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特集 Special Part 1

褥瘡の病態と分類

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はじめに

褥瘡は外力による虚血性皮膚潰瘍と定義されるが，褥瘡の病態は非常に多様で，それらが複雑に絡み合っている．故に，悪化要因と治癒阻害要因を明らかにし，各患者に応じた対策や治療が行われる必要がある．しかし，褥瘡の多様性を解析する診療体系の医学的基盤は十分に確立されていない．

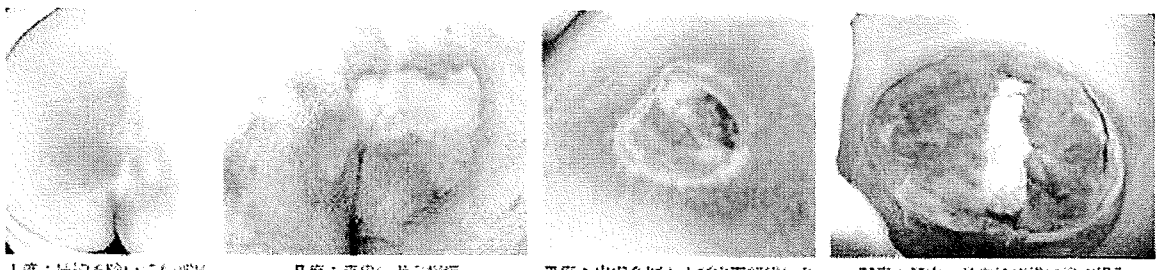
本稿では，褥瘡の多様な病態を概説する．さまざまな視点から病態，分類を挙げるが，それは，褥瘡診療における複眼的な視点が必要だからである．創傷治癒過程では，褥瘡と急性創傷との違いを認識し，それとともに，いったん褥瘡形成されてからの治癒過程では，さまざまな要素が異なることを認識する必要がある．

深さによる分類

褥瘡は，その治癒機転から浅い褥瘡と深い褥瘡に分類することが重要である．「褥瘡の予防・治療ガイドライン」では[表1]にかかげるように，I～IV度に分類されている．深さがI度，II度の褥瘡，すなわちDESIGN分類(26頁の表1，2参照)でd2までの褥瘡は，いわゆる「浅い褥瘡」であり，III度，IV度の褥瘡は「深い褥瘡」と定義されている．

II度褥瘡では通常，皮膚付属器(汗腺，皮脂腺，毛)から表皮細胞が増殖し，瘢痕を残さず治癒する．また，I度，II度では真皮という創性を持つ組織が残存することも重要である．治癒後は点状の色素沈着を残すことが特徴である．一方，III度，IV度，すなわちDESIGN分類でのD3，D4ではI度，II度の褥瘡と経過を異にする．つまり，肉芽組織を形成した後，治癒する経過をとる．

褥瘡において，これらの治癒機転を理解することはケアに直接関与することであり，重要である．理想的には，褥瘡をこの段階に食い止めるような治療とケアが必要であるが，深部から発症する褥瘡の存在も知ら



I度：発赤を除いても消退しない皮膚，紅斑　II度：真皮に及ぶ損傷　III度：皮膚全層および皮下組織に至る深在性潰瘍に及ぶ損傷　IV度：筋肉・骨支持組織に及ぶ損傷

図1　褥瘡の深さによる違い

れるようになってきている。外用薬による薬物療法は，深い褥瘡に対して不可欠で，基剤の性質を熟知した薬剤師の役割が重要である。

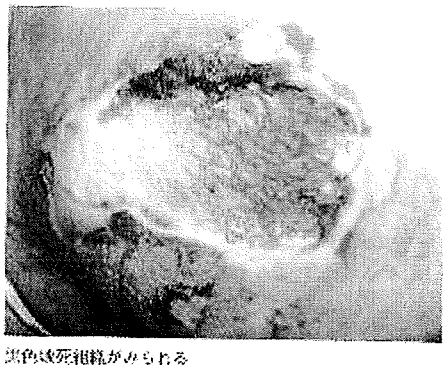
✓ 経過による分類

1．急性期褥瘡

褥瘡の急性期（図2）では，紅斑に引き続き水疱や紫斑などの所見がみられる。紫斑は皮膚の出血を意味し，III度以上の褥瘡に発展することを示唆する。一般的に，循環障害による組織障害は，発症の時点から明らかな組織障害の程度が確定するまでには2～3週間の時間経過がある。その間に褥瘡は日を追うごとに変化する。また，この時期には感染の併発の検索が必要とされる。この時点では，刻々と変化する創傷を評価しつつ経過を観察することが重要である。

2．慢性期褥瘡

一般に慢性期褥瘡と称されるものの，病態的には急性期を経過した褥瘡と捉えた方がよいと思われる。慢性期褥瘡では，まず壊死組織除去が必要である。壊死組織が固着した状態は，治癒機転が全く起こらないばかりか，感染症の母地となる。これを取り除くのがデブリードマン（創縁切除）である。通常は健常組織との境界が形成されたころから開始するが，通常，壊死組織は経過を追って明瞭になってくるので，数回のデブリードマンを必要とすることが多い。理論的には，デブリードマンが終了すれば赤色の肉芽組織が増生し，上皮化に向かうはずだが，実際はそうでないことも少なくなく，臨床病態は複雑である。



黒色壊死組織がのられる

図2　急性期仙骨部褥瘡

✓ 色調分類

福井基成氏らによって提唱された色調分類は，黒色期，黄色期，赤色期，白色期といった経過による分類で，褥瘡の治癒へ向かう時間経過をよく反映している。今どのような病期であるかを簡便に把握し，褥瘡診療におけるデブリードマンや外用薬の使い方について検討するのに一定の役割を果たした。しかし実際は，このような経過をとるのはIII度以上の深い褥瘡に限られる。

✓ DESIGN分類

DESIGN分類については，26頁の表1，2を参照されたい。この分類は，日本褥瘡学会によって作成され，褥瘡診療において注目点を明らかにするとともに，重症度を一定の範囲で規定するものである。さらに「科学的根拠に基づく褥瘡局所治療ガイドライン」では，各指標をそれぞれに改善させるという視点で，統計学的な一定のエビデンスに従って作成された。しかし，実際の臨床現場において，慢性に経過した肉芽組織の改善やポケットの解消などは，経験則によることが多いそのため，この分類は治療法の選択に使うのは難しいことも多い。

✓ 原疾患による分類

褥瘡において，発症の原因となった原疾患を把握することは非常に重要である。すなわち，褥瘡は外力による虚血性皮膚潰瘍であるため，その外力を発生させた，もしくは発生させている原疾患を把握することが重要である。しかし，それは簡単ではなく，患者の実際の生活を観察して初めて原疾患が明らかになること

3：肉芽表面に起こる不顕性感染：いわゆるバイオフィルム感染

このうち，迅速な対応が必要なのは3である．褥瘡に合併した感染では，壊死組織が嫌気的環境を作り出し，さらに嫌気性菌との混合感染によって全身症状を伴って急速に進行する．デブリードマンによる壊死組織の除去と，嫌気性菌をターゲットとした抗菌療法（ペネム系薬剤など）が必要である．

1は通常，ブドウ球菌などの感染が多く，第2世代のセフェム系抗生剤で治療可能である．2も時に遭遇するが，徹底した洗浄が効果的である場合も多い．また，時に切開を必要とする．4に対しては原則的に腐骨除去が必要である．その場合，周囲の腱などの組織を外科的に除去することで施行できることもある．5は炎症性の病態を示唆することが多く，外用薬，特に基剤の使い方が重要である．

鑑別診断と周囲の皮膚病変

褥瘡の診断は決して容易ではない．筆者らの施設において，皮膚科医以外から褥瘡として依頼を受けた患者のうち，褥瘡以外の診断をした疾患として，単純疱疹，リウマチ性血管炎，刺激性接触皮膚炎，皮膚カンジダ症，熱傷（II度）を経験した．また，周囲にうっ滞性皮膚炎，刺激性接触皮膚炎，カンジダ症をしばしば合併するので，その鑑別も重要である．

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Evidence That PI3K, Rac, Rho, and Rho Kinase Are Involved in Basic Fibroblast Growth Factor-Stimulated Fibroblast–Collagen Matrix Contraction

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Abstract Fibroblast–collagen matrix contraction has been used as a model system to study how cells organize connective tissue. Previous work showed that lysophosphatidic acid (LPA)-stimulated floating collagen matrix contraction is independent of Rho kinase while platelet-derived growth factor (PDGF)-stimulated contraction is Rho kinase-dependent. The current studies were carried out to determine the signaling mechanisms of basic fibroblast growth factor (bFGF)-stimulated fibroblast–collagen matrix contraction. Both bFGF and LPA promoted equally collagen matrix contraction well. Three different inhibitors, LY294002 for phosphatidylinositol-3-kinase (PI3K), C3 exotransferase for Rho and Y27632 for Rho kinase, suppressed the bFGF-stimulated fibroblast–collagen matrix contraction. With bFGF stimulation, fibroblasts spread with prominent stress fiber network formation and focal adhesions. In the presence of Rho kinase inhibitor, focal adhesions and stress fibers were mostly lost. We demonstrated that bFGF stimulation for fibroblast caused transient Rac and Rho activation but did not activate Cdc42. In addition, bFGF enhanced fibroblast migration in wound healing assay. The present study implicates PI3K, Rac, Rho, and Rho kinase as being involved in bFGF-stimulated collagen matrix contraction. The elucidation of bFGF-triggered signal transduction may be an important clue to understand the roles of bFGF in wound healing. J. Cell. Biochem. 102: 1290–1299, 2007.

Key words: wound healing; basic fibroblast growth factor (bFGF); lysophosphatidic acid; Rho; Rac; phosphatidylinositol-3-kinase

Recently, human recombinant basic fibroblast growth factor (bFGF) has been available for the treatment of non-healing skin ulcer [Robson et al., 1992]. Although its significant effects for cutaneous wound healing have been already confirmed in daily clinical practice, little is known about its biological effects on dermal fibroblasts. Fibroblasts synthesize extracellular matrices and organize connective tissues during matrix development and in response to injury. The motile mechanisms by which fibroblasts remodel the extracellular matrices during the morphogenetic processes have been studied using cultured cells in three-dimensional collagen matrices [Tomasek et al., 2002; Grinnell, 2003].

As fibroblasts exert force on and move collagen fibrils of the matrix, collagen concentration can be measured as a decrease in the diameter of free matrices or as a decrease in the height of restrained matrices. During contraction of restrained matrices, collagen fibrils become oriented in the same plane as the restraint, and then mechanical loading develops. In floating matrices, on the other hand, contraction occurs without collagen fibrils developing a particular orientation, and the matrix remains mechanically unloaded [Brown et al., 1998; Tranquillo, 1999; Cukierman et al., 2002; Tomasek et al., 2002; Grinnell, 2003].

The signaling mechanisms in fibroblasts which direct them to regulate collagen matrix contraction depend on whether the cells are mechanically loaded or unloaded at the time that contraction is initiated as well as on the growth factor added to initiate contraction. For example, stimulation of fibroblasts with lysophosphatidic acid (LPA) but not with

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Evidence That PI3K, Rac, Rho, and Rho Kinase Are Involved in Basic Fibroblast Growth Factor-Stimulated Fibroblast–Collagen Matrix Contraction

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platelet-derived growth factor (PDGF) generates robust force in restrained matrices [Kolodney and Elson, 1993], whereas both LPA and PDGF stimulate floating matrix contraction (FMC) equally [Grinnell et al., 1999; Shreiber et al., 2001]. FMC has exhibited something of an enigma since LPA stimulation of fibroblasts in floating matrices causes activation of the small G protein Rho (GTP loading) [Grinnell et al., 2003], but blocking Rho kinase with Y27632 does not inhibit matrix contraction [Lee et al., 2003]. The current studies were carried out to determine the signaling mechanisms of bFGF-stimulated fibroblast–collagen matrix contraction.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and trypsin solution were obtained from Nihon Seiyaku (Tokyo, Japan). Bovine serum albumin (BSA) and LPA were obtained from Sigma, Steinheim (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Cytosystems (Castle Hill, NSW, Australia). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG H + L and goat anti-rabbit IgG H + L antibodies were obtained from ICN Biomedicals, Inc. (Aurora, OH). Enhanced chemiluminescence (ECL) Western blotting reagent was obtained from Amersham Pharmacia Biotechnologies (Piscataway, NJ). Vitrogen "100" collagen was obtained from Cohesion (Palo Alto, CA). Rac activation assay kit and Cdc42 activation assay kit were obtained from Upstate Biotechnology (Lake Placid, NY). G-LISA™ RhoA activation assay biochem kit was obtained from Cytoskeleton (Denver, CO). Lipofectin was obtained from Invitrogen (Carlsbad, CA). C3 exotransferase, Y27632 and LY294002 were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Mouse anti-vinculin antibodies were purchased from Sigma Chemicals. RITC-conjugated phalloidin and FITC-conjugated goat anti-mouse IgG H + L antibodies were obtained from Molecular Probes, Inc. (Eugene, OR). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corp. (Bedford, MA). Western blotting reagent was obtained from Amersham Pharmacia Biotechnologies. Fluoromount G was obtained from Southern Biotechnology Associates (Birmingham, AL). Chemotaxicell, 8-μm-pore polycarbonate filter, was obtained from Kurabo (Osaka, Japan).

Monolayer and Collagen Matrix Culture

After informed consent was given, dermal fibroblasts were obtained from five healthy volunteers. Skin specimens were cut into small pieces, and outgrown fibroblasts were trypsinized and grown in DMEM supplemented with 10% FBS at 37°C in 5% CO_2 and 95% humidified air incubator. The culture medium was changed every 3 days. Cells were used within 10th passages. Cells were harvested by 0.25% trypsin for 1 min, followed by treatment with DMEM with 10% FBS. For monolayer culture experiments, harvested cells were seeded at a density of 4×10^4 cells on 22 mm^2 glass coverslips (Fisher Scientific, Chicago, IL), which were coated with collagen (50 μg/ml for 30 min), and then incubated in DMEM containing 5 mg/ml BSA, the respective growth factors and inhibitors, as indicated.

Collagen matrix cultures were prepared using Vitrogen "100" collagen as previously described [Abe et al., 2003; Grinnell et al., 2003]. Briefly, neutralized collagen solution (1.5 mg/ml) containing harvested cells (10^6 cells/ml unless indicated otherwise) were prewarmed to 37°C for 4 min, and then aliquots (200 μl) were placed on an area outlined by a 12-mm-diameter circular score within a well of 24-well-culture plates (Greiner Bio-one, Frickenhausen, Germany) and allowed to polymerize for 1 h at 37°C in a 5% CO_2 humidified incubator. To initiate FMC, matrices were gently released from the culture dish with a spatula into 0.8–1.0 ml of DMEM containing 5 mg/ml BSA (DMEM/BSA), the respective growth factors and inhibitors, as indicated. For stressed matrix contraction (SMC), polymerized matrices were cultured 24 h in 1.0 ml of DMEM/10% FBS containing 50 μg/ml ascorbic acid before release.

Matrix contraction was carried out for the times shown in the text, after which the samples were fixed for 10 min at room temperature with 3% paraformaldehyde in phosphate-buffered saline (PBS) (150 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4, pH 7.2). To quantify contraction, fixed matrices were washed, placed on a flat surface, and the diameters were measured. Contraction data were presented as the change in diameter (starting-final) in millimeters. All experiments were carried out in

duplicate, and every experiment was repeated three times or more. Data points and error bars in the figures represent averages and standard deviations. Where error bars cannot be seen, the data points overlapped.

To load fibroblasts with C3 exotransferase (C3), Lipofectin was used as a delivery system. Lipofectin/C3 was prepared in 120 μl of DMEM and diluted with additional DMEM after 1 h at 22°C to give a final concentration of 10 μg/ml Lipofectin and 5 μg/ml C3. Subsequently, the cells were incubated with the Lipofectin/C3 mixture or Lipofectin prepared identically except without C3 for 30 min at 37°C. Following treatment with Lipofectin/C3 or Lipofectin alone, the cells were rinsed and further incubated with DMEM and 10% FBS for 60 min at 37°C before harvesting.

SDS–PAGE and Immunoblotting

Rac and Cdc42 activation were assessed by the respective assay kit. SDS–PAGE and immunoblotting were performed following instructions. Briefly, cells were extracted in Mg^{2+} lysis buffer. Samples were clarified by centrifugation for 10 min at 16,000*g* (Beckman Microfuge), and the supernatants were either incubated with reagents for the Ras-GTP pull-down assay or else dissolved in 1× reducing sample buffer (250 mM Tris, 2% sodium dodecyl sulfate (SDS), 40% glycerol, 20% mercaptoethanol, 0.04% bromphenol blue) and boiled for 5 min. Equal amounts of protein were subjected to SDS–PAGE electrophoresis using 10% acrylamide minislab gels. Transfer to PVDF membranes was carried out at 100 V for 1 h. The membranes were blocked with 5% milk (for anti-Rac antibody) in TTBS (0.1% Tween-20, 150 mM NaCl, 20 mM Tris, pH 7.5) or 3% BSA (for anti-Cdc42 antibody) in TTBS, and then incubated with anti-Rac antibody (1:500) or anti-Cdc42 antibody (1:500) in blocking solution at 4°C for 12 h. After washing with TTBS, membranes were incubated with either HRP-conjugated goat anti-mouse IgG antibody in 5% milk in TTBS (for anti-Rac antibody) or HRP-conjugated goat anti-rabbit IgG antibody in 3% BSA in TTBS (for anti-Cdc42 antibody) for 1.5 h. After washing with TTBS, membranes were visualized by the ECL system.

RhoA Activation Assay

RhoA activation was assessed by G-LISA™ RhoA activation assay biochem kit. Luminescence assay was performed following according to the instructions. Data were read at 100 gain, 100 ms integration time on luminometer. In each experiment, the Luminescence was calculated from the values of triplicate wells.

Immunofluorescence Microscopy

Cells in matrices or on coverslips were fixed for 10 min with 3% paraformaldehyde in PBS at room temperature, blocked with 2% glycine/1% BSA in DPBS (150 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.2) for 30 min, and then permeabilized for 15 min with 0.5% triton X-100 in DPBS. Subsequently, the samples were washed with DPBS and treated for 10 min with 1% BSA in DPBS. Primary antibody against vinculin was diluted in 1% BSA in DPBS (1:200) and reacted with cells for 1 h at 37°C. After washing with DPBS, samples were treated for 10 min with 1% BSA in DPBS, and then FITC-conjugated goat anti-mouse IgG antibody in 1% BSA in DPBS (1:300) was added to cells for 30 min at 37°C. After washing with DPBS, samples were treated for 10 min with 1% BSA in DPBS, and then incubated with RITC-conjugated phalloidin in 1% BSA (8 U/ml) for 30 min at 37°C. After additional washes, samples were mounted on glass slides with Fluoromount G. Observed images were collected with an Olympus DP70 camera and Olympus DP Controller system.

Cell Migration Assay

Fibroblast migration was quantified by a modification of Boyden chamber technique using Chemotaxicell. Briefly, cells were suspended in DMEM/BSA at 2×10^5 cells/200 μl and placed in the top well. The bottom well was filled with 600 μl of DMEM/BSA containing bFGF and was separated from the top well by Chemotaxicell. The chambers were incubated for 12 h at 37°C in a moist 5% CO_2 atmosphere. The number of cells that had migrated into the lower compartment was then counted in triplicate by phase-contrast microscopy, and expressed as a percentage of the total number of cells added to the upper well.

Wound Healing Assay

Cells were grown in 60 mm culture dishes until confluent, and the cell monolayer was then wounded by a sterile plastic 200 μl micropipette tip. In the case of treatment with Y27632 or LY294002, cells were preincubated with the

compound for 20 min before wounding. The cells were washed twice with DMEM and incubated under a humidified atmosphere of 5% CO_2 for 24 h at 37°C in DMEM in the presence or absence of bFGF. Then, they were observed under phase-contrast microscopy at indicated time points.

Statistical Analysis

Data were expressed as mean ± SD. Statistical analyses of the experiments about the effects of bFGF on the RhoA activation were performed with software program Statview (version 4.0; Abacus Concepts, Berkeley, CA). The group data were subjected to analysis of variance testing to determine the overall impact of sample treatments within an experiment, with additional post hoc testing by using the Fisher protected least significant difference (PLSD) test to determine the statistical significance of individual sample treatments on the parameters in question. The analysis of variance results is reported as significant only if both the analysis of variance and the Fisher PLSD tests yielded a probability (P) value of 0.05 or lower.

RESULTS

bFGF and Collagen Matrix Contraction

Figure 1A shows the representative result of floating collagen matrices 4 h after contraction in the presence or absence of bFGF. The diameter of the matrices was 10.5 mm at the time contraction was initiated. In DMEM alone, the matrix scarcely contracted. The matrix diameter markedly decreased when fibroblasts were stimulated with 1 ng/ml of bFGF. Figure 1B shows the results of matrix contraction at the various concentrations of bFGF. The FMC rate was highest around 1 ng/ml of bFGF. On the other hand, bFGF did not promote SMC at any concentration (data not shown).

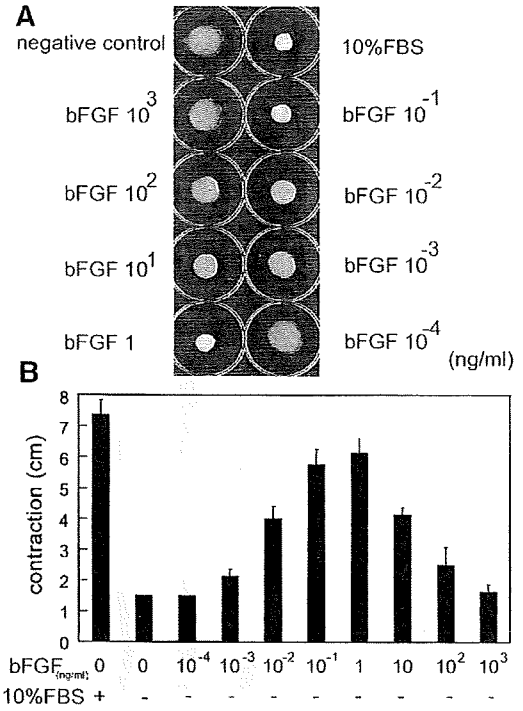

Fig. 1. Dose-dependence study of matrix contraction in the presence of various concentrations of bFGF. Collagen matrices containing fibroblasts were released from culture dishes to initiate contraction immediately after polymerization. Contraction medium was DMEM/BSA containing bFGF at indicated concentrations. At the end of the incubations, 4 h for floating matrices, samples were fixed and the extent of matrix contraction was measured as a decrease in matrix diameter (A). The rates of floating matrix contraction were highest around 1 ng/ml of bFGF (B).

PI3K Inhibitor, LY294002, Rho Inhibitor, C3 Exotransferase and Rho Kinase Inhibitor, Y27632, Blocked bFGF-Stimulated Floating Matrix Contraction

Studies were carried out to elucidate the mechanisms of FMC by using two different kinase inhibitors, LY294002 for phosphatidylinositol-3-kinase (PI3K) and Y27632 for Rho kinase. Figure 2A shows the results of experiments measuring the collagen matrix contraction stimulated with 1 ng/ml of bFGF in the presence or absence of LY294002. LY294002 completely inhibited bFGF-stimulated matrix contraction. Figure 2B shows the effect of Y27632 in the same experimental system. Y27632 also completely inhibited bFGF-stimulated matrix contraction. Consistent with previous studies [Lee et al., 2003], addition of Rho kinase inhibitor, Y27632, had little effect on LPA-stimulated contraction of floating matrices. To study the effect of C3 exotransferase, a specific inhibitor of Rho activity [Narumiya et al., 1988], on contraction of floating collagen matrices, C3 was loaded into fibroblasts in monolayer culture using Lipofectin. Fibroblasts treated with Lipofectin/C3, but not Lipofectin or C3 alone, showed arborized spreading morphology (data not shown). Figure 2C presents a typical experiment showing that cells treated with Lipofectin/C3 were unable to contract floating collagen matrices

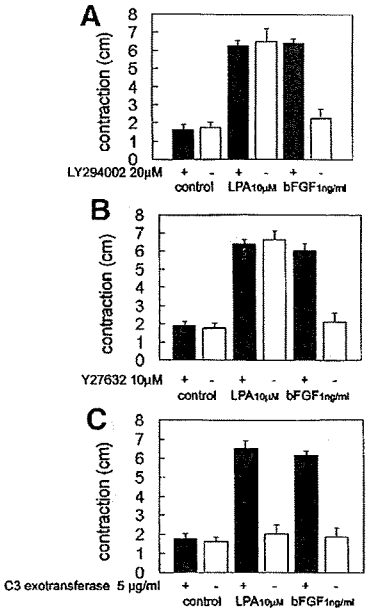

Fig. 2. Phosphatidylinositol-3-kinase dependence, Rho and Rho kinase dependence of collagen matrix contraction. Polymerized collagen matrices containing cells were released from culture dishes and incubated 15 min in DMEM/BSA and 10 μM LPA or 1 ng/ml bFGF as shown. Where indicated 20 μM LY294002 (A) or 10 μM Y27632 (B) were added 15 min before the stimulation of growth factors. For study the effect of C3 exotransferase (C) on contraction of floating collagen matrices, fibroblasts in monolayer culture were incubated for 30 min at 37 C with Lipofectin (10 μg/ml) mixed with or without C3 (5 μg/ml) as indicated. Subsequently, the cells were tested for contraction. At the end of incubations, samples were fixed and the extent of matrix contraction was measured as a decrease in matrix diameter.

regardless of whether bFGF or LPA was used to stimulate contraction, whereas cells treated with Lipofectin alone were unaffected. Therefore, the small G-protein Rho appeared to be required for bFGF-stimulated matrix contraction.

bFGF Accelerated Cell Migration

The results indicated that bFGF-stimulated FMC was Rho kinase-dependent. Since, Rho kinase has been reported to play a role in cell migration as well as fibroblast–collagen matrix contraction [Ehrlich et al., 1991; Parizi et al., 2000; Takayama and Mizumachi, 2001], cell migration assay was performed. Figure 3 shows that cell migration stimulated with bFGF was enhanced in a dose-dependent manner. Figure 4A shows the representative results of wound healing assay in the presence or absence of bFGF. These two results may indicate that bFGF accelerate cell migration around at 100 ng/

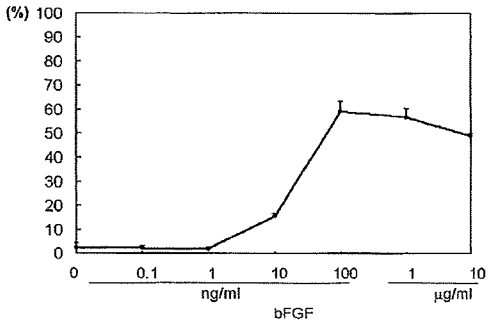

Fig. 3. Cell migration stimulated with bFGF in dose-dependent manner. Cells were stimulated with various concentrations of bFGF using the Boyden chamber technique.

ml of bFGF. Figure 4B shows the typical findings of wound healing assay to examine the effects of LY294002 and Y27632 in the

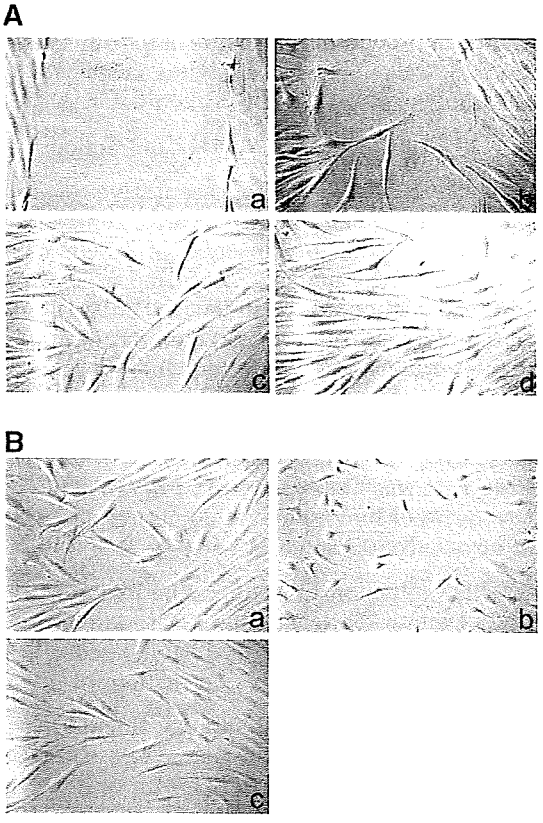

Fig. 4. Wound healing assay. A: Effects of bFGF on cell migration in an assay of wound healing. Monolayers of fibroblasts were wounded and then cultured for 0 (a) or 24 h in the presence of 1 (b), 10 (c) or 100 (d) ng/ml of bFGF. B: Effects of LY294002 or Y27632 on bFGF-promoted cell migration in an assay of wound healing. Monolayers of fibroblasts were wounded and then cultured with 100 ng/ml bFGF in the absence (a) or presence of 10 μM Y27632 (b) or 20 μM LY294002 (c).

presence of 100 ng/ml of bFGF. Addition of LY294002 had little effect on bFGF-stimulated cell migration although addition of Y27632 was markedly inhibited.

bFGF and Cell Spreading on Collagen-Coated Coverslips

Further experiments were carried out to examine whether or not the bFGF-stimulated Rho kinase-dependent mechanism of force generation also could be demonstrated during cell spreading on collagen-coated coverslips following LPA stimulation. Figure 5 shows that in bFGF-containing medium, fibroblasts spread with prominent stress fibers (actin staining). In the presence of Rho kinase inhibitor (+Y27632), stress fiber formation was abolished. Instead of broad lamellae, cells revealed long dendritic extensions. In the presence of PI3K inhibitor (+LY294002), fibroblasts spread and elongated, and instead of broad lamellae, cells had long dendritic extensions. Fibroblasts incubated on collagen-coated coverslips in LPA-containing medium also developed stress fibers, but tended to be much less polarized than those in bFGF-containing medium. Fibroblasts in medium containing LPA and Rho kinase inhibitor (LPA + Y27632) became more polarized but, unlike cells in bFGF medium, often retained regions of broad lamellae and also developed stress fibers. In the presence of PI3K inhibitor, fibroblasts spread and elongated similar to fibroblasts under bFGF stimulation. Fibroblasts incubated on collagen-coated coverslips in LPA-containing medium also developed stress fibers, but tended to be much less polarized than cells in bFGF-containing medium.

Figure 6 shows that in bFGF-containing medium, fibroblasts spread with prominent stress fibers and focal adhesions (vinculin). In the presence of Rho kinase inhibitor (+Y27632), focal adhesions and stress fibers were mostly lost, instead of broad lamellae, cells had long, dendritic extensions. However, in the presence of PI3K inhibitor (+LY294002), focal adhesions were maintained. Thus, fibroblasts on collagen-coated surfaces in bFGF-containing medium are proved to require Rho kinase to generate tractional force for flattening and for formation of stress fibers and focal adhesions.

bFGF and Rac Activation

It is reasonable to consider that, in floating matrices, the Rac signaling pathway may be involved in the upstream of Rho kinase. Consequently, subsequent studies focused on Rac, a member of the Rho family. Pull-down assays to

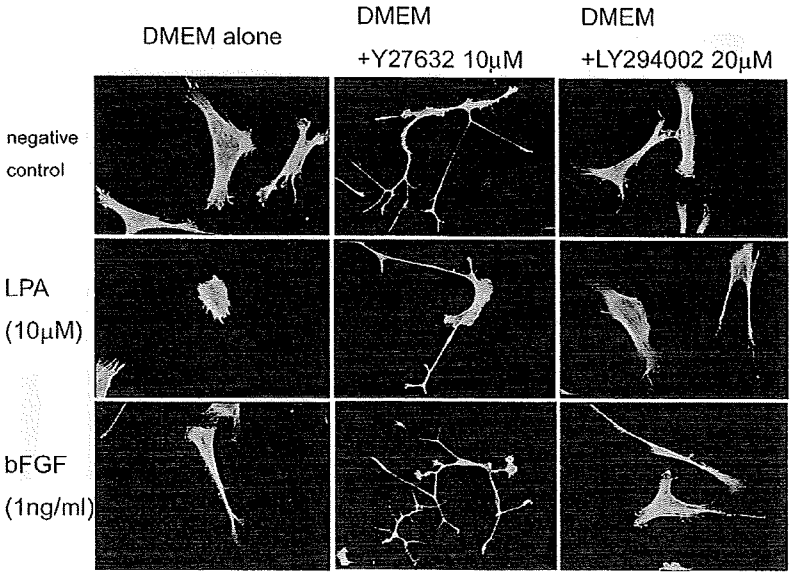

Fig. 5. Phosphatidylinositol-3-kinase dependence and Rho kinase dependence of fibroblast spreading on collagen coated coverslips. Cells were incubated on collagen-coated coverslips in DMEM and 5 mg/ml BSA for 15 min with or without 10 μM Y27632 or 20 μM LY294002 as indicated, and then stimulated with 10 μM LPA or 1 ng/ml bFGF. After a 4 h incubation, samples were fixed and stained to visualize actin.

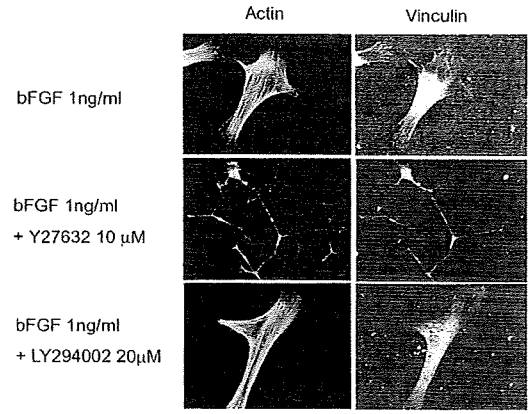

Fig. 6. Phosphatidylinositol-3-kinase dependence and Rho kinase dependence of bFGF promoted fibroblast spreading on collagen-coated coverslips. Cells were incubated on collagen-coated coverslips in DMEM and 5 mg/ml BSA for 15 min with or without 10 μM Y27632 or 20 μM LY294002 as indicated, and then stimulated with 1 ng/ml bFGF. After a 4 h incubation, samples were fixed and stained to visualize actin and vinculin.

detect GTP-loaded Rac were carried out to know whether or not the Rac activation was involved in the bFGF-mediated collagen matrix contraction. Figure 7A shows a representative result from three experiments. bFGF stimulation caused transient Rac activation as compared with unstimulated cells. In parallel experiments, Figure 7B shows a representative result of GTP-loaded Cdc42. bFGF did not activate Cdc42 in human dermal fibroblasts.

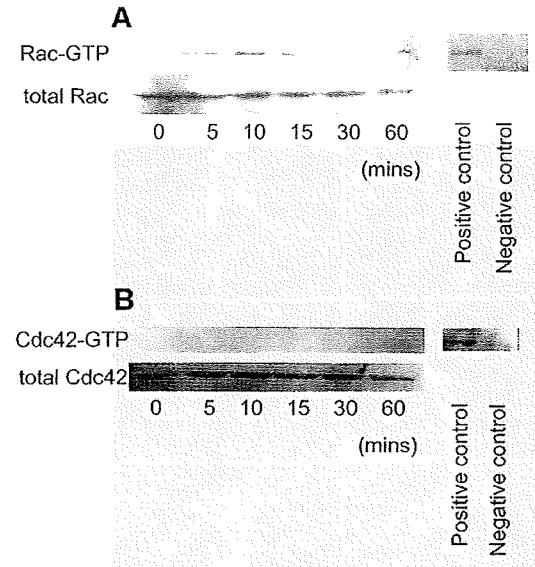

Fig. 7. GTP-loading of Rac or Cdc42 in fibroblasts stimulated with bFGF. Fibroblasts were cultured on collagen-coated coverslips in DMEM and 5 mg/ml BSA for 30 min and then stimulated with 1 ng/ml bFGF. At the times indicated, cell extracts were prepared and subjected to pull-down assay analyses. Samples of the extracts and pull-down pellets were analyzed by SDS–PAGE and immunoblotting for Rac (**A**) and Cdc42 (**B**).

bFGF and RhoA Activation

Final experiments were carried out to make a quantitative analysis of Rho activation stimulated with bFGF by G-LISA™. Data shown in Figure 8 were read at 100 gain, 100 ms integration time on a luminometer. bFGF stimulation caused significant Rho activation as compared with unstimulated cells.

DISCUSSION

Form and function of multicellular organisms depend on cell proliferation, migration, and differentiation. Fibroblasts synthesize and degrade extracellular matrices to organize and maintain connective tissues during matrix development and in response to injury and fibrotic diseases. Studies on cells in three-dimensional matrices also suggest that reciprocal and adaptive mechanical interactions play a role in the regulation of morphogenesis. Therefore, fibroblasts cultured in collagen matrices have been used as a model system to study how cells organize connective tissue. Previous studies have shown that LPA-stimulated floating collagen matrix contraction is independent of Rho kinase whereas PDGF-stimulated contraction is Rho kinase-dependent [Abe et al., 2003].

Recently, human recombinant bFGF has been available for the treatment of non-healing skin ulcer [McGee et al., 1988]. The effect of

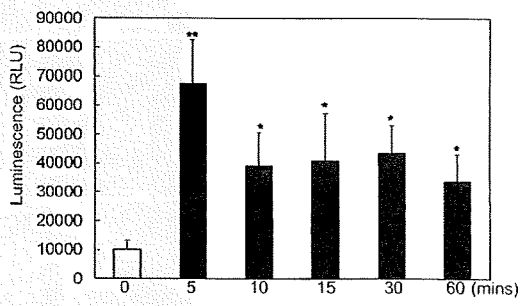

Fig. 8. Quantitative analysis of GTP-loading of Rho in fibroblasts stimulated with bFGF. Fibroblasts were cultured on collagen-coated coverslips in DMEM and 5 mg/ml BSA for 30 min and then stimulated with 1 ng/ml bFGF. At the times indicated, cell extracts were prepared and analyzed bFGF by G-LISA™. Each column represents mean ± SD from five separate experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ versus 0 min.

bFGF for cell proliferation is well documented in previous studies [Gospodarowicz et al., 1987; Gospodarowicz, 1988; Rifkin and Moscatelli, 1989]. However, little is known about its biological effects in wound healing processes [Grinnell et al., 1999; Ono, 2002; Spyrou and Naylor, 2002; Akasaka et al., 2004; Ono et al., 2004]. In particular, only limited information is available on the signal transduction pathways that regulate dermal wound healing promoted by bFGF. The present studies aimed to learn more about the effects of bFGF on wound healing using the fibroblast–collagen matrix contraction assay system. bFGF promoted FMC but not SMC, which is similar to those effects under PDGF. Contraction of floating and stressed collagen matrices may also reflect different aspects of cell motility. Fibroblasts in floating collagen matrices are round-shaped during contraction [Grinnell et al., 1999], whereas cells in stressed matrices assume a spread morphology with prominent stress fibers and withdraw their extensions during contraction [Mochitate et al., 1991; Tomasek et al., 1992; Lee et al., 1993]. Under FMC, contraction probably depends on tractional forces that accompany the protrusion of cell extensions [Harris et al., 1981]. Under SMC, the stress fibers themselves can contract once there is no longer a rigid substratum to maintain isometric tension [Burridge, 1981]. Our results using three inhibitors suggest that bFGF-stimulated FMC respectively utilizes PI3K, Rac, Rho and Rho kinase-dependent mechanisms.

Although cell migration is indispensable to cutaneous wound healing, the details of wound healing promoted by bFGF are still unclear. We could confirm the effect of bFGF for fibroblast migration by two different methods, wound healing assay and Boyden chamber technique. It is of note that Rho kinase inhibitor suppressed bFGF-stimulated fibroblast motility, but PI3K inhibitor had little effect. Taken together, the results suggest that bFGF may stimulate fibroblast motility through Rho kinase activation, but not by PI3K. There is no doubt that other signal transduction pathways may be involved in bFGF-stimulated fibroblast motility. There are large differences between in bFGF concentrations required for FMC and cell migration. However, we have already reported that collagen matrix contraction and cell behaviors are completely different depending on stimulators [Abe et al., 2003].

The results imply that human fibroblast spreading on collagen-coated coverslips in the presence of bFGF may have a Rho kinase-dependent mechanism for exerting force. Blocking Rho kinase in the presence of bFGF caused loss of most stress fibers and focal adhesions, and the cells fanned out in one direction suggesting the induced formation of lamellipodia by bFGF. Activation of Cdc42 triggers actin polymerization and bounding to form either filopodia or shorter cell protrusions called microspikes [Aspenstrom et al., 2004; Begum et al., 2004; Kurokawa et al., 2004; Hall, 2005]. Activation of Rac promotes actin polymerization at the cell periphery leading to the formation of sheet-like lamellipodial extensions and membrane ruffles [Aspenstrom et al., 2004; Begum et al., 2004; Kurokawa et al., 2004; Hall, 2005]. Activation of Rho promotes both the bundling of actin filaments with myosin II filaments into stress fibers and associated proteins to form focal contacts ruffles [Aspenstrom et al., 2004; Kurokawa et al., 2004; Hall, 2005]. Therefore, we asked whether or not bFGF could activate Rac and Rho in human dermal fibroblast. Rac-GTP pull-down and Rho-GTP chemiluminescence assays demonstrated that transient Rac and RhoA activation occurred in fibroblasts with bFGF stimulation, but Cdc42 was not activated. Ras superfamily GTPases not only regulate growth and differentiation, but also link cell surface receptors to actin cytoskeleton. These GTPases regulate actin dynamics and regulate fundamental processes such as cell movement, cell cycle progression, cytokinesis as well as gene expression in the nucleus [Hall, 1998; Giancotti and Ruoslahti, 1999]. Rac and Cdc42 are wound-activated and are respectively linked to the actin-polymerized structures "lamellipodia" and "filopodia" formation [Aspenstrom et al., 2004; Begum et al., 2004; Kurokawa et al., 2004]. Rac is essential for the formation of leading edge protrusions, and necessary for forward movement in the wound; Cdc42 is important for maintaining the polarized phenotype of migrating cells, whereas Ras is involved in stress fiber formation and focal adhesion turnover and is also required for cell movement [Chotani et al., 2000]. Thus, these small GTPases coordinately regulate cell movement [Nobes and Hall, 1999]. The present study strongly suggests that bFGF is involved in this fundamental process.

The present study demonstrated that PI3K, Rac, Rho, and Rho kinase are involved in bFGF-stimulated collagen matrix contraction. Moreover, bFGF accelerate dermal fibroblast motility via Rac activation but does not activate Cdc42. Clarification of downstream effectors on signal transduction may be an important clue to understand the role of bFGF in wound healing.

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