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Signal Transduction in Matrix Contraction and the Migration of Vascular Smooth Muscle Cells in Three-Dimensional Matrix

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Key Words
Extracellular matrix · Force generation · Migration · Rho · Signal transduction · Smooth muscle cells · Three-dimensional matrix

Abstract
The interaction of vascular smooth muscle cells (SMCs) and extracellular matrix plays important roles in vascular remodeling. We investigated the signaling pathways involved in SMC-induced matrix contraction and SMC migration in three-dimensional (3D) collagen matrix. Matrix contraction is inhibited by the disruption of actin filaments but not microtubules. Therefore, we investigated the roles of signaling pathways related to actin filaments in matrix contraction. SMC-induced matrix contraction was markedly blocked (–80%) by inhibiting the Rho-p160ROCK pathway and myosin light chain kinase, and was decreased to a lesser extent (30–40%) by a negative mutant of Rac and inhibitors of phosphatidylinositol 3-kinase (PI 3-kinase) or p38 mitogen-activated protein kinase (MAPK), but it was not affected by the inhibition of Ras and Cdc42-Wiskott-Aldrich syndrome protein (WASP) pathways. Inhibition of extracellular-signal-regulated kinase (ERK) decreased SMC-induced matrix contraction by only 15%. The migration speed and persistence of SMCs in the 3D matrix were decreased by the inhibition of p160ROCK, PI 3-kinase, p38 MAPK or WASP to different extents, and p160ROCK inhibitor had the strongest inhibitory effect. Our results suggest that the SMC-induced matrix contraction and the migration of SMCs in 3D matrix share some signaling pathways leading to force generation at cell-matrix adhesions and that various signaling pathways have different relative importance in the regulations of these processes in SMCs.

Introduction

The interaction between smooth muscle cells (SMCs) and extracellular matrix (ECM) plays important roles in vascular remodeling under physiological and pathological conditions [1, 2]. Vascular remodeling such as restenosis involves the proliferation and migration of SMCs, the synthesis of ECM, and the inward constrictive remodeling of blood vessel walls [3, 4]. While the molecular events leading to SMC proliferation and matrix synthesis during restenosis have been extensively studied, the mechanisms involved in the constrictive remodeling and SMC migra-

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tion in three-dimensional (3D) matrix are less understood. From the perspective of vascular tissue engineering, artificial blood vessels can be reconstructed with collagen and vascular cells [5, 6], and it is necessary to understand the interactions between SMCs and ECM in 3D collagen matrix.

Unlike membrane ruffling in two-dimensional (2D) models, cells in 3D matrix extend pseudopodia following matrix fibrils (contact guidance). SMCs in 3D collagen matrix had less stress fibers, less focal adhesions and cell spreading, and a lower level of tyrosine phosphorylation of focal adhesion kinase than SMCs in 2D culture [7]. During migration, SMCs can exert forces on ECM molecules through integrins to facilitate migration, and at the same time regulate ECM assembly and contraction [8–10]. The interactions between SMCs and ECM may contribute to the constrictive remodeling of the vessel wall. However, the signal transduction pathways leading to the forces exerted on ECM by SMCs and SMC migration in 3D matrix are not well characterized.

Small GTPase Rho regulates cell contractility and the formation of focal adhesions and actin stress fibers [11–14]. Although there are studies on the roles of Rho and its downstream effector p160ROCK in Ca^{2+} sensitization and vasoconstriction (an immediate response within seconds or minutes [15–17]) there is a lack of study on the role of Rho-mediated signaling in SMC-induced matrix contraction (a long-term event involving hours or days of matrix remodeling). Other Rho family small GTPases, e.g. Rac and Cdc42, have differential functions in regulating the actin-based cytoskeletal structure and cell migration [13, 14]. Rac induces lamellipodia formation and membrane ruffles, as well as peripheral actin structure [18–20]. Cdc42 regulates the filopodia formation [21, 22]. Many downstream effectors of Rho family GTPases that regulate cytoskeletal organization have been identified. Phosphatidylinositol 3-kinase (PI 3-kinase) can stimulate Rac activity [23] and mediate the Rac-induced cell migration [24]. Wiskott-Aldrich syndrome protein (WASP) can be activated by Cdc42, and it regulates actin branching and elongation [25–27]. Small GTPase Ras relays the signals from cell surface receptors (e.g. integrins and receptor tyrosine kinases) to mitogen-activated protein kinases (MAPKs) to regulate cell growth, differentiation, migration, and apoptosis [28–30]. It has been shown that inhibition of Ras decreases the proliferation of SMCs after vascular injury [31–33]. However, the roles of Rac, Cdc42 and Ras in SMC-induced matrix contraction and SMC migration in 3D matrix remain to be determined.

MAPKs, including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAPK, play critical roles in cell proliferation, apoptosis, and migration [34–36]. MAPKs are important mediators of signal transduction induced by Ras and Rho GTPases. For example, ERK can be activated by Ras and MAPK kinase (MEK) to regulate cell proliferation [34–36]. ERK can also regulate the phosphorylation of myosin light chain (MLC) and the fibroblast-induced matrix contraction [37]. JNK and p38 MAPK can be activated by Cdc42 and Rac [38–40] to mediate the activity of transcriptional factors. Activation of p38 MAPK also leads to phosphorylation of heat shock protein 27 (HSP27), which enhances actin polymerization [41].

In this study, we used various negative mutants and inhibitors to interfere with the signaling pathways involved in the regulation of actin cytoskeleton, and demonstrated the differential roles of Rho family GTPases, Ras and MAPKs in SMC-induced collagen matrix remodeling and SMC migration in 3D collagen matrix.

Materials and Methods

Cell Culture

To isolate bovine aortic SMCs (BASMCs), the inner surface of bovine aortas was scraped with a surgical knife to denude the endothelial cells, and small pieces of the underlying tissue (~1 mm thick) were removed for culture in 6-well plates to enable the emigration of SMCs. Immunostaining showed that these cells expressed smooth muscle (SM) α-actin, SM myosin heavy chain and calponin (data not shown). Cell culture reagents were obtained from GibcoBRL (Grand Island, N.Y., USA) unless otherwise specified. The cells were cultured in a complete medium that included Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and 1 m*M* penicillin-streptomycin. Cell cultures were maintained in a humidified 95% air-5% CO_2 incubator at 37°C. All experiments were conducted with cultures prior to passage 8 (with split ratio 1:3 for each passage).

3D Culture and Measurement of Matrix Contraction

Collagen gel contraction is an accepted in vitro model for studying matrix remodeling. In most of our experiments, SMCs were cultured in 0.1% collagen gels with 10% FBS. Collagen gels were prepared by mixing 25% of 4 mg/ml rat tail collagen I (Fisher Scientific), 5% of 0.1 *M* NaOH, 40% of 2× DMEM, 8% of FBS, 2% water, and 20% of complete medium with SMCs (~1 million cells/ml collagen gel). Collagen gels without FBS were prepared by replacing FBS with DMEM. The mixture of collagen gels and SMCs was cast into 12-well plates (0.5 ml/well). For gel contraction assay, the culture wells were pre-blocked with 1% bovine serum albumin (BSA) to facilitate the detachment of collagen gels. After polymerization for 30 min at 37°C, the collagen gels were allowed to undergo free contraction in the presence or absence of inhibitors. The collagen gel images were recorded with a CCD camera and NIH Image software. The areas of the gels were measured with the NIH Image software, and the ratio of

contraction was calculated as (Ao – A)/Ao, where Ao is the area before contraction and A is the area after contraction. For statistical analysis, ANOVA was performed to determine whether there was a significant difference between groups, followed by post-hoc tests.

DNA Constructs and Recombinant Proteins

The uses of adenoviruses carrying the negative mutant Ras(N17) and β-galactosidase gene (LacZ) were described previously, and the infection efficiency was >90% for SMCs [32]. Rho inhibitor C3 exoenzyme was from CalBiochem (La Jolla, Calif., USA). Plasmids containing GST-linked Rac1(N17) and Cdc42(N17) were kindly provided by Dr. Anne Ridley (Ludwig Institute for Cancer Research, London, UK). Recombinant GST-proteins were expressed in *Escherichia coli* and purified as described [11, 18]. LipofectAmine (GibcoBRL) was used to facilitate the recombinant proteins to penetrate the cell membrane [42]. Briefly, the recombinant proteins were preincubated with LipofectAmine (5 μl LipofectAmine/5 μg recombinant proteins per 10 cm^2 of culture area) for 30 min in DMEM, and then applied to the SMCs for 4 h. The cells were allowed to recover in the complete medium with 10% FBS for 1 h prior to the experiments. With this method, we can achieve >90% efficiency, and the effects of the recombinant proteins on cell morphology (same as those reported previously) [11, 18] lasted for at least 20 h (data not shown).

Chemical Inhibitors

Cytochalasin D and colchicine were from Sigma. PI 3-kinase inhibitors Ly294002 and wortmannin, N-WASP inhibitor, MEK inhibitor PD98059, p38 MAPK inhibitor SB202190, JNK inhibitor II, tyrosine kinase inhibitor genistein and calcium chelators EGTA and BAPTA/AM were from CalBiochem (San Diego, Calif., USA). Specific p160ROCK inhibitor Y27632 was kindly provided by Yoshitomi Pharmaceutical Industries, Japan. In pilot experiments, different concentrations of inhibitors (based on the inhibitory concentrations recommended by the manufacturers) were used to test their effects on cell morphology, cytoskeleton and protein activity. The effective concentrations used in our experiments were similar to those used in other studies on SMCs in the literature.

Immunoblotting

The cells in collagen matrix were lysed in a 4× lysis buffer containing 80 m*M* Tris, pH 7.4, 0.6 *M* NaCl, 4% Triton X-100, 0.4% SDS, 4 m*M* PMSF, 40 μg/ml leupeptin, 40 m*M* NaF, and 4 m*M* Na_3VO_4. The collagen gels were homogenized with douncers. The lysates were centrifuged at 12,000 rpm for 10 min, and the supernatants were loaded onto a gel in 2% SDS sample buffer, and separated by SDS-polyacrylamide gel electrophoresis. Proteins in the gel were transferred to a nitrocellulose membrane. The membrane was blocked with 3% nonfat milk followed by incubation with the primary antibody in TTBS (50 m*M* Tris-HCl, pH 7.4, 120 m*M* NaCl, and 0.05% Tween 20) containing 0.1% BSA. The bound primary antibody was detected using an anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and the ECL detection system (Amersham, Arlington Heights, Ill., USA). The monoclonal antibody against phospho-specific p44/42 MAPK (ERK1 and ERK2) was from New England Biolabs (Beverly, Mass., USA). The polyclonal antibodies against actin and ERK2/ERK1 were from Santa Cruz Biotechnology.

Actin Staining and Confocal Microscopy

The cells in collagen matrix were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 10 min. For actin staining, the specimens were stained with FITC-conjugated phalloidin (5 U/ml, Molecular Probes, Eugene, Oreg., USA) for 1 h. The images of actin structure were collected as Z-series sections by using a Leica confocal microscopy system with argon and He/Ne laser sources, a TCL-SL scanner, and a Leica DM IRB microscope. Multiple sections (0.5-μm thick for each section) were projected onto one plane for presentation. FITC was excited at a wavelength of 488 nm and detected within a band between 506 and 538 nm.

Cell Migration in 3D Matrix

SMC migration in 3D matrix was monitored by time-lapse phase-contrast microscopy using a Nikon inverted fluorescent microscope (TE300) with 10× objectives. A temperature hood was built around the microscope to maintain it at 37°C during experiments. Cell culture medium was replaced with CO_2-independent DMEM (from GibcoBRL) with 10% FBS in the absence or presence of inhibitors. The CO_2-independent DMEM was composed of mono- and di-basic sodium phosphate and a small amount of sodium bicarbonate. Phase-contrast images were collected using a Hamamatsu Orca100 cooled digital CCD camera at 10-min intervals and transferred directly from a frame grabber to computer storage using C-Imaging System software (Compix, Cranberry Township, Pa., USA). A scanning stage with controls in X, Y and Z directions allowed the collection of images from different areas of the samples at different focus planes automatically. Dynamic motion of individual cells was analyzed by using the Dynamic Image Analysis System (DIAS; Solltech, Oakdale, Iowa, USA). Dividing cells and cells in contact with each other during migration were excluded. The cells that went out of focus plane were also excluded. By using the DIAS program, the XY centroids of the cells were determined from the cell boundaries in the phase-contrast images. The path of each cell was generated from the XY centroids at different time points, and the migration distance, migration speed and migration direction of cells were quantified. The persistence of cell migration was defined as migration distance/change of direction/time interval.

Results

The SMC-Induced Matrix Contraction Is Serum Dependent

A piece of collagen gel after contraction is shown in figure 1a as an example. The time course of gel contraction is shown in figure 1b. The rate of gel contraction was serum dependent. DMEM with 10% FBS induced 80% contraction (by area) after 8 h. Thereafter, the contraction rate diminished and the degree of contraction reached a plateau. The decrease in contraction rate may be related to the rise in contraction resistance due to the increase in density of collagen fibrils after contraction. In the following experiments, we examined collagen gel contraction in the presence of 10% FBS, and the gel contraction was

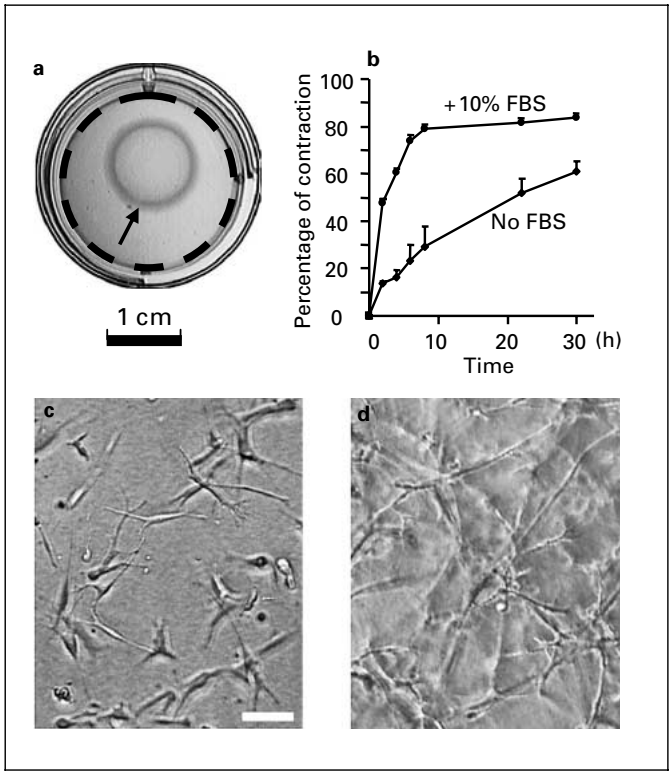

Fig. 1. The contraction of collagen gels by SMCs. **a** Contraction of a collagen gel. The dashed line indicates the original size of the gel. The arrow indicates the gel after contraction. **b** The time course of collagen gel contraction in the presence and absence of serum. Same number of cells was cultured in the collagen gels with media containing 10% or no FBS, and the percentage of gel contraction was monitored for 30 h. Data represent means ± SD from three experiments. **c** BASMCs cultured in collagen matrix before contraction. **d** BASMCs cultured in collagen matrix after contraction. **c**, **d** Bar = 30 μm.

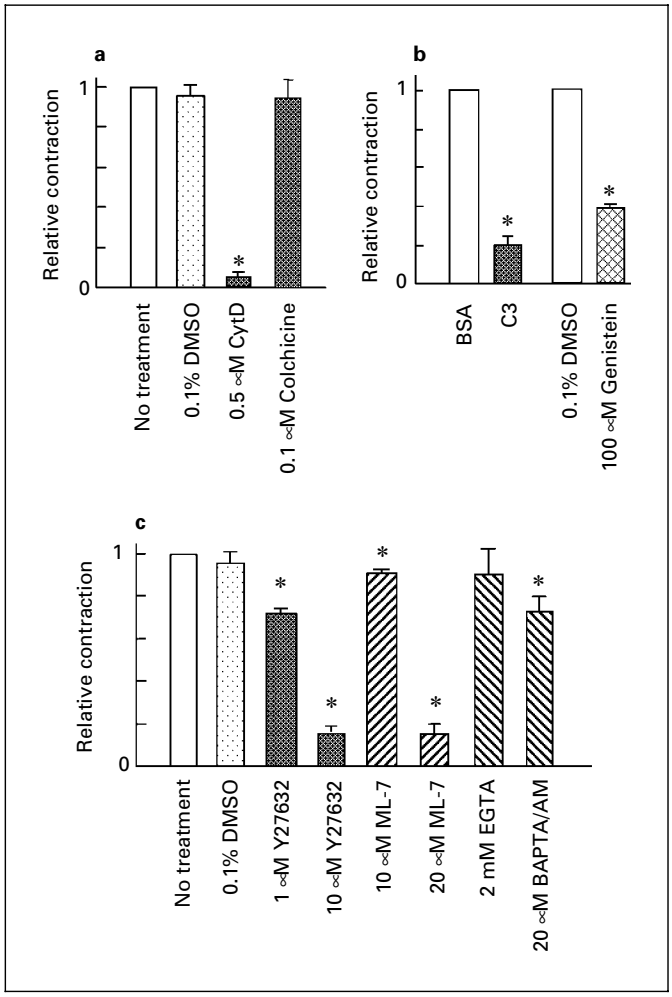

Fig. 2. Roles of cytoskeleton, Rho-p160ROCK, MLCK and calcium in the SMC-induced contraction of collagen matrix. **a** SMCs in 3D matrix were treated with cytochalasin D (CytD), colchicine or DMSO (solvent control), and SMC-induced matrix contraction was determined after 8–12 h. **b** BASMCs treated with C3 exoenzyme or BSA (as control) were cast into collagen gels. In parallel, SMCs in 3D matrix were treated with tyrosine kinase inhibitor genistein or DMSO. The SMC-induced matrix contraction was measured after 8–12 h. **c** SMCs in 3D matrix were treated with p160ROCK inhibitor Y27632, MLCK inhibitor ML-7, calcium chelators EGTA or BAPTA/AM, or DMSO, and SMC-induced matrix contraction was determined after 8–12 h. **a**–**c** Bars represent means ± SD from three experiments. The degree of contraction was normalized with the mean area of the control samples in the respective experiments. * $p < 0.05$ vs. control samples.

measured after 8–12 h of contraction. Representative microphotographs of SMCs cultured in collagen matrix before and after contraction are shown in figure 1c and 1d, respectively.

Rho-p160ROCK and MLC Kinase Mediate the Force Generation through Actin Filaments during Matrix Contraction

To determine the cytoskeleton components required for matrix contraction, actin filaments and microtubules were disrupted by cytochalasin D and colchicine, respectively (fig. 2a). Cytochalasin D, but not colchicines, blocked the SMC-induced matrix contraction by 90% (compared with DMSO-treated control), suggesting that force generation through actin filaments is necessary for matrix contraction. Since Rho-mediated signaling exerts major influences on actin assembly and contractility, we determined the role of Rho in the SMC-induced matrix contraction by treating BASMCs with C3 exoenzyme, a specific Rho inhibitor. C3 exoenzyme disrupted actin

stress fibers and the cells had elongated filopodia (data not shown) in comparison with control (cells treated with BSA). As shown in figure 2b, C3 exoenzyme blocked the contraction by 80%, suggesting that Rho plays a major role in regulating the SMC-induced matrix contraction. Tyrosine kinases have been shown to regulate Rho activity and mediate Rho function [43]. Genistein, a general tyrosine kinase inhibitor, decreased the SMC-induced matrix contraction by 60%, suggesting that tyrosine kinases may be involved in the Rho-induced matrix contraction.

Rho controls the actin cytoskeleton through the downstream effectors p160ROCK and mDia proteins [13, 16, 44]. Ca^{2+}-independent activation of p160ROCK inhibits MLC phosphatase, thus enhances MLC phosphorylation by increasing Ca^{2+} sensitization in SMCs [15–17]. Treatment of BASMCs in collagen gels with Y27632, a specific inhibitor for the Rho effector p160ROCK, inhibited matrix contraction by 80% at 10 μM (fig. 2c). These results suggest that Rho may function through p160ROCK and the actin cytoskeleton to regulate matrix contraction.

In parallel with Rho-p160ROCK pathway, MLC kinase (MLCK) regulates the phosphorylation of MLC and the contractility of stress fibers in a Ca^{2+}-dependent or Ca^{2+}-independent manner [45]. ML-7, an MLCK inhibitor, inhibited matrix contraction by 80% at 20 μM (fig. 2c). This result, together with the data on the Rho-p160ROCK pathway, suggests that MLC phosphorylation is a key event in the force generation and matrix contraction. We further determined whether extracellular and/or intracellular calcium was required in matrix contraction. EGTA, an extracellular calcium chelator, had no significant effect on matrix contraction (fig. 2c). A cell-permeable calcium chelator BAPTA/AM attenuated matrix contraction. These results suggest that intracellular but not extracellular calcium is involved in the SMC-induced matrix contraction. Since the inhibitory effect by BAPTA/AM was not dramatic, intracellular Ca^{2+} concentration may not be the limiting factor, and some calcium-independent pathway (e.g. Rho-p160ROCK) may be more important in matrix contraction.

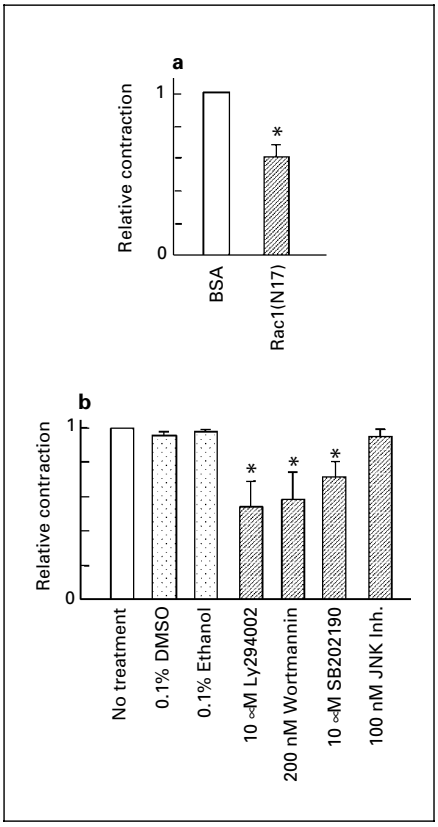

Fig. 3. Roles of Rac, PI 3-kinase, p38 MAPK and JNK in SMC-induced matrix contraction. **a** BASMCs treated with dominant negative mutant Rac1(N17) or BSA (as control) were cast into collagen gels. The SMC-induced matrix contraction was measured after 8–12 h. **b** SMCs in 3D matrix were treated with PI 3-kinase inhibitors Ly294002 or wortmannin, p38 MAPK inhibitor SB202190, JNK inhibitor (Inh) II, or vehicle solvents (DMSO or ethanol). SMC-induced matrix contraction was determined after 8–12 h. **a**, **b** Bars represent means ± SD from three experiments. The degree of contraction was normalized with the mean area of the control samples in the respective experiments. * $p < 0.05$ vs. control samples.

PI 3-Kinase, Rac and p38MAPK Are Involved in the SMC-Induced Matrix Contraction

To determine the role of Rac-mediated signaling in the SMC-induced matrix contraction, BASMCs were treated with recombinant protein Rac1(N17) or BSA (as control) in the presence of LipofectAmine (to transiently permeabilized the cell membrane). By treating SMCs with FITC-BSA (from Sigma), we showed that more than 90% of the cells had FITC-BSA in the cytosol (data not shown). As shown in figure 3a, the negative mutant Rac1(N17) decreased the SMC-induced matrix contraction by 40%, suggesting that Rac plays a significant role in the SMC-induced matrix contraction. PI 3-kinase has been shown to stimulate Rac activity [23]. In concert with our finding that negative mutant of Rac inhibited matrix contraction, inhibition of PI 3-kinase with Ly294002 or wortmannin decreased SMC-induced matrix contraction by approximately 40% (fig. 3b). In contrast, inhibition of Cdc42 and N-WASP by Cdc42(N17) and an N-WASP inhibitor caused a decrease in filopodia at cell periphery, but had

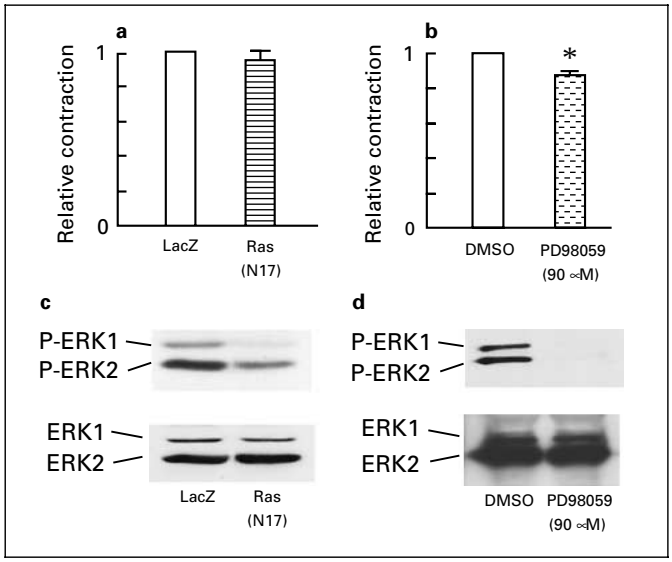

Fig. 4. Roles of Ras and ERK in the SMC-induced contraction of collagen matrix. **a** Role of Ras in matrix contraction. The same number of BASMCs expressing LacZ (as control) or Ras(N17) were cast into collagen gels, and the matrix contraction was measured after 8–12 h. **b** Role of ERK in matrix contraction. BASMCs were cast into collagen gels and allowed to contract in the presence of DMSO (vehicle control) or the MEK inhibitor PD98059 (90 μM). Matrix contraction was measured after 8–12 h. **c**, **d** Cells from each sample were lysed and used for immunoblotting with either an antibody against phospho-specific p44(ERK1)/p42(ERK2) MAPK or an antibody against ERK2/ERK1. **a**, **b** Bars represent means ± SD from three experiments. The degree of contraction was normalized with the mean area of the control samples in the respective experiments. * $p < 0.05$ vs. control samples.

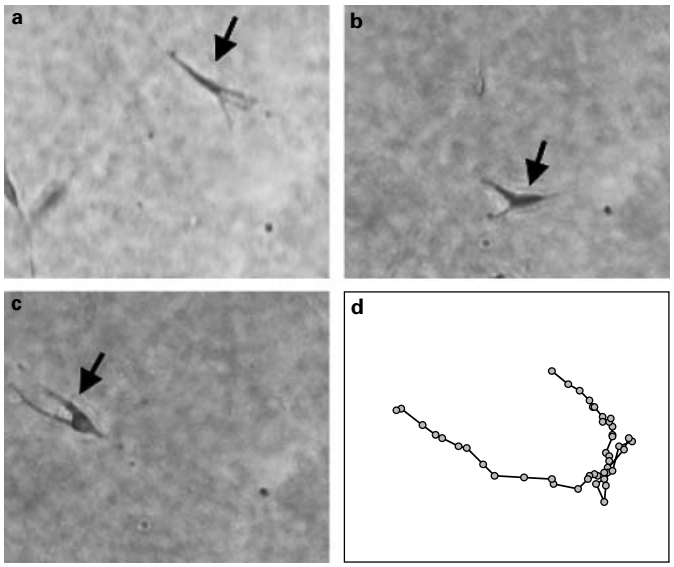

Fig. 5. Monitoring SMC migration in 3D matrix. SMCs were cultured in 3D adherent collagen matrix for 3 h, and SMCs were kept in CO_2-independent DMEM with 10% FBS. Phase-contrast images were taken at 10-min intervals. **a**–**c** Images of a migrating cell taken at t = 0, 12 and 15 h, respectively. The centroid position of the cell at each moment was determined and the path of the cell migration was reconstituted using DIAS software. **d** Path of the cell shown in **a**–**c**. Each dot represents the centroid position of the cell at the moment, and the time interval between the adjacent two dots is 20 min.

no effect on matrix contraction (data not shown), indicating that the Cdc42/N-WASP-induced actin branching is not required in matrix contraction.

p38 MAPK can be activated by Rac to induce the phosphorylation of HSP27 and enhancement of actin polymerization [40, 41]. Inhibition of p38 MAPK with SB202190 attenuated the SMC-induced matrix contraction by ~30% (fig. 3b), suggesting that p38 MAPK is involved in SMC-induced matrix contraction. In contrast, inhibition of JNK had no effect on matrix contraction (fig. 3b).

Inhibition of ERK but Not Ras Slightly Decreased Matrix Contraction

Ras has been shown to be involved in SMC proliferation and restenosis [31–33, 46]. Ras can also function through Rac to induce membrane ruffling and transformation of fibroblasts [18, 47, 48], and PI 3-kinase could mediate the signaling from Ras to Rac [23, 49–51]. To determine the role of Ras in matrix contraction, BASMCs were infected with adenovirus carrying the negative mutant Ras(N17) or LacZ (as a control), which had >90% infection efficiency [32]. ERK phosphorylation was suppressed by Ras(N17) (fig. 4c), indicating the effectiveness of the expressed exogenous proteins. SMC-induced matrix contraction was not affected by Ras(N17) (fig. 4a), suggesting that Ras is not necessary for SMC-induced matrix contraction.

To determine whether ERK plays a role in the SMC-induced matrix contraction, BASMCs were treated with the MEK inhibitor PD98059. As shown in figures 4b and 4d, although PD98059 completely blocked ERK phosphorylation, it decreased the matrix contraction by only 15%, suggesting that ERK only plays a minor role in the SMC-induced matrix contraction, which is in contrast to the fibroblasts-induced matrix contraction [37].

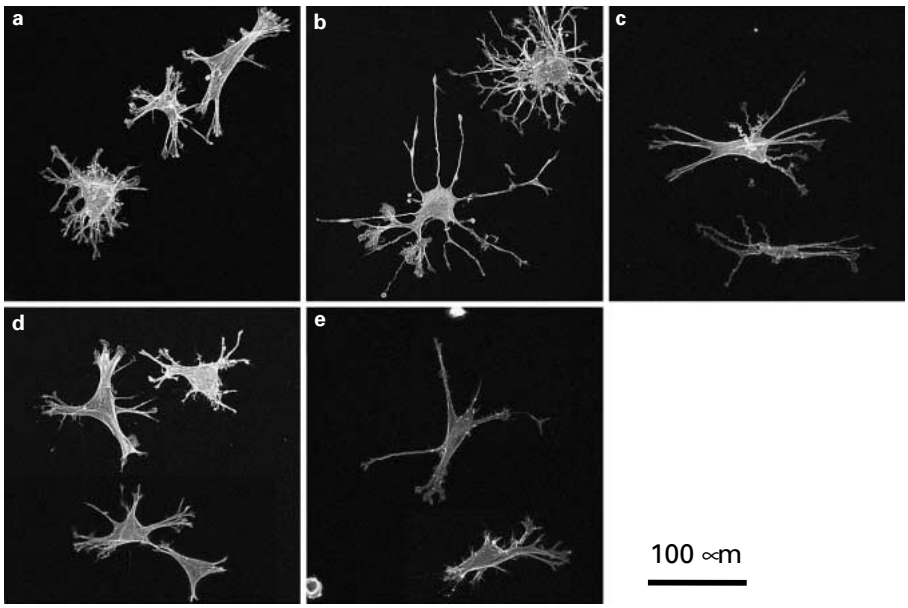

Fig. 6. Effects of inhibitors on the actin structure of SMCs in 3D matrix. SMCs were cultured in 3D matrix in the absence or presence of inhibitors for 16 h. Then the cells were fixed and stained for actin with FITC-phalloidin. The actin structure was visualized by confocal microscopy. **a** SMCs in 3D matrix without inhibitor. **b** SMCs treated with p160ROCK inhibitor Y27632 at 10 μ*M*. **c** SMCs treated with PI-3K inhibitor Ly294002 at 10 μ*M*. **d** SMCs treated with p38 MAPK inhibitor SB202190 at 10 μ*M*. **e** SMCs treated with N-WASP inhibitor at 5 μ*M*. SMCs treated with DMSO or ethanol as solvent controls did not show significant difference compared with **a** (not shown).

p160ROCK, PI 3-Kinase, p38 MAPK and N-WASP Are Involved in SMC Migration in 3D Matrix

Rho-, Rac- and Cdc42-mediated signaling has been shown to regulate cell migration [14, 52, 53]. We selected representative signaling molecules from each pathway to study their roles in SMC migration in 3D matrix. SMC migration in 3D matrix was monitored by time lapse phase-contrast microscopy. As shown in figure 5, the path of a cell was reconstituted from the image series, and the speed and persistence of cell migration can be calculated.

We used inhibitors of p160ROCK, PI 3-kinase, p38 MAPK and N-WASP to interfere with their respective signaling pathways. SMCs in 3D matrix had less spreading but more filopodia (fig. 6a) when compared with those on 2D matrix [54]. p160ROCK inhibitor Y27632 inhibited central stress fibers, but the cells showed more filopodia elongating into different directions (fig. 6b). Inhibition of PI 3-kinase decreased cell spreading but enhanced filopodia extension (fig. 6c), while p38 MAPK inhibitor did not induce significant change in actin structure or cell morphology (fig. 6d). The N-WASP inhibitor decreased filopodial extension at cell periphery (fig. 6e).

SMC migration in 3D matrix showed different characteristics from that on 2D matrix. SMC had multiple filopodial protrusions in many directions during migration, and many of the filopodia detached from the cell body during migration (data not shown), suggesting that the detachment at the rear might be critical during SMC migration in 3D matrix. Indeed, among the inhibitors, p160ROCK inhibitor had the most dramatic inhibition on SMC migration speed (–50%) and persistence (–75%; fig. 7). These data suggest that stress fibers and cell contractility may be necessary for the retraction of filopodia at the rear. Inhibition of PI 3-kinase, p38 MAPK or N-WASP decreased migration speed and persistence by 20–30%, suggesting that cell spreading and filopodial protrusion are involved, but they play less important roles in the SMC migration in 3D matrix. We also showed that the inhibition of ERK and JNK did not significantly affect SMC migration in 3D matrix (data not shown).

Discussion

Matrix contraction and cell migration are two coupled processes. SMCs exert forces on ECM at cell-ECM adhesions through the actin cytoskeleton, which retracts matrix fibrils and at the same time drives SMC migration. Thus, some of the signaling pathways leading to force generation through the actin cytoskeleton are shared by matrix contraction and cell migration. The differential roles of Rho family GTPases, Ras and MAPKs in SMC-induced matrix contraction and SMC migration in 3D matrix are summarized in figure 8.

Inhibition of Rho and p160ROCK with either recombinant proteins or chemical inhibitor had the strongest

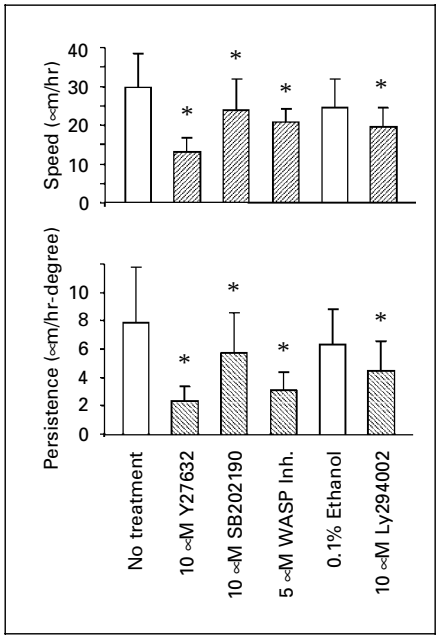

Fig. 7. Roles of p160ROCK, PI-3K, p38 MAPK and N-WASP in SMC migration in 3D matrix. SMCs were cultured in 3D adherent collagen matrix in the presence or absence of the inhibitors, and SMC migration was monitored and quantified as described in Materials and Methods. The average speed and persistence of each cell during 15 h were calculated, and at least 20 cells from each sample were used for statistical analysis. Bars represent means ± SD. * $p < 0.05$ vs. control samples.

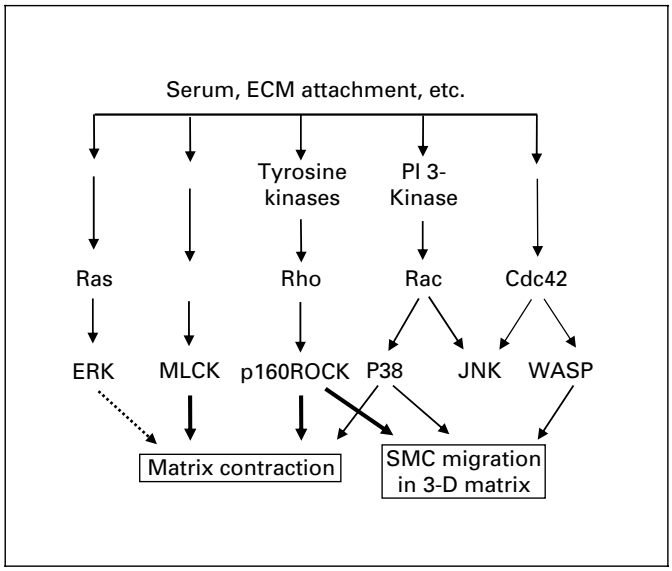

Fig. 8. Summary of the differential roles of Rho family GTPases, Ras and MAPKs in SMC-induced matrix contraction and SMC migration in 3D matrix.

inhibitory effects on matrix contraction (–80%) and SMC migration (–50%; fig. 2, 7), suggesting that the Rho-p160ROCK pathway plays a major role in both matrix contraction and SMC migration in 3D matrix. Together with the data on MLCK (fig. 2), our results indicate that the MLC phosphorylation and actin-myosin contraction induced by Rho-p160ROCK and MLCK are responsible for most of the forces exerted on ECM. The inhibition of cell migration in 3D matrix by p160ROCK inhibitor is different from some of the findings on cell migration on 2D matrix. For example, inhibition of Rho-p160ROCK decreased cell adhesion and enhanced cell migration in fibroblasts [55]. In 3D matrix, SMCs have multiple protrusions in different directions, and Rho-p160ROCK activity may help to pull back these protrusions to allow directional migration.

Since the Rho-p160ROCK pathway plays a major role in both matrix contraction and SMC migration in 3D matrix (fig. 2, 7), it could be a therapeutic target to prevent the constrictive remodeling of blood vessels during restenosis. A recent study has shown that the phosphorylation of myosin phosphatase and MLC increases in injured rat arteries in a Y27632-sensitive manner, suggesting that neointimal formation involves an augmentation in ROCK activity [56]. The neointimal formation of balloon-injured carotid arteries is significantly suppressed in Y27632-treated rats, and this has been attributed to the anti-proliferative activity of Y27632 [56], but the effects of Y27632 and Rho inhibition on the constrictive remodeling of blood vessels and SMC migration in vivo remain to be determined.

SMCs regulate vascular activity and matrix contraction; both processes use the Rho-p160ROCK pathway and the actin cytoskeleton to exert forces. However, unlike the acute regulation of the SMC-induced vascular constriction and relaxation, the chronic contraction of matrix is not fully reversible. We found that treatment of contracted collagen gel with cytochalasin D only partially reversed the matrix contraction (data not shown), suggesting that SMCs not only pull the matrix together mechanically, but also modify the matrix assembly. Consistent with our observations, Rho has been shown to regulate matrix assembly in other cell types. For example, fibroblasts regulate the assembly of fibronectin fibrils through Rho, cytoskeleton and integrins [57–59].

In addition to the Rho-p160ROCK pathway, PI 3-kinase, Rac and p38 MAPK are also involved in both matrix contraction and SMC migration (fig. 3, 7). It is likely that PI 3-kinase, Rac and p38 MAPK function in a common pathway to enhance actin polymerization, cell

spreading and protrusions [23, 24, 38–41]. The 30–40% inhibition of matrix contraction and SMC migration by the inhibitors of PI 3-kinase, Rac and p38 MAPK suggest that the actin filaments at cell periphery induced by Rac-mediated signaling also contribute to the forces exerted on ECM by cells and cell protrusions during migration, although to a lesser extent than Rho-mediated signaling. The different extents of inhibitory effects by Rac (30–40%) and Rho (70–80%) on matrix contraction and SMC migration imply that Rac and Rho do not function in a linear pathway. The regulation of Rho by Rac [21] and the counter effects of Rac and Rho in contractility regulation [60] could be either restricted to a particular part of the actin structure (e.g. cell periphery) or cell type specific. It is also possible that Rac and Rho regulate different parts of the actin structure or different stages of focal adhesion formation, as reported in other cell types [19, 61, 62].

Interestingly, the Cdc42-N-WASP pathway is involved in SMC migration in 3D matrix (fig. 7), but not required for matrix contraction (data not shown). These findings suggest that the Cdc42/N-WASP-mediated actin branching and filopodia formation may be involved in the path finding during migration, but do not contribute significantly to the forces exerted on ECM. It is possible that newly formed actin filaments at cell protrusions are less stable and have not assembled into bundles to effectively generate forces. Alternatively, the new protrusions controlled by Cdc42/N-WASP may not have stable adhesion anchorage on ECM to transduce the force generated by actin filaments. Although Cdc42 could also regulate p38 MAPK in parallel to Rac [40, 41], the lack of any effect of Cdc42 on matrix contraction suggests that Cdc42 is not required for the activation of p38 MAPK in matrix contraction and SMC migration.

It is somewhat surprising that Ras is not required for matrix contraction (fig. 4). It has been shown that Ras can function through Rac to induce membrane ruffling and transformation of fibroblasts [18, 47, 48] and that PI 3-kinase could mediate the signaling from Ras to Rac [23, 49–51]. Since inhibition of PI 3-kinase and Rac1 decreased matrix contraction (fig. 3), but the negative mutant of Ras did not (fig. 4), it is possible that PI 3-kinase and Rac can be regulated by molecules other than Ras in the SMC-induced matrix contraction. ERK only plays a minor role in the SMC-induced matrix contraction (fig. 4) and is not required for SMC migration in 3D matrix (data not shown). In contrast to the present findings, ERK has been shown to regulate the fibroblast-induced matrix contraction [37]. These results suggest that the role of the ERK pathway in matrix contraction is cell type dependent.

Besides the forces exerted on ECM by cells, matrix metalloproteinases (MMPs) are required for collagen matrix contraction induced by epithelial cells, endothelial cells and fibroblasts [63–65]. Although MMPs are upregulated in injured blood vessels [66], MMP-2, a major MMP expressed by SMCs, was downregulated during matrix contraction and not required for the constrictive remodeling of matrix [unpubl. observation]. These results suggest that the role of MMPs in matrix contraction is cell type dependent. It is possible that SMCs exert stronger forces on ECM than other cell types, and thus the pulling force plays a predominant role in matrix contraction.

In this study, we have dissected the signaling pathways involved in SMC-induced matrix contraction and SMC migration in 3D matrix. SMC-induced matrix contraction is mainly due to force generation by actin filaments and related signaling pathways. Small GTPase- and MAPK-mediated signaling differentially regulates the SMC-induced matrix contraction and SMC migration, in some cases in a cell type-dependent manner. This study demonstrates the relative importance of several signaling pathways in matrix remodeling and SMC migration. The findings can provide a rational basis for potential therapeutic approaches in vascular diseases and for the control of tissue remodeling in artificial vascular grafts.

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