Spike Formation by Fibroblasts Adhering to Fibrillar Collagen I Gel

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ABSTRACT. The fibrillar collagen I gel induced the formation of numerous dendritic cell-like protrusions (cell spikes) from the cell body, whereas monomeric collagen I induced typical cell spreading with filopodia and lamellipodia in skin fibroblasts. Peripheral, not central stress fibers appeared upon adhesion to fibrillar collagen gel, whereas both types of fibers were evident upon adhesion to monomeric collagen. Microtubules and vimentin filaments were elongated inside stress fibers along the terminal tip of cell spikes. Spike formation was totally inhibited by nocodazole and severely delayed by cytochalasin D. This suggests that cell spike formation is dependent on microtubules rather than on F-actin. We then investigated the intracellular signaling responsible for cytoskeleton organization to identify the key factor that induces cell spike morphology. During cell spike formation, FAK and CAS were activated. More CAS was activated in cells on fibrillar collagen gel than on the monomeric form, whereas FAK was activated to the same level on either. At 90 min of culture, Rac1 was activated in cells on monomeric collagen I, whereas Cdc42, Rac1 and RhoA were activated in cells on fibrillar collagen gel. These results suggest that microtubule organization via CAS and small GTPases is important for the cell spike formation that is involved in collagen gel contraction and in wound retraction in skin.

Key words: collagen/cell spike/actin/tubulin/integrin

The importance of collagen I in tissue engineering, has led to extensive studies on the signaling from fibrillar collagen via integrin and the discoidin domain receptor. When dermal fibroblasts are cultured on monomeric form (2-dimensional collagen) or fibrillar collagen gel (3-dimensional collagen), signaling from monomeric and fibrillar collagen gels induces different types of cell behavior (Sato et al., 2000a), with the results that the shapes of cells adhering to monomeric and fibrillar collagen gel are different. Endothelial cells develop lamellipodia on monomeric collagen but become tubular on fibrillar collagen gel (Kuzuya et al., 1999). Many other types of cells, including dermal fibroblasts and hepatic stellate cells, also become dendritic on fibrillar collagen gel (Sato et al., 2000a; Grinnell, 2000; Banyard et al., 2000; Kojima et al., 1998). In addition, cell proliferation is enhanced on monomeric collagen, but arrested at G1 on fibrillar collagen gel (Nishiyama et al., 1990; Koyama et al., 1996), and some cells, such as endothelial cells and keratinocytes undergo apoptosis (Kuzuya et al., 1999; Fujisaki and Hattori, 2002). Focal adhesion is not evident in cells cultured in collagen gel (Ivaska et al., 1999) because the mechanical strength of the collagen gel affects the targeting state of adhesion molecules (Pelham and Wang, 1997). Cells cultured on flexible collagen gel form irregular and highly dynamic focal adhesions.

The collagen receptors, integrin α1β1, α2β1, and αvβ3, are expressed in human foreskin fibroblasts (Klein et al., 1991). Integrin α1β1 mediates cell proliferation (Pozzi et al., 1998) and negatively regulates collagen I synthesis.
(Ivaska et al., 1999). Discoidin domain receptor 2 also mediates cell proliferation (Labrador et al., 1999; Olaso et al., 2002). Integrin α2β1 is the most important fibrillar collagen receptor for collagen gel contraction via cell-cell contact formation and actin stress fiber formation (Langholz et al., 1995; reviewed by Heino, 2000). The integrin α2 cytoplasmic domain regulates the cell branching morphogenesis and the force development (Zutter et al., 1999). Notably, endothelial cells invade the inside of collagen gels through integrin αvβ3 which recognizes the RGD motif in fibrillar collagen I (Kuzuya et al., 1999; Kanda et al., 2000). Integrin β1-deficient GD25 cells adhere to collagen and induce collagen gel contraction via integrin αvβ3 (Cooke et al., 2000). Integrin αvβ3 may function as a fibrillar collagen receptor in GD25 and endothelial cells.

Focal adhesion kinase (FAK) signaling mediates RhoA activation in the integrin signaling cascade of cells on fibronectin. Crk associated substrate (CAS) activation seems to be important for invasion into collagen gel (Banyard et al., 2000), and the activation of CAS and its downstream Rac1 induces MT1-MMP expression (Zhu and Xu, 2001). As downstream molecules of integrin signaling, Rho GTPases play a role in cytoskeletal organization. The Rho GTPases, Cdc42, Rac and Rho, are essential for invading collagen gel and for generating protrusions from the cell body (Banyard et al., 2000). Downstreams of Cdc42, Rac1, and RhoA in cytoskeletal reorganization have been investigated in detail. Cdc42 leads to filopodia formation via p21-activated kinase 1 (PAK1) and neural Wiscott-Aldrich syndrome protein (N-WASP). Rac leads to lamellipodia formation via PAK and WASP-family verprolin-homologous protein (WAVE). Cdc42 mediates microtubule assembly and cell polarization (Etienne-Manneville and Hall, 2001). Reorientation of the microtubule-organizing center that occurs during wound healing depends on the activation of Cdc42/dynein/dynactin pathway independent of Rho-regulated microtubule stabilization (Palazzo et al., 2001a). RhoA organizes actin stress fibers including central and peripheral stress fibers, which mediate ROCK and myosin light chain kinase (MLCK), respectively (Katoh et al., 2001). Rho also organizes and stabilizes microtubules that mediates the FH2 domain of mDia (Ishizaki et al., 2001; Palazzo et al., 2001b). Moreover, growing microtubules target Rho-dependent focal adhesion and regulate their turn-over (Kaverina et al., 2002). Vimentin intermediate filament is organized by Cdc42 and PAK (Chan et al., 2002; Goto et al., 2002), and is disassembled through phosphorylation by ROCK that is a downstream target of Rho (Nakamura et al., 2000).

We previously reported that skin fibroblasts form lamellipodia and spread on monomeric collagen, but form long spikes and meshwork on fibrillar collagen gel (Sato et al., 2000a). However, the molecular mechanism underlying cell spike formation has yet to be clarified. Spike formation is regarded as the first stage of wound healing for fibroblasts, and is essential to induce gel contraction with sufficient force, because the HT-1080 fibrosarcoma and endothelial cells produce short cell protrusions but they cannot contract collagen gel. We observed that the spike formation begun from 15 min after fibroblast adhesion to fibrillar collagen gel, therefore the selection of cell morphology must be determined before 15 min. The aim of the present study is to elucidate the molecular mechanism underlying the different types of cell morphology, cell spreading on monomeric collagen and cell spike formation on collagen gel, and determine the molecules that regulate the cell morphological change. We compared the morphology, actin stress fibers, microtubules, and vimentin filaments in cells cultured on monomeric and fibrillar collagen I gels. To identify whether actin or tubulin is important for the morphology, cells were incubated with cytochalasin D or nocodazole. We also examined the signaling pathways from collagen I via integrin β1, FAK, CAS and the cytoskeletal organizer, Rho GTPases.

**Materials and Methods**

**Materials**

Cytochalasin D and nocodazole (Sigma; St. Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO), stored at −20°C, and diluted with culture medium immediately before use. The antibodies used were: anti-Cdc42 (P1), anti-CAS (C-20), anti-discoidin domain receptor 1 (DDR1) (C-20), anti-discoidin domain receptor 2 (DDR2) (C-19), anti-Dia1 (V-20), anti-FAK (C-20), anti-proline-rich tyrosine kinase 2 (PYK2) (C-19), anti-PAK (C-19), anti-protein kinase N (PKN) (H-234), anti-Rac1 (C-14), anti-RhoA (26C4), and anti-ROCK (H-85) polyclonal antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), anti-integrin αvβ3 function inhibitory monoclonal antibody (mAb) (LM609) from Chemicon (Temecula, CA, USA), anti-vimentin mAb (V9) from NeoMarkers (Fremont, CA, USA), anti-paxillin mAb from Transduction Laboratories (Lexington, CA, USA), anti-phosphotyrosine mAb (4G10) from Upstate Biotechnology (Lake Placid, NY, USA) and anti-β-tubulin I mAb (4G5) from Sigma. Anti-integrin β1 function inhibitory mAb P4C10 was a gift from Dr. William G. Carter at the Fred Hutchinson Cancer Research Center, Seattle, WA, USA.

**Collagen preparation and papain digestion**

Acetic acid-soluble type I collagen purified from neonatal bovine skin was digested with papain as described (Sato et al., 2000b) to remove telopeptide and induce maximal cell spike formation (Sato et al., unpublished data).

**Cell culture of human neonatal foreskin fibroblasts**

Culture dishes (35 mm) were coated with monomeric collagen I (0.1 mg/ml in 5 mM acetic acid) for at least one hour up to overnight at room temperature. To prepare fibrillar collagen I gel,
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dishes were coated with collagen I (0.1 mg/ml in Dulbecco’s modified Eagle’s medium (DMEM) containing 1 mg/ml bovine serum albumin (BSA)) and left for at least one hour up to overnight at 37°C in a 5% CO₂ atmosphere to polymerize collagen I. Human neonatal foreskin fibroblasts were maintained in DMEM containing 10% fetal bovine serum. Confluent cells were dispersed in phosphate-buffered saline (PBS (–)) containing 0.05% bovine trypsin and 1 mM EDTA, and re-suspended in DMEM containing 1 mg/ml BSA.

Cytochalasin D and nocodazole treatment

Cells in suspension were incubated with 0.1% DMSO or 1 μM cytochalasin D or 2 μM nocodazole for 15 minutes at room temperature, plated at a density of 2–3×10⁵ cells/35-mm dish, and cultured for the indicated periods.

Adhesion assay

Cells in suspension were incubated with 20 μg/ml of the integrin inhibitory mAb, P4C10 (β1 function inhibitory) or LM609 (αβ3 function inhibitory), for 15 minutes at room temperature, plated at a density of 5×10⁴ cells/well in triplicate in 96-well plates coated with collagen I monomeric or fibrillar form, and cultured for the indicated times. The numbers of cells that adhered to collagen were measured using a Cell Counting Kit-8 (Dojin-Wako, Tokyo, Japan). The rates of adhesion were calculated taking the value of unseeded wells as 100%.

Immunoprecipitation and immunoblotting

Cells were allowed to adhere to monomeric or fibrillar collagen gel for the indicated periods. The cells were then washed once with PBS (–), lysed for 15 minutes on ice in RIPA buffer (1% NP-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM NaF, 0.5 mM PMSF, and 20 μM leupeptin), harvested, and centrifuged at 15,000 rpm (15,000×g) for 10 minutes. Anti-FAK, PYK2, CAS, DDR2, PAK1, Dia1, PKN, or ROCK antibody (1 μg/ml) was added to the supernatant and the mixture was incubated for 2 hours on ice. Protein G-Septarose beads (Pharmacia) were added, and the mixture was then rotated for an additional 2 hours at 4°C. The beads were collected by centrifugation at 7,000 rpm (3,200×g) for 2 minutes. The supernatant was aspirated and the beads were washed three times with RIPA buffer. Immunoprecipitates solubilized from the beads with Laemmli’s SDS sample buffer were boiled for 5 minutes and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% or 12.5% acrylamide gels. Separated proteins were transferred to PVDF membranes (Millipore). Non-specific binding was blocked with TBS-T (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% non-fat skim milk. The membranes were then incubated overnight with primary antibody at 4°C, washed three times with TBS-T, and probed with horseradish peroxidase-conjugated secondary antibody (1:1,000 dilution). Tyrosine phosphorylation was first detected with anti-phosphotyrosine mAb (4G10) and horseradish peroxidase-conjugated antibody, and the membranes were then incubated with antibodies against FAK, PYK2, and DDR2 overnight at 4°C or room temperature for 1 h, and probed with alkaline phosphatase-conjugated rabbit anti-rabbit or anti-goat secondary antibody. Proteins were visualized using the ECL Western Blot Detection System (Amersham Pharmacia) or an alkaline phosphatase detection kit (Vector Laboratories, Inc.) according to the protocols supplied with the kits.

Immunofluorescence microscopy

Fibroblasts cultured on collagen-coated glass cover slips (14 or 15 mm in diameter) for 3 hours in DMEM containing 1 mg/ml BSA were washed once with Heps-buffered saline (20 mM Hepes-NaOH, pH 7.2, and 150 mM NaCl) (HBS), fixed for 30 minutes with 3.7% paraformaldehyde, washed again with HBS, and incubated in RIPA buffer for 1 minute at room temperature. Cells were washed again with HBS, blocked with 1% BSA in HBS, and incubated with antibodies against paxillin, tubulin, and vimentin. Secondary antibody (fluorescein-5-isothiocyanate (FITC)-labeled anti-mouse IgG), tetramethylrhodamine-5-isothiocyanate (TRITC)-conjugated phallolidin, and 4',6-diamidino-2-phenylindole (DAPI) were added, then the cells were incubated for 1 hour at room temperature and observed under a Zeiss indirect immunofluorescence microscope. Images were photographed using a cooled CCD camera (ATTO imaging system) and analyzed using Adobe Photoshop 3.0.

Results

Cell spreading on monomeric collagen and spike formation on fibrillar collagen gel

The morphology of adherent fibroblasts differed depending on the form of collagen I. Fibroblasts formed lamellipodia after adhesion to monomeric collagen (Fig. 1A), but formed a dendritic shape with extended long protrusions (cell spikes) after adhesion to fibrillar collagen gel (Fig. 1B). On both types of collagen, fibroblasts started to extend after 15 minutes, and cell spikes and lamellipodia continued to grow for 6 hours on fibrillar and monomeric collagens, respectively. To study these cytoskeletal reorganization systems, we examined the organization of actin stress fibers in the early stage after cell adhesion. Fibroblasts that adhered to monomeric collagen formed filopodia (Fig. 1C, yellow arrowheads) after 15 minutes at the cell periphery. After 3 hours, cells formed filopodia (Fig. 2A, yellow arrowheads) and lamellipodia (Figs. 1C and 2A, white arrowheads), typical central fibers (Fig. 1C, white arrows), and peripheral stress fibers (Fig. 1C, blue arrowheads) at the cell periphery.

Upon adhesion to fibrillar collagen gel, irregular actin condensations sometimes formed ring-like structures, or podosomes, at the cell central area (Fig. 1D, green arrowheads). At the same time, lamellipodia and a few filopodia (Fig. 1D, white and yellow arrowheads) were primarily
Fig. 1. Lamellipodia formation on monomeric collagen I and spike formation on fibrillar collagen I gel. Fibroblasts were dispersed using trypsin, washed twice, and resuspended in DMEM containing 1 mg/ml BSA. Suspended fibroblasts were plated on monomeric (A) or fibrillar (B) collagen I and cultured for 15 minutes, 90 minutes and 3 hours, and then photographed under a phase contrast microscope. Suspended fibroblasts were plated on monomeric (C) or fibrillar (D) collagen I and cultured for 15 minutes (left) and 3 hours (right), then F-actin stained with TRITC-phalloidin for observation by indirect immunofluorescence microscopy. Small yellow arrowheads indicate filopodia, green arrowheads indicate podosomes, and white arrowheads indicate lamellipodia. White arrows and blue arrowheads indicate central and peripheral stress fibers, respectively.
formed at the cell periphery by 15 minutes. The sheath of peripheral stress fibers (blue arrowheads) was intensely stained around the cell spikes, but central stress fibers did not appear until 3 hours of culture (Fig. 1D right).

We examined the organization of other cytoskeletons, microtubules and vimentin filaments during cell spreading and the extension of cell spikes by immunofluorescence microscopy (Fig. 2). In spreading cells, microtubules were distributed from around the nucleus to the edges of cells along the inside of peripheral stress fibers but they did not extend into filopodia and lamellipodia (Figs. 2A and 2B). At the cell spike terminals, numerous fine branches formed a broom-like shape but lamellipodia were absent (Fig. 2D, yellow arrowheads). The thin branches in cell spikes may be the same as those in filopodia. In the other spikes, lamellipodia often accompanied filopodia at the terminal tip as in neurite outgrowth (data not shown). Microtubules extended inside of peripheral sheath of stress fibers to the tip of cell spikes (Figs. 2E and F). The distribution of vimentin was similar to that of microtubules in cells that adhered to both monomeric and fibrillar collagen gel (Figs. 2G and H). Vimentin filaments were distributed from around the nucleus to the cell edge on monomeric collagen (Fig. 2G), and extended from the inside of actin stress fibers to the terminal tip of cell spikes on fibrillar collagen gel (Fig. 2H).

**Fig. 2.** Staining of F-actin, tubulin and vimentin in cells on monomeric and fibrillar collagen I. Trypsinized fibroblasts were plated on monomeric (A, B, C, and G) or fibrillar (D, E, F, and H) collagen I and cultured for 3 hours. Cells were stained with TRITC-phalloidin (A and D), anti-tubulin mAb followed by FITC-conjugated secondary antibody (B and E), or anti-vimentin mAb followed by FITC-conjugated secondary antibody (G and H), and observed by indirect immunofluorescence microscopy. Images are merged in C and F. Small yellow arrowheads in A and D indicate filopodia, and white arrowheads lamellipodia. Bar, 30 μm.

**Cytochalasin D and nocodazole inhibit cell spike formation**

We examined the mechanisms of filamentous actin (F-actin) and microtubule formation in cells with the morphology...
described above. Cytochalasin D and nocodazole that inhibit actin and tubulin polymerization, respectively, were added to the cultured cells, and the cell morphology and actin/microtubules were then observed.

Cytochalasin D (1 μM) inhibited cell spreading at 90 minutes (Figs. 3B and E). Cell spikes continued to be formed after 17 hours in culture, but the process was obviously delayed compared with the control (Figs. 3J and K). This suggests that F-actin plays an important role in the formation of both lamellipodia and cell spikes. Instead of cell spreading, irregular cell spikes were formed after adhesion to monomeric collagen in the presence of cytochalasin D at 17 hours (Fig. 3H). Nocodazole (2 μM) did not disturb lamellipodia formation at 90 minutes on monomeric collagen (Fig. 3C). However, it significantly disturbed cell elongation to form a spindle-like, typical fibroblastic morphology for up to 17 hours (Figs. 3G and I). In addition, nocodazole totally inhibited cell spike formation for up to 17 hours compared with the DMSO control in cells adhering to fibrillar collagen gel (Figs. 3J and L). This shows that microtubule is essential for the formation of cell spikes, but not of lamellipodia.

Fig. 3. Effects of cytochalasin D and nocodazole on cell morphology. Trypsinized fibroblasts were incubated with control 0.1% DMSO (A, D, G, and J), 1 μM cytochalasin D (B, E, H, and K), or 2 μM nocodazole (C, F, I, and L) for 15 minutes at room temperature. Cells were then plated on monomeric (A–C and G–I) or fibrillar (D–F and J–L) collagen I and cultured for 90 minutes (A–F) or 17 hours (G–L). Cells were photographed under a phase contrast microscope.
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Fig. 4.
We examined the effects of cytochalasin D and nocodazole on the formation of actin stress fibers and microtubules (Fig. 4). Cytochalasin D disturbed the reorganization of actin stress fibers in cells on monomeric collagen, and phalloidin staining revealed irregular protrusions at the terminal tips (Fig. 4C). Microtubules were found in all of the irregular cell spikes (Fig. 4C). The morphology of cells on monomeric and fibrillar collagen gels was similar in the presence of cytochalasin D. In addition, microtubules were also formed in all spikes of cells on the fibrillar collagen gel (Fig. 4D). These observations suggest that microtubules support the structure of both normal and irregular cell spikes in the absence and presence of cytochalasin D, respectively. In the presence of nocodazole, stress fibers were thinner and more numerous, but microtubules were absent in cells on monomeric collagen (Fig. 4E). This thinner F-actin is also observed in transfected cells with constitutively active mDia1 mutant in which ROCK activity is relatively low (Watanabe et al., 1999). Actin stress fibers and microtubules were not evident in cells exposed to nocodazole on fibrillar collagen gel (Fig. 4F). The reason why actin polymerization is perturbed in cells on fibrillar collagen gel in the presence of nocodazole remains obscure. However, these results suggest that microtubules are essential for the formation of cell spikes on fibrillar collagen gel and for that of irregular cell spikes in the presence of cytochalasin D.

These results using cytochalasin D suggest that the destruction of F-actin induces the rearrangement of signaling for cell spike formation. Therefore, the activation levels of integrin-associated molecules, FAK and CAS, and Rho family were examined.

**FAK, PYK2, and CAS activation**

To identify the key molecules that lead to the formation of spikes on collagen gel rather than lamellipodia, we examined the activation states of the intracellular signaling molecules associated with integrin, namely, FAK, PYK2, and CAS. Tyrosine phosphorylation of FAK was detected from 15 minutes in cells adhering to collagens but not in cells in suspension, and the expression level was the same in cells on monomeric and fibrillar collagens (Fig. 5). Tyrosine phosphorylation of CAS was detected from 15 minutes and sustained for 90 minutes in cells on monomeric collagen, while more CAS was phosphorylated, although transiently so, at 15 minutes in cells on fibrillar collagen gel. Protein levels of immunoprecipitated FAK and CAS were not changed. This means that the degradation of FAK and CAS by cytoplasmic proteases such as calpain was not prominent. The tyrosine phosphorylation of proline-rich tyrosine kinase 2 (PYK2) was below the detectable levels, and was at least 20-fold lower than that of FAK in skin fibroblasts.

**Anti-integrin β1 antibody inhibits FAK and CAS phosphorylation**

Cells respond to collagen I using integrins α1β1, α2β1, and αvβ3 (Klein et al., 1991; Langholz et al., 1995; Pozzi et al., 1998; Ivaska et al., 1999; Zutter et al., 1999; Kanda et al., 2000; Cooke et al., 2000; Heino, 2000), and DDR2 (Olaso et al., 2002). Skin fibroblasts use α2β1 to adhere onto collagen I (Klein et al., 1991; Langholz et al., 1995; Ivaska et al., 1999). To understand the signaling cascade from monomeric and fibrillar collagens, we examined the effects of function inhibitory mAbs against β1 (P4C10) and αvβ3 (LM609) on cell adhesion, tyrosine phosphorylation of signaling molecules, and formation of actin stress fibers. Fibroblasts immediately adhered to monomeric and fibrillar collagen gel within 10 minutes in the presence of normal mouse IgG and LM609, whereas P4C10 significantly inhibited adhesion at 10 minutes (Figs. 6A and B). This means that integrin β1, mainly α2β1, mediates cell binding to the monomeric and fibril forms of collagen in skin fibroblasts. P4C10 inhibited FAK and CAS phosphorylation in cells on monomeric and fibrillar collagen (Figs. 6C and D). This indicates that FAK and CAS phosphorylation depends on β1 integrin activation. DDR2 phosphorylation was not inhibited by P4C10 or by LM609, suggesting that DDR2 signaling is independent of these integrins (data not shown).

The formation of both lamellipodia and stress fibers in cells on monomeric and fibrillar collagen were inhibited by the anti-β1 integrin mAb (Figs. 6E and F, middle panels) but not by the anti-αvβ3 integrin mAb (Figs. 6E and F lower panels), suggesting that integrin β1, but not αvβ3, is responsible for actin organization in skin fibroblast. The cell spikes, though short and irregular in shape, were formed in the presence of anti-β1 integrin mAb, and had microtubules but no peripheral stress fiber (Fig. 6F, middle panel), suggesting that the microtubules may elongate and support the cell spikes. Among the integrin β1 species, α2β1 rather than α1β1 is responsible for cell spike formation and for the generation of actin-mediated contractile force in fibroblasts (Langholz et al., 1995).

LM609 did not inhibit cell adhesion to monomeric and fibrillar collagens (Figs. 6A and B), but lamellipodia formation was enhanced during the early phase on monomeric collagen (data not shown). In parallel with lamellipodia formation...
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Tyrosine phosphorylation of FAK, PYK2 and CAS induced by adhesion to collagens. Trypsinized fibroblasts in DMEM containing 1 mg/ml BSA were reattached to monomeric (m) or fibrillar (f) collagen I and cultured for 15 or 90 minutes. Cells were lysed with RIPA buffer containing 1% NP-40, and FAK, PYK2 and CAS in cell lysates were immunoprecipitated using respective specific antibodies and purified over protein G-agarose. Immunoprecipitates were extensively washed, dissolved in SDS sample buffer, resolved by SDS-PAGE (7.5%), and immunoblotted with anti-phosphotyrosine antibody (4G10). Proteins were detected by Western blotting using enhanced chemiluminescence (upper panels). PVDF membranes were washed and reprobed with anti-FAK, anti-PYK2 and anti-CAS antibodies. Proteins were detected using alkaline phosphatase (lower panels). Two independent experiments were performed and the same results were obtained.

**Fig. 5.**

**Cdc42, Rac1 and RhoA activation**

Rho GTPases, Cdc42, Rac1 and RhoA, are key molecules to the organization of actin, tubulin and vimentin filaments. To examine the activation of Rho GTPases in cells on monomeric and fibrillar collagens, we compared the activation levels of Rho GTPases after immunoprecipitation as the complexes with their endogenous downstream substrates (Fig. 7A). The substrates were PAK1 for Cdc42 and Rac1, and Dia1, ROCK and PKN for RhoA (see Materials and Methods). After immunoprecipitation, the amounts of associated Cdc42, Rac1, and RhoA were determined by immunoblotting using their respective antibodies. The results showed that Cdc42 and RhoA were transiently activated early on (20 minutes) and inactivated after 90 minutes in cells on monomeric collagen (Fig. 7A). However, more Rac1 activation was observed at 90 minutes, when Cdc42 and RhoA were inactivated, than at 20 minutes. The activation levels of Cdc42 and Rac1 were parallel to filopodia and lamellipodia formation, respectively (Fig. 1C). In cells on fibrillar collagen gel, Cdc42, Rac1 and RhoA were activated for up to 90 minutes. Activated RhoA bound Dia1, PKN, and ROCK in a similar manner (Fig. 7A). These findings showed that all of the three downstream signals of RhoA and the downstream signal of Cdc42 were strongly activated and sustained in cells adhering to fibrillar collagen gel but not in cells adhering to monomeric collagen, while Rac signal was sustained in both cells adhering to monomeric and fibrillar collagens.

Finally, we examined the effects of cytochalasin D and nocodazole on Rho GTPase activation in cultured fibroblasts adhering to monomeric collagen (Fig. 7B). Cdc42 and Rac1 activation was not inhibited by either cytochalasin D or nocodazole. In fact, Cdc42 activation was rather enhanced by nocodazole, whereas that of Rac1 was enhanced by cytochalasin D. These results indicate that the disruption of actin or tubulin filaments in cells adhering to monomeric collagen induces Rac1 and Cdc42 activation, respectively.

**Discussion**

The present study investigated the morphology, cytoskeletal organization and integrin signaling of human skin fibroblasts adhering to monomeric and fibrillar forms of collagen I. The characteristic features of cells on fibrillar collagen gel are a dendritic cell-like shape, the formation of peripheral stress fibers without a central stress fiber, and the absence of lamellipodia among neighboring cell spikes.

Inhibition experiments using cytochalasin D and nocodazole revealed how cell spikes are formed (Figs. 3 and 4). Spike formation in the presence of cytochalasin D was delayed on fibrillar collagen gel, and completely inhibited by nocodazole. Anti-integrin β1 mAb, P4C10, inhibited the peripheral stress fiber formation but not the terminal tip actin condensation and microtubule formation (Fig. 6F). These findings indicate that microtubules construct and support the cell spike structure. Furthermore, the results in Fig. 4F suggest that actin reorganization is prevented in cells on...
Fig. 6. Effect of integrin-inhibitory antibodies on adhesion, tyrosine phosphorylation, and F-actin/microtubule formation in fibroblasts on monomeric and fibrillar collagen I. Fibroblasts were dispersed with trypsin, resuspended in DMEM containing 1 mg/ml BSA. Cells were incubated for 30 minutes with normal mouse IgG (mlgG), P4C10 (β1 integrin-inhibitory mAb), LM609 (αvβ3 integrin-inhibitory mAb), or P4C10 combined with LM609. (A and B) Thereafter, cells were reattached to 0.1 mg/ml collagen I-coated plastic wells (A) or 0.1 mg/ml fibrillar collagen I gel (B) in triplicate, incubated for 10 minutes, and washed with DMEM containing 1 mg/ml BSA. The numbers of cells that adhered to collagen were measured using a Cell Counting Kit-8. (C and D) Cells were incubated for 30 minutes with normal mouse IgG (lane 1), P4C10 (lane 2) or LM609 (lane 3), reattached to monomeric (m) or fibrillar collagen I gel (f) for 90 minutes, and lysed. Tyrosine phosphorylation of FAK (C) and CAS (D) was examined as described in the legend to Fig. 5. (E and F) Incubated cells were reattached for 3 hours to monomeric (E) or fibrillar (F) collagen I in the presence of normal mouse IgG (upper), P4C10 (middle) or LM609 (lower). Cells were stained with TRITC-phalloidin or anti-tubulin mAb followed by FITC-conjugated secondary antibody, and observed by indirect immunofluorescence microscopy.
fibrillar collagen gel, because nocodazole prevented not only the formation of microtubules but also that of actin stress fibers. The low level of mechanical stress in collagen gel (0.1 mg/ml) might initiate the formation of peripheral, rather than central stress fibers. These results indicate that the mechanism of cytoskeleton organization leading to cell spike formation is as follows. Primarily F-actin condenses at the tip of cell spikes and forms podosomes. An actin sheath is assembled from the podosome, and microtubules then elongate inside the actin sheath to maintain the cell spike morphology. However, it remains unknown whether F-actins that were stained with phalloidin at the terminal tip of cell spike in cytochalasin D-treated cells and integrin β1 inhibitory mAb-treated cells have any function such as spike elongation (Figs. 4C and 6F).

To understand the difference in molecular signaling in cells on monomeric and fibrillar collagens, we investigated the activation of signaling molecules (Fig. 5–7). CAS was transiently activated and FAK activation was sustained in cells on fibrillar collagen I gel, while CAS and FAK were activated in parallel on monomeric collagen I. However, the activation of the Rho GTPases, Cdc42 and RhoA, differed from that of FAK and CAS (Figs. 5 and 7A). The activation of Cdc42 and RhoA decreased at 90 minutes while high levels of FAK and CAS activation were sustained in cells on monomeric collagen. Cytoskeletal signaling might modulate Cdc42/RhoA signaling when integrin signaling is switched “ON” by adhering to monomeric collagen using integrins. Rho signaling would not be activated if integrin signal was switched “OFF”, because the formations of lamellipodia and stress fibers were blocked when the integrin signal was turned “OFF” by the β1 integrin-inhibitory mAb (Figs. 6G and H). Cytochalasin D and nocodazole enhanced Rac1 and Cdc42 activation, respectively, when the integrin signal was “ON” (Fig. 7B). These results suggested that cytoskeletal signaling affects the cytoskeletal rearrangement signal via these small GTPases. We speculated that Cdc42 in cells on fibrillar collagen gel is activated not only by integrin signaling but also by the rearrangement of the cytoskeletons that involves microtubules. Activations of Cdc42 were observed both in nocodazole-treated cells and in cells adhering to collagen gel, but the cell spike was prominent only in cells adhering to collagen gel (Figs. 4B and 4E, Fig. 7B upper panel). This suggested that the activation of RhoA is needed for cell spike formation in addition to Cdc42 activation.

Other reports indicate that CAS activates Rac but not Cdc42 and Rho (Gu et al., 2001; reviewed by Bouton et al., 2001). The CAS/Rac pathway might be a switch in cell migration and invasion which works by reorganizing membrane protrusion and expression of MT1-MMP (Banyard et al., 2000, Zhuge and Xu, 2001). MT1-MMP and CD44 locate at the membrane protrusion terminal tip, where they may guide the directions of cell spikes on the cell membrane (Nakahara et al., 1997; Bourguignon et al., 2001).

Fig. 7. Rho GTPase activation on lamellipodia and cell spike formation. (A) Suspended fibroblasts (2×10⁵ cells) (sus) were plated on monomeric (m) or fibrillar (f) collagen I and cultured for 15 or 90 minutes. The cells were lysed and immunoprecipitated with anti-Pak1 antibody. The immunoprecipitates were equally separated to 2 samples (10⁵ cells/lane), resolved by SDS-PAGE (12.5%), and immunoblotted with anti-Cdc42 or anti-Rac1 antibody. To detect the activated RhoA, cell lysates were immunoprecipitated with anti-Dia, anti-PKN, or anti-ROCK antibody and immunoblotted with anti-RhoA antibody after SDS-PAGE (10⁵ cells/lane). Co-immunoprecipitated activated Cdc42, Rac1 and RhoA are indicated by arrowheads on the right. (B) Fibroblasts were cultured on monomeric collagen I (m) for 90 minutes in the presence of cytochalasin D (CD), nocodazole (N) or vehicle (–). Other cells were similarly cultured on fibrillar collagen I gel for 90 minutes (f). Activated Cdc42, Rac1, and RhoA were detected as described in (A).
The formation of membrane protrusions and the enhanced cell motility via p190Rho GTPase-activating proteins may also be implicated in cell spike formation (Arthur and Burridge, 2001; Nakahara et al., 1998). We speculate that Cdc42 might be located in this terminal position where it function to elongate microtubules.

We showed that fibrillar collagen gel induces dendritic morphology whereas monomeric collagen (on plastic dishes) induces typical cell spreading. Figure 8 summarizes our findings on the signal transduction mechanism on the different forms of collagen I. Skin fibroblasts may contract circumstantial collagen fibrils to gain an appropriate level of mechanical strength and concentration (number of fibrils/volume) in collagen gels, and this is likely to occur also in skin in vivo. Bershadsky’s group indicated that the mechanosensor is the focal complex via ROCK and mDia (Geiger and Bershadsky, 2001; Riveline et al., 2001). Further study is required to determine how the mechanical strength of collagen gels affects the activation of signaling molecules such as CAS/Rac, the disassembly of focal adhesions, the switching of cell morphology to dendritic shape, and invasion. In addition, a similar cell spike, termed “invadopodia”, was reported in melanoma (Nakahara et al., 1997; and Nakahara et al., 1998). This type of cell protrusion is characterized by its invasive degradation of the fibronectin-coated gelatin matrix, and is similar to our cell spike formed from the podosome. These similar types of cell protrusions are ubiquitous among the various cell types such as interstitial cells (skin fibroblasts) and epithelial cells (melanoma), but their functions are different with one being involved in wound healing and the other in cancer cell invasion.

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References


Cell Spike Formation on Collagen I Gel


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