

A Contraction Assay System Using Primary Cultured Mouse Bronchial Smooth Muscle Cells

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Key Words

Asthma · Bronchial smooth muscle · Bronchoconstriction

Abstract

Background: Contraction of bronchial smooth muscle is the main mechanism of asthmatic responses. Inflammatory cells such as eosinophils and mast cells produce chemical mediators that induce smooth muscle contraction. To investigate the mechanisms of IgE-independent asthmatic response in murine asthma models, a novel in vitro assay system using primary cultured mouse bronchial smooth muscle cells (BSMC) was explored. **Method:** Trachea and bronchi were taken from young mice and underwent primary culture. BSMC were expanded in culture and then embedded in a collagen gel. The well-known constrictors leukotriene D₄ (LTD₄), methacholine and histamine were applied to the BSMC gels. The gel images were captured by an image analyzer and contractile responses were evaluated. **Results:** LTD₄ and methacholine significantly induced the gel contraction in a dose-dependent manner, but histamine did not. Montelukast, a CysLT type II receptor antagonist, and atro-

pine, a muscarinic acetylcholine receptor antagonist, inhibited the contractile responses in an agonist-specific manner.

Conclusion: A contraction assay system using cultured mouse BSMC was successfully established for the first time. It may go a long way toward identifying bronchoconstricting mediators involved in murine asthma models.

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Introduction

Bronchial asthma is characterized by reversible airway obstruction which is mainly caused by the contraction of bronchial smooth muscle [1–3]. Two types of asthmatic responses, immediate asthmatic response (IAR) and late asthmatic response (LAR), are induced upon antigen inhalation by atopic asthmatics [4, 5]. IAR is triggered by mast cell degranulation and is totally dependent on CysLTs and histamine [6], whereas LAR is not affected by antihistamine and only partly suppressed by CysLT type 1 receptor antagonist [7, 8]. To further analyze mechanisms of LAR, we developed a T-cell-dependent asthma

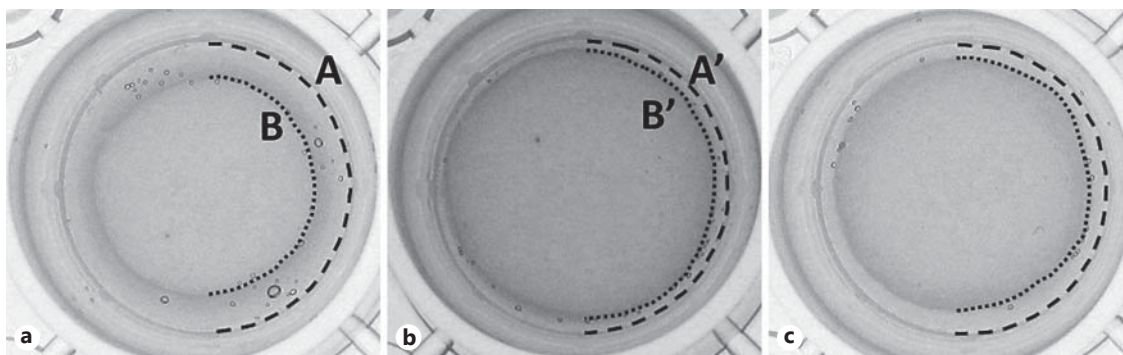


Fig. 1. Contraction of mouse BSMC collagen gels. LTD₄ (10 μM) was added to the collagen gel embedded with mouse BSMC (a) or the gel was left unstimulated (b). c The gel was preincubated with montelukast (10 μM). The gel images were captured by ImageMas-

ter VDS-CL 15 min after the addition of LTD₄. The surface area of the gel top (B) and that of the base layer (A) were automatically calculated by NIH image software.

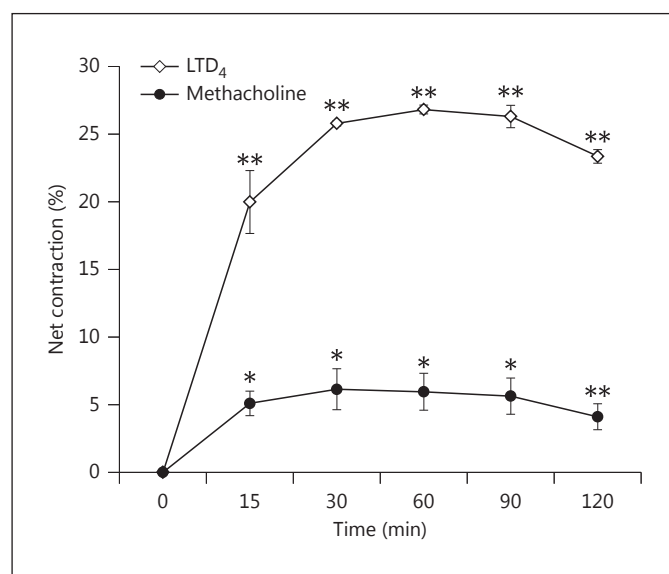


Fig. 2. Contraction of BSMC gels by LTD₄ and methacholine. LTD₄ (10 μM, n = 4) and methacholine (100 μM, n = 5) were applied to the BSMC gels. Gel contraction was analyzed 15, 30, 60, 90 and 120 min after the addition of the agents. Data are presented as the mean ± SEM. To test differences between individual groups, the Student t test was performed, and * p < 0.05 and ** p < 0.01 were considered statistically significant.

model in which T helper (Th) clones were adoptively transferred into unprimed mice [9]. In addition to eosinophilic inflammation, airway hyperresponsiveness and remodeling [10, 11], bronchoconstriction was induced in this model [11, 12]. An ovalbumin (OVA)-reac-

tive Th clone, T6-2, conferred LAR, while another Th clone, BF7, induced airway hyperresponsiveness upon OVA challenge [12]. These distinctive asthmatic reactions stemmed from the characteristics of transferred Th clones, but humoral immune response was not involved. Previous researchers used collagen gel assays embedded with cultured smooth muscle cells of various origins to study smooth muscle contraction in vitro [13–15]. However, murine bronchial smooth muscle cells (BSMC) are not commercially available yet, and so it is difficult to further clarify the role of Th cells in IgE-independent asthmatic response in terms of the nature of T-cell-derived mediators. In this study, we tried to establish murine BSMC and utilize them to assay the constriction activity in vitro.

Material and Methods

Murine BSMC

Preparation and culture of primary murine BSMC was performed according to the method reported by Oldenburg et al. [16] with some modifications. Briefly, trachea and bronchi were obtained from 3-week-old BALB/c mice (Sankyo-labo Service Co., Inc.), washed 3 times with Hanks' Balanced Salt Solution (HBSS) and minced with a scalpel on the culture dish. Under a microscope, the minced organ containing bronchial smooth muscle was selected, then placed in a 15-ml tube, suspended in 1 ml HBSS containing 1 mg/ml type I collagenase (Wako), and incubated at 37°C for 3.5 h. The digestion reaction was stopped by adding HBSS containing 5% fetal bovine serum (FBS). The resulting material was washed twice, suspended in DMEM/F-12 medium (Sigma) supplemented with 10% FBS plus penicillin-streptomycin, and then seeded in a 24-well culture plate (BD Biosciences). Cells were cultured in a humidified 5% CO₂ incubator at 37°C. When the BSMC culture reached

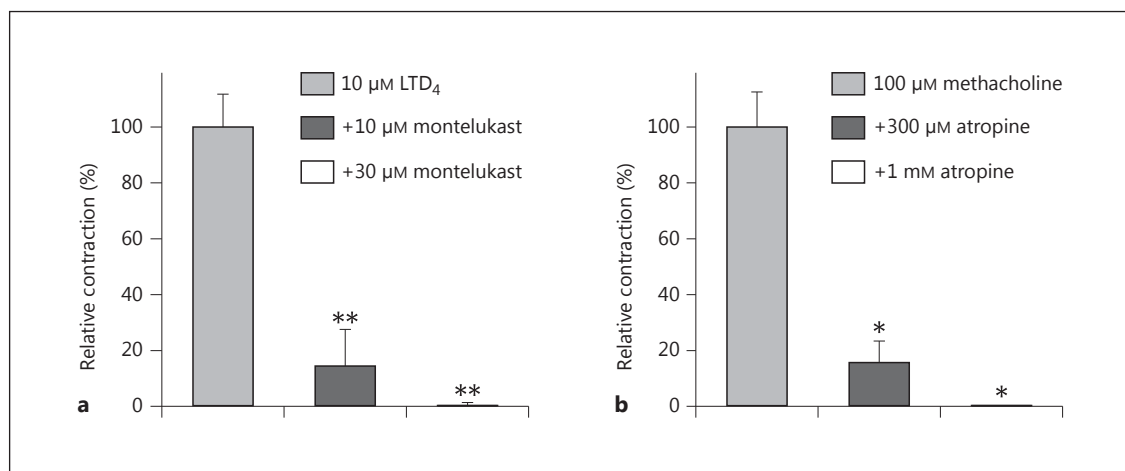


Fig. 3. Inhibition of contractile activity of the specific antagonists. **a** Montelukast (10 and 30 µM) was preincubated for 30 min before the addition of constrictors and then LTD₄ (10 µM) was added (n = 3). **b** Atropine (300 and 1,000 µM) was preincubated for 30 min before the addition of constrictors and then methacholine

(100 µM) was added (n = 3). Data were presented as the mean ± SEM of triplicate experiments. To test differences between individual groups, the Student t test was performed, and * p < 0.05 and ** p < 0.01 were considered statistically significant.

confluent, the plates were washed twice with HBSS, and treated with 0.005% trypsin plus 20 µM EDTA. The detached cells were resuspended in a new medium, and cultured in a 100-mm culture dish.

Preparation of Collagen Gels and Contraction Assays

Preparation of embedded-BSMC gels and contraction assay were performed as previously described [13, 17]. Briefly, confluent BSMC were harvested and suspended in Cellmatrix Type I-A (Nitta-Gelatin Inc.) supplemented with 1 × (final concentration) HBSS, 20 mM HEPES, 5 mM NaOH and 26.2 mM NaHCO₃ at 4 × 10⁵ cells/ml. Cell suspension (0.5 ml) was layered onto the base layer containing 0.3 ml Cellmatrix only. The preparations were then kept at 37°C for 30 min to form the gel. The embedded BSMC were cultured for 4 days with culture medium to allow cell growth in a formed gel matrix and with serum-free DMEM for the last 2 days. For the contraction assay, medium was exchanged for Krebs buffer, and the gel wall was detached from the culture plate using a 27-gauge needle. LTD₄ (Cayman Chemical, Colo., USA), methacholine (Kanto-chemical), and histamine (Sigma) were added to the wells at the designated concentrations. For inhibition assays, atropine (Sigma) and montelukast (kindly donated by Merck) were added into the culture 30 min before the addition of constrictors. The gel images were captured with Image-Master VDS-CL (GE Healthcare) at the designated time points. Contraction of the gel was then evaluated by measuring its surface area with the use of image analysis software (NIH image).

Immunohistochemical Staining

BSMC were seeded in a 35-mm culture dish after 1–2 passages. Confluent cells on the dish were then fixed with 4% formaldehyde (Wako) for 15 min and treated with 0.25% Triton X-100 (Roche Applied Sciences) in phosphate-buffered saline (PBS) for 15 min. Immunohistochemical (IHC) staining was performed with monoclonal anti-caldesmon, a smooth muscle cell marker antibody (Sig-

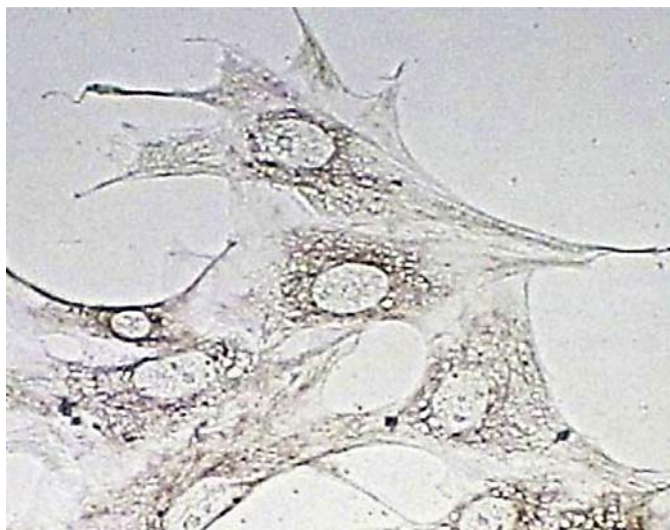
ma, C4562). Purified mouse IgG1 (BD Pharmingen, 550878) was used as a control antibody. Antibody solution (60 µg/ml in PBS containing 0.05% Triton X-100 and 0.5% BSA; PBST) was applied to the fixed cells and incubated at room temperature for 1.5 h. The dish was then washed twice with PBST- and HRP-conjugated goat anti-mouse IgG antibody (Betyl, A90-216P, 1:10,000 dilution in PBST) was applied. After washing, 0.5 mg/ml DAB substrate was added.

Statistics

Statistical analysis was performed using a paired t test. p < 0.05 was considered to be statistically significant.

Results and Discussion

To analyze the bronchoconstricting activities involved in murine asthma models, BSMC were cultured from the trachea and bronchi of 3-week-old mice, and the contractile activity of well-known mediators was analyzed by using the BSMC-embedded collagen gels. LTD₄ (10 µM), methacholine (100 µM) and histamine (10 µM) were applied to the BSMC gels first. Measurement of the contraction activity started 15 min after the addition of constrictors. Gel contraction started 15 min after the addition of LTD₄ and reached a maximum after 30 min (fig. 1, 2). The constriction continued until 120 min after the start of the reaction. Methacholine also induced significant constriction. The time course of the constriction was similar to that induced by LTD₄, but the magnitude was much



Color version available online

Fig. 4. IHC staining of mouse BSMC. IHC staining was performed using anti-caldesmon antibody. Caldesmon is localized in the actomyosin domains of smooth muscle cells.

smaller: the maximum net contraction induced by LTD₄ was approximately 25%, whereas that induced by methacholine was 8% (fig. 2). Histamine did not induce significant responses (data not shown).

Next, the specificity of the response was confirmed. Montelukast, a CysLT₁ antagonist, was added prior to the addition of LTD₄ (fig. 3a). Either 10 or 30 μ M montelukast significantly abrogated the contraction induced by LTD₄, but not that induced by methacholine (data not shown). When atropine was added prior to the addition of metha-

choline, the net contraction was almost completely inhibited (fig. 3b).

To investigate the nature of cultured BSMC in detail, IHC staining was performed to test the specific marker of smooth muscle cells. Almost all the cultured cells were stained with anti-caldesmon antibody (fig. 4), although the possibility that some part of the cells might be comprised of myofibroblasts could not be excluded at this particular moment.

Our result clearly indicated that primary BSMC were successfully established from very young mice, and collagen gels embedded with murine BSMC worked in response to the well-known bronchoconstrictors LTD₄ and methacholine (fig. 2). As we reported earlier, murine Th clones conferred LAR to unprimed mice. Our novel in vitro assay system will go a long way towards the understanding of the nature of bronchoconstricting mediators involved in T-cell-dependent LAR in mice.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of this article.

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