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ORIGINAL ARTICLE

Involvement of Fibronectin and Matrix Metalloproteinases in Airway Smooth Muscle Cell Migration for the Process of Airway Remodeling

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ABSTRACT

Background: Airway remodeling is a repair process occurring after airway injury; its primary histopathological features are subepithelial fibrosis and smooth muscle thickening of the bronchi. These histopathological changes are considered to occur due to bronchial smooth muscle cells (bSMC) that secrete extracellular matrix (ECM) proteins, which work as chemoattractants and influence cell migration. Therefore, we examined the interaction between bSMCs and ECM proteins *in vitro* for understanding the remodeling process in the bronchi.

Methods: bSMCs were cultured to collect a bSMC-conditioned medium. Using the bSMC-conditioned medium thus obtained, we performed a cell migration assay, characterized β integrin expression, and identified ECM proteins and matrix metalloproteinases by western blotting and gelatin zymography, respectively.

Results: The response of bSMC migration to bSMC-conditioned medium increased with time in culture, and fibronectin (FIB) was detected as a chemoattractant for bSMCs in bSMC-conditioned medium by western blot analysis and a cell migration assay using anti-FIB antibodies. The involvement of β1 integrin in the migration of bSMCs toward FIB contained in bSMC-conditioned medium was demonstrated by inhibition of cell migration using anti-β1 integrin antibodies. Expression of β1 integrin on bSMCs was confirmed by using a β-integrin-mediated cell adhesion array. In addition, metalloproteinases detected in bSMC-conditioned medium by gelatin zymography were suggested to be matrix metalloproteinase-1 and 2 by western blotting and amino acid sequencing.

Conclusions: Our results suggest that FIB and matrix metalloproteinases secreted from bSMCs might play major roles in bSMC migration in the process of airway remodeling.

KEY WORDS

airway remodeling, cell migration, fibronectin, matrix metalloproteinases, smooth muscle cells

ABBREVIATIONS

BSA, Bovine serum albumin; bSMC, bronchial smooth muscle cell; CBB, Coomassie brilliant blue; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; FBS, fetal bovine serum; FIB, fibronectin; HBSS, Hanks' balanced salt solution; hEGF, human epidermal growth factor; hFGF-B, human fibroblast growth factor-basic; HPF, high power field; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; PBS, phosphate buffered saline; PBST, PBS containing 0.1% Tween 20; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SMC, smooth muscle cell; TIMP, tissue inhibitor of metalloproteinases; uPA, urokinase plasminogen activator.

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INTRODUCTION

In chronic and severe asthma, persistent narrowing occurs in the airway due to poorly reversible structural changes in the airway wall. These changes are called "airway remodeling" and are associated with epithelial injury and subsequent impaired healing.[1] Despite advances in understanding the development and progression of airway inflammation, there is relatively little understanding of cellular and molecular mechanisms underlying the structural changes observed in such remodeling. The bronchi are located in the lower regions of the airway and consist of epithelium, basement membrane, connective tissue, and smooth muscle, in succession from the lumen. Histological characteristics of the bronchial remodeling comprise increases in smooth muscle mass and subepithelial fibrosis accompanied by epithelial injury.[2] This remodeling process may be associated with migration of smooth muscle cells (SMCs) into connective tissue. It is known that intimal thickening in the aortic wall caused by endothelial injury is deeply involved with the migration of vascular SMCs; moreover, their increased migration can lead to atherosclerosis.[3,4] Therefore, several investigators have pointed out the potential role of the migration of bronchial SMCs (bSMCs) in airway remodeling.[5-7] However, it is still unclear whether the behavior of bSMCs is similar to that of vascular SMCs in the wound healing processes.

Johnson *et al*. measured the relative amounts of extracellular matrix (ECM) protein produced by bSMCs in a medium with nonasthmatic serum and identified a wide variety of ECM proteins, including fibronectin (FIB), collagen type I, and laminin-β1, -γ1, -β2, and -α1 chains.[8] They reported that the amount of FIB was greater than the amounts of collagen type I and laminin chains. Furthermore, they determined that the addition of asthmatic serum to the medium caused a significant increase in the production of FIB from bSMCs. In histological studies of asthmatic bronchi, increased FIB was located in not only interstitial and subepithelial connective tissues but also smooth muscle.[9] However, it is not clear whether FIB secreted by bSMCs is involved in bSMC migration. It has been shown that integrin β1 and β3 subunits are involved in the migration of vascular SMCs stimulated by FIB.[10] As β1 and β3 integrins have been reported to be expressed on bSMCs,[11] these integrins might play a role in bSMCs migration in response to FIB produced by bSMCs.

The increase in ECM in the asthmatic airway could be due to the following reasons: (i) increased *de novo* synthesis of ECM proteins, (ii) decreased activity of matrix metalloproteinases (MMPs), or (iii) upregulation of tissue inhibitors of metalloproteinases (TIMPs).[12] MMP-1, 2, 3, 9, 10, and 12 as well as MT1-MMP are expressed by airway SMCs.[13-16] Furthermore, several studies on airway SMC migration have suggested that the upregulation of MMP-2 and membrane-type matrix metalloproteinases-1 (MT1-MMP) are correlated with increased cell migration,[17] and that MMP inhibitors reduce cell migration.[18]

In the present study, we investigated whether bSMCs migrate in response to FIB in a bSMC-conditioned medium via a mechanism involving their own β integrins. Furthermore, we examined the possibility of the existence of MMPs in a bSMC-conditioned medium.

METHODS

MATERIALS

Dulbecco's modified Eagle medium (DMEM) and Hanks' balanced salt solution (HBSS) were purchased from Life Technologies Corp., CA, USA. Bovine serum albumin (BSA) and polyclonal anti-human FIB (F3648), anti-human albumin (A0433), anti-human MMP-1 (hinge region; M4177), and anti-human MMP-2 antibodies (hinge region; M4677) were obtained from Sigma-Aldrich Corp., MO, USA. Monoclonal anti-human β1 integrin (IG6061) and anti-human β3 integrin antibodies (IG6062) were from Enzo Life Sciences International, PA, USA. All other materials, unless specified otherwise, were obtained from Wako Pure Chemical Industries, Osaka, Japan.

CELL CULTURE

Human bSMCs were purchased from Lonza Walkersville, MD, USA and cultured in SmGM-2 medium containing 0.5 ng/ml human epidermal growth factor (hEGF), 2 ng/ml human fibroblast growth factor-basic (hFGF-B), 5 μg/ml insulin, 50 μg/ml gentamicin, 50 ng/ml amphotericin, and 5% fetal bovine serum (FBS) (Lonza Walkersville). In brief, the cells were seeded at a density of 2.8×10^4 cells/ml in 100-mm dishes and grown to a subconfluent density at 37℃ in 5% CO_2/95% air. Then, the cells were washed with 30 mmol/L HEPES (Lonza Walkersville) and harvested by treatment with 0.025% trypsin/0.01% EDTA (Lonza Walkersville). Subsequently, trypsin-neutralizing solution (Lonza Walkersville) was added to the cells, and they were centrifuged at 1,000 rpm for 5 minutes. The pellet was then resuspended in the same medium and reseeded in the same conditions. The cells from the 5^{th}-11^{th} passages were used for the following experiments.

COLLECTION OF CELL-CONDITIONED MEDIUM

We cultured bSMCs (2.8×10^4 cells/ml) in SmGM-2 medium containing hEGF, hFGF-B, insulin, gentamicin, amphotericin, and 5% FBS to a confluent monolayer at 37℃ in 5% CO_2/95% air. The confluent monolayer of bSMCs was washed twice with HBSS and then cultured in DMEM without FBS for the indicated times (0-72 hours) according to the method de-

scribed by Shoji *et al*.[19] The medium was collected at 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 3, 6, 12, 24, 48, and 72 hours from the beginning of the culture in DMEM and centrifuged at 2,000 rpm for 5 minutes. Each of the supernatants was used as a bSMC-conditioned medium for performing cell migration assay, gelatin zymography, and western blotting.

CELL MIGRATION ASSAY

We measured bSMC migration toward bSMC-conditioned medium using a modified Boyden chamber assay.[20] bSMC-conditioned medium or FIB was placed in the lower compartment of a 48-well Boyden chamber (Neuro Probe, MD, USA). DMEM was used as a negative control. bSMC suspension (1.0×10^6 cells/ml in DMEM) was added to the upper compartment of the chamber. A polyvinylpyrrolidone-free polycarbonate membrane with 8-μm pore size (Whatman plc, Kent, UK) was placed between the upper and lower compartments. After 6-hours incubation at 37℃ in 5% CO_2/95% air, the cells on the upper surface of the membrane were removed by scraping, and the cells that migrated through the membrane were stained with Diff-Quik® (Sysmex Corp., Hyogo, Japan) and counted in randomized 10 high-power fields (10 HPF, 400× magnification) for each of the triplicate membranes using a light microscope (Nikon Corp., Tokyo, Japan). Cell migration was expressed as the number of migrated cells per 10 HPF in which the background (DMEM: negative control) number of migrating cells per 10 HPF was not subtracted from the total number of migrating cells per 10 HPF.

QUANTITATIVE ASSAY FOR THE CHARACTERIZATION OF β INTEGRIN EXPRESSION

The expression of β integrins on the surface of bSMCs was determined using β integrin-mediated cell adhesion array kit (CHEMICON International, CA, USA). In brief, an aliquot (100 μl) of bSMCs (1.0×10^6 cells/ml in DMEM) were added to each well of the array coated with various β integrin monoclonal antibodies through goat anti-mouse coating antibodies. The negative control wells contained only the coating antibodies. After incubation for 1 hour at 37℃ in 5% CO_2/95% air, these wells were washed 3 times with the assay buffer (200 μl) from the kit. bSMCs that were attached to the wells were stained with a cell staining solution (100 μl) from the kit for 5 minutes at room temperature. Subsequently, bSMCs were washed 4 times with deionized water, and bSMC-bound dyes were completely solubilized by the extraction buffer (100 μl) provided. The amount of solubilized dyes was determined by the absorbance at 540 nm using ImmunoMini NJ2300 (Nalge Nunc International Corp., NY, USA).

INHIBITION BY ANTI-FIB OR ANTI-INTEGRIN ANTIBODIES OF CELL MIGRATION TOWARD THE CELL-CONDITIONED MEDIUM

To determine whether FIB in bSMC-conditioned medium acts as a chemoattractant for bSMCs, bSMC-conditioned medium was preincubated with anti-FIB antibodies (25 μg/ml) for 1 hour at 37℃ and added to the lower compartment of the Boyden chamber, while the bSMC cell suspension (1.0×10^6 cells/ml in DMEM) was placed in the upper compartment of the chamber.

Furthermore, to examine whether integrins are involved in bSMC migration in response to bSMC-conditioned medium, bSMC suspension (0.5×10^6 cells/ml in DMEM) was preincubated with anti-β1 or anti-β3 integrin antibodies (10 μg/ml) for 1 hour at 37℃ and placed in the upper compartment of the Boyden chamber, while the bSMC-conditioned medium was added to the lower compartment of the chamber.

GELATIN ZYMOGRAPHY

The gelatinolytic activity of the bSMC-conditioned medium was analyzed by gelatin zymography, according to a modification of the method of Hibbs *et al*.[21] In brief, bSMC-conditioned medium was concentrated to one-fifth of its original volume and electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (7.5%) containing 1 mg/ml gelatin. After electrophoresis, the gel was washed twice in 50 mM Tris-HCl (pH 7.6) containing 5 mM $CaCl_2$ and 2.5% Triton X-100 for 30 minutes at room temperature and incubated with the same buffer containing 5 mM $CaCl_2$ and 150 mM NaCl for 20 hours at 37℃. Then, the gels were stained with 0.05% Coomassie brilliant blue (CBB) and destained with 20% methanol in 10% acetic acid, and the gelatinolytic activity was identified as transparent bands in the CBB-stained background. The molecular weights of the bands were estimated through the use of molecular-weight markers (Daiichi Pure Chemicals Corp., Tokyo, Japan).

WESTERN BLOTTING

To identify FIB and MMPs in the bSMC-conditioned medium, western blotting was performed. In brief, the bSMC-conditioned medium was concentrated to one-fifth or one-hundredth of its original volume for FIB or MMPs detection, respectively, and electrophoresed on SDS-polyacrylamide gels (7.5%). Bands on the gel were transferred onto nitrocellulose membranes using a semidry blotting apparatus (ATTO Corp., Tokyo, Japan); then, the membranes were incubated in blocking solution of phosphate buffered saline (PBS) containing 0.1% Tween 20 and 2% BSA, washed in 0.1% Tween 20/PBS (PBST) and allowed to react with anti-FIB, anti-MMP-1, and anti-MMP-2 polyclonal antibodies diluted at 1 : 1000, 1 : 20, and 1 : 20, respectively, in blocking solution for 1 hour at

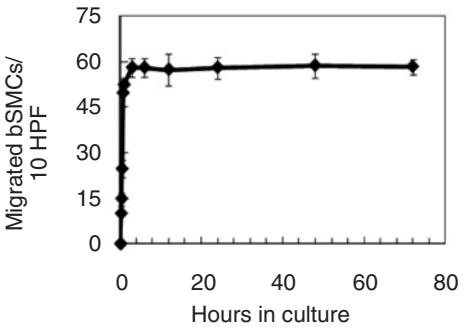

Fig. 1 Bronchial smooth muscle cell (bSMC) migration toward bSMC-conditioned medium. Migrated bSMCs were counted in randomized 10 high-power fields (HPF, 400× magnification). bSMC-conditioned medium was collected at 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 3, 6, 12, 24, 48, and 72 hours after the beginning of the culture in serum-free Dulbecco's modified Eagle medium (DMEM) and used for the migration assay. Background (DMEM: negative control) migration was 2 cells per 10 HPF. Values are represented as mean ± SD (n = 3). Similar results were obtained in three independent experiments.

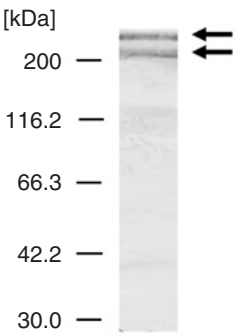

Fig. 2 Analysis of fibronectin (FIB) in bSMC-conditioned medium by western blotting. bSMC-conditioned medium was obtained after 48-hours of incubation in serum-free DMEM, concentrated to one-fifth of its original volume, electrophoresed on 7.5% SDS-polyacrylamide gels, and analyzed using anti-FIB polyclonal antibodies. Similar results were obtained in three independent experiments.

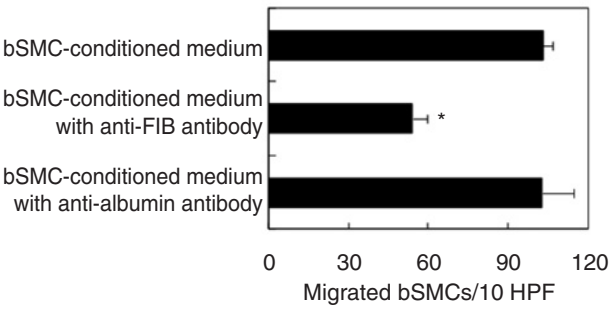

Fig. 3 bSMC migration toward bSMC-conditioned medium treated with anti-FIB or anti-albumin antibodies. The concentration of these antibodies was 25 μg/ml. bSMC-conditioned medium was obtained after 48-hours of incubation in serum-free DMEM. Asterisk (*) indicates a significant difference from the value at bSMC-conditioned medium (P < 0.01, t-test). Background (DMEM: negative control) migration was 28 cells per 10 HPF. Values are represented as mean ± SD (n = 3). Similar results were obtained in three independent experiments.

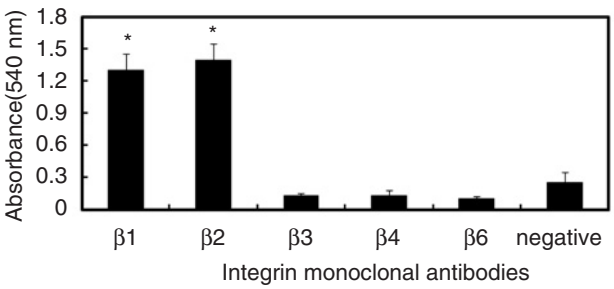

Fig. 4 Expression of β integrins on bSMCs. Aliquots (100 μl) of bSMCs (1.0×10^6 cells/ml) were incubated at 37°C/5% CO_2 in the array of wells coated with various β integrin monoclonal antibodies. After incubation for 1 hour, bSMCs that attached on the array of wells were stained, and the dyes that bound to bSMCs were solubilized and determined by absorbance at 540 nm. Asterisk (*) indicates a significant difference from the value at negative control (goat anti-mouse antibodies only; P < 0.01, t-test). Values are represented as mean ± SD (n = 4).

room temperature. After washing in PBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, PA, USA) diluted at 1 : 1000 or 1 : 160 in PBST for 1 hour at room temperature. After further washing with PBST, visualization of the blots was achieved by incubating with 3,3'-diaminobenzidine (Tokyo Chemical Industry Corp., Tokyo, Japan) in PBS containing 0.3% hydrogen peroxide.

N-TERMINAL AMINO ACID ANALYSIS

The bSMC-conditioned medium was concentrated to one-five hundredth of its original volume and electrophoresed on SDS-polyacrylamide gels (7.5%). Bands on the gel were transferred onto polyvinylidene difluoride membranes using the semidry blotting apparatus. After staining with Ponceau S, the corresponding bands were excised and analyzed on a gas-phase protein sequencer (Life Technologies Corp., CA, USA).

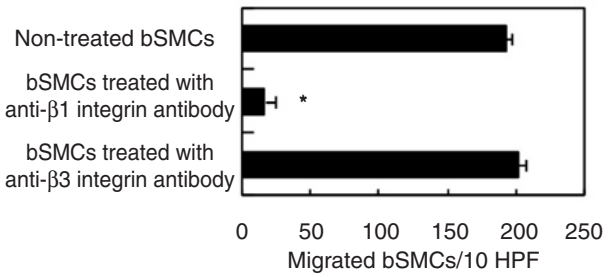

Fig. 5 Migration of bSMCs treated with anti-β1 or anti-β3 integrin antibodies toward bSMC-conditioned medium. The concentration of these antibodies was 7.5 μg/ml. bSMC-conditioned medium was obtained after 48-hours of incubation in serum-free DMEM. Asterisk (*) indicates a significant difference from the value at bSMC-conditioned medium ($P < 0.01$, *t*-test). Values are represented as mean ± SD ($n = 3$). Similar results were obtained in three independent experiments.

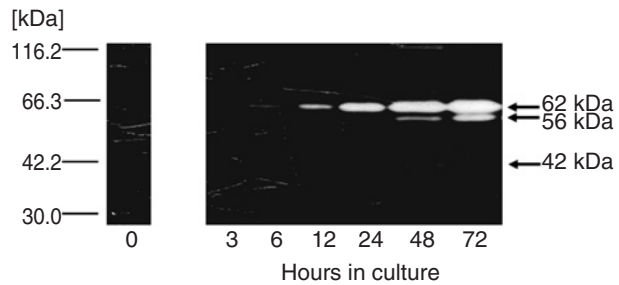

Fig. 6 Analysis of proteases in bSMC-conditioned medium by gelatin zymography. bSMC-conditioned medium was collected at 3, 6, 12, 24, 48, and 72 hours after the initiation of culture in serum-free DMEM, concentrated to one-fifth of its original volume, and electrophoresed on SDS-polyacrylamide gels (7.5%) containing 1 mg/ml gelatin. Bands with gelatinolytic activity were identified as transparent bands on the background of gels stained with Coomassie brilliant blue (CBB, arrows). Similar results were obtained in three independent experiments.

STATISTICAL ANALYSIS

All results were obtained by at least three independent experiments and represented as mean ± SD. Statistical significance was evaluated using Student's *t*-test. A value of $P < 0.01$ was considered statistically significant.

RESULTS

bSMC MIGRATION TOWARD bSMC-CONDITIONED MEDIUM

The bSMC migration toward bSMC-conditioned medium obtained at definite time intervals in culture is shown in Figure 1. bSMC migration toward bSMC-conditioned medium increased with time and, after 3 hours, reached a constant.

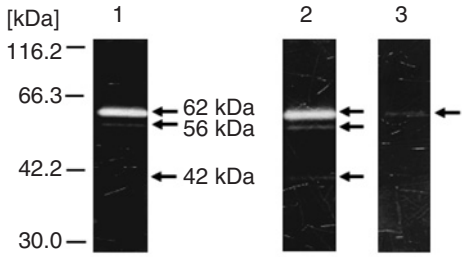

Fig. 7 Classification of proteases in bSMC-conditioned medium by gelatin zymography using protease inhibitors. bSMC-conditioned medium was obtained after 48-hours of incubation in serum-free DMEM, concentrated to one-fifth of its original volume and applied on gelatin zymography in the absence (Lane 1) and presence of inhibitors: phenylmethylsulfonyl fluoride (PMSF, Lane 2) and EDTA (Lane 3). Bands with gelatinolytic activity were identified as transparent bands on the background of the gels stained with CBB (arrows). Similar results were obtained in three independent experiments.

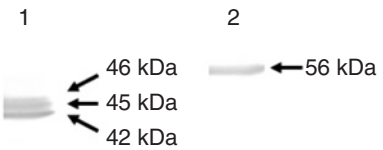

Fig. 8 Analysis of matrix metalloproteinases in bSMC-conditioned medium by western blotting. bSMC-conditioned medium was obtained after 48-hours of incubation in serum-free DMEM, concentrated to one-hundredth of its original volume, electrophoresed on 7.5% SDS-polyacrylamide gels, and analyzed using anti-matrix metalloproteinase (MMP)-1 (Lane 1) and anti-MMP-2 (Lane 2) polyclonal antibodies. Similar results were obtained in three independent experiments.

FIB AS A CHEMOATTRACTANT IN bSMC-CONDITIONED MEDIUM

FIB was detected in a bSMC-conditioned medium incubated for 48 hours in a serum-free medium by western blotting as shown in Figure 2. To examine whether FIB detected in the bSMC-conditioned medium is a chemoattractant for bSMCs, a blocking approach by using anti-FIB antibodies was performed. The concentration of the antibodies added to the bSMC-conditioned medium was examined over a range of 2.5-100 μg/ml; the optimal concentration was determined to be 25 μg/ml because at this level, the antibodies inhibited bSMC migration without nonspecific binding. As shown in Figure 3, bSMC migration to the bSMC-conditioned medium was significantly inhibited by pretreatment of the bSMC-conditioned medium with anti-FIB antibodies at 25 μg/ml ($P < 0.01$) but not by pretreatment with anti-

albumin antibodies at the same concentration.

IDENTIFICATION OF β INTEGRINS ON bSMCS

The quantitative expression of β integrins on bSMC was assayed using a β integrin-mediated cell adhesion array. As shown in Figure 4, significant expression of β integrins such as β1 and β2 was identified ($P < 0.01$), while the expression of β3, β4, and β6 integrins was not identified.

INVOLVEMENT OF β1 INTEGRIN IN bSMC MIGRATION

To examine the involvement of integrins in the migration of bSMCs induced by the bSMC-conditioned medium, we performed blocking by anti-integrin antibodies. The concentration of the antibodies added to the bSMC-conditioned medium was examined over a range of 1-10 μg/ml; the optimal concentration was determined to be 7.5 μg/ml because at this level, the antibodies inhibited bSMC migration without nonspecific binding. As shown in Figure 5, preincubation of bSMCs with anti-β1 integrin antibodies at 7.5 μg/ml markedly abolished their ability to migrate in response to the bSMC-conditioned medium ($P < 0.01$). However, there was no influence on bSMC migration following exposure of the bSMCs to anti-β3 integrin antibodies.

GELATIN ZYMOGRAPHY FOR PROTEASE ANALYSIS

The gelatinolytic activity in bSMC-conditioned medium was analyzed by gelatin zymography. Figure 6 showed two major bands corresponding to 62 and 56 kDa and a minor band corresponding to 42 kDa. The 62-kDa band appeared as a strong active band in bSMC-conditioned medium after 6-hours of incubation and enhanced with time, while the 56-kDa band appeared as a moderate active band at 24 hours and enhanced with time. The 42-kDa band appeared as a fairly weak active band in bSMC-conditioned medium incubated for 48 hours.

The effects of proteinase inhibitors on gelatinolytic activities corresponding to the 42-, 56-, and 62-kDa bands were shown in Figure 7. These activities were inhibited by the metalloproteinase inhibitor, EDTA (lane 3), but not by the serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF, lane 2). These results demonstrate that these 42-, 56-, and 62-kDa proteases are metalloproteinases. Moreover, these gelatinolytic activities were inhibited by another metalloproteinase inhibitor, 1,10-phenanthroline, but not by another serine proteinase inhibitor, diisopropyl fluorophosphate (DFP, data not shown).

WESTERN BLOT ANALYSIS FOR METALLOPROTEINASES

As shown in Figure 8, western blot analysis for metalloproteinases in bSMC-conditioned medium revealed that three metalloproteinases corresponding to 42, 45, and 46 kDa and a metalloproteinase corresponding to 56 kDa were detected by anti-MMP-1 (lane 1) and anti-MMP-2 (lane 2) polyclonal antibodies, respectively. From Figure 7, 8, it is clear that the 45- and 46-kDa metalloproteinases detected by anti-MMP-1 antibodies were gelatinolytically inactive in this experimental system.

N-TERMINAL AMINO ACID SEQUENCING OF 62- AND 56-kDa METALLOPROTEINASES

The N-terminal sequence of the metalloproteinase corresponding to 56 kDa in the bSMC-conditioned medium was Ala-Pro-Leu-Pro-Ile-Ile-Lys-Phe-Pro-Gly, which had a high homology with residues 30-39 of human MMP-2 (Ala-Pro-Ser-Pro-Ile-Ile-Lys-Phe-Pro-Gly). Furthermore, the N-terminal sequence of the metalloproteinase corresponding to 62 kDa in the bSMC-conditioned medium was Ala-Pro-Leu-Pro and in agreement with the N-terminal sequence of 56-kDa metalloproteinase. The determination of only these four amino acid sequences may be due to the fact that the amount of 62-kDa metalloproteinase was too small to perform amino acid sequence analysis, despite the bSMC-conditioned medium being concentrated to one-five hundredth of its original volume.

DISCUSSION

In the present study, we suggested that FIB and MMPs produced from bSMCs are involved in bSMC migration involved in the process of airway remodeling.

bSMCs were cultured to collect bSMC-conditioned medium for up to 72 hours. As shown in Figure 1, bSMC migration toward bSMC-conditioned medium increased with time and, after 3 hours, reached a constant. However, as given in Figure 6, proteinases corresponding to 42, 56, and 62 kDa were all detected in the bSMC-conditioned medium cultured for 48 hours. Therefore, we used the bSMC-conditioned medium cultured for 48 hours in the present experiments.

First, FIB was detected as a chemoattractant in the bSMC-conditioned medium that induced bSMC migration (Fig. 1-3). Two bands detected in Figure 2 may be derived by the cleavage of S-S bonds that associate two polypeptides to form the FIB molecule. These two bands have also been detected in western blot analysis of fibronectin synthesized by human dermal fibroblasts.[22]

The expression of β1 and β2 integrins on bSMCs was detected by the β integrin-mediated cell adhesion array as shown in Figure 4. It was reported that integrins α1β1, α2β1, α3β1, α4β1, α5β1, αvβ1, and αvβ3 that express on airway SMCs bind to fibronectin.[23] Freyer and others[24] confirmed that α5, αv, and β1 integrin subunits were universally expressed on airway SMCs, and that nearly half of identical cells were α6-positive. Less than one third of

cells had detectable levels of α1, α3, and α4 integrins. On the other hand, Nguyen and others[25] reported that subconfluent growth-arrested airway SMCs expressed low (<30%) levels of α1 and α6 integrins, intermediate (30-70%) levels of α4 and αv integrins, and high (>70%) levels of α2, α5, β1 and β2 integrins. A subpopulation of cells (～10%) expressed high levels of α3, but in the remainder, the expression was low, while β3 and β4 integrin expression was not detected. We used ～70-80% confluent bSMCs for performing the migration assay and the detection of integrins.

It is probable that the bSMCs used in this experiment are synthetic phenotypes because of subculture with trypsin treatment and serum stimulation. In addition, these bSMCs are derived from human fetal lineages and preserved for shipments after 3 or 4 passages. It is speculated that transformed SMCs from contractile to synthetic phenotype have high migratory and proliferative activities, leading to diseases with epithelium or endothelium injury such as asthma and arteriosclerosis.

bSMC migration toward the bSMC-conditioned medium was inhibited by pretreatment with anti-β1 integrin antibodies but not by anti-β3 integrin antibodies (Fig. 5), suggesting that β3 integrin may not contribute to bSMC migration induced by FIB in the bSMC-conditioned medium because of its low expression. Present data also indicated that bSMC migration toward the bSMC-conditioned medium was inhibited almost completely by pretreatment with anti-β1 integrin antibodies; however, ～70% inhibition was noted following pretreatment with anti-FIB antibodies. These facts suggest that chemoattractants other than FIB, which are recognized by β1 integrin, may be present in the bSMC-conditioned medium. Since it has been reported that β1 integrin recognizes not only FIB but collagen type I and laminin as well,[23] the detection of collagen type I and laminin in bSMCs-conditioned medium were attempted by western blotting. However, there was little or no detection of these chemoattractants in the bSMC-conditioned medium (data not shown). It has been demonstrated that airway SMC migration is elicited by IL-1β, IL-8, and PDGF-BB.[26,27] As IL-1β, IL-8, and PDGF-BB are expressed by airway SMCs,[16] these immunomodulatory molecules might be present in the bSMC-conditioned medium.

FAK and p42/44 MAPK are known to be involved in vascular SMC migration stimulated by FIB.[28] p42/44 MAPK, Rho, and p38 signals have been shown to be crucial for airway SMC migration by urokinase plasminogen activator (uPA).[29] These signal molecules may play an important role in bSMC migration toward bSMC-derived FIB.

Proteinases corresponding to 42, 56, and 62 kDa detected in the bSMC-conditioned medium were determined to be metalloproteinases (Fig. 7), and anti-MMP-1 and anti-MMP-2 antibodies recognized the 42- and 56-kDa metalloproteinases, respectively (Fig. 8). However, these antibodies did not recognize the 62-kDa metalloproteinase, despite the bSMC-conditioned medium being highly concentrated. This strongly active 62-kDa metalloproteinase might also be MMP-2, the amount of which was lesser than that of the 56-kDa metalloproteinase in the bSMC-conditioned medium. It is known that MMP-2 is activated by autolysis at a rate that is concentration-dependent, processing not only the N-terminal domain but also the C-terminal domain.[30,31] Atkinson *et al*. reported that human proMMP-2 was partially cleaved by the cell membrane to yield the 59-kDa form via the 62-kDa form, but they were unable to determine the precise cleavage site.[32] Lindstad *et al*. showed that trypsin-mediated activation of human proMMP-2 resulted in the formation of 62-, 56-, 52-, and 50-kDa forms.[33] In addition, they suggested that the 62-kDa form was generated by truncating the N-terminal propeptide domain of 72-kDa proMMP-2; subsequently, 56-, 52- and 50-kDa forms were generated by truncating the C-terminal hemopexin domain of the 62-kDa form. However, our study proposes that the 62- and 56-kDa metalloproteinases contained in the bSMC-conditioned medium might be due to 10- and 16-kDa cleavages in the hemopexin domain of proMMP-2, respectively.

This proposal was verified by western blotting using antibodies that recognize the propeptide domain of MMP-2 (M5928, Sigma) and by N-terminal amino acid sequencing of both 56- and 62-kDa metalloproteinases. In western blotting analysis of the bSMC-conditioned medium, the 56-kDa metalloproteinase was immunoreactive for anti-MMP-2 antibodies that recognize a hinge region located between the catalytic and C-terminal hemopexin domains (Fig. 8). Furthermore, this 56-kDa metalloproteinase was immunoreacted with anti-MMP-2 antibodies that recognize the propeptide domain (data not shown). In the analysis of N-terminal amino acid sequences, the 56- and 62-kDa metalloproteinases in the highly concentrated bSMC-conditioned medium were compared with that of human MMP-2 in a public database (NCBI P08253). The N-terminal sequence of 10 amino acid residues of the 56-kDa metalloproteinase was in fair agreement with residues 30-39 of human MMP-2. Moreover, the N-terminal sequence analysis of the 62-kDa metalloproteinase exactly coincided with that of 56-kDa metalloproteinase.

The disagreement between the N-terminal sequences of these two metalloproteinases and residues 30-39 within human MMP-2 was observed at residue 32, where Ser was replaced by Leu. This disagreement of MMP-2 might be induced by a genetic mutation. Ser32 within human MMP-2 is encoded by the DNA sequence TCG (405-407, NCBI NM_004530.4) whereas Leu is encoded by TTG. It is known that a G=C → A=T point mutation in the DNA

molecule is the most frequent one in human cells and cancer.[34,35]

It has been reported that cell adhesion to fibronectin through integrin induces the production of MMP-2 as well as the downregulation of TIMP-2.[36] This finding also suggests that the adhesion to fibronectin transduced both stimulatory (through Src-type tyrosine kinases) and inhibitory signals (through Ras/MAPKinase signaling pathways) for MMP-2 expression and that their relative predominance was regulated by additional stimuli related to cell adhesion, motility, and growth.

These findings suggest that FIB and metalloproteinases containing MMPs-1 and 2 produced from bSMCs lead to the stimulation of bSMC migration, implying that this behavior may be an important process in airway remodeling in asthma. Suppression of bSMC migration by a β1 integrin antagonist or MMP-1 and -2 inhibitors might offer a new therapeutic direction against bronchial remodeling in asthma.

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