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ORIGINAL ARTICLE

Reference Ranges for Exhaled Nitric Oxide Fraction in Healthy Japanese Adult Population

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ABSTRACT

Background: The measurement of the exhaled nitric oxide fraction (FENO) is proposed as a useful marker of airway inflammation. In healthy adults, there have been a few studies of the reference ranges for FENO in Caucasians. A community study in other regions may reveal any possible ethnic differences in the FENO levels.
Methods: A total of 240 healthy adults aged between 18 to 74 years were recruited from four medical centers in Japan. Current smokers and subjects having a history of atopic disease were not included. FENO was measured using an online electrochemical nitric oxide analyzer according to the current guidelines. The reference ranges for FENO were estimated using two different statistical methods recommended by International Federation of Clinical Chemistry and Laboratory Medicine.
Results: The mean FENO was 16.9 ppb (parts per billion) with a 95% prediction interval (2.5 to 97.5 percentiles) of 6.5 to 35.0 ppb in healthy Japanese adults. Normality assumptions were met for the logarithm-transformed FENO. The geometric mean FENO was 15.4 ppb with a mean ± two standard deviations of 6.5 to 36.8 ppb. Age, gender, height, and past smoking history were not associated with the FENO levels.
Conclusions: The reference ranges for FENO in healthy Japanese adults were similar to those of Caucasians. It seems reasonable that the upper limit of FENO for healthy adults should be set at approximately 36.0 ppb irrespective of ethnic differences.

KEY WORDS

airway inflammation, asthma, atopy, ethnic difference, smoking

ABBREVIATIONS

BMI, Body mass index; FENO, Exhaled nitric oxide fraction; ppb, Parts per billion.

INTRODUCTION

The measurement of the exhaled nitric oxide fraction (FENO) has been proposed as a useful marker of airway inflammation.[1] FENO levels are elevated in inflammatory lung diseases, such as asthma.[1-3] Establishing reference ranges in healthy subjects would be useful for the interpretation of FENO measurements. Although the measurement procedures have been standardized, the normal upper limits of FENO levels have not been specified.[1]

Previous studies have demonstrated that there are several determinants of FENO, such as age, gender, atopy, smoking status, and diet.[1,4-17] It has been reported that the FENO levels in Asian children are significantly higher than those in Caucasian children.[15-17] However, in healthy adults, there have been a few studies of the reference ranges for FENO in Caucasians.[4-6] A community study in other regions may reveal any possible ethnic differences in the FENO levels.

In the present study, the reference ranges for FENO in healthy Japanese adults were estimated using two different statistical methods recommended

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Table 1 Demographics of study subjects

Number	240
Gender (Female/Male)	131/109
Smoking status (Nonsmoker/Ex-smoker)	197/43
Age (years)	39.4 ± 13.6
Height (m)	1.63 ± 0.08
Body weight (kg)	59.6 ± 11.5
Body mass index (kg/m²)	22.2 ± 3.1

Values are Mean ± SD.

Table 2 Geometric mean and reference ranges for logarithm-transformed FENO

Group	Geometric mean (SD)	90% confidence interval	Mean ± 2 SD
All	15.44 (1.54)	14.75, 16.17	6.49, 36.76
Female	14.64 (1.59)	13.69, 15.66	5.79, 37.04
Male	16.46 (1.47)	15.48, 17.51	7.60, 35.68
Nonsmoker	15.34 (1.56)	14.55, 16.17	6.27, 37.57
Ex-smoker	15.91 (1.44)	14.49, 17.48	7.67, 35.02

Definition abbreviation: SD, standard deviation.

by International Federation of Clinical Chemistry and Laboratory Medicine,[18] and the results were compared with those of Caucasians. Furthermore, factors influencing the FENO levels were also investigated.

METHODS

STUDY SUBJECTS

A general population sample of people aged 18 years or older was randomly selected from the population register in Wakayama, Fukuoka, Kanagawa, and Tokyo, Japan. All participants were interviewed by physicians and a total of 240 healthy adults were recruited. The study was approved by the local ethics committee and informed consent was obtained from each subject. To avoid the influence of the pollen season, the enrollment was performed from May to July 2009. The study subjects had neither history of atopic rhinitis, atopic dermatitis, food allergy, nor history of asthma, or other lung diseases. We did not include those subjects who reported having symptoms of either asthma or rhinitis based on guidelines.[19,20] Subjects were not included if they were current smokers or ex-smokers with more than 20 pack-years, had had an airway infection or were taking any form of corticosteroids in the 4 weeks preceding the study. Baseline demographics of the subjects are presented in Table 1.

STUDY DESIGN

This was a multi-center cross-sectional study. The subjects attended the outpatient clinic on one occasion for physical examination and FENO measurements.

FENO MEASUREMENTS

FENO was measured by an online electrochemical nitric oxide analyzer (NIOX MINO; Aerocrine AB, Solna, Sweden). This nitric oxide analyzer has been approved by the U.S. Food and Drug Administration for clinical use. Measurements of FENO were performed asking the subjects to empty their lungs and then to inhale to total lung capacity through the mouthpiece and finally exhale into device at a constant flow rate of 50 mL/s; the software within the device automatically checks that the breathing manoeuvre is performed according to American Thoracic Society/European Respiratory Society guidelines.[1] The calibration of the analyzer is automatically performed by the software. The sensor on the device was changed periodically according to the manufacture's guidance. Repeated exhalations were performed to obtain two acceptable measurements that agreed within 10% deviation, and the average of these two values was registered. All subjects were fasted for one hour before the FENO measurements.

STATISTICAL ANALYSIS

The reference ranges for FENO were estimated using two different statistical methods recommended by International Federation of Clinical Chemistry and Laboratory Medicine: 1) 95% prediction interval (2.5 to 97.5 percentiles) of FENO values; 2) Since normality assumptions were met for the logarithm of FENO, the logarithm-transformed value was used to calculate the mean ± two standard deviations (SD) and then back-transformed values were estimated.[18]

The factors influencing the FENO values were examined in a regression tree-based model with the logarithm-transformed FENO as the response variable in relation to the different explanatory variables, age, gender, height, and past smoking history. Comparisons of mean FENO values between the groups (gender and smoking status) were performed by unpaired t tests. Pearson's correlation coefficients were calculated to determine the correlation between the FENO values and continuous data (age and height). All data were expressed as mean ± SD and significance was defined as a p value of less than 0.05.

RESULTS

The reproducibility of FENO measurements was expressed as the limit of agreement (intraclass correlation coefficient = 0.97). The mean FENO was 16.9 ppb (parts per billion) with a 95% prediction interval of 6.5 to 35.0 ppb. Normality assumptions were met for the logarithm-transformed FENO (Kolmogorov-Smirnov test, $p = 0.43$) and back-transformed values were used in the subsequent analysis. The geometric mean FENO for all subjects was 15.4 ppb with a mean ± 2 SD of 6.5 to 36.8 ppb in healthy Japanese adults (Table 2).

The mean FENO was 1.13 times higher in males,

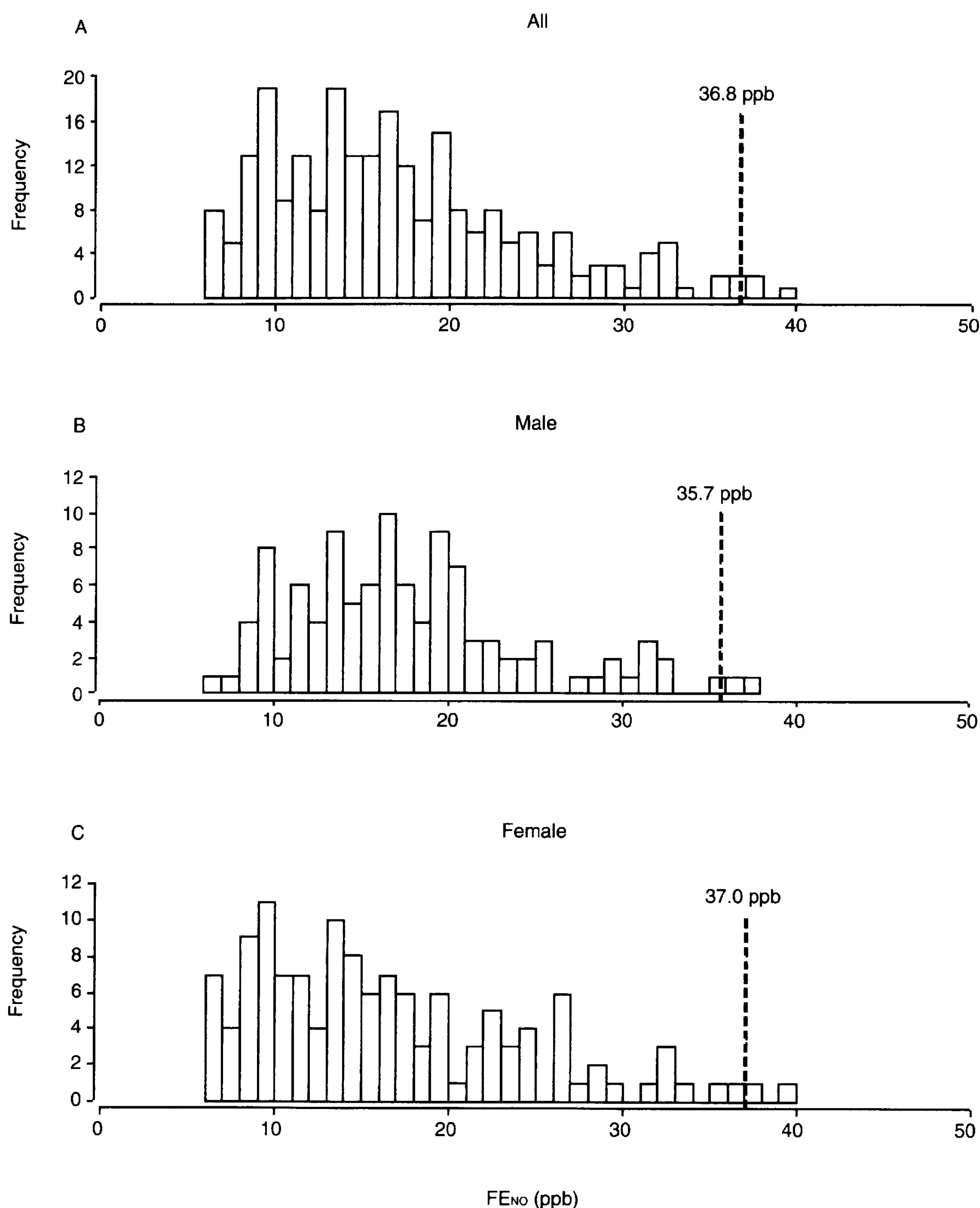

Fig. 1 The distribution of the FENO levels in all subjects (**A**), males (**B**), and females (**C**). The dotted lines correspond to the upper limits of the reference range for the logarithm-transformed FENO (mean + 2 standard deviation) in each group.

16.5 ppb, compared to the 14.6 ppb in females ($p < 0.05$), and that was 1.04 times higher in ex-smokers, 15.9 ppb, compared to the 15.3 ppb in nonsmokers ($p = 0.62$). However, the mean ± 2 SDs of FENO values were similar in each group although it was not statistically analyzed (Table 2). The distribution of the FENO levels in all subjects, males, and females, and the upper limits of FENO in each group are shown in Figure 1. In a linear regression analysis, no correlations were found between FENO and the values of age (r = 0.12, $p = 0.07$) or height (r = -0.02, $p = 0.82$). The regression tree analysis showed that age, gender, height, and past smoking history were not significant factors influencing the FENO levels.

DISCUSSION

The present study is one of the largest that investigated the FENO levels in a community sample of adults. We have shown that the reference range for FENO was approximately 6.5 to 36.0 ppb in healthy Japanese adults.

For healthy Caucasian adults, a few reports have shown the reference ranges for FENO measurements according to the current guidelines.[4-6,8] Travers *et al.* recruited 193 normal subjects categorized as follows: no physician diagnosis of lung disease, no symptoms of lung disease, no inhaled medication, and no allergic rhinitis.[5] The criteria were similar to ours although 19 current smokers were included in the study population. The geometric mean FENO was 17.9 ppb with a 90% confidence interval of 7.8 to 41.1 ppb.[5] In another study comprising 30 healthy non-atopic subjects, the mean FENO was 16.3 ppb with SD of 8.4 ppb in adults.[7] On the basis of this report, a recent review suggested 33.1 ppb (mean + 2 SD) as the upper limit of FENO for adults.[8] Olin *et al.* recruited healthy adults from the general population, and 1,131 never smokers, including 845 non-atopic and 286 atopic subjects, were selected for the study.[6] Subjects with physician-diagnosed asthma or asthma symptoms, and those using inhaled steroids were excluded. Using a reference equation based on multiple regression modeling, they proposed upper limits of FENO ranging from 24.0 ppb to 54.0 ppb depending on age and height.[6] This upper limit of FENO seems definitely higher than those reported in previous studies and ours. In their study, the subjects having symptoms of rhinitis were not excluded, and this population comprised about 45% of the study subjects. The high prevalence of subjects with nasal symptoms may explain the increased FENO levels. In the present study, we estimated the reference ranges for FENO using two different statistical methods recommended by the guidelines,[18] and this study demonstrated approximately 36.0 ppb as a reasonable upper limit of FENO for healthy Japanese adults, a value that is within previously suggested limits for Caucasians.

It has been shown that the mean FENO levels in healthy Asian children are significantly higher than those in healthy Caucasian children.[15-17] Several factors, such as dietary differences, environmental differences, and genetic variation have been proposed to explain the ethnic differences.[15-17] However, these studies included less than 70 subjects who were ethnic minorities, and the reference ranges for FENO in each ethnic group were not estimated. In addition, FENO was measured according to the current guidelines in only one of these studies.[17] Further study with large samples using standardized methods will be necessary to clarify ethnic differences in the FENO levels of children.

The normal range for FENO is influenced by patient factors. Previous studies have shown that there are several determinants of FENO levels, such as age, gender, height, atopy, smoking, and diet.[1,4-17] The finding of a higher FENO in subjects with atopy has been reported previously,[5,9-11] while chronically reduced levels of FENO have been demonstrated in current smokers.[5,9,12] Therefore, current smokers and subjects having a history of atopic disease were not included in this study. Furthermore, the FENO level has been found to be elevated after the intake of a nitrate-rich meal.[13,14] Thus, the subjects were fasted for one hour before the FENO measurements. In the present study, the mean FENO level for males was significantly higher than that for females, which is consistent with previous reports.[4,5,7,15] However, the upper limits of FENO were similar in each gender group as shown in Figure 1. In addition, age, gender, height, and past smoking history were not significant predictors for FENO. Although the association between FENO and age, gender, height is still controversial in adults,[1,4-9] the present results suggested that these demographics are not critical factors in establishing the normal range for FENO.

In the present study, subjects were recruited based on interview. Although the subjects with a history of atopic disease were carefully excluded according to the guidelines,[19,20] we made no attempt to validate information by skin prick testing. However, a detailed interview is a basic approach to investigate individual demographics, and it will not always be practical for clinicians to perform skin prick testing before FENO measurements. In clinical practice, the proposed reference data would be useful for interpretation of the FENO measurements as a reasonably approximate value. Additionally, the reference ranges for FENO in Caucasians adults were analyzed by different measurement system, chemiluminescene analyzer.[5-7] However, it has been shown that FENO values measured by the electrochemical analyzer are reproducible, reliable, and in agreement with the chemiluminescene analyzer.[21-24]

In summary, the reference ranges for FENO in healthy Japanese adults were similar to those of Caucasians. It seems reasonable that the upper limits of FENO for healthy adults should be set at approximately 36.0 ppb irrespective of ethnic differences.

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Differentiation of embryonic stem cells into fibroblast-like cells in three-dimensional type I collagen gel cultures

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Abstract Fibroblasts are heterogeneous mesenchymal cells that play important roles in the production and maintenance of extracellular matrix. Although their heterogeneity is recognized, progenitor progeny relationships among fibroblasts and the factors that control fibroblast differentiation are poorly defined. The current study was designed to develop a reliable method that would permit in vitro differentiation of fibroblast-like cells from human and murine embryonic stem cells (ESCs). Undifferentiated ESCs were differentiated into embryoid bodies (EBs) with differentiation media. EBs were then cast into type I collagen gels and cultured for 21 d with basal media. The spindle-shaped cells that subsequently grew from the EBs were released from the gels and subsequently cultured as monolayers in basal media supplemented with serum. Differentiated cells showed a characteristic spindle-shaped morphology and had ultrastructural features consistent with fibroblasts. Immunocytochemistry showed positive staining for vimentin and alpha-smooth muscle actin but was negative for stage-specific embryonic antigens and cytokeratins. Assays of fibroblast function, including proliferation, chemotaxis, and contraction of collagen gels demonstrated that the differentiated cells, derived from both human and murine ESCs, responded to transforming growth factor-β1 and prostaglandin E_2 as would be expected of fibroblasts, functions not expected of endothelial or epithelial cells. The current study demonstrates that cells with the morphologic and functional features of fibroblasts can be reliably derived from human and murine ESCs. This methodology provides a means to investigate and define the mechanisms that regulate fibroblast differentiation.

Keywords Embryonic stem cell differentiation · Fibroblasts · Three-dimensional collagen gel cultures

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Introduction

Fibroblasts represent a heterogeneous population of mesenchymal cells that play important roles in the production and maintenance of extracellular matrix (Raghow 1994; Ohnishi et al. 1998; Phan 2008). In addition, fibroblasts have important regulatory roles modulating the function of many other cell types (Knight 2001; Nanki et al. 2001; Rennard 2001; Hay 2005). While fibroblast heterogeneity is clearly recognized, progenitor progeny relationships among fibroblasts and the factors that control fibroblast differen-

tiation are poorly defined. This is, to a significant degree, complicated by a lack of surface markers to define differentiated fibroblast phenotypes. Fibroblasts, therefore, are, at the present time, best characterized by morphology, ultrastructure, and function supported by molecular marker expression (Powell et al. 1999a; Powell et al. 1999b; Fireman et al. 2001; Eyden 2004; Eyden 2005).

The ultimate progenitor cell is the embryonic stem cell (ESC), which in in vitro culture systems, ECSs can differentiate in to cells of many lineages. Interestingly, the culture of ESCs is most commonly accomplished by co-culture with fibroblast feeder layers, which provide undefined but necessary cofactors. Because xenogenic fibroblasts present a number of theoretical and technical problems, several investigators have described methods to prepare autogenic and syngenic fibroblasts from ESCs to use as feeder layers for ESCs (Xu et al. 2004; Stojkovic et al. 2005; Yoo et al. 2005; Choo et al. 2008; Chen et al. 2009). However, these studies have not demonstrated that the fibroblast-like cells function like fibroblasts, which would be a necessary step toward an experimental system to delineate the differentiation pathways leading to heterogeneous populations of fibroblasts. The current study, therefore, was designed to develop a reliable methodology that would permit in vitro differentiation of fibroblasts from human and murine ESCs. The development of this methodology provides a means for delineating the mechanisms that control fibroblast differentiation and that lead to functionally heterogeneous mature cell populations.

Materials and Methods

Materials. Native type I collagen (rat tail tendon collagen [RTTC]) was extracted from rat-tail tendons by a previously published method (Elsdale and Bard 1972). Commercially available reagents were obtained as follows: transforming growth factor (TGF)-β1 was from R&D Systems (Minneapolis, MN); prostaglandin E_2 (PGE_2), monoclonal anti-α-smooth muscle actin (SMA), anti-pan cytokeratin monoclonal, anti-vimentin monoclonal antibodies, anti-mouse IgG FITC (fluorescein isothiocyanate stain-green immunofluorescence) conjugate, propidium iodide and 2-mercaptoethanol were from Sigma (St. Louis, MO); ESGRO® (leukemia inhibitory factor; LIF), anti-stage specific embryonic antigen (SSEA)-1 and 4 monoclonal antibodies were from Chemicon International (Temecula, CA); Dulbecco's modified eagle's medium (DMEM), fetal calf serum (FCS), DMEM/F12 [1:1 mixture], KnockOut™ serum replacement, KnockOut™ DMEM, non-essential amino acids, L-glutamine, basic fibroblast growth factor (bFGF), collagenase type IV, and 0.05% Trypsin-EDTA were from Invitrogen (Carlsbad, CA).

Cell culture and differentiation. Human ESCs Culture. The National Institutes of Health-approved human embryonic stem cell line H9.2 (passages 45–65; WiCell Research Institute, Madison, WI) was used in this study with the approval of the Institutional Review Board and Embryonic Stem Cell Research Oversight committee of the University of Nebraska Medical Center. Undifferentiated human ESCs were cultured on irradiated mouse embryonic fibroblasts (MEF) in six-well plates with human ESC culture medium containing 80% DMEM/F12, 20% KnockOut™ serum replacement, 1% non-essential amino acids, 1 mmol/lL-glutamine, 0.1 mmol/l 2-mercaptoethanol, and 4 ng/ml bFGF. Colonies were mechanically dissected with finely pulled glass micropipettes (1.0 mm OD; Clark Electromedical Instruments, Reading, UK) every 7 d and transferred to a freshly prepared MEF layer.

Culture of embryoid bodies in type I collagen gels. To prepare embryoid bodies (EBs), human ESCs from four- to five-wells of a six-well plate were treated with 1 mg/ml collagenase and cells were collected by centrifugation at 200×*g* for 2 min. The pellet was resuspended in differentiation medium containing 90% DMEM/F12, 10% Knockout serum replacement, 1% non-essential amino acids, and 1 mmol/lL-glutamine without 2-mercaptoethanol and bFGF (Schuldiner et al. 2000). Cells were then placed into a Petri dish (Sarstedt, Nümbrecht, Germany) and cultured for 4–5 d. Floating EBs from the Petri dish were collected into a 50 ml polypropylene conical tube (Falcon; Becton-Dickinson Labware, Franklin Lakes, NJ) and precipitated without centrifugation.

Collagen gels were prepared as described previously (Mio et al. 1996). Briefly, RTTC, distilled water and 4× concentrated DMEM were combined so that the final mixture resulted in 0.75 mg/ml collagen, with a physiologic ionic strength of 1× DMEM at pH 7.4. EBs from a Petri dish were then suspended in the neutralized collagen solution. Aliquots (1.0 ml/well) of the mixture of EBs in collagen were then cast into each well of a 12-well tissue culture plate (Falcon) and allowed to polymerize. After polymerization was completed, normally within 20 min at room temperature, basal medium (1:1 mixture of differentiation medium and DMEM/F12) was added on the top of the gels in a 12-well plate (1.0 ml/well). The basal medium was changed every 2–3 d and EBs were cultured for 21 d in type I collagen gels.

Murine ESCs and EBs culture. The murine embryonic stem cell line (CRL-11632) was obtained from the American Type Culture Collection (Rockville, MD). KnockOut™ DMEM with 20% KnockOut serum replacement, 1% non-essential amino acid, 1 mmol/lL-glutamine, 0.1 mmol/l 2-mercaptoethanol and 10^3 units/ml LIF was used for

culture medium, and KnockOut™ DMEM with 2% FCS for basal medium. Murine ESCs and EBs were cultured using the same methods as human cells.

Differentiated fibroblast culture. The gels in a 12-well culture plate were dissolved with 1 mg/ml collagenase at 37°C in a 5% CO_2 atmosphere for 1 h. The resulting cells were resuspended with DMEM containing 10% FCS (10% FCS-DMEM) and centrifuged at 200×*g* for 5 min. The cells, containing EBs, were cultured in a 100 mm tissue culture dish (Falcon) with DMEM containing 10% FCS, 45 units/ml penicillin, 45 μg/ml streptomycin, and 1 μg/ml amphotericin B. When near confluent, the cells were trypsinized gently to prevent EBs from detaching and the cells were passaged in 10% FCS-DMEM (Fig. 9). Cultures were routinely inspected using phase contrast microscopy and cells were assessed after 4–5 passages.

Collagen gel contraction assay. Collagen gels were prepared as described previously (Mio et al. 1996). Differentiated fibroblasts were trypsinized and mixed with the neutralized collagen solution so that the final cell density in the collagen solution was 3×10^5 cells/ml. Aliquots (0.5 ml/well) of the mixture of cells in collagen were cast into each well of 24-well tissue culture plates (Falcon) and the mixture was allowed to polymerize. After polymerization was completed, the gels were gently released from the 24-well tissue culture plates and transferred into 60-mm tissue culture dishes (three gels in each dish) which contained 5 ml of freshly prepared serum-free DMEM (SF-DMEM) with or without 10^{-10} mol/l TGF-β1 or 10^{-7} mol/l PGE_2. The gels were then incubated at 37°C in a 5% CO_2 atmosphere for 5 d. Gel contraction was quantified using an Optomax V image analyzer (Optomax, Burlington, MA) daily. Data were expressed as percentage of the initial gel size.

Chemotaxis assay. Cell migration was assessed using the Boyden blindwell chamber (Neuroprobe Inc., Gaithersburg, MD) as previously described (Boyden 1962). Briefly, 26 μl of SF-DMEM containing human fibronectin (20 μg/ml) was placed into the bottom wells. Eight-micrometer pore polycarbonate membranes (Neuroprobe Inc.), which were precoated with 5 μg/ml gelatin in 0.1% acetic acid, were employed. Cells were trypsinized and suspended with 10% FCS-DMEM to stop the trypsin. Cells were then pelletted and re-suspended in SF-DMEM at a density of 1×10^6/ml. Fifty microliters of the cell suspension supplemented with or without TGF-β1 (10^{-10} mol/l) or PGE_2 (10^{-7} mol/l) were then added into each top well. Cells were allowed to migrate at 37°C in a 5% CO_2 atmosphere for 12 h. Cells that had not migrated were scraped off the upper surface of the membrane, and the membranes were air-dried. Cells were then stained with PROTOCOL (Fisher Scientific,

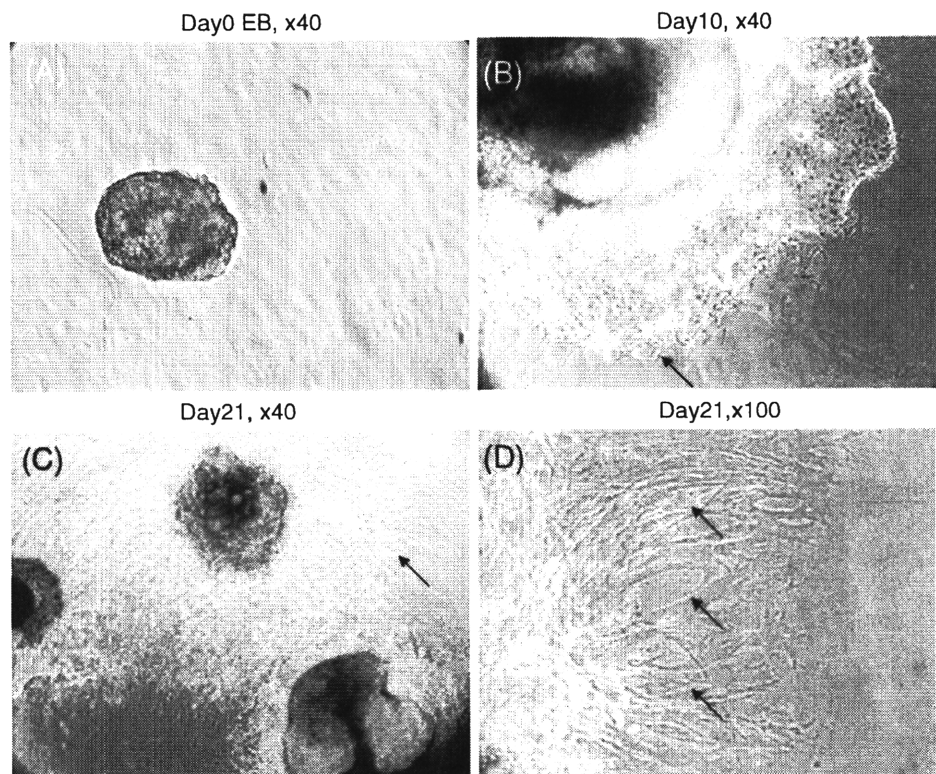


Figure 1. Morphology of human EBs differentiating in type I collagen gel culture. EBs were cast into type I collagen gels and allowed to differentiate for 21 d. (*A*) Day 0, (*B*) day 10, (*C*) day 21 (original magnification, ×40), (*D*) higher magnification (×100) demonstrating spindle-shaped cells surrounding differentiated EBs (*arrows*).

Swedesboro, NJ) and mounted on a glass microscope slide. Chemotaxis was assessed by counting the number of cells in five high-power fields.

Proliferation assay. Cells were plated into 12-well plates (10^5 cells per each well) in 10% FCS-DMEM with or without 10^{-10} mol/l TGF-β1 or 10^{-7} mol/l PGE_2. Cells were fed with fresh 10% FCS-DMEM every 2 d. Cell numbers from three separate wells were determined after 24, 72, and 120 h using a Coulter electronic cell counter (Beckman Coulter Inc., Fullerton, CA).

Immunohistochemistry. Differentiated EBs in type I collagen gels were fixed in 4% paraformaldehyde for 30 min. Differentiated fibroblasts were cultured until sub-confluent in eight chamber slides (Nunc Inc, Naperville, IL) in 10% FCS-DMEM and fixed in 4% paraformaldehyde for 30 min at passage 4. Cells were washed briefly with phosphate buffered saline followed by permeabilization with 0.1% Triton in sodium citrate buffer at 4°C for 5 min. After blocking with horse serum, the cells were incubated with monoclonal anti-α-SMA (1:200 dilution), anti-pan cytokeratin (1:200), anti-vimentin (1:200), anti-SSEA-1 (1:100), or anti-SSEA-4 (1:100) antibodies at 4°C overnight. After washing, cells were then incubated with FITC-conjugated anti-mouse IgG antibody followed by nuclear staining with propidium iodide. Stained cells were visualized and photographed using a Nikon Eclipse TE300 microscope (Nikon, Tokyo, Japan) equipped with a DP71 digital camera (Olympus, Tokyo, Japan).

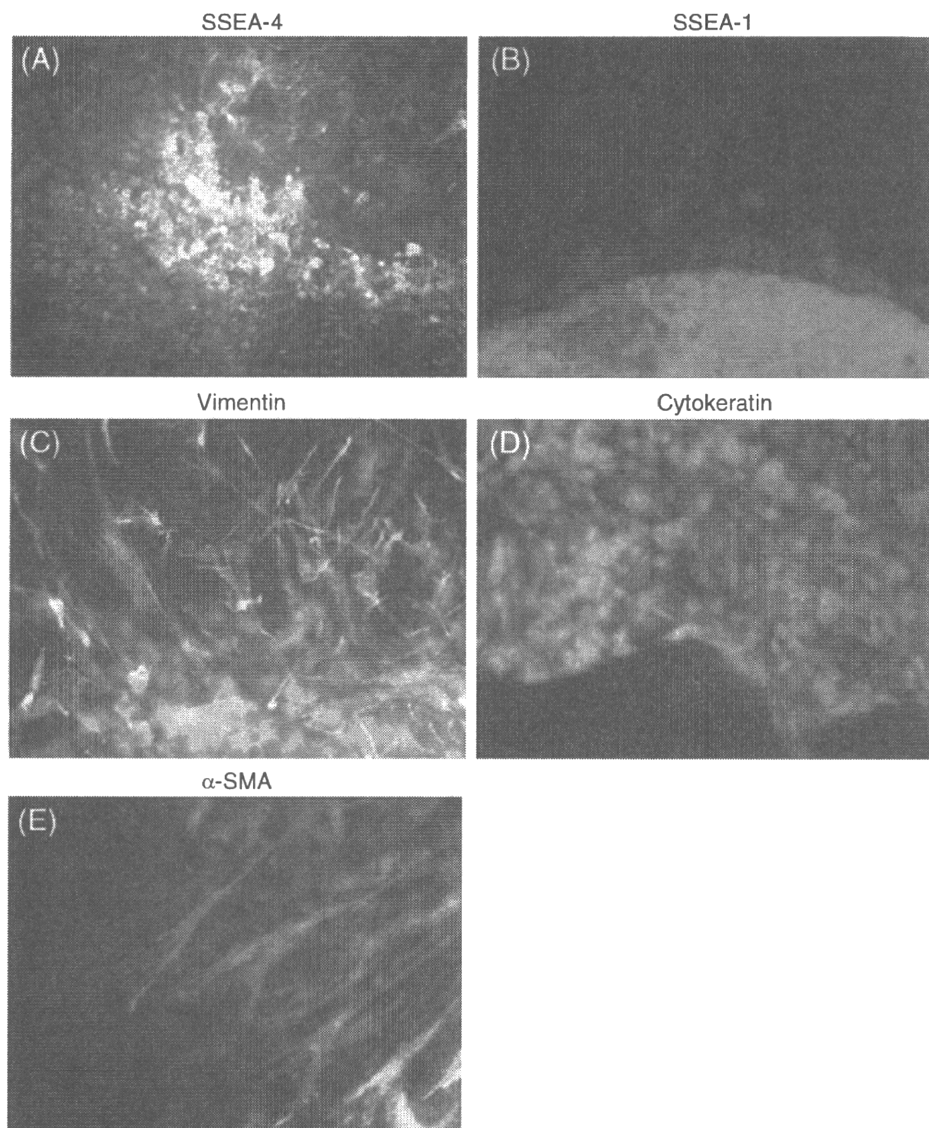


Figure 2. Immunocytochemistry of differentiating human EBs in type I collagen gel culture. EBs were cast into three-dimensional collagen gels and allowed to differentiate. On day 21, cellular biomarkers were evaluated by immunocytochemistry. (*A*) SSEA-4, (*B*) SSEA-1, (*C*) vimentin, (*D*) cytokeratin, (*E*) α-SMA (original magnification, ×200).

Electron microscopic examination. Cells were cultured on Thermanox™ coverslips (Thermo Fisher Scientific, Rochester, NY) and fixed in 2% glutaraldehyde, 2% paraformaldehyde, and 0.5% acrolein. After washing with 0.1 mol/l Sorenson's phosphate buffer, samples were post-fixed in 1% osmium tetroxide. Samples were then washed with buffer and dehydrated in a graded ethanol series. After dehydration samples were embedded in Araldite. Thin sections were stained with 2% uranyl acetate and Reynolds lead citrate, and examined using a Philips 410LS transmission electron microscope (Philips Electronics, Eindhoven, The Netherlands) operated at 60Kv. Images were acquired with an Advanced Microscopy Techniques digital imaging system (Danvers, MA).

Statistical analysis. Data were expressed as means± standard error of the mean (SEM). Experiments with multiple comparisons were evaluated using one-way analysis of variance followed by Bonferroni's test. For all comparisons, significance was determined using separate experiments performed on different occasions. Probability values of <0.05 were considered significant.

Results

Differentiation of human ESCs into fibroblast-like cells in three-dimensional type I collagen gel culture. Generally, spindle-shaped cells appeared surrounding human EBs after

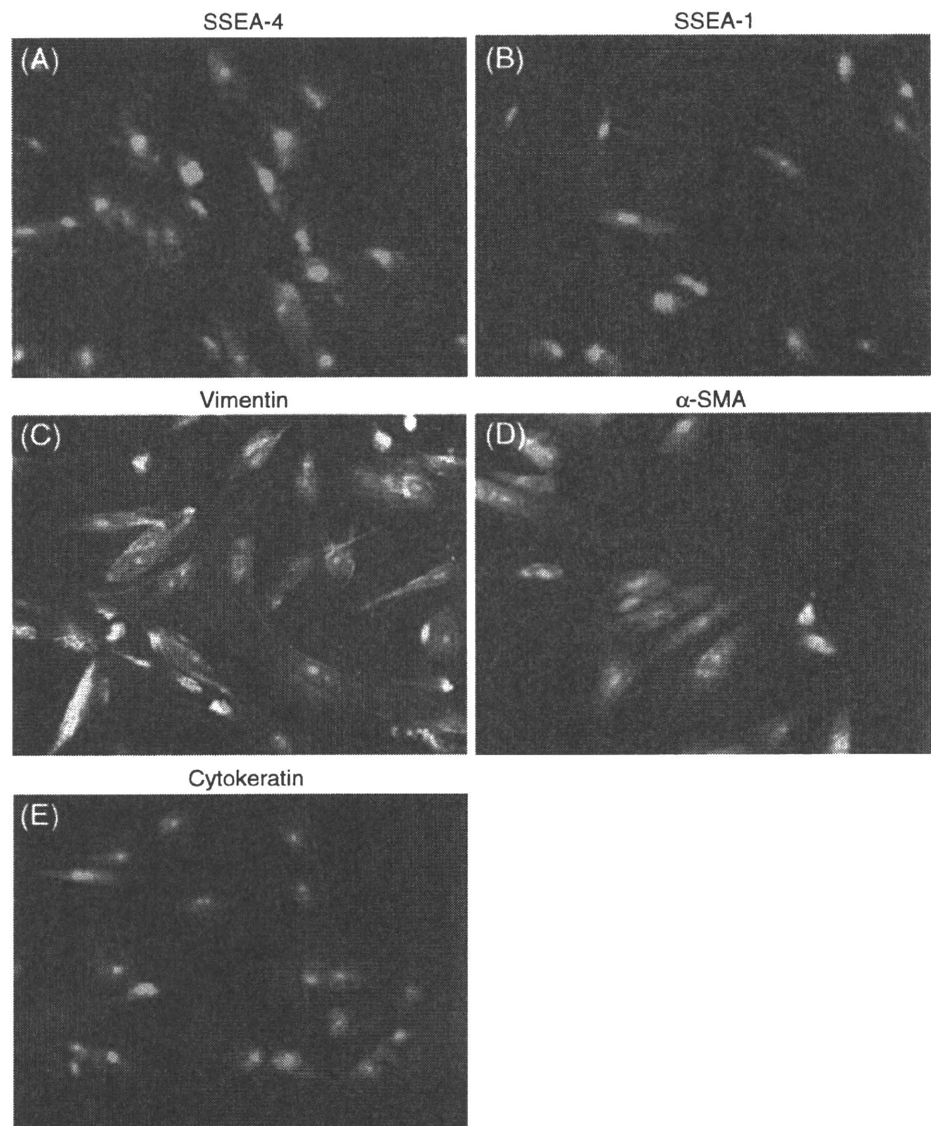


Figure 3. Immunocytochemistry of differentiated fibroblasts in monolayer culture. Collagen gels in which EBs had been cultured for 21 d were dissolved with collagenase and the cells were passaged into monolayer culture. At passage 4, cellular biomarkers were assessed by immunocytochemistry. (*A*) SSEA-4, (*B*) SSEA-1, (*C*) vimentin, (*D*) cytokeratin, (*E*) α-SMA (original magnification, ×200).

7–10 d of culture in three-dimensional type I collagen gels, and were increasingly prominent with further culture to day 21 (Fig. 1). The spindle-shaped cells were collected following collagenase treatment and re-plated into tissue culture plates in 10% FCS-DMEM. The EBs were also released from the collagen gel, but remained floating and were lost with serial feeding and passaging. The fibroblast-like cells were used at passage 4–6 for subsequent characterization.

To evaluate the differentiation of human EBs in three-dimensional type I collagen gels, we first assessed several markers of cell differentiation by immunocytochemistry. EBs in collagen gels were SSEA-4 positive, a marker for undifferentiated embryonic stem cells, but SSEA-1 negative (Fig. 2*A*, *B*). Spindle-shaped cells surrounding the EBs were positive for vimentin and negative for cytokeratin (Fig. 2*C*, *D*). The leading edge of the spindle-shaped cells showed positive staining for α-SMA (Fig. 2*E*). We also stained fibroblast-like cells in monolayer culture. These cells were negative for both SSEA-4 and SSEA-1 (Fig. 3*A*, *B*). In contrast, fibroblast-like cells in monolayer culture showed positive staining for vimentin in 96% of cells (Fig. 3*C*) and for α-SMA in 92% of cells (Fig. 3*D*). They were entirely negative for cytokeratin (Fig. 3*E*). We further assessed the ultra-structural feature of the fibroblast-like cells derived from human ESCs by transmission electron microscopy. Cells showed a characteristic spindle-shaped morphology with prominent rough endoplasmic reticulum and stress fibers, which were consistent with fibroblasts and myofibroblasts (Figs. 4*A*, *B*; Dell'Orbo et al. 1992).

Characteristics of differentiated fibroblast-like cells. To assess the functional features of the fibroblast-like cells, we evaluated cell proliferation, chemotaxis, and contraction of three-dimensional type I collagen gels mediated by the differentiated fibroblasts. In addition, we investigated the ability of exogenous TGF-β1 or PGE_2 to modulate each function.

Differentiated fibroblasts slowly grew in 10% FCS-DMEM with longer than 48 h of doubling time (Fig. 5). Exogenous TGF-β1 significantly stimulated cell proliferation, whereas PGE_2 significantly suppressed proliferation (Fig. 5). Ability of cell migration was assessed by the chemotaxis assay using fibronectin as a chemoattractant. Consistent with our previous reports on lung fibroblast chemotaxis (Kohyama et al. 2002), ESC-differentiated fibroblasts migrated towards fibronectin. Furthermore, exogenous PGE_2 significantly inhibited, while TGF-β1 slightly but (not significant) augmented chemotaxis of these cells towards fibronectin (Fig. 6). These differentiated fibroblasts could also contract type I collagen gels when they were cast into the gels and cultured in SF-DMEM, which is considered as an in vitro model of tissue repair. Collagen gel contraction was significantly augmented by TGF-β1 but inhibited by PGE_2 at all time points assessed (Fig. 7).

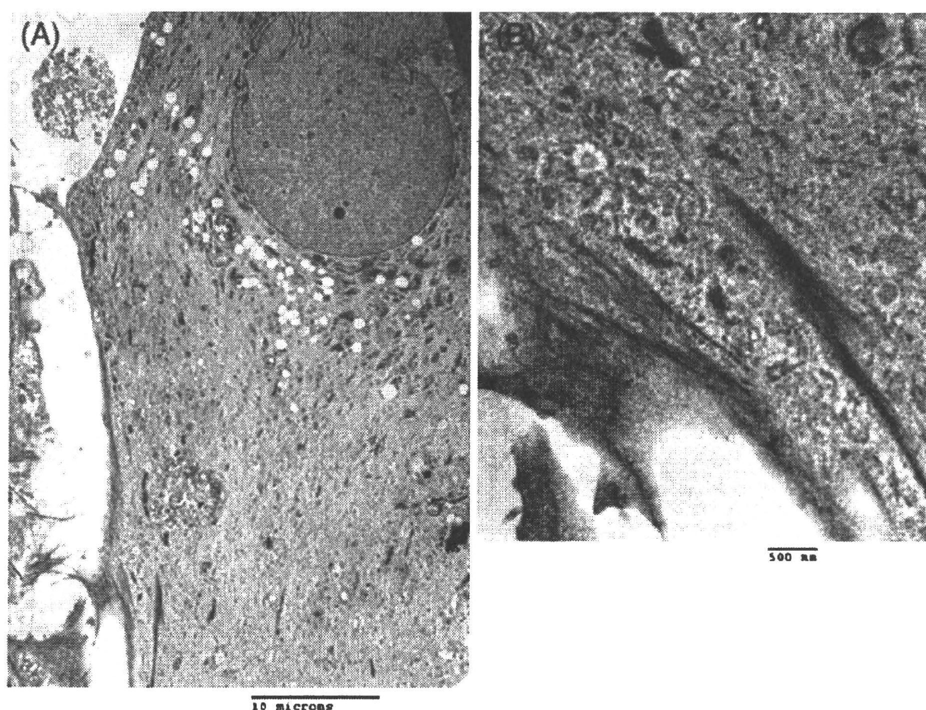


Figure 4. Ultra-structure of fibroblasts derived from human ESCs. Cells were cultured on Araldite coverslips for transmission electron microscopy. Cells derived from human ESCs showed a characteristic spindle-shaped morphology with a prominent rough endoplasmic reticulum and stress fibers (*A*: magnification, ×2,400; (*B*) magnification ×14,000).

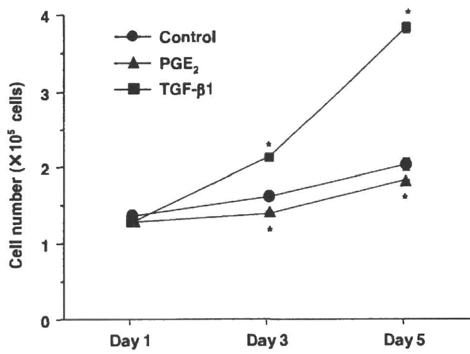


Figure 5. Effects of TGF-β1 and PGE2 on proliferation of fibroblasts derived from human ESCs. Fibroblasts were cultured in monolayers in 10% FCS-DMEM with or without PGE_2 (10^{-7} mol/l) or TGF-β1 (10^{-10} mol/l). Cells were detached with trypsin/EDTA and cell numbers were determined using a Coulter electronic cell counter. *Vertical axis* cell number (×10^5 cells/ml); *horizontal axis*, time (d). Each *point* shows mean±SEM of three separate experiments, each of which included triplicated wells. *Circles* control, *triangles* PGE_2, *squares* TGF-β1; *p<0.05, compared with control group. SEMs are not evident as they are within the plot symbols.

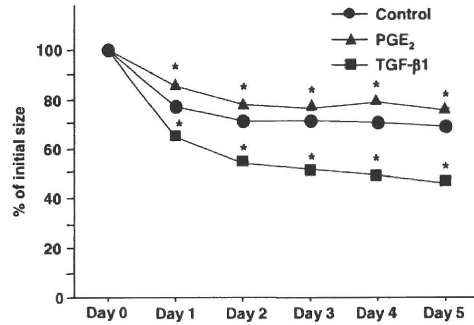


Figure 7. Contraction of three-dimensional collagen gels by fibroblasts derived from human ESCs. Fibroblasts were cast into three-dimensional collagen gels. The gels were released into SF-DMEM supplemented with or without either 10^{-10} mol/l TGF-β1 or 10^{-7} mol/l PGE_2. Gel size was measured daily by an image analyzer. *Vertical axis* gel size (percentage of initial area), *horizontal axis* time (d). Each *point* represents mean±SEM of five separate experiments, each performed in triplicate gels. *Circles* control, *triangles* PGE_2, *squares* TGF-β1; *p<0.01, compared with control group. SEMs are not evident as they are within the plot symbols.

Differentiation of murine ESCs into fibroblast-like cells. Having demonstrated that culture of human ESCs in three-dimensional type I collagen gels led to differentiation of fibroblast-like cells, we next sought to determine if similar results would be obtained with murine ESCs.

Spindle-shaped cells appeared after 10–14 d of culture of murine EBs in three-dimensional collagen gels (Fig. 8*A–D*). To evaluate the phenotype of these fibroblast-like cells, we performed immunocytochemical staining for vimentin and cytokeratin in monolayer culture. As expected, these cells showed positive staining for vimentin, but were negative for cytokeratin (Fig. 8*E*, *F*). It was also demonstrated that exogenous TGF-β1 significantly augmented chemotactic activity toward fibronectin and collagen gel contraction of the fibroblast-like cells derived from murine ESCs, whereas PGE_2 significantly inhibited both chemotaxis and gel contraction (Fig. 8*G*, *H*).

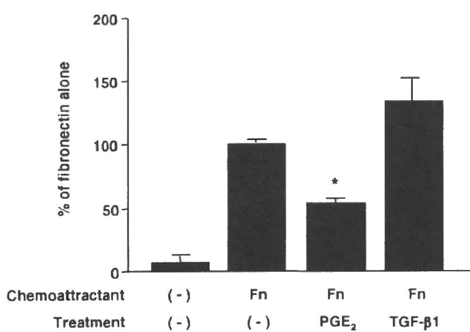
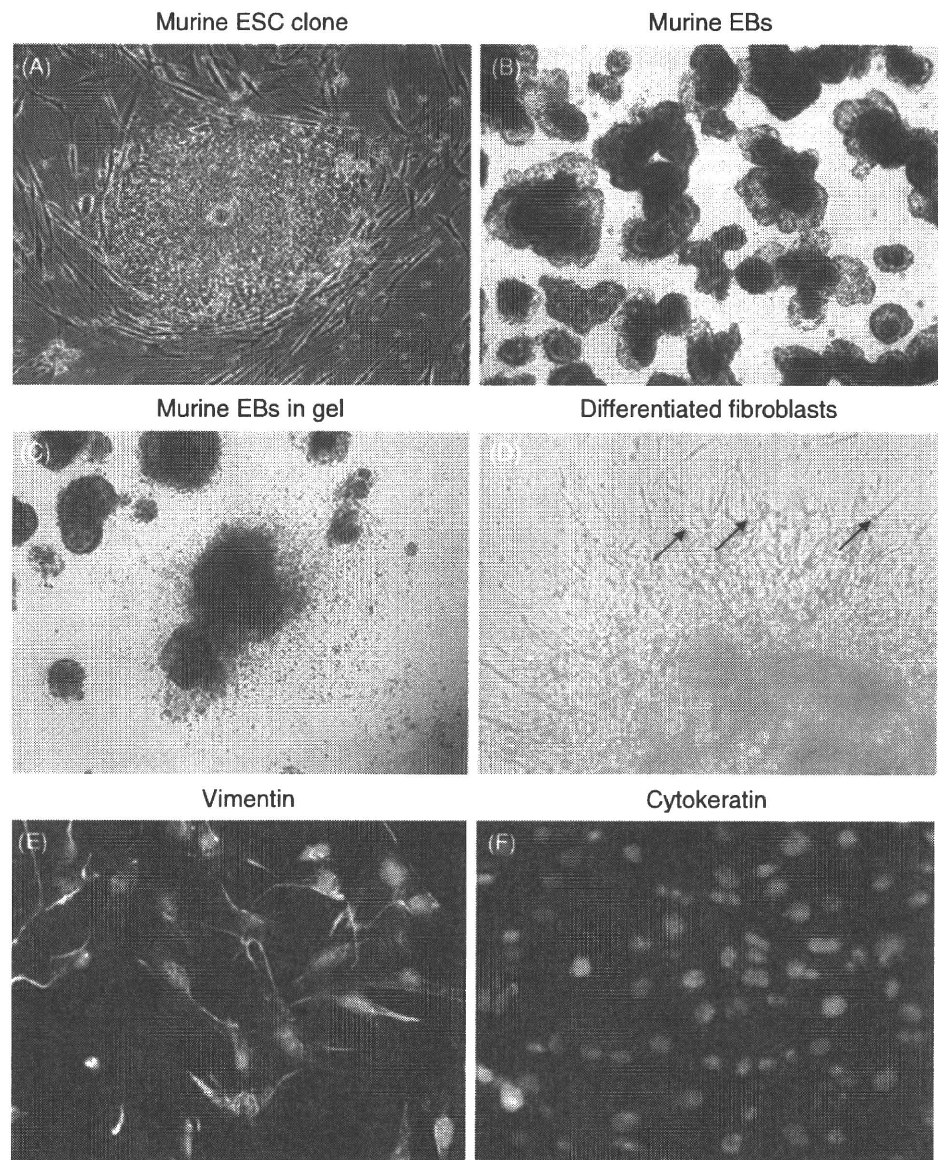


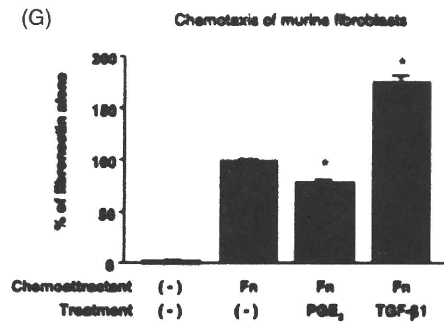
Figure 6. Chemotaxis of fibroblasts derived from human ESCs. Fibroblasts derived from differentiated EBs were trypsinized and chemotaxis toward fibronectin (10 μg/ml) was assessed in the presence or absence of either 10^{-7} mol/l TGF-β1 or 10^{-7} mol/l PGE_2. *Vertical axis* percentage of fibronectin alone, *horizontal axis* conditions. Each *point* represents the mean±SEM of three replicates in four separate experiments; *p<0.05, compared to fibronectin alone (control group).

Discussion

Fibroblasts represent a heterogeneous population of cells present in mesenchymal connective tissues (Fries et al. 1994; Powell et al. 1999a; Powell et al. 1999b; Phan 2008). These cells are thought to be the major cells responsible for the production and maintenance of extracellular matrix (Raghow 1994; Ohnishi et al. 1998). In addition, fibroblasts produce mediators that regulate epithelial and endothelial cell proliferation and functions (Roberts and Sporn 1989; Vignola et al. 1997). Fibroblasts, moreover, can also regulate inflammatory cell recruitment and activation (Glaros et al. 2009).

Cytokines can modulate fibroblast functions (Scotton and Chambers 2007). TGF-β, for example, induces the expression of α-SMA and increases fibroblast production of extracellular matrix (Ronnov-Jessen and Petersen 1993; Desmouliere and Gabbiani 1994). Cells with these features are sometimes termed "myofibroblasts" as the increased expression of α-SMA containing fibers resembles the fibers present in smooth muscle cells (Hinz et al. 2007). In addition to mediator-induced modulation of structure and function, which may be transient, heterogeneous populations of fibroblasts with stable phenotypes have been described in many tissues (Fries et al. 1994; Cassiman et al. 2002). Alterations in populations of differentiated fibro-





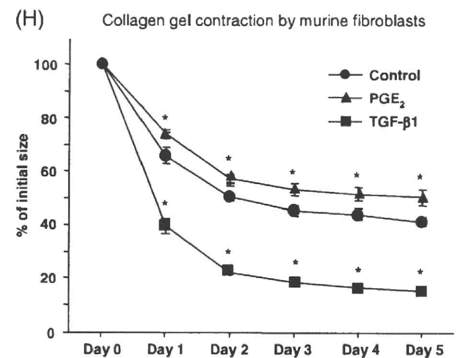


Figure 8. Differentiation of murine ESCs into fibroblasts in three-dimensional collagen gel culture. (*A*) Murine ESCs in monolayer culture with feeder layer (original magnification, ×400). (*B*) Murine EBs were ready to cast into gels (original magnification, ×200). (*C*) Murine EBs in three-dimensional collagen gels (original magnification, ×200). (*D*) Spindle-shaped cells surrounding murine EBs as indicated by the *arrows* (original magnification, ×400). (*E* and *F*) Immunocytochemistry of differentiated murine fibroblasts in monolayer culture stained for vimentin (*E*) and cytokeratin (*F*) (original magnification, ×200). (*G*) Chemotaxis toward fibronectin. Chemotaxis of murine fibroblasts toward fibronectin was assessed in the presence or absence of either 5×10^{-11} mol/l TGF-β1 or 10^{-7} mol/l PGE_2. (*H*) Contraction of three-dimensional collagen gels by fibroblasts derived from murine ESCs. Collagen gels containing fibroblasts were floated in the media with or without 10^{-10} mol/TGF-β1 or 10^{-7} mol/l PGE_2. *Circles* control, *triangles* PGE_2, *squares* TGF-β1; $*p<0.05$, compared with control group.

blasts, moreover, have been described in a number of disease states (Kahari 1993; Fireman et al. 2001; Holz et al. 2004; Sugiura et al. 2007; Togo et al. 2008; Sato et al. in press).

The origin of differentiated fibroblasts is poorly understood. Recent evidence suggests that circulating cells may contribute as progenitors of tissue fibroblasts (Lama and Phan 2006; Hinz et al. 2007; Hong et al. 2007; Phan 2008). Little is known, however, about the mechanisms that control differentiation of stem/progenitor cells into stable populations of fibroblasts. The current study provides a method for the evaluation of fibroblast differentiation from embryonic stem cells as illustrated in Fig. 9.

Several other investigators have reported the derivation of fibroblast-like cells from ESCs (Xu et al. 2004; Stojkovic et al. 2005; Yoo et al. 2005; Choo et al. 2008; Chen et al. 2009). Both differentiation from embryoid bodies (Xu et al. 2004; Choo et al. 2008), as described in the current report, and direct differentiation from ECS were used. All these studies were designed to prepare non-xenogenic fibroblast-like cells to use as feeder layers. The utility of the various types of derived cells for this purpose was well established. Much less attention has been given to demonstrating that the derived cells function as fibroblasts. Yoo et al. (2005) described their cells as being keratin negative and positive for the enzyme prolyl hydroxylase, which plays a role in collagen biosynthesis. Stojkovic et al. assessed a number of histochemical markers, but the derived cells differed from foreskin fibroblasts, which are a specific type of differentiated cell.

Several lines of evidence support describing the fibroblast-like cells prepared by the method described in the current report as fibroblasts. First, these cells have the characteristic spindle-shaped morphology of fibroblasts. In addition, the ultrastructure of the cells is consistent with that of fibroblasts/myofibroblasts. Finally, cells cultured in the present study expressed the cytoskeletal protein vimen-

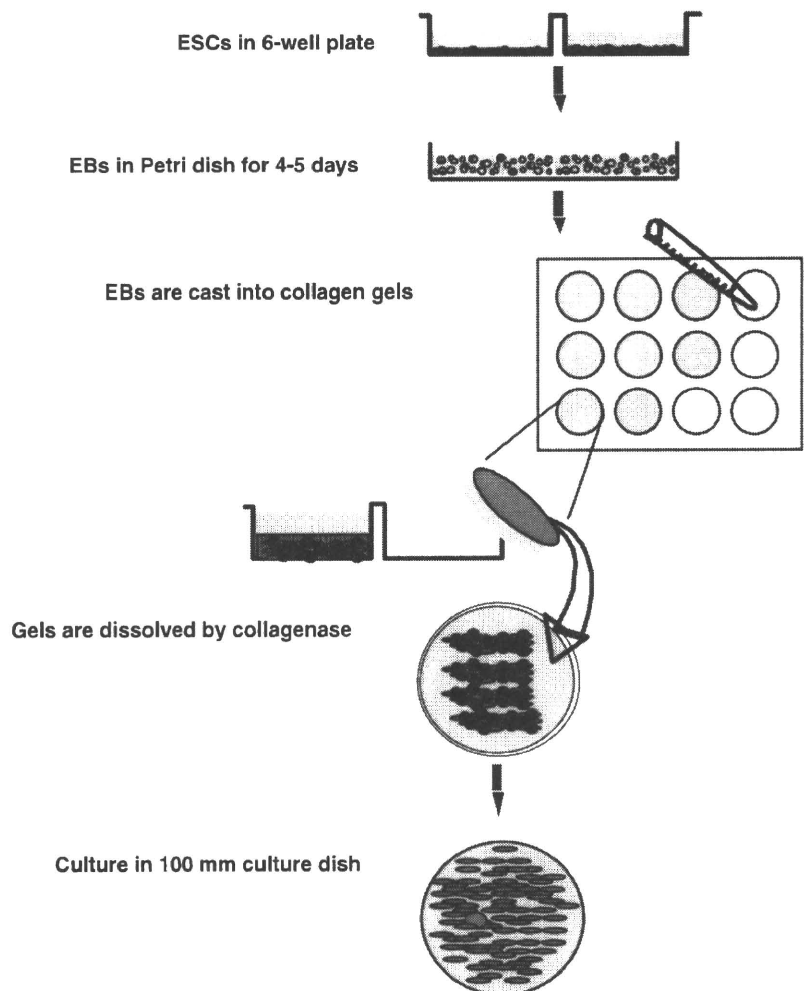


Figure 9. Schematic illustration of the method for differentiation of ESCs into fibroblasts in three-dimensional type I collagen gel culture. Undifferentiated ESCs are cultured on MEF feeder layer in six-well plate. ESCs are detached with collagenase and re-suspended with differentiation medium. Cells are then placed into a Petri dish and cultured for 4–5 d to allow formation of EBs. EBs are cast into type I collagen gels in a 12-well plate (1.0 ml/well) and cultured for 21 d three-dimensionally in collagen gels with basal medium. Gels are dissolved by collagenase and the cells are suspended in 10% FCS-DMEM. Differentiated fibroblasts are cultured in 100 mm culture dishes. EBs are lost with serial feeding and passaging.

tin, which is characteristically present in fibroblasts, and lacked the cytoskeletal protein cytokeratin, which is characteristically present in epithelial cells.

A major distinction between fibroblasts on the one hand and epithelial cells and endothelial cells on the other are their responses to PGE_2 and TGF-β (Liu et al. 2000; Umino et al. 2000; Zhu et al. 2000). In general, TGF-β inhibits epithelial cell proliferation and migration (Vignola et al. 1997). In contrast, TGF-β augments proliferation and migration of fibroblasts (Kurosaka et al. 1998; Togo et al. 2008). Similarly, PGE_2 characteristically stimulates proliferation and migration of epithelial cells while it inhibits proliferation and migration of fibroblasts (Zhu et al. 2000; Huang et al. 2007; Stenson 2007; Togo et al. 2008). Cells cultured in the current study responded to PGE_2 and TGF-β in a manner characteristic of fibroblasts. In addition, fibroblasts cultured in three-dimensional collagen gels attached to collagen and exerted mechanical tension, resulting in gel contraction. This property is thought to be a model of tissue reorganization (Mio et al. 1996). Characteristically, TGF-β augments and PGE_2 inhibits this process in fibroblasts (Campbell et al. 2004; Togo et al. 2008) as was observed in the cells prepared in the current study. In contrast, the ability of endothelial and epithelial cells to contract collagen gels differs markedly. Endothelial cells are unaffected by TGFß or PGE (Liu et al. 2000) and epithelial cells contract gels, but only when plated on the surface and PGE has no effect (Liu et al. 1998; Umino et al. 2000).

In summary, the current study demonstrates that cells with the morphologic and functional features of fibroblasts can be reliably derived from human and murine ESCs. The development of this methodology provides a means to define the mechanisms that regulate fibroblast differentiation.

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ガイドラインのワンポイント解説

喘息予防・管理ガイドライン2009
—薬物療法のポイント—

和歌山県立医科大学内科学第三講座

一ノ瀬正和

Key words: asthma therapy —— controller medications —— exhaled nitric oxide —— inhaled corticosteroids —— pulmonary function test

はじめに

「喘息予防・管理ガイドライン2009」は，日本アレルギー学会ガイドライン委員会/喘息ガイドライン専門部会（部会長：大田健）を構成する内科5名，小児科2名の委員を中心に，内科47名，小児科13名の作成委員によって2009年10月29日に刊行された[1]．今回のガイドラインは2006年版を基にした改訂ということであったが，近年の治療薬の進歩による治療効果の大きな向上から，管理目標が従来より高く設定されている．さらに，治療（長期管理）の段階的薬物療法を，2006年版の「重症度に沿ったステップ」から「治療の強度に基づいたステップ」に変え，喘息治療の国際ガイドライン(GINA, Global Initiative for Asthma)[2]と整合性を持たせた点が大きな変更点である．また，エビデンスの強さに関しても，A(無作為化比較試験大規模データ)，B（無作為化比較試験小規模データ)，C（非無作為化試験)，D（パネルコンセンサス）の4段階で示され，参考になる．本稿では，この新しいガイドラインに関し，薬物療法に焦点を絞り解説する．

尚，本稿で用いた表は全て「喘息予防・管理ガイドライン2009」[1]からの引用である．

喘息の治療目標

一般的に言って疾患の治療目標は，「疾患の可逆性と現存の治療薬の有効性」によって変わりうる．喘息に関しては吸入ステロイドの薬効向上やデバイスの改良により治療効果が大きく改善している．こういった状況から今回のガイドラインでは「治療目標は症状，増悪がなく，呼吸機能も正常なレベルに薬剤の副作用なく持ち込むことである」としている（**表1**）．もちろん，気道のリモデリングがある患者では呼吸機能が正常値まで改善し得ない例もあるので，患者の自己最良値に基づいて判定する．

具体的には，喘息症状，発作治療薬使用，運動を含む活動制限が無く，増悪も無い状態で，且つ呼吸機能(FEV1及びPEF)が正常範囲内で，PEFの日（週）内変動が20％未満を目指す．これまでのガイドラインに比べ項目が整理されわかりやすくなっている．

治療の原則

喘息治療が奏効するか否かは，初期治療がいかに有効であるかにかかっている．よって，「まず喘息症状の改善と安定を図る」と新ガイドラインは明記している．薬物療法に加えて，感作アレルゲ

利益相反（conflict of interest）に関する開示：著者は本論文の研究内容について他者との利害関係を有しません．

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表1　喘息コントロール状態の評価

	コントロール良好（すべての項目が該当）	コントロール不十分（いずれかの項目が該当）	コントロール不良
喘息症状（日中および夜間）	なし	週1回以上	コントロール不十分の項目が3つ以上当てはまる
発作治療薬の使用	なし	週1回以上	
運動を含む活動制限	なし	あり	
呼吸機能（FEV_1 および PEF）	正常範囲内	予測値あるいは自己最高値の80%未満	
PEF の日（週）内変動	20%未満	20%以上	
増悪	なし	年に1回以上	月に1回以上*

*増悪が月に1回以上あれば他の項目が該当しなくてもコントロール不良と評価する.

ン（ダニ，真菌，ゴキブリ，動物，花粉など）の回避，受動・能動喫煙，過労などをも含めた増悪因子の回避，除去の重要性も指摘されている．アレルギー性鼻炎などの併存疾患が喘息症状の悪化に結びつくこともあることから，そういった併存疾患の管理も併せて行うことが望ましいとされる．

喘息治療の4ステップ

従来のガイドラインでは重症度にステップという表現を用いていたが，新ガイドラインでは「治療の強弱に沿ったステップ」を採用した（**表2**）．これは先に述べたGINA[2]と同様のアプローチである．一番弱い治療ステップ1から最強のステップ4までの4段階ある．さらに長期管理薬の項目はこれまでひとつであったが，今回のガイドラインでは有用性の順位付けを行い，上位の基本治療薬と下位の追加治療薬に整理して記載した．基本治療薬は吸入ステロイド（ICS，inhaled corticosteroids），長時間作用性β2刺激薬（LABA，long-acting beta2 agonists），ICS/LABA配合剤，ロイコトリエン受容体拮抗薬（LTRA，leukotriene receptor antagonists），テオフィリン徐放製剤，抗IgE抗体，経口ステロイド薬であり，追加治療薬はLTRA以外の抗アレルギー薬があてはまる（**表4**）．

発作治療薬としては作用発現が速やかな短時間作用性β2刺激薬（SABA，short-acting beta2 agonists）が各治療ステップで用いられる．長期管理の基本薬としては，すべての治療ステップでICSがファーストラインとして推奨されている．各治療ステップでの使用ICS量の目安は**表3**のとおりである．これに治療ステップに応じ他剤をエビデンスに応じ単剤或いは多剤併用していく．

治療の実際

受診時，喘息に関し未治療患者においては，**表5**に示したような症状を目安にして治療ステップを選択する．つまり，**表5**に示す軽症間欠型相当の症状であれば治療ステップ1，軽症持続型相当の症状なら治療ステップ2，中等症持続型の症状であれば治療ステップ3，重症持続型の症状であれば治療ステップ4といった具合である．

受診時すでに喘息に対し，薬物治療を受けている患者であれば，**表1**に示したコントロール状態の評価項目を参考にし，「コントロール良好」に相当すれば現在の治療をそのまま継続，あるいは良好な状態が3〜6カ月持続していればステップダウンを考慮する．「コントロール不十分」にあてはまるなら現行の治療ステップを1段階アップ，「コントロール不良」に相当すれば現行の治療ステップを2段階アップする．

治療ステップ4で経口ステロイドが記載されているがその使用法に関して，「短期間の間欠投与を原則とし，他の薬剤で治療内容を強化する」旨，記載されている．これは，十分な治療薬の追加を

表2 喘息治療ステップ

		治療ステップ1	治療ステップ2	治療ステップ3	治療ステップ4
長期管理薬	基本治療	吸入ステロイド薬（低用量）	吸入ステロイド薬（低～中用量）	吸入ステロイド薬（中～高用量）	吸入ステロイド薬（高用量）
		上記が使用できない場合以下のいずれかを用いる LTRA テオフィリン徐放製剤 （症状が稀であれば必要なし）	上記で不十分な場合に以下いずれか一剤を併用 LABA （配合剤の使用可） LTRA テオフィリン徐放製剤	上記に下記のいずれか1剤，あるいは複数を併用 LABA （配合剤の使用可） LTRA テオフィリン徐放製剤	上記に下記の複数を併用 LABA （配合剤の使用可） LTRA テオフィリン徐放製剤 上記のすべてでも管理不良の場合は下記のいずれかあるいは両方を追加 抗IgE抗体[2] 経口ステロイド薬[2]
	追加治療	LTRA以外の抗アレルギー薬[1]	LTRA以外の抗アレルギー薬[1]	LTRA以外の抗アレルギー薬[1]	LTRA以外の抗アレルギー薬[1]
発作治療[4]		吸入SABA	吸入SABA	吸入SABA	吸入SABA

LTRA：ロイコトリエン受容体拮抗薬，LABA：長時間作用性β_2刺激薬，SABA：短時間作用性β_2刺激薬．

1）抗アレルギー薬とは，メディエーター遊離抑制薬，ヒスタミンH_1拮抗薬，トロンボキサンA_2阻害薬，Th2サイトカイン阻害薬を指す．

2）通年性吸入抗原に対して陽性かつ血清総IgE値が30～700IU/mLの場合に適用となる．

3）経口ステロイド薬は短時間の間欠的投与を原則とする．他の薬剤で治療内容を強化し，かつ短期間の間欠投与でもコントロールが得られない場合は，必要最小量を維持量とする．

4）軽度の発作までの対応を示し，それ以上の発作については7-2を参照．

表3 各吸入ステロイド薬の治療ステップ別推奨量

薬剤名	治療ステップ1～2 低用量	治療ステップ3 中用量	治療ステップ4 高用量
BDP-HFA	100～200μg/日	200～400μg/日	400～800μg/日
FP-HFA	100～200μg/日	200～400μg/日	400～800μg/日
CIC-HFA	100～200μg/日	200～400μg/日	400～800μg/日
FP-DPI	100～200μg/日	200～400μg/日	400～800μg/日
BUD-DPI	200～400μg/日	400～800μg/日	800～1,600μg/日
MF-DPI	100～200μg/日	200～400μg/日	400～800μg/日

せずに安易に経口ステロイドの長期投与を行うことへの警鐘である．**表4**に示したように，現在は作用機序の異なる有効性の高い薬剤が多数あり，以前とは異なり経口ステロイドの長期投与を必要とする喘息患者は少ないと考えられる．勿論，Churg-Strauss症候群のような経口ステロイドが必要な患者の存在は念頭に置くべきであるが，そうでない場合の喘息患者で経口ステロイド長期使用患者に関しては，**表2，4**を参考にし，他の治療薬の追加で経口ステロイドの減量，離脱を図るべ

表4 喘息長期管理薬

1. 副腎皮質ステロイド薬
   1) 吸入ステロイド薬
      i) ベクロメタゾンプロピオン酸エステル
      ii) フルチカゾンプロピオン酸エステル
      iii) ブデソニド
      iv) シクレソニド
      v) モメタゾンフランカルボン酸エステル
   2) 経口ステロイド薬
2. 長時間作用性 β_2 刺激薬
   1) 吸入薬
      サルメテロールキシナホ酸塩
   2) 貼付薬
      ツロブテロール
   3) 経口薬
      プロカテロール塩酸塩
      クレンブテロール塩酸塩
      ホルモテロールフマル酸塩
      ツロブテロール塩酸塩
      マブテロール塩酸塩
3. 吸入ステロイド薬/吸入長時間作用性 β_2 刺激薬配合剤
   1) フルチカゾンプロピオン酸エステル/サルメテロールキシナホ酸塩配合剤
   2) ブデソニド/ホルモテロール配合剤
4. ロイコトリエン受容体拮抗薬
   i) プランルカスト水和物
   ii) ザフィルルカスト
   iii) モンテルカストナトリウム
5. テオフィリン徐放製剤
6. 抗 IgE 抗体
   オマリズマブ
7. ロイコトリエン受容体拮抗薬以外の抗アレルギー薬
   1) メディエーター遊離抑制薬
      クロモグリク酸ナトリウム，トラニラスト，アンレキサノクス，レピリナスト，イブジラスト，タザノラスト，ペミロラストカリウム
   2) ヒスタミン H_1 受容体拮抗薬
      ケトチフェンフマル酸塩，アゼラスチン塩酸塩，オキサトミド，メキタジン，エピナスチン塩酸塩
   3) トロンボキサン阻害薬
      i) トロンボキサン A_2 合成阻害薬
         オザグレル塩酸塩
      ii) トロンボキサン A_2 受容体拮抗薬
         セラトロダスト
   4) Th2 サイトカイン阻害薬
      トシル酸スプラタスト
8. その他の薬剤・療法（漢方薬，特異的免疫療法，非特異的免疫療法）

表5 未治療患者の症状と目安となる治療ステップ

	治療ステップ1	治療ステップ2	治療ステップ3	治療ステップ4
対象となる症状	（軽症間欠型相当） ・症状が週1回未満 ・症状は軽度で短い ・夜間症状は月に2回未満	（軽症持続型相当） ・症状が週1回以上，しかし毎日ではない ・月1回以上日常生活や睡眠が妨げられる ・夜間症状は月2回未満	（中等症持続型相当） ・症状が毎日ある ・短時間作用性吸入 β_2 刺激薬がほぼ毎日必要・週1回以上日常生活や睡眠が妨げられる ・夜間症状が週1回以上	（重症持続型相当） ・治療下でもしばしば増悪 ・症状が毎日ある ・日常生活が制限される ・夜間症状がしばしば

きである．

コントロールの状態の評価に関しても，注意を要する．患者は一般的に言って，自分の症状を過小評価・申告しがちである[3]．**表6**に喘息患者評価に有用な検査項目を示すが，症状の聞き取りに加え呼吸機能（スパイロメトリやピークフロー）を逐次行うことが重要である．さらに，喘息気道の炎症評価法として呼気一酸化窒素（NO，nitrogen oxide）濃度測定も新ガイドラインでは紹介されている（**表6**）．呼気 NO 濃度は喘息患者で特異的に上昇し[4][5]，喘息診断に有用で且つ治療の効果判定にも有用性を示し[6][7]，今後の一般臨床応用の広がりは喘息治療の向上につながると考えられる．