SE for 4 separate experiments. Media were assayed for total TGF-β_1 by ELISA (*E*). All values are expressed as means ± SE for 4 separate experiments. $*P < 0.05$, $**P < 0.01$, compared with the values of control. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, compared with the values of vehicle-pretreated peroxynitrite-exposed group.

with 10^{-5} M peroxynitrite, the translocation of NF-κB p65 was clearly enhanced at 60 min, and the fluorescence intensity of NF-κB p65 in the nucleus was diminished at 120 min (Fig. 5*A*). The enhanced nuclear translocation of NF-κB p65 was also confirmed by Western blotting. The amount of NF-κB p65 in the nucleus was significantly increased at 60 min compared with the control (2.7-fold increase; $P < 0.01$; Fig. 5, *B* and *C*) and decreased at 120 min (Fig. 5, *B* and *C*). To investigate whether NF-κB is related to α-SMA expression in HFL-1 cells, the effects of MG132, a proteasomal inhibitor and CAPE, a specific NF-κB inhibitor, on the α-SMA expression were evaluated. The peroxynitrite-augmented α-SMA expression was completely inhibited by both inhibitors (Fig. 6, *A–D*). Furthermore, CAPE completely inhibited the peroxynitrite-augmented TGF-β_1 release (at 3 μg/ml, 315 ± 28 vs. 99 ± 21 pg/ml; $P < 0.01$; Fig. 6*E*).

To clarify the mechanistic role of TGF-β_1 in peroxynitrite-mediated α-SMA expression in HFL-1 cells, we investigated the effect of neutralizing anti-TGF-β antibody on α-SMA expression. Neutralizing anti-TGF-β antibodies significantly reduced the peroxynitrite-augmented α-SMA expression compared with the control IgG-treated group (at 10^{-5} M, 2.4-fold increase vs. 1.3-fold increase; $P < 0.01$; Fig. 7, *A* and *B*).

To determine whether peroxynitrite stimulates the production of ECM proteins, the release of fibronectin and expression of collagen I were assessed by ELISA and Western blotting, respectively. Peroxynitrite significantly augmented the fibronectin (at 10^{-5} M, 2,178 ± 176 vs. 698 ± 41 ng/ml; $P < 0.01$; Fig. 8*A*) and collagen I production (at 10^{-5} M, 2.0-fold increase; $P < 0.01$; Fig. 8, *B* and *C*) in a concentration-dependent manner. Neutralizing anti-TGF-β antibodies significantly inhibited the peroxynitrite-augmented production of fibronectin (at 10^{-5} M, 1,958 ± 65 vs. 816 ± 49 ng/ml; $P <$

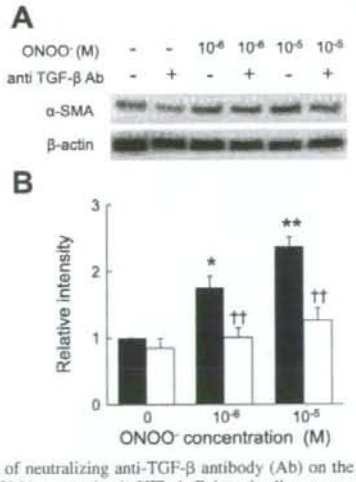


Fig. 7. Effect of neutralizing anti-TGF-β antibody (Ab) on the peroxynitrite-augmented α-SMA expression in HFL-1. Cultured cells were treated with 10^{-6} to 10^{-5} M peroxynitrite in the presence of neutralizing anti-TGF-β antibody (filled bars) or control IgG (open bars) for 48 h. α-SMA expression was analyzed by Western blotting (*A*) and quantified by densitometry (*B*). Each α-SMA band intensity was normalized with the corresponding β-actin band intensity. All values are expressed as means ± SE for 4 separate experiments. $*P < 0.05$, $**P < 0.01$, compared with the values of control. $\dagger\dagger P < 0.01$, compared with the values of control IgG-treated group.

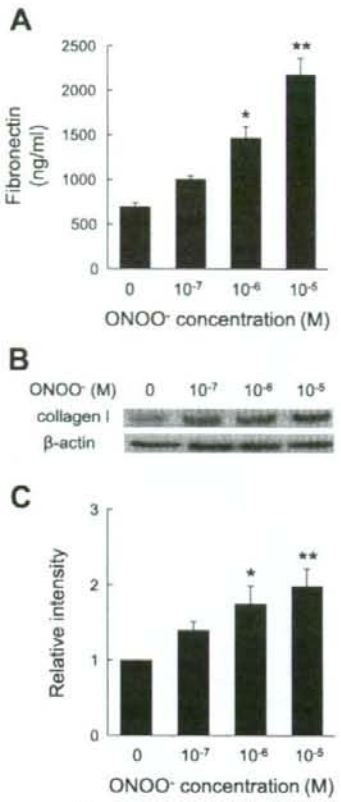


Fig. 8. Effect of peroxynitrite on fibronectin release and collagen I expression in HFL-1. Cultured cells were treated with various concentrations of peroxynitrite for 48 h. After 48 h, media and cells were harvested. Media were assayed for fibronectin by ELISA (*A*). Collagen I expression was analyzed by Western blotting (*B*) and quantified by densitometry (*C*). Each collagen I band intensity was normalized with the corresponding β-actin band intensity. All values are expressed as means ± SE for 5 separate experiments. $*P < 0.05$, $**P < 0.01$, compared with the values of control.

0.01; Fig. 9*A*) and collagen I (at 10^{-5} M, 2.1-fold increase vs. 1.3-fold increase; $P < 0.01$; Fig. 9, *B* and *C*) compared with the control IgG-treated group.

DISCUSSION

The present study demonstrated that peroxynitrite significantly augmented α-SMA expression in HFL-1 and NHLF cells. Peroxynitrite enhanced TGF-β_1 release by HFL-1 and NHLF cells and also promoted the translocation of NF-κB p65 into the nucleus. A proteasomal inhibitor, MG132, and a specific NF-κB inhibitor, CAPE, completely inhibited the peroxynitrite-augmented α-SMA expression. In addition, CAPE diminished the peroxynitrite-augmented TGF-β_1 release. Neutralization of TGF-β significantly inhibited the peroxynitrite-augmented α-SMA expression. Furthermore, fibronectin and collagen I production were significantly enhanced by peroxynitrite, which was inhibited by neutralizing anti-TGF-β antibody. These data suggest that RNS can induce the differentiation of lung fibroblasts to myofibroblasts and the excessive production of ECM protein via a NF-κB-TGF-β_1-dependent pathway.

It has been reported that the differentiation of lung fibroblasts to myofibroblasts plays a pivotal role in the development of airway remodeling in asthma (14). In fact, Schmidt et al. (30) also described that the accumulation of circulating fibrocytes was observed in the airways of asthmatic patients and that these were precursors of bronchial myofibroblasts. Brewster et al. (6) showed that more myofibroblasts were observed in the subepithelial basement membrane-collagen layer of asthmatic airways compared with healthy subjects. In the current study, we clearly showed that peroxynitrite, which is one of the RNS and is an excessively produced NO-related molecule in the airways of asthmatic patients, augmented the differentiation of lung fibroblasts to myofibroblasts.

TGF-β_1 is reported to be a key mediator of airway remodeling (17, 22), and the gene expression of TGF-β_1 is reportedly regulated by NF-κB (7, 18, 19) and AP-1 (11, 19). Broide et al.

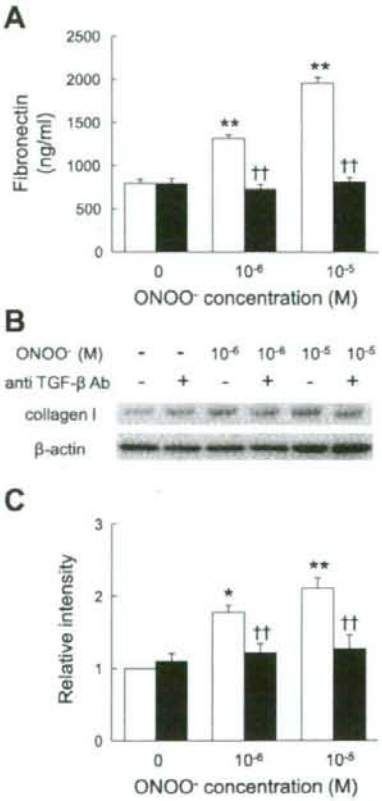


Fig. 9. Effect of neutralizing anti-TGF-β antibody on peroxynitrite-augmented fibronectin release and collagen I expression in HFL-1. Cultured cells were treated with 10^{-6} to 10^{-5} M peroxynitrite in the presence of neutralizing anti-TGF-β antibody (filled bars) or control IgG (open bars). After 48 h, media and cells were harvested. Media were assayed for fibronectin by ELISA (*A*). Collagen I expression was analyzed by Western blotting (*B*) and quantified by densitometry (*C*). Each collagen I band intensity was normalized with the corresponding β-actin band intensity. All values are expressed as means ± SE for 5 separate experiments. $*P < 0.05$, $**P < 0.01$, compared with the values of control. $††P < 0.01$, compared with the values of control IgG-treated group.

(7) reported that TGF-β_1 release was increased in ovalbumin-challenged mice, whereas the TGF-β_1 level in IκB kinase knockout mice was decreased compared with wild-type mice, and the airway remodeling was ameliorated. These findings suggest that NF-κB plays a key role in the regulation of TGF-β_1 gene expression. In a variety of cells, peroxynitrite is reported to enhance NF-κB DNA binding activity (2, 3, 16, 21). In the current study, peroxynitrite promoted the translocation of NF-κB p65 into the nucleus in HFL-1 cells and stimulated TGF-β_1 release, which was inhibited by a NF-κB inhibitor. Taken together, peroxynitrite could augment TGF-β_1 release by lung fibroblasts through NF-κB activation.

The possible mechanism for the activation of NF-κB by peroxynitrite is as follows: according to a previous study, peroxynitrite nitrates tyrosine 181 residues of IκBα, which consequently leads to the dissociation of intact IκBα from NF-κB (38). Peroxynitrite itself has also been reported to activate NF-κB without preactivation by proinflammatory mediators such as TNF-α and LPS (2, 3, 16, 21). However, the regulation of NF-κB by nitrative stress has not been fully elucidated. Therefore, further study is needed.

Excessive deposition of ECM proteins crucially contributes to airway remodeling. It has been reported that myofibroblasts can produce greater amounts of ECM proteins compared with undifferentiated fibroblasts (13). However, the precise effect of RNS on ECM production in HFL-1 cells remains unknown. TGF-β reportedly induces myofibroblast differentiation and subsequent ECM protein synthesis (20). The current study demonstrated that peroxynitrite augmented the α-SMA expression and ECM production by HFL-1 cells. This augmentation was inhibited by neutralizing anti-TGF-β antibody, and our data support the previous study (1). Taken together, RNS can enhance the myofibroblast differentiation and ECM production via a TGF-β_1-dependent pathway.

Although peroxynitrite has a very short half-life (~1.5 s), TGF-β_1 was accumulated in the media in a time-dependent manner (data not shown). The possible reason is as follows: the gene expression of TGF-β_1 was triggered after NF-κB activation, and subsequently the produced TGF-β_1 induced myofibroblast differentiation. Differentiated myofibroblasts can produce greater amounts of TGF-β_1, and this positive feedback would be related to TGF-β_1 accumulation in the media.

Because fetal lung fibroblasts may not respond to peroxynitrite the same way as postnatal lung fibroblasts, we investigated the effects of peroxynitrite on the α-SMA expression and TGF-β_1 release in NHLF. As shown in Fig. 4, peroxynitrite had similar effects on the protein production in fetal fibroblasts and adult fibroblasts. These results are compatible with those of our previous study (34).

High doses of peroxynitrite have cytotoxic effects. In this study, we used noncytotoxic doses of peroxynitrite (~10^{-5} M) as shown in Supplemental Fig. 1*C*. A concentration of 10^{-5} M peroxynitrite has no proliferative effect (Supplemental Fig. 1*D*). Furthermore, ebselen, a peroxynitrite scavenger, diminished the peroxynitrite-mediated profibrotic response. These data suggest that peroxynitrite directly altered lung fibroblasts to the profibrotic phenotype.

Peroxynitrite can enhance the production of IL-8 and TNF-α via NF-κB activation (16, 21). Recently, we showed that 3-nitrotyrosine, a footprint of RNS production, was excessively produced in the sputum cells from refractory asthmatic patients compared with well-controlled asthmatic patients (32). In addition, the amount of 3-nitrotyrosine was well-correlated with the degree of airflow limitation in the asthmatic patients. Although the possible mechanisms by which RNS are related to the pathogenesis of refractory asthma are still unclear, peroxynitrite-mediated tissue remodeling may be involved in the refractoriness of asthma.

In conclusion, the current study shows that RNS can enhance NF-κB activation and TGF-β_1 release in HFL-1 cells, and consequently myofibroblast differentiation and excessive ECM production are induced. Therefore, the NF-κB-TGF-β_1 pathway is thought to play a pivotal role in the differentiation of lung fibroblasts to myofibroblasts. Our data suggest that the modulation of this pathway may have therapeutic potential for airway remodeling.

ACKNOWLEDGMENTS

We thank Brent Bell for reading this manuscript. We thank Satoru Fukinbara for excellent assistance in statistical analysis.

REFERENCES

1. **Alcorn JF, Rinaldi LM, Jaffe EF, van Loon M, Bates JH, Janssen-Heininger YM, Irvin CG.** Transforming growth factor-beta1 suppresses airway hyperresponsiveness in allergic airway disease. *Am J Respir Crit Care Med* 176: 974–982, 2007.
1a. **American Thoracic Society.** Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. *Am J Respir Crit Care Med* 162: 2341–2351, 2000.
2. **Bar-Shai M, Hasnis E, Wiener-Megnazi Z, Reznick AZ.** The role of reactive nitrogen species and cigarette smoke in activation of transcription factor NF-kappaB and implication to inflammatory processes. *J Physiol Pharmacol* 57: 39–44, 2006.
3. **Bar-Shai M, Reznick AZ.** Peroxynitrite induces an alternative NF-kappaB activation pathway in L8 rat myoblasts. *Antioxid Redox Signal* 8: 639–652, 2006.
4. **Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA.** Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 87: 1620–1624, 1990.
5. **Blobe GC, Schiemann WP, Lodish HF.** Role of transforming growth factor beta in human disease. *N Engl J Med* 342: 1350–1358, 2000.
6. **Brewster CE, Howarth PH, Djukanovic R, Wilson J, Holgate ST, Roche WR.** Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 3: 507–511, 1990.
7. **Broide DH, Lawrence T, Doherty T, Cho JY, Miller M, McElwain K, McElwain S, Karin M.** Allergen-induced peribronchial fibrosis and mucus production mediated by IkappaB kinase beta-dependent genes in airway epithelium. *Proc Natl Acad Sci USA* 102: 17723–17728, 2005.
8. **Busse WW, Banks-Schlegel S, Wenzel SE.** Pathophysiology of severe asthma. *J Allergy Clin Immunol* 106: 1033–1042, 2000.
9. **Busse W, Elias J, Sheppard D, Banks-Schlegel S.** Airway remodeling and repair. *Am J Respir Crit Care Med* 160: 1035–1042, 1999.
10. **Carroll NG, Perry S, Karkhanis A, Harji S, Butt J, James AL, Green FH.** The airway longitudinal elastic fiber network and mucosal folding in patients with asthma. *Am J Respir Crit Care Med* 161: 244–248, 2000.
11. **Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A.** IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat Med* 12: 99–106, 2006.
12. **Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS, Barnes N, Robinson D, Kay AB.** Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J Clin Invest* 112: 1029–1036, 2003.
13. **Gabbiani G.** The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 200: 500–503, 2003.
14. **Holgate ST, Davies DE, Lackie PM, Wilson SJ, Puddicombe SM, Lordan JL.** Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin Immunol* 105: 193–204, 2000.

15. **Ichinose M, Sugiura H, Yamagata S, Koarai A, Shirato K.** Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. *Am J Respir Crit Care Med* 162: 701–706, 2000.
16. **Jozsef L, Khreiss T, El Kebir D, Filep JG.** Activation of TLR-9 induces IL-8 secretion through peroxynitrite signaling in human neutrophils. *J Immunol* 176: 1195–1202, 2006.
17. **Kenyon NJ, Ward RW, Last JA.** Airway fibrosis in a mouse model of airway inflammation. *Toxicol Appl Pharmacol* 186: 90–100, 2003.
18. **Lee KY, Ho SC, Lin HC, Lin SM, Liu CY, Huang CD, Wang CH, Chung KF, Kuo HP.** Neutrophil-derived elastase induces TGF-beta1 secretion in human airway smooth muscle via NF-kappaB pathway. *Am J Respir Cell Mol Biol* 35: 407–414, 2006.
19. **Lee KY, Ito K, Hayashi R, Jazrawi EP, Barnes PJ, Adcock IM.** NF-kappaB and activator protein 1 response elements and the role of histone modifications in IL-1beta-induced TGF-beta1 gene transcription. *J Immunol* 176: 603–615, 2006.
20. **Makinde T, Murphy RF, Agrawal DK.** The regulatory role of TGF-beta in airway remodeling in asthma. *Immunol Cell Biol* 85: 348–356, 2007.
21. **Matata BM, Galinanes M.** Peroxynitrite is an essential component of cytokines production mechanism in human monocytes through modulation of nuclear factor-kappa B DNA binding activity. *J Biol Chem* 277: 2330–2335, 2002.
22. **McMillan SJ, Xanthou G, Lloyd CM.** Manipulation of allergen-induced airway remodeling by treatment with anti-TGF-beta antibody: effect on the Smad signaling pathway. *J Immunol* 174: 5774–5780, 2005.
23. **Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, Hamid Q.** Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 17: 326–333, 1997.
24. **Morishima Y, Nomura A, Uchida Y, Noguchi Y, Sakamoto T, Ishii Y, Goto Y, Masuyama K, Zhang MJ, Hirano K, Mochizuki M, Ohtsuka M, Sekizawa K.** Triggering the induction of myofibroblast and fibrogenesis by airway epithelial shedding. *Am J Respir Cell Mol Biol* 24: 1–11, 2001.
25. **Natarajan K, Singh S, Burke TR Jr, Grunberger D, Aggarwal BB.** Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. *Proc Natl Acad Sci USA* 93: 9090–9095, 1996.
27. **Plante S, Semlali A, Joubert P, Bissonnette E, Laviolette M, Hamid Q, Chakir J.** Mast cells regulate procollagen I (alpha 1) production by bronchial fibroblasts derived from subjects with asthma through IL-4/IL-4 delta 2 ratio. *J Allergy Clin Immunol* 117: 1321–1327, 2006.
28. **Sadeghi-Hashjin G, Folkerts G, Henricks PA, Verheyen AK, van der Linde HJ, van Ark I, Coene A, Nijkamp FP.** Peroxynitrite induces airway hyperresponsiveness in guinea pigs in vitro and in vivo. *Am J Respir Crit Care Med* 153: 1697–1701, 1996.
29. **Saleh D, Ernst P, Lim S, Barnes PJ, Giaid A.** Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. *FASEB J* 12: 929–937, 1998.
30. **Schmidt M, Sun G, Stacey MA, Mori L, Mattoli S.** Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *J Immunol* 171: 380–389, 2003.
31. **Sugiura H, Ichinose M, Oyake T, Mashito Y, Ohuchi Y, Endoh N, Miura M, Yamagata S, Koarai A, Akaike T, Maeda H, Shirato K.** Role of peroxynitrite in airway microvascular hyperpermeability during late allergic phase in guinea pigs. *Am J Respir Crit Care Med* 160: 663–671, 1999.
32. **Sugiura H, Komaki Y, Koarai A, Ichinose M.** Nitrative stress in refractory asthma. *J Allergy Clin Immunol* 121: 355–360, 2008.
33. **Sugiura H, Liu X, Duan F, Kawasaki S, Togo S, Kamio K, Wang XQ, Mao L, Ahn Y, Ertl RF, Bargar TW, Berro A, Casale TB, Rennard SI.** Cultured lung fibroblasts from ovalbumin-challenged "asthmatic" mice differ functionally from normal. *Am J Respir Cell Mol Biol* 37: 424–430, 2007.
34. **Sugiura H, Liu X, Kobayashi T, Togo S, Ertl RF, Kawasaki S, Kamio K, Wang XQ, Mao L, Shen L, Hogaboam CM, Rennard SI.** Reactive nitrogen species augment fibroblast-mediated collagen gel contraction, mediator production, and chemotaxis. *Am J Respir Cell Mol Biol* 34: 592–599, 2006.
35. **van der Vliet A, Eiserich JP, Shigenaga MK, Cross CE.** Reactive nitrogen species and tyrosine nitration in the respiratory tract: epiphenomena or a pathobiologic mechanism of disease? *Am J Respir Crit Care Med* 160: 1–9, 1999.
36. **Vignola AM, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, la Rocca AM, Bellia V, Bonsignore G, Bousquet J.** Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 156: 591–599, 1997.
37. **Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z.** Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol* 166: 1321–1332, 2005.
38. **Yakovlev VA, Barani IJ, Rabender CS, Black SM, Leach JK, Graves PR, Kellogg GE, Mikkelsen RB.** Tyrosine nitration of IkappaBalpha: a novel mechanism for NF-kappaB activation. *Biochemistry* 46: 11671–11683, 2007.

気道炎症を評価する③

—呼気凝縮液を用いた検討—

The utility of exhaled breath condensate analysis to evaluate the airway inflammation

和歌山県立医科大学医学部内科学第3講座講師 **松永 和人** *Kazuto Matsunaga*
和歌山県立医科大学医学部内科学第3講座 **市川 朋宏** *Tomohiro Ichikawa*
和歌山県立医科大学医学部内科学第3講座教授 **一ノ瀬正和** *Masakazu Ichinose*

Key words
気管支喘息，サイトカイン，ケモカイン，成長因子，気流制限，気道過敏性

Summary

喘息やCOPDの病態生理には気道炎症が関連しており，簡便な気道炎症評価方法の確立は，これらの疾患の病態生理の解明に有用である。呼気凝縮液は安静換気時の呼気を冷却し，凝縮した液体を採取したものであり，非侵襲的な気道炎症のバイオマーカーとして注目されている。Protein array法を用いた呼気凝縮液分析では，喘息患者ではIL-4，IL-8，IL-17，TNF-α，RANTES，IP-10，TGF-β，MIP-1αおよびMIP-1βの発現が，健常者と比較して有意に亢進していた。呼気凝縮液におけるRANTESの発現レベルは，気流制限の生理学的指標である1秒量や呼吸抵抗と相関が認められた。またTNF-αとTGF-βの発現レベルは，気道反応性およびピークフロー変動性と相関が認められた。さらに呼気凝縮液を用いた炎症関連物質の分析において，唾液成分の混入は重要な影響を与える因子とはならないことが示された。呼気凝縮液を用いた炎症関連物質の解析は，喘息気道の状態をモニタリングする方法として有用であり，喘息の病態生理の解明や治療効果の判定に応用できる可能性がある。

はじめに

喘息や慢性閉塞性肺疾患(chronic obstructive pulmonary disease：COPD)は気道の慢性炎症性疾患であり，その炎症には種々の炎症細胞や気道構築細胞，さらに細胞から産生されるサイトカイン，ケモカイン，成長因子など多くの炎症性メディエーターが関与する[1)2)]。これらの疾患では，気道炎症に関連した気流制限や気道過敏性亢進などの生理的機能障害が生じ，呼吸困難や喘鳴などの呼吸器症状が惹起される[1)]。そのため，非侵襲的な気道炎症評価法を開発し，臨床応用することは疾患管理上，有用と考えられる。

従来，気道炎症は，気管支鏡を用いて採取する気管支生検組織・気管支肺胞洗浄液(bronchoalveolar lavage fluid：BALF)や，高張食塩水の吸入後に採取する誘発喀痰などの検体を用いて評価されてきた。これらの方法は細胞由来の情報が得られるが，侵襲的であるため，同一症例に反復して行うことや

重症例に適用することは困難であった。そのため最近では，簡便で非侵襲的な気道炎症の検査法として，呼気一酸化窒素(nitric oxide；NO)濃度測定や，呼気凝縮液(exhaled breath condensate；EBC)分析が注目されている。本稿では，気管支喘息の気道炎症評価におけるEBC分析の有用性について，われわれの検討結果を踏まえて述べていきたい。

I EBC検査

呼気成分の大部分は水蒸気であるが，呼吸により気道に生じる乱流により，エアロゾル化された気道被覆液の成分も微量ながら含有される[3]。EBCとは，安静換気時の呼気を冷却し，凝縮した液体を採取したものである。EBCは約15分程度の安静換気で1～3mL程度の検体が非侵襲的に採取可能である。採取装置には据え置き型のもの(図1)に加え，携帯性に優れたポータブルタイプ(図2)もあり，在宅や外来での採取も可能である。EBC検査の長所は簡便で非侵襲的なことであり，喘息発作時や重症度の高い症例においても実施が可能である。また反復して採取することが容易であるため，気道炎症のモニタリングへの応用も期待されている。さらに，呼気NO濃度測定と異なり，換気パターンの影響を受けることがない。一方，短所としては検体中の物質含有量が微量であること，各物質が気道のどの部分に由来するかが特定できないこと，などが挙げられる[3]。

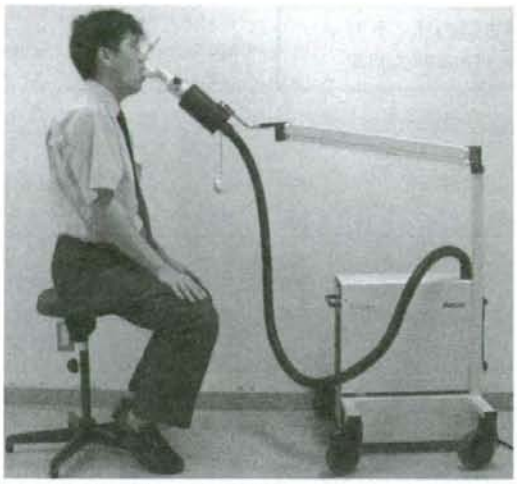


図1．据え置き型のEBC採取装置エコスクリーン®(Jaeger社，ドイツ)

喘息においては，これまで脂質メディエーターや酸化・窒素化ストレスのマーカーなどがEBCを用いて測定されている。喘息気道における好酸球遊走や，気管支平滑筋収縮に関与する脂質メディエーターであるleukotoriene(LT)$C_4/D_4/E_4$のEBC中の濃度は，喘息重症度に関連して増加することが報告されている[4][5]。また酸化ストレスのマーカーであるH_2O_2(hydrogen peroxide)や8-isoprostaneは，喘息患者より採取したEBCにおいて増加しており[6][7]，EBC中のH_2O_2発現量は喀痰中の好酸球比率や気道過敏性と相関することが示されている[5]。喘息の気道炎症における炎症細胞間のネットワークにおいて，炎症性メディエーターは重要な役割を果たしているが[1]，EBC中のサイトカインについては含有量が微量なこともあり，これまで十分に検討されていない。そこでわれわれは，喘息患者より採取したEBC中に含有されるサイトカイン，ケモカイン，成長因子をprotein array法を用いて網羅的に解析し，炎症関連物質の発現レベルと生理学的パラメーターとの関連について検討した[8]。

図2．ポータブルタイプのEBC採取装置(RTube™，Respiratory research Inc.，アメリカ)

Ⅱ 喘息気道のサイトカイン発現と肺機能の関連

未治療の安定期喘息患者16例および非喫煙健常者10例を対象に，据え置き型のEBC採取装置エコスクリーン®(Jaeger社，ドイツ)を用いて検体を採取した。喘息患者においては，スパイロメトリーによる努力性肺活量(forced vital capacity；FVC)と1秒量(forced expiratory volume in one second；FEV_1)，およびアストグラフ(Chest Co. 日本)を用いたメサコリンに対する気道過敏性の閾値を測定した。気道過敏性の指標には，呼吸抵抗が初期呼吸抵抗の2倍となるまでのメサコリン累積濃度(PD_{200}；cumulative provocative dose of methacholine causing a 100% increase in respiratory resistance)を用いた。また2週間以上のピークフロー(PEF)モニタリングを行い，PEF変動性についても評価した。EBC中の炎症関連物質の発現はCytokine Protein Array (Human Inflammatory Antibody Ⅲ；Ray Biotech Inc. アメリカ)を用いて，40種類のサイトカイン，ケモカイン，増殖因子を網羅的に測定した。各炎症関連物質は化学発光法を用いて測定し，positive controlの発現レベルに対する各炎症関連物質の相対的な発現レベルを算出した。まず健常者と比べ，喘息気道において増加している炎症関連物質の同定を行い，さらに喘息患者における炎症関連物質の発現レベルと生理学的パラメーターとの関連性について検討した。

喘息患者のEBCにおいては，interleukin(IL)-4，IL-8，IL-17，tumor necrosis factor(TNF)-α，regulated upon activation, normal T cell expressed and secreted (RANTES)，inducible protein(IP)-10，transforming growth factor(TGF)-β，macrophage inflammatory protein(MIP)-1αおよびMIP-1β発現が健常者と比較して有意に亢進していた(図3)。喘息患者のEBC中におけるRANTESの発現レベルは，気流制限の生理学的指標であるFEV_1と呼吸抵抗の程度と相関していた(図4)。またTNF-αとTGF-βの発現レベルは，気道過敏性(図5)およびPEF変動率(図6)の程度と相関していた[8]。

Ⅲ EBC分析における唾液成分混入の影響

呼吸による気流が層流となるか乱流となるかはレイノルズ数に依存しているが，乱流は上気道から下気道まですべての気道において生じるため，EBCは口腔，咽喉頭，気道，肺胞などすべての気道由来のエアロゾル粒子を含有する[9]。これまでの検討で，EBCには唾液中に検出されないタンパク質が存在すること[10]や，EBCと唾液とでは電解質のプロファイルが異なること[9]より，EBC成分の主たる由来は下気道であり，唾液成分混入は分析に重大な影響を与える因子ではないと考えられている[3]。しかしながら，EBCを用いた炎症関連物質の分析において唾液成分の混入が及ぼす影響については十分に検討されていない。

そこでわれわれは10例の喘息患者よりEBCと唾液を同時に採取し，各々の検体におけるタンパク濃度および炎症関連物質の発現を測定し，比較検討を行った。さらに気管支鏡検査を施行する患者からinformed consentを

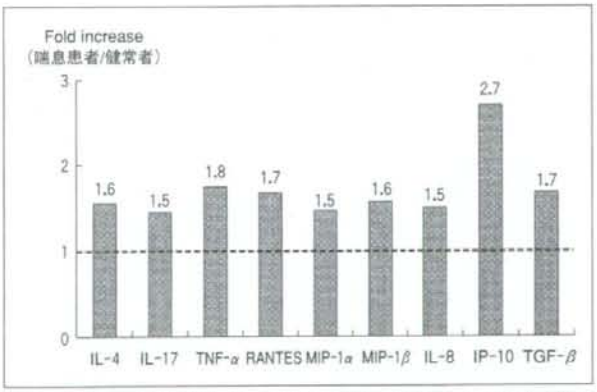


図3．喘息患者より採取したEBCにおいて発現が亢進している炎症関連物質

図で示した9種の炎症関連物質のEBC中の発現は健常者(controls)と比べ，喘息患者(asthma)で1.5～2.7倍，有意に亢進していた。

(文献8より引用・一部改変)

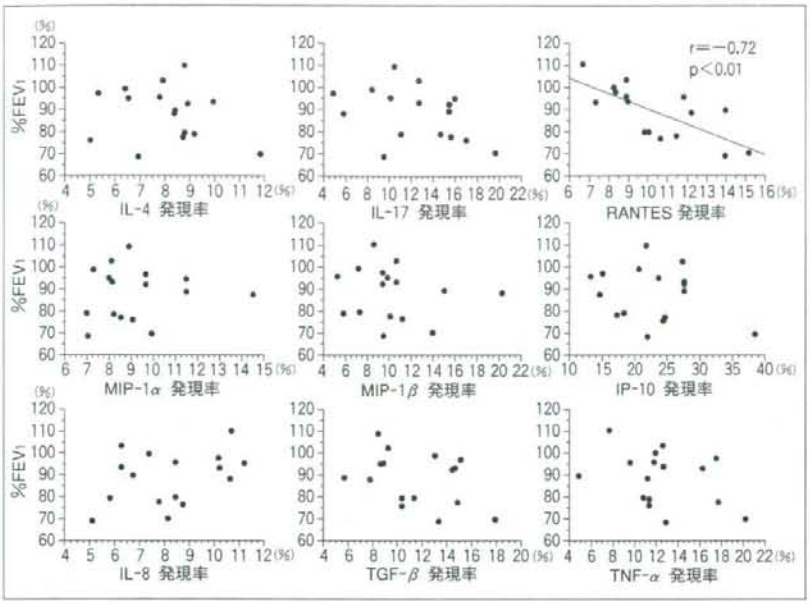


図4．喘息におけるEBC中のサイトカイン発現レベルと気流制限の関連
喘息患者のEBC中におけるRANTESの発現レベルは，気流制限の生理学的指標であるFEV1の程度と相関が認められた。
（文献8より引用，一部改変）

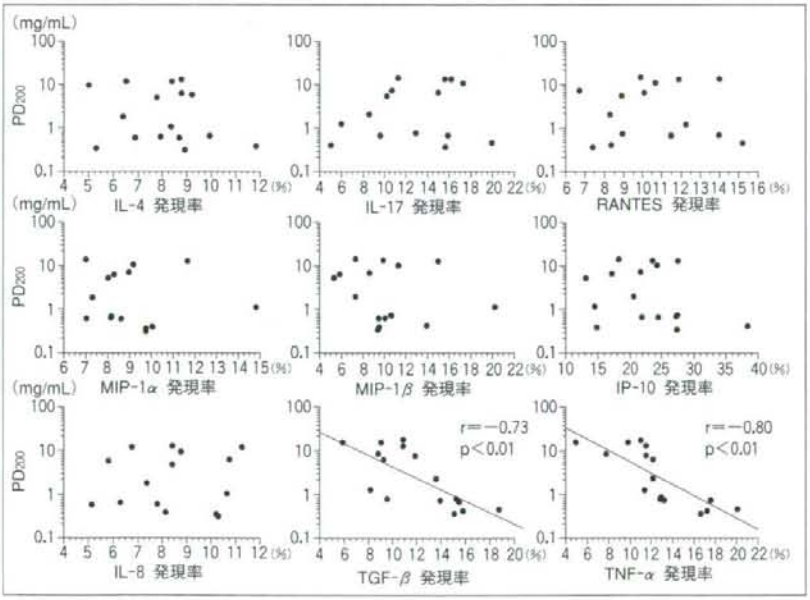


図5．喘息におけるEBC中のサイトカイン発現レベルと気道過敏性の関連
喘息患者のEBCにおけるTNF-αとTGF-βの発現レベルは，気道過敏性の程度と相関が認められた。
（文献8より引用，一部改変）

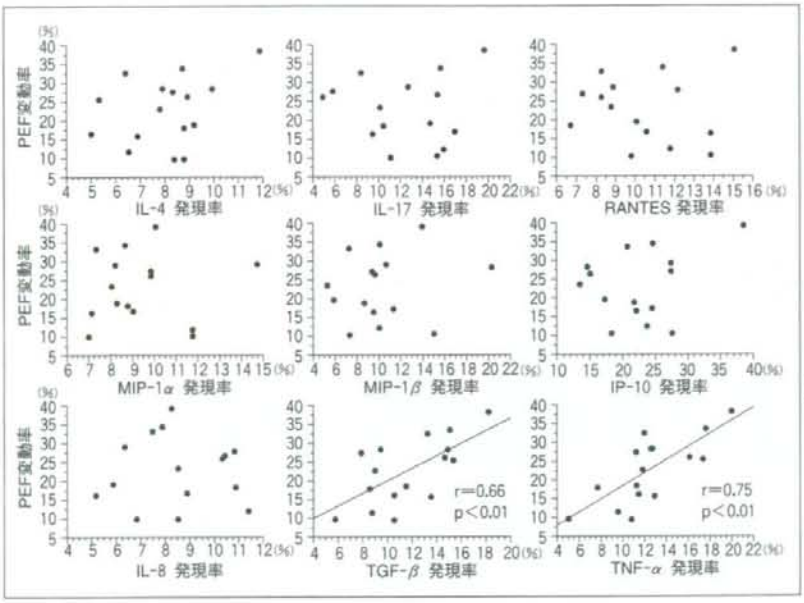


図6．喘息におけるEBC中のサイトカイン発現レベルと気道不安定性の関連
喘息患者のEBCにおけるTNF-αとTGF-βの発現レベルは，気道不安定性の指標であるPEF変動率の程度と相関が認められた。
（文献8より引用，一部改変）

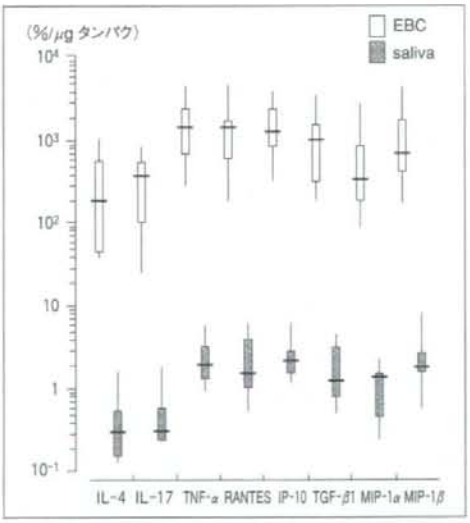


図7．タンパク補正後のEBCと唾液における炎症関連物質の発現レベル
唾液（saliva）中の炎症関連物質の発現レベルは，EBCの1/200以下の水準であった。
（文献11より引用）

得て，気管支の気道被覆液を採取し，タンパク濃度の測定を行った[11]。EBCのタンパク濃度は4.6μg/mLであり，EBC：唾液：気道被覆液のタンパク濃度の比率は，約1：500：3,000であった。また，EBCと唾液のサイトカイン発現プロファイルは全く異なっており，IL-1βやIL-8などはEBCよりも唾液で発現が亢進していたが，唾液のタンパク濃度がEBCより500倍程度高いのにもかかわらずエオタキシン，顆粒球単球コロニー刺激因子（granulocyte macrophage colony-stimulating factor；GM-CSF）など，EBCにおいて唾液よりも発現レベルが高い炎症性分子も認められた。タンパク濃度の比率から，EBCは水蒸気などにより気道被覆液が約1/3,000程度に希釈されたものと推測されたため，唾液も同

程度に希釈されるものと仮定し，EBCと唾液のサイトカイン発現レベルをタンパク濃度で補正し比較検討した。タンパク濃度で補正した場合，すべての炎症関連物質は唾液よりもEBCにおいて発現が亢進しており，健常者との比較で喘息患者のEBCにおいて有意な発現の亢進を認めた炎症関連物質についても，唾液中の分子の発現レベルはEBC中の発現レベルに比べて約1/200以下であった（**図7**）。これらの結果より，EBC分析により検出される炎症関連物質は主に下気道由来であり，唾液成分混入は分析に重要な影響を与える因子とはならないと考えられた[11]。

おわりに

今回の検討で，非侵襲的に採取可能なEBCにおける炎症関連物質の発現は網羅的に測定が可能であり，健常者と比較して喘息患者において発現が亢進している9種の炎症関連物質が同定された。またEBC中の炎症関連物質の分析において，唾液の混入は重要な影響を与える因子とはならないことが示された。喘息気道におけるこれらの炎症関連物質の発現亢進は，BALFを解析した従来の報告とも合致しており，今回の検討においてEBC中のサイトカイン発現レベルと生理学的パラメーターとの間に関連が認められたことからも，EBCを用いた炎症関連物質の解析は，喘息気道の状態をモニタリングする方法として有用と考えられた。今後，EBCを用いた気道炎症の評価が，喘息の病態生理の解明や薬物療法の効果判定に応用できる可能性がある。

文　献

1）Global initiative for asthma. global strategy for asthma management and prevention. Bethesda : National Institutes of Health. National Heart, Lung, and Blood Institute ; updated 2006.
2）Global initiative for chronic obstructive lung disease (GOLD). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease NHLBI/WHO workshop report. Bethesda : U.S. Department of Health and Human Services. Public Health Services. National Institute of Health. National Heart, Lung, and Blood Institute. updated 2006.
3）Horvath I, Hunt J, Barnes PJ, et al : Exhaled breath condensate : methodological recommendations and unresolved questions. Eur Respir J 2005 ; **26** : 523-48.
4）Montuschi P, Barnes PJ : Exhaled leukotrienes and prostaglandins in asthma. J Allergy Clin Immunol 2002 ; **109** : 615-20.
5）Antczak A, Montuschi P, Kharitonov SA, et al : Increased exhaled cysteinyl-leukotrienes and 8-isoprostane in aspirin-induced asthma. Am J Respir Crit Care Med 2002 ; **166** : 301-6.
6）Hovarth I, Donnely LE, Kiss A : Combined use of exhaled hydrogene peroxide and nitric oxide in monitoring asthma. Am J Respir Crit Care Med 1998 ; **158** : 1042-6.
7）Montuschi P, Corradi M, Ciabattoni G, et al : Increased 8-isoprostane, a marker of oxidative stress, in exhaled breath condensate of asthma patients. Am J Respir Crit Care Med 1999 ; **160** : 216-20.
8）Matsunaga K, Yanagisawa S, Ichikawa T, et al : Airway cytokine expression measured by means of protein array in exhaled breath conden sate : correlation with physiologic properties in asthmatic patients. J Allergy Clin Immunol 2006 ; **118** : 84-90.
9）Effros RM, Hoagland KW, Bosbous M : Dilution of respiratory solutes in exhaled breath condensates. Am J Respir Crit Care Med 2002 ; **65** : 663-9.
10）Griese M, Noss J, von Bredow C : Protein pattern of exhaled breath condensate and saliva. Proteomics 2002 ; **2** : 690-6.
11）Ichikawa T, Matsunaga K, Minakata Y, et al : Possible impact of salivary contamination on cytokine analysis in exhaled breath condensate. Analytical Chemistry Insights 2007 ; **2** : 85-92.

松永　和人
平成3年 和歌山県立医科大学卒業
現在，和歌山県立医科大学内科学
第3講座講師
専門分野：呼吸器内科学，アレルギー内科学

治療法に関するエビデンス

喘息治療効果のモニタリング法

柳澤 悟，一ノ瀬正和 ●和歌山県立医科大学第三内科
Yanagisawa Satoru　Ichinose Masakazu

Point

- 本稿では，一般的に用いられているモニタリング法を示すとともに，最近になり有用性が明らかとなった呼気 NO や呼気凝縮液にもふれ，各検査法の特徴と今後の展望について示す．
- ピークフローメーターは日常診療で最も普及し，また知見が集積されている検査法であり，病状の把握や患者教育において簡便な指標として必要性が高い．
- 誘発喀痰中の好酸球数は喘息の重症度と相関を示し，急性増悪の予見・予防にも役立つ．
- 呼気 NO 濃度は喘息の種々の指標と有意な相関を示し，診断や病態の把握に有用である．ポータブル型呼気 NO 測定装置により，日常診療への普及が進むものと考えられる．
- 呼気凝縮液採取法は，低侵襲で，幅広い患者に応用できることに加えて，気道環境における炎症病態を直接評価することが可能である．
- ピークフローメーターや喀痰好酸球による評価に加えて，呼気 NO や呼気凝縮液といった低侵襲の検査を組み合わせることで，より個々の患者に応じた喘息管理が可能となる．

喘息治療においては，臨床症状のみならず，その病態の中心をなす気道炎症・気道過敏性・構築変化（remodeling）の各項目を適切に評価しながら，治療の step-up/step-down を行うことが重要である．治療効果に客観性をもたせることは，経験則に陥りがちな診療に普遍性を与え，同時に患者に主体的な治療への参加を促すことを可能にする．本稿では，一般的に用いられているモニタリング法を示すとともに，最近になり有用性が明らかとなってきた呼気 NO や呼気凝縮液にも触れ，現実応用可能なモニタリング法の展望について概説する．

ピークフローメーター（PEF）

Peak Expiratory Flow（PEF）は閉塞性障害の簡便な指標となり，安価であるという利点から，臨床現場で最も広く普及しているモニター法である．1日1〜4回，努力呼気を行って呼気流速を計測し，① 自己最良値との比較，② 予測値（年齢・身長から算出）との比較，および ③ 日内変動の程度，の3点を鑑みて重症度や管理状態を判定していく．「PEF の自己最良値はどの程度か？」ということがしばしば問題になるが，一般的に，①1日2〜4回の測定を2〜3週間記録し，なおかつ ② 十分な治療による症状安定後に得られた最良値を，治療効果モニターの基準値とする[1]（Level 5）．使用する PEF 測定器によって得られる PEF の値が少しずつ変わること，また気管支拡張薬の有無によっても PEF が変化することを踏まえ，できるだけ条件をそろえた状態で過去の値と比較していくことが望ましいといえる[2]．

ピークフローを指標とした管理群と，症状に基

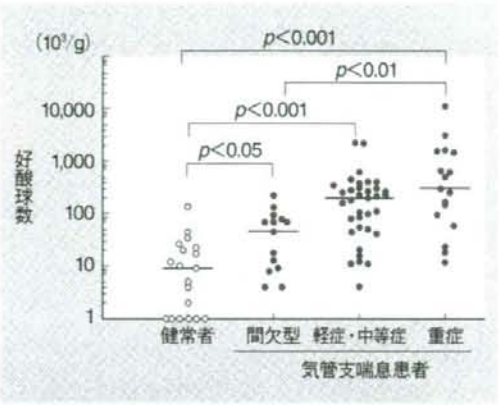
づく管理群を比較し，両者で包括的マネジメントに有意差を認めなかったとするいくつかの報告もある[3,4]．しかし，これらは比較的診断からの時間が経ち，病状への理解が得られている症例群における検討であり，気管支喘息を診断した病初期には，やはり一定期間，継続的なPEFモニタリングが必要であるといえる．

誘発喀痰

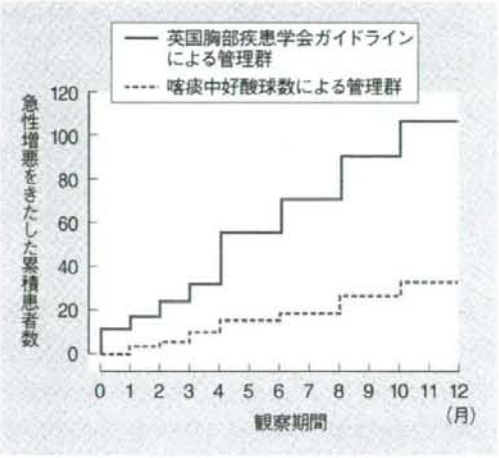
気管支喘息では気道における好酸球の増加が特徴的であり，誘発喀痰中の好酸球数と気管支喘息の重症度は相関を示す（❶）[5]（Level 3）．事実，健常非喫煙者の喀痰中好酸球は0.4～1.1％程度であるのに対し，ステロイド未治療・喘息患者の80％，ステロイド治療・喘息患者でも50％において好酸球数が正常範囲を超える．喀痰中好酸球1％を気管支喘息診断のcut-offとすると感度80％以上・特異度95％とされるが，現実的には喀痰中好酸球が3％以上で「好酸球数増多」と判断しているのが臨床の実情と思われる．

喀痰中の好酸球はステロイド治療により減少し，気流閉塞や気道過敏性の改善とも相関するため，治療効果の有用な指標となる[6]（Level 4）．一方で，気管支喘息が明らかに存在する患者であっても，喀痰中の好酸球が同定できない患者も25～55％おり，こういった患者ではステロイド反応性が悪いことがわかっている．このため治療前の喀痰中好酸球がステロイド反応性・治療反応性を反映する最も鋭敏な指標とする報告もある[7]（Level 4）．

加えて，急性発作が発症する前に喀痰中の好酸球数が増加することが報告されている[8,9]（Level 1）．このため，喀痰中の好酸球を正常範囲にするように治療的介入を行うことで，急性増悪の頻度や入院回数を減少させることができ，コストの削減にもつながる（❷）．すなわち，急性増悪の予見，およびその予防においても喀痰中の好酸球のモニタリングが有用である．



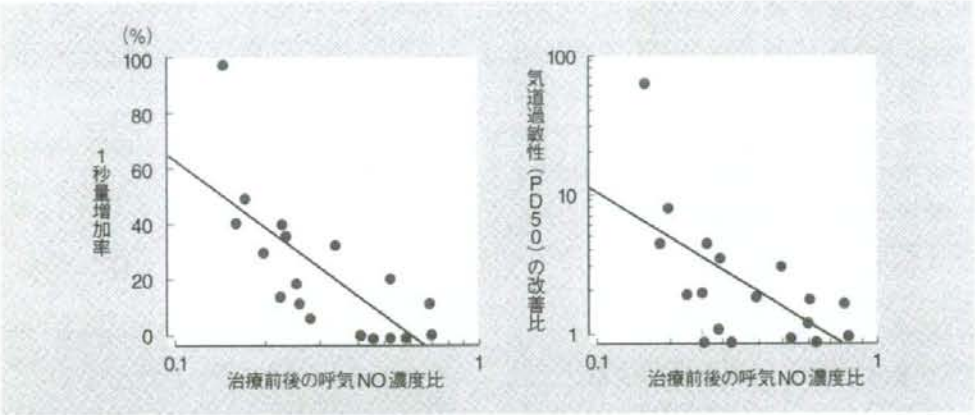
❶ 気管支喘息患者における誘発喀痰中好酸球数
健常者に比べて，気管支喘息患者では有意に誘発喀痰中の好酸球数の増加を認め，重症ではより顕著な好酸球数の増加が認められる．
(Louis R, et al. Am J Respir Crit Care Med 2000; 161: 9-16[5]より引用)



❷ 英国胸部疾患学会ガイドラインによる管理と喀痰による管理における気管支喘息急性増悪累積患者数の比較
気管支喘息を喀痰中の好酸球を指標に管理した群では，英国胸部疾患学会ガイドラインに沿った管理群に比べて，急性増悪累積患者数が有意に少ない（35人 vs 109人，$p=0.01$）
(Green RH, et al. Eur Respir J 2006; 27: 1144-51[8]より引用)

呼気ガス―呼気NOは気道炎症を反映

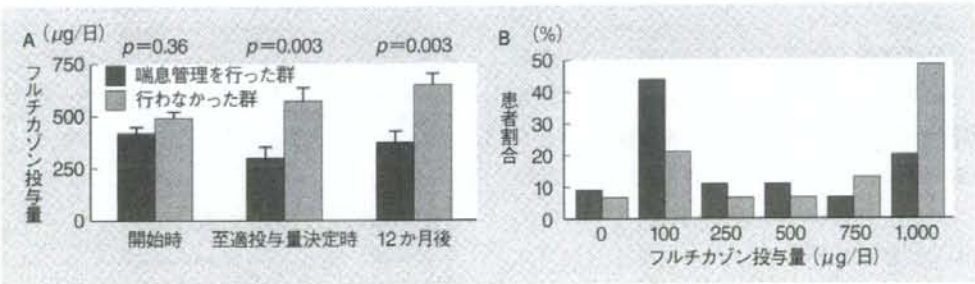
呼気ガス分析は侵襲が低く，以前より呼気中の



❸呼気NO濃度と閉塞性障害・気道過敏性の関連

気管支喘息患者ではステロイド治療により呼気NO濃度が減少するが，その減少の程度は閉塞性障害の改善（1秒量の増加）や気道過敏性の改善の程度と有意に相関する．

(Ichinose M, et al. Eur Respir J 2000; 15: 248-53[6] より引用)



❹呼気NOを目安として喘息管理を行った場合と，そうでない場合の比較

Aに示したように呼気NOを目安として治療した群のほうが最終観察日での吸入ステロイド量は少なくてすむ．Bは呼気NOモニタリング群と対照群との治療最終日の吸入ステロイド量の分布を示したもの．

(Smith AD, et al. N Engl J Med 2005; 352: 2163-73[13] より引用)

一酸化炭素（CO）や一酸化窒素（NO）をはじめ，さまざまな揮発性物質が気道炎症を反映することが報告されてきた．なかでも，NOは喘息の炎症関連物質の一つとしても重要であり，気道上皮や炎症細胞が炎症時に産生する誘導型・一酸化窒素合成酵素（iNOS）により産生される．気管支喘息患者の呼気NO濃度は健常人に比べて有意に増加しており[10,11]（Level 3），①気流制限や，②気道過敏性の程度，③喀痰中好酸球浸潤の程度，および④喘息の重症度と有意な相関を示し，疾患の診断や病態把握に有用である（❸）[6]（Level 4）．最近の報告では，呼気NOが47 ppbを超える気管支喘息患者では，より良好なステロイド反応性が期待できることがわかっており[12]（Level 3），また，ステロイド治療により呼気NOが減少することから，治療反応性の指標としても呼気NOが有用である可能性も示されている．実際，呼気NO濃度を指標として気管支喘息の治療を行った場合，GINA2002（Global Initiative for Asthma 2002）に基づく治療と比べ，より少ない吸入ステロイドで同等の患者管理が得られたとの報告もあり，ステロイド治療を行う際の客観的な指標になると考えられている（❹）[13]（Level 1）．

従来，測定機器が高価であるために測定可能施

設が限られていたが，近年，ポータブル型の呼気NO測定器（NIOX MINO®；Aerocrine社製）が低価格で開発・発売された．NIOX MINO®は，フィルター付きの測定部をくわえ深吸気・深呼気を1回ずつ行うだけで呼気NO濃度の測定が可能であるため，肺機能検査や気道過敏性検査への協力が難しい小児でも施行可能であり，臨床現場に普及しつつある．

呼気凝縮液―より侵襲の低い検査

呼気時には，気道の分岐部などで生じる乱流により気道被覆液が巻き上げられてエアロゾル化される．この呼気に混じって体外に排出されてくるエアロゾルを急速に冷却・液化して回収する方法が呼気凝縮液検査法である．呼気凝縮液の採取法は，一方向弁付きのチューブをくわえて15分程度の安静換気を行うのみで，患者の協力が不十分でも施行可能なことから，より幅広い年代・病状の患者から検体を採取することが可能である．呼気凝縮液には揮発性物質のみならず，気道被覆液に含まれる種々の液性分子が含まれ，*in vivo*に近い状況で，気道環境や気道の炎症病態を直接評価することが可能であると考えられている．本法の特徴を❺に示す．その操作の簡便性・再現性の高さから多くの炎症関連物質の測定がなされており，❻に示すような種々の物質が喘息患者において増加していることが示されている[14]（Level 3）．最近，われわれは気管支喘息患者における呼気凝縮液中のサイトカインや増殖因子を蛋白アレイによって網羅的に解析したが，喘息患者ではこれらの発現は健常者に比べて有意に増加しており，喘息の本態である気道過敏性や気道閉塞の程度とも有意な相関を示すことを見出した[15]（❼）（Level 3）．なお，吸入ステロイド治療により，これらの発現は有意に低下することから，難治性喘息との鑑別や，治療効果判定の手法としての応用が期待される．

❺ 呼気凝縮液の特徴

長所	短所
操作が簡便・非侵襲的	由来する気道部位が不明
増悪期や小児で施行可能	得られる物質が微量
反復検査や在宅で検査可能	測定に濃縮が必要な場合もある
再現性が喀痰よりも高い	
換気パターンに影響されない	

❻ 喘息患者呼気凝縮液変動

		安定期	不安定期
脂質メディエーター	LTB4, LTC4/D4/E4	↑	↑↑
酸化ストレス	H_2O_2	↑	↑↑
	8-イソプロスタン	↑	↑↑↑
窒素化ストレス	NO_2^-/NO_3^-	↑	↑↑
	ニトロタイロシン	↑	?
サイトカイン	IL-4, TNF-α, IL-6	?	?

LT: leukotriene
IL: interleukin
TNF-α: tumor necrosis factor-α
（Kharitonov SA, et al. Curr Opin Allergy Clin Immunol 2001; 1: 217-24[14]より引用）

質問紙法による評価法

上記にあげた評価法は，いずれもなんらかの検査器具を必要とするのに対して，より簡便に患者の実生活におけるADL（activities of daily living）を複合的に評価する方法として，下記に示すような質問紙法による方法が開発・検討されている（Level 3）．

- Asthma Control Test
- Asthma Control Questionnaire
- Asthma Therapy Assessment
- Asthma Control Scoring System

これらの評価法により，患者の日常生活の評価・把握が可能となるのみならず，その簡便性から治療到達目標が明らかになること，患者自身による治療への自己評価・治療への動機づけに寄与すること，非専門医での治療経過が可能になること，などが期待されている．しかし，喘息患者は自ら