Collagen Gel Contraction Assay Using Human Bronchial Smooth Muscle Cells and Its Application for Evaluation of Inhibitory Effect of Formoterol

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Collagen gel contraction assay is a method for evaluating contraction of cells embedded in collagen gel matrices through measuring the gel size. In the present study, we established a protocol for collagen gel contraction assay using human bronchial smooth muscle cells obtained commercially, and applied it for evaluation of inhibitory effect of formoterol on histamine-induced contraction. Human bronchial smooth muscle cells were embedded in collagen gel in wells of 24-well plates, and gel contraction against histamine or acetylcholine was observed. Gel size was measured at an interval of 10 min for 60 min from the addition of a stimulant. Both acetylcholine and histamine caused gel contraction in a concentration-dependent manner, and the contraction by histamine was apparently potent than that by acetylcholine. Formoterol at concentrations of $10^{-10}$–$10^{-7} \text{M}$ inhibited collagen gel contraction caused by histamine concentration-dependently. Pre-treatment with fluticasone at a concentration of $10^{-8} \text{M}$ apparently potentiated the inhibitory effect of formoterol at $10^{-10}$ and $10^{-8} \text{M}$ on collagen gel contraction by histamine. Prolonged pre-treatment with $10^{-8} \text{M}$ formoterol abolished the inhibitory effect of $10^{-8} \text{M}$ formoterol. Furthermore, 4 h simultaneous pre-treatment with $10^{-8} \text{M}$ formoterol and fluticasone partially but significantly recovered the inhibitory effect of $10^{-8} \text{M}$ formoterol. Present results indicate that the collagen gel contraction assay using human bronchial smooth muscle cells is useful for evaluating the effects of bronchodilating drugs, and that fluticasone potentiates the inhibitory effect of formoterol on histamine-induced collagen gel contraction.

Key words human bronchial smooth muscle cell; contraction assay; histamine; formoterol; fluticasone

Pathology of bronchial asthma is a complex chronic airway inflammation characterized by airway narrowing and airway hyperresponsiveness. Continuous airway inflammation leads to airway wall remodeling as well as airway hyperresponsiveness, resulting in thickening of basement membrane, collagen deposition, smooth muscle hypertrophy and goblet cell hyperplasia. These changes cause airway narrowing, and difficulty in breathing is induced upon asthmatic attack through airway smooth muscle contraction and mucus hypersecretion in narrowed airways.

$\beta_2$-Adrenoceptor agonists are effective bronchodilators due to their ability to relax airway smooth muscle, suggesting that contribution of airway smooth muscle contraction is important for the development of difficult breathing in asthma. Fast- and short-acting drugs are useful for on demand usages to relieve bronchoconstriction and long-acting drugs are beneficial for the prophylactic control of asthma symptoms. However, frequent usages of these drugs, especially long-acting drug monotherapy, are pointed out to increase the risk of asthma mortality, and long-acting drugs are recommended to use in combination with an inhaled corticosteroid. Recently, benefit of combination therapy with an inhaled corticosteroid and a long-acting $\beta_2$-adrenoceptor agonist have been established. It has also been shown that the addition of long-acting $\beta_2$-adrenoceptor agonist to inhaled corticosteroid in patients with persistent asthma symptoms provides greater clinical benefit than doubling the dosage of the inhaled corticosteroid. Furthermore, it is also reported that the efficacy of fluticasone propionate and salmeterol inhaled from a single inhaler at the same time (combination therapy) was superior than the concurrent use of the same doses of the 2 drugs from separate inhalers. It is well known that prolonged or repeated treatment of bronchial smooth muscle with $\beta_2$-adrenoceptor agonists leads to functional desensitization and $\beta_2$-adrenoceptor down-regulation, and that these changes could be reversed by the treatment with corticosteroids.

Although these phenomena seem to be involved in the combination therapy of an inhaled corticosteroid and a long-acting $\beta_2$-adrenoceptor agonist, precise mechanisms of synergistic effects on bronchial smooth muscle in the combination therapy have not yet been fully elucidated.

In the study of bronchodilators, human bronchial tissues have been sometimes employed for the functional analyses. However, the human specimens are usually difficult to obtain. Recently, human bronchial smooth muscle cells are available commercially.

Collagen gel contraction assay is a method for evaluating contraction of cells embedded in collagen gel matrices through measuring the gel size. Tissue contraction is a dynamic process characterized by cellular and extracellular components in the tissue. The cell-populated collagen gel matrices provide a model tissue useful for examining tissue contraction. The assay could be applied for a variety of contracting cells, and for examining tissue remodeling but has rarely been applied for the contraction by human bronchial smooth muscle cells. In the present study, therefore, we established a protocol for evaluating collagen gel contraction by human bronchial smooth muscle cells obtained commercially according to the previous reports and evaluated the inhibitory effects of formoterol, a long-acting $\beta_2$-adrenoceptor agonist, with...
or without treatment with fluticasone,\textsuperscript{23} an inhaled corticosteroid, on the gel contraction caused by histamine.

MATERIALS AND METHODS

Cells and Reagents Normal human bronchial smooth muscle cells (BSMCs) were purchased from Lonza, Visp, Switzerland. Smooth Muscle Growth Media-2 (SmGM-2) BulletKit consisting of basal medium and supplemental factors (SingleQuots) and ReagentPack consisting of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline solution, trypsin/ethylenediaminetetraacetic acid (EDTA) and trypsin neutralizing solution were also obtained from Lonza. SmGM-2 medium was prepared using the BulletKit according to the manufacturer’s instruction. Dulbecco’s modified Eagle’s medium (DMEM, GIBCO, Life Technologies, Carlsbad, CA, U.S.A.), Cellmatrix Type I-A (3.0 mg/mL solution of type I collagen derived from porcine tendon, Nitta Gelatin, Osaka, Japan), reconstituting buffer and the cell suspension were mixed carefully on ice at a ratio of 7:2:1:1. The final cell concentration was $4 \times 10^5$ cells/mL. The mixture at a volume of 500 $\mu$L containing $2 \times 10^5$ cells was placed in each well of 24-well plates and gelated in an incubator at 37°C for 30 min. After gelation, 500 $\mu$L of SmGM-2 medium was added onto the gel in each well and the medium was replaced by fresh medium 24 h later. Then, cells in gel were further cultured for 3 d at 37°C in a 5% CO$_2$ atmosphere.

After the culture for 3 d, medium in each well was removed by aspiration and the gel was detached from well wall using a spatula carefully. Then, 450 $\mu$L of HEPES buffered saline solution and 50 $\mu$L of a stimulant solution were added to each well, and collagen gel contraction was observed for 60 min. The images of gels were scanned from the bottom of plates using a scanner (Epson Scan GT-X750, Seiko Epson, Suwa, Japan), and the images were analyzed using Image J program (National Institutes of Health, Bethesda, MA, U.S.A.) to measure the gel area. The gel was scanned after the addition of a stimulant at an interval of 10 min for 60 min. Results were expressed as a change in gel area and its area under the curve (AUC). Typical images of the collagen gel in a well are shown in Fig. 1.

Statistical Analysis Experiments were repeated at least 3 times and the results were expressed as the mean±S.E.M. of repeated experiments. Statistical difference between two groups was evaluated by Student’s t-test or Aspin–Welch’s t-test after confirming the variance of data by F-test. To evaluate the statistical difference among three or more groups, parametric or non-parametric Dunnett’s multiple comparison test was used after confirming the variance of data by Kruskal–Wallis test. A $p < 0.05$ was considered to be significant.

RESULTS

Gel Contraction Caused by Acetylcholine and Histamine Gel contraction by acetylcholine and histamine was observed. As shown in Fig. 2A, acetylcholine at concentrations of $10^{-5}$–$10^{-2}$M caused concentration-dependent gel contraction. The contraction was developed very slowly and reached its plateau at around 30 min after the stimulation. The gel size was reduced by 50% in the case of $10^{-2}$M acetylcholine. Results of histamine are shown in Fig. 2B. Histamine at concen-

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**Fig. 1. Typical Images of Collagen Gels in the Well**

Upper drawings show images of a floating gel in a well, and lower pictures show images of a collagen gel in a well from the bottom of the plate. (A) Immediately after detachment of gel from well wall (0 min), contraction 0%. (B) Sixty minutes after addition of a stimulant, contraction 45%. White dotted circle in the right picture shows the gel area.
Concentrations of $10^{-7}$–$10^{-4}$M also contracted the gel concentration-dependently. Similar to the case of acetylcholine, the gel contraction was developed very slowly and reached its plateau at around 30 min after the stimulation. The gel size was reduced by over 50% in the case of $10^{-4}$M histamine.

In the following experiments, gel contraction was caused by $10^{-5}$M histamine.

**Effect of Formoterol on the Histamine-Induced Gel Contraction**

Effect of formoterol on the gel contraction caused by $10^{-5}$M histamine was observed. Formoterol was added to the well at the same time with histamine. Results are shown in Fig. 3. Histamine caused a potent gel contraction and the gel size reduced by about 40%. Formoterol at concentrations of $10^{-10}$–$10^{-7}$M inhibited the gel contraction almost concentration-dependently and the inhibition by $10^{-8}$ and $10^{-7}$M was statistically significant. The inhibition by $10^{-7}$ and $10^{-8}$M for-
Fluticasone was almost comparable.

**Effect of Fluticasone on the Inhibition of Gel Contraction by Formoterol**  Effect of pre-treatment with fluticasone on the inhibition of gel contraction by formoterol was examined. Fluticasone at a final concentration of $10^{-8}$M was added to the well 12 or 4 h before detachment of gel from the well wall. We employed $10^{-8}$M fluticasone for the pre-treatment according to our preliminary experiment. The medium containing fluticasone was removed just before the detachment of gel, and then fresh medium was added and stimulated with histamine. Formoterol was added at the same time with histamine. As shown in Fig. 4, although $10^{-10}$M formoterol failed to inhibit gel contraction, it apparently inhibited the gel contraction after treatment with fluticasone for 12 and 4 h. Furthermore, although $10^{-8}$M formoterol inhibited the gel contraction, pre-treatment with fluticasone for 12 or 4 h apparently potentiated the inhibition. Fluticasone pre-treatment did not affect the gel contraction.

**Effect of Treatment with Formoterol on the Inhibition of Gel Contraction by Formoterol**  Effect of prolonged pre-treatment with $10^{-8}$M formoterol on the inhibition of gel contraction by $10^{-8}$M formoterol was examined. Formoterol at a final concentration of $10^{-8}$M was added to the well 48 or 24 h before detachment of gel from the well wall. The medium containing formoterol was removed just before the detachment of gel, and then fresh medium was added and stimulated with histamine. Formoterol was again added at the same time with histamine. As shown in Fig. 5, prolonged pre-treatment with $10^{-8}$M formoterol for 48 or 24 h apparently reduced the inhibitory effect on the gel contraction by formoterol.

**Effect of Simultaneous Treatment with Formoterol and Fluticasone on the Inhibition of Gel Contraction by Formoterol**  Effect of simultaneous treatment with formoterol and fluticasone on the inhibition of gel contraction by formoterol was examined. Fluticasone and formoterol at a final concentration of $10^{-8}$M each were added to the well 4 h before detachment of gel from the well wall. We selected the pre-treatment period according to the results shown in Fig. 4. The medium containing fluticasone and formoterol was removed just before the detachment of gel, and then fresh medium was added and stimulated with histamine. Formoterol was again added at the same time with histamine. Results are shown in Fig. 6. Pre-treatment with $10^{-8}$M formoterol for 4 h abrogated the inhibition of gel contraction by $10^{-8}$M formoterol. In contrast, simultaneous pre-treatment with formoterol and fluticasone partially but significantly recovered the inhibition of gel contraction.

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Fig. 4. Effect of Pre-treatment with Fluticasone on the Inhibition of Gel Contraction by Formoterol

Collagen gel was treated with $10^{-7}$M fluticasone for 12 or 4 h before the addition of $10^{-7}$M histamine and $10^{-10}$ or $10^{-8}$M formoterol. Results are shown as change in gel size for 60 min (A) and AUC (B). Values are the mean and S.E.M. for 3 experiments. FF: formoterol, FP: fluticasone, ‖: spontaneous, □: control (without FP pre-treatment), ▲: FF $10^{-10}$M (without FP pre-treatment), ●: control (pre-treated with FP for 12 or 4 h), ▲: FF $10^{-8}$M (pre-treated with FP for 12 or 4 h), ■: FF $10^{-3}$M (pre-treated with FP for 12 or 4 h). **$p<0.01$ by t-test (against histamine (control) without FP pre-treatment), #*$p<0.05$, **$p<0.01$ by Dunnett’s test (against histamine (control) without FP pre-treatment, histamine with FP pre-treatment for 12 h, or histamine with FP pre-treatment for 4 h).
DISCUSSION

In the present study, we established a protocol for a collagen gel contraction assay using human bronchial smooth muscle cells according to the previous reports and manufacturer's instructions, and applied the assay for the evaluation of inhibitory effects of formoterol on the gel contraction with or without fluticasone treatment.
At first, we compared the contraction induced by acetylcholine and histamine, potent constrictors for tracheal smooth muscles. Both stimulants caused apparent gel contraction concentration-dependently, and the highest concentrations examined reduced the gel area by 50% or more. However, the concentrations enough for collagen gel contraction seem to be very high when comparing to those for smooth muscle contraction. One reason is that the contraction of collagen gel is produced secondarily through smooth muscle cell contraction buried in the gel. Furthermore, contracting activity of acetylcholine was apparently weaker than that of histamine as reported by Kitamura et al.,20 although the reason has not yet been defined. The collagen gel contraction was developed very slowly after the addition of a stimulant and reached its plateau at around 30 min after the stimulation. The gel contraction is induced through contraction of buried smooth muscle cells in the collagen gel matrices, and it may be a time-requiring process. This may suggest, therefore, that this assay could not be applied for evaluation of a quick relaxing effect of smooth muscle relaxants as well as a quick constricting effect of smooth muscle constricting agents. As a collagen gel gelated in well is stretched by the attaching well wall, it reduced its size over 10% for 60 min spontaneously after detaching from the wall. However, taking the spontaneous contraction into account, the size change by the constricting agent seems to be large enough for evaluating the effects of smooth muscle relaxing agents.

Formoterol is a long-acting β2-adrenoceptor agonist useful for prophylactic control of asthma symptoms in conjunction with inhaled corticosteroids.4,5,24 In the present study, it inhibited histamine-induced collagen gel contraction almost concentration-dependently, and the inhibition was significant at concentrations of 10−8 and 10−7 M. The inhibition at the concentrations was comparable and reached about 50%. Although the onset of action of formoterol is observed within 3 min clinically,20 its rapid relaxing effect in pre-contracted collagen gel could not be observed in our preliminary experiment (data not shown), because the assay may not be applicable for detecting a quick change as mentioned above.

Next, we examined the effects of fluticasone pre-treatment on the inhibition of histamine-induced gel contraction by formoterol. The concentrations of formoterol examined were 10−10 and 10−8 M, the former was without apparent inhibitory effect and the latter exhibited apparent effect. Formoterol at a concentration of 10−10 M failed to inhibit the gel contraction, it apparently inhibited the contraction after the pre-treatment with fluticasone. Furthermore, fluticasone pre-treatment apparently potentiated the inhibition of gel contraction by 10−8 M formoterol. These results clearly indicate that fluticasone potentiates relaxing effect of formoterol, although it did not affect the gel contraction under the present experimental condition. A complex of glucocorticoid receptor and CCAAT/enhancer binding protein α (C/EBPα) inhibits smooth muscle cell proliferation,23,26 and fluticasone inhibits contractile protein expression induced by transforming growth factor-β in bronchial smooth muscle.27 Furthermore, dexamethasone increases high-affinity isoproterenol binding sites and Gsα protein expression in bovine tracheal smooth muscle.28 Although these reports suggest that glucocorticoids augment the relaxing effect of β2-adrenoceptor agonists, the mechanism involved in the potentiation by fluticasone of formoterol action observed in the present study has to be elucidated.

It is well known that β2-adrenoceptor agonists induce receptor desensitization involving uncoupling of the receptor from Gs following β-arrestin binding, internalization and down-regulation of the receptors,29 and that the desensitization is reversed by glucocorticoids.30 However, human airway smooth muscle seems to be relatively resistant to the desensitization because of its large β2-adrenoceptor reserve.31 In the present results, prolonged pre-treatment with formoterol for 48 or 24 h abrogated the inhibitory effect of formoterol on histamine-induced collagen gel contraction. Furthermore, formoterol inhibition of gel contraction was diminished even after 4 h pre-treatment with formoterol. Uncoupling and internalization of the receptors may occur in a relatively short period. Therefore, formoterol may easily induce desensitization in human bronchial smooth muscle in the present assay system. Although the 4 h pre-treatment with formoterol abrogated the inhibition, simultaneous pre-treatment with formoterol and fluticasone for 4 h partially but significantly recovered the inhibition. The collagen gel contraction assay may be useful for examining the mechanisms of synergistic effects of β2-adrenoceptor agonists and inhaled corticosteroids.

In summary, present results indicate that the collagen gel contraction assay using human bronchial smooth muscle cells is useful for evaluating the effects of bronchodilating drugs, and that fluticasone potentiates the inhibitory effect of formoterol on histamine-induced collagen gel contraction.

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