



# Fibroblast growth factor 2 uses PLC- $\gamma$ 1 for cell proliferation and PI3-kinase for alteration of cell shape and cell proliferation in corneal endothelial cells

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**Purpose:** Fibroblast growth factor 2 (FGF-2) induces endothelial-mesenchymal modulation in corneal endothelial cells, including stimulation of cell proliferation and cell shape change and induction of fibrillar collagen. In the present study, we investigated whether FGF-2 uses distinct signaling pathways for individual biological activities.

**Methods:** Specific metabolic inhibitors were used to block cell proliferation, while reversion of cellular morphology (modulated with FGF-2) was determined using specific antibodies and inhibitors. Immunocytochemical analysis was performed to identify any changes observed in the cytoskeleton in relation to cell shape. Association of cytoskeleton molecules with phosphatidylinositol 3-kinase was determined using co-precipitation. Cell proliferation was assayed using a colorimetric method for determining the number of viable cells.

**Results:** The fibroblastic morphology induced by FGF-2 reverted to a polygonal shape in cells treated with anti-FGF-2 antibody, anti-phosphatidylinositol 3-kinase antibody, LY294002, and genistein, while anti-phospholipase C  $\gamma$ 1 antibody did not to reverse the modulated cell morphology. Cell proliferation mediated by FGF-2 was blocked by metabolic inhibitors (genistein, LY294002 and wortmannin); genistein inhibited FGF-mediated cell proliferation in a dose-response manner and had a maximum inhibition of 80% at 100  $\mu$ M, while inhibitors of phosphatidylinositol 3-kinase had less inhibitory effect than did genistein. When cytoskeleton proteins were examined, the characteristic punctated staining profiles of vinculin observed in normal cells were maintained in fibroblastic corneal endothelial cells treated with FGF-2. The inhibitors that cause reversion of cell shape also demonstrated the punctated staining potential. Likewise, the staining profiles of  $\alpha$ -actinin and smooth muscle  $\alpha$ -actin were not altered, regardless of cell shape. Filamentous actin and  $\alpha$ -actinin were co-localized to the cytoskeleton and phosphatidylinositol 3-kinase was associated with the cytoskeleton, regardless of cell shape.

**Conclusions:** These findings indicate that FGF-2 uses distinct and/or dual signaling pathways for individual biological activities.

The corneal endothelium is a monolayer of differentiated cells located in the posterior portion of the cornea. It is essential for maintaining corneal transparency, but its capacity for regeneration after injury is severely limited in humans, primates, and cats [1,2]. In response to certain pathological conditions, corneal endothelial cells (CECs) *in vivo* may respond by converting to fibroblast-like cells. These morphologically modulated cells then resume their proliferation ability and start to produce fibrillar collagens, leading to the formation of a fibrillar extracellular matrix. A clinical example of this process is the development of a retrocorneal fibrous membrane [3,4], the presence of which blocks vision, thereby causing blindness. In our previous studies [5,6], we found that corneal endothelium modulation factor (CEMF) secreted by polymorphonuclear leukocytes (PMNs), fibroblast growth factor-2 (FGF-2), or a combination of the two factors modulates phenotypes of CECs, leading to a modulation similar to that observed *in vivo* (up-regulation of cell proliferation, cell shape changes, and collagen phenotype alteration). We further found that CEMF could induce *de novo* synthesis of FGF-2 and that the newly produced FGF-2 was the direct mediator for the modulation of CECs [7].

FGF-2, a member of the fibroblast growth factor family, is a multifunctional regulator of cell development, differentiation, regeneration, senescence, proliferation, and migration [8-10]. In normal cornea, FGF-2 is a component of Descemet's membrane that may be necessary for wound repair [7,11-13]. The biological actions of FGF-2 are mediated through transmembrane cell surface receptors that possess tyrosine kinase activity [14,15]. One of the early cellular events induced by the binding of FGF-2 to its receptor is the stimulation of phospholipase C $\gamma$ 1 (PLC- $\gamma$ 1), which hydrolyzes inositol phospholipid and generates the second messengers diacylglycerol and inositol 1,4,5-phosphate [16,17]. PLC- $\gamma$ 1 has been a major substrate of FGF receptor 1 (FGFR-1) [18] and is known to be responsible for mitogenesis [19,20]. In our previous studies, we demonstrated that PLC- $\gamma$ 1 associated with the cytoskeleton (vinculin and actin) plays a role in mitogenesis, whereas the PLC- $\gamma$ 1 complex has no effect on cell shape changes mediated by FGF-2 [21]. Furthermore, PLC- $\gamma$ 1 is involved in carcinogenesis or tumor progression [22-24]. Likewise, phosphatidylinositol (PI) 3-kinase, another intracellular effector molecule, is implicated in a variety of cellular processes, including mitogenesis, transformation, membrane ruffling, actin polymerization, and vesicle transport [25-27]. Nevertheless, limited information is available concerning the existence of a specific signal transduction pathway for individual cellular responses in a given growth factor-bound receptor system.

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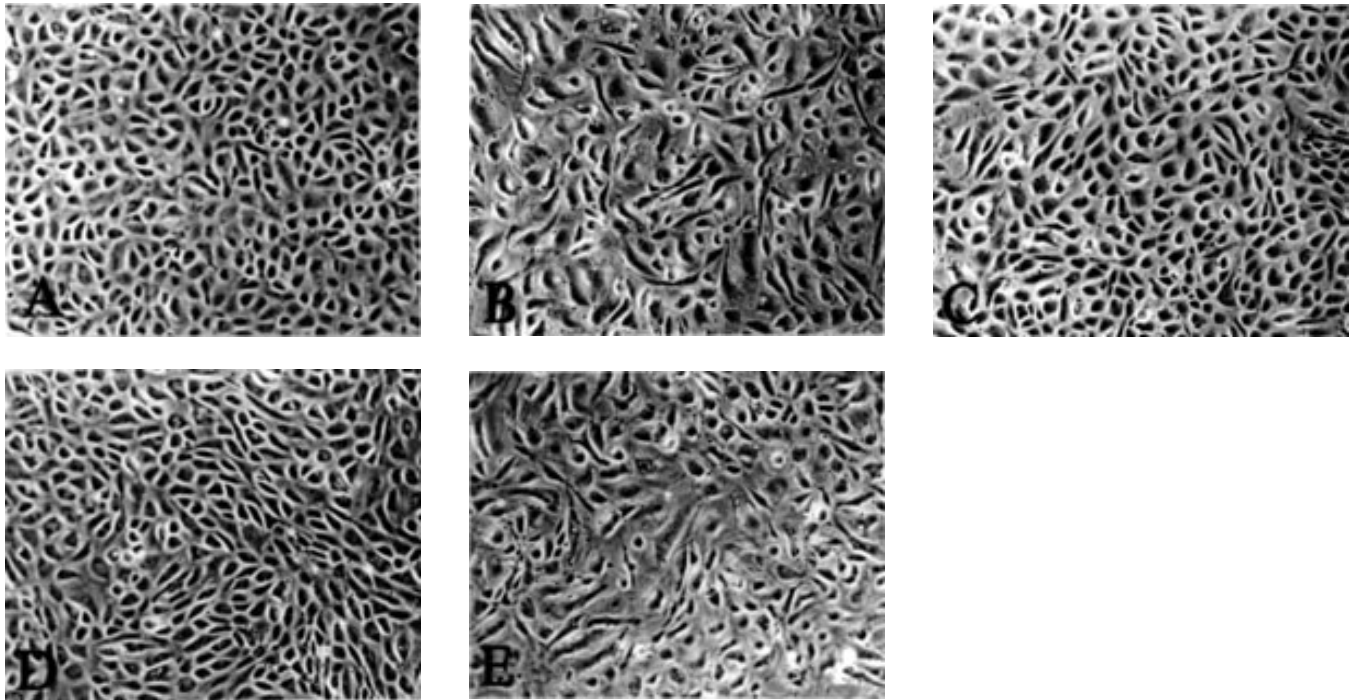


Figure 1. Effect of neutralizing antibodies on cell morphology of corneal endothelial cells modulated with FGF-2 (10 ng/ml) supplemented with heparin (10 µg/ml). The confluent first passage CECs were treated with different conditions for 24 h (x150). (A) Normal CECs, (B) FGF-2, (C) FGF-2 and anti-FGF-2 antibody, (D) FGF-2 and anti-PI3-kinase, (E) FGF-2 and anti-PLC-γ1.

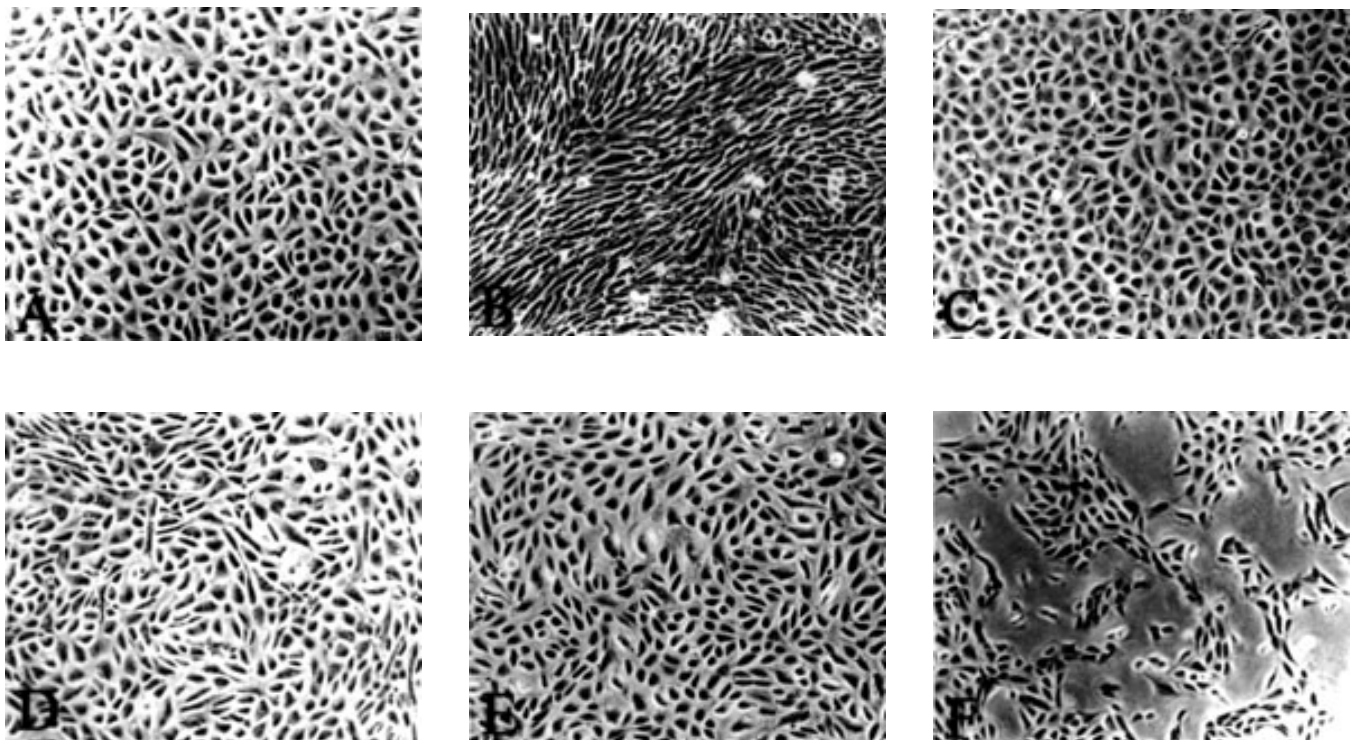


Figure 2. The effect of inhibitors on cell morphology of CECs modulated with FGF-2. The first passage CECs were treated with FGF-2 (10 ng/ml) supplemented with heparin (10 µg/ml) upon plating. On day 4, in which cellular morphology was changed to elongated fibroblastic shape, the cells were exposed to inhibitors for 48 h. (x150). (A) normal CECs, (B) CECs treated with FGF-2, (C) CECs treated with FGF-2 and 20 µM LY294002, (D) CECs treated with FGF-2 and 10 µM genistein, (E) CECs treated with FGF-2 and 100 nM wortmannin, (F) CECs treated with FGF-2 and 40 µM LY294002.

Although we demonstrated that PLC- $\gamma$ 1 was responsible for mitogenesis mediated by FGF-2 in CECs, the pathway leading to cell shape change remains undefined. It may be that different forms of phosphoinositides are involved in differential cellular responses [28]. In this respect, it is likely that PLC- $\gamma$ 1 and PI3-kinase are responsible for differential cellular responses mediated by FGF-2. In this report, we show that there are indeed differential signaling pathways for mitogenesis and modulation of cell shape mediated by FGF-2. PI3-kinase is involved in the modulation of cell shape and in mitogenesis, in part, whereas PLC- $\gamma$ 1 is responsible for mitogenesis, but is not involved in cell shape change.

### METHODS

**Cell Cultures:** Isolation and establishment in culture of rabbit CECs were performed as previously described [5]. Briefly, Descemet's membrane-corneal endothelium complex was treated with 0.2% collagenase and 0.05% hyaluronidase for 90 min at 37 °C. Cultures were maintained in Dulbecco's

modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 50  $\mu$ g/ml of gentamicin (DMEM-10) in a 5% CO<sub>2</sub> incubator. This procedure has been shown to promote cell proliferation during the early phase of culture and to maintain the culture as a contact-inhibited monolayer when the cells reach confluency. First passaged CECs were used for all experiments. Modulated CECs were prepared by treating the cells with FGF-2 (10 ng/ml) and heparin (10  $\mu$ g/ml); the duration of the treatment of cells with FGF-2 varied in experiments.

**Treatment with Inhibitors and Neutralizing antibodies:** When the modulated cells reached confluency with elongated cell morphology, the cells were treated with genistein (Sigma, St. Louis, MO), LY294002 (Sigma), anti-FGF-2 antibody (Upstate Biotechnology Inc, UBI, Lake Placid, NY), anti-PI3-kinase antibody (Transduction Laboratories, Lexington, KY), or anti-PLC- $\gamma$ 1 antibody (UBI). For reversion of cellular morphology, the inhibitors were used at a concentration of 10  $\mu$ M for genistein and 20  $\mu$ M for LY294002; for the inhibitory ac-

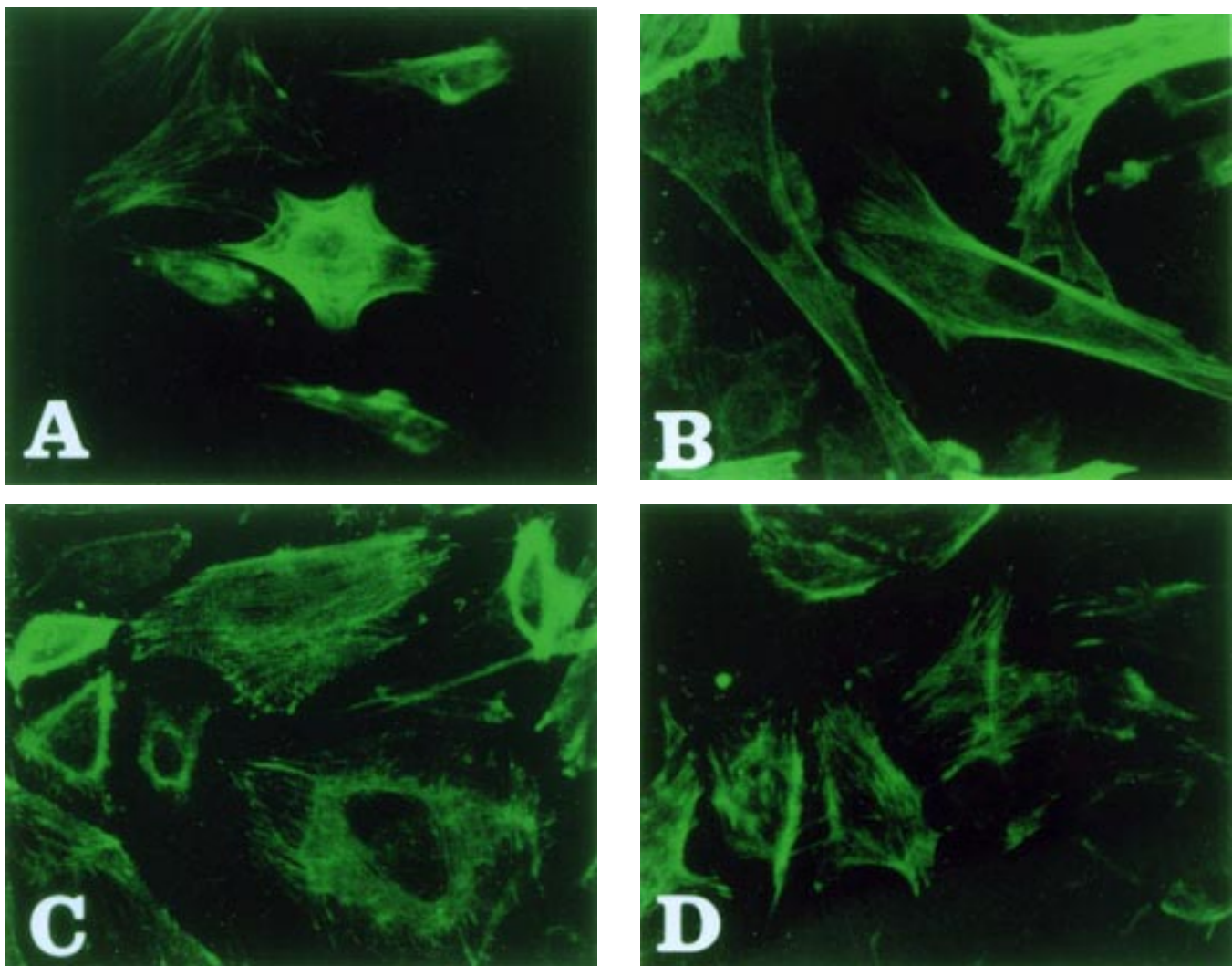


Figure 3. Immunofluorescent analysis of smooth muscle  $\alpha$ -actin in CECs. CECs modulated with FGF-2 (10 ng/ml and 10  $\mu$ g/ml heparin) were treated with inhibitors in the presence of FGF-2 for 24 h. Cells were stained with anti-smooth muscle  $\alpha$ -actin antibody, processed and analyzed on confocal laser microscope as described in the text. (X 400). (A) normal CECs, (B) cells treated with FGF-2, (C) cells treated with FGF-2 and LY294002 (20  $\mu$ M), (D) cells treated with FGF-2 and genistein (10  $\mu$ M).

tion of the inhibitors on cell proliferation mediated by FGF-2, a range of concentrations was used. Neutralizing antibody was added to the culture in the amount of  $5 \mu\text{g}$  per  $1 \times 10^6$  cells.

**Cell Proliferation Assay:** Normal and modulated CECs ( $5 \times 10^3$ ) were plated in 96-well tissue culture plates. When the cells reached approximately 80% confluency, they were treated with respective inhibitors for 24 h. At the end of the incubation period,  $100 \mu\text{l}$  of CellTiter 96<sup>®</sup>AQueous One Solution Cell Proliferation Assay reagent (Promega, Madison, WI) was added to the wells. The plates were incubated for 1 h at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere after which the absorbance was read at 490 nm using a 96 well plate reader. This step was repeated at the end of the 2- and 3-hour incubations. Absorbance readings at the end of the 2-hour incubation were similar to those following the 3-hour incubation, therefore, a 2-hour incubation was used.

**Immunofluorescent Staining :** Normal and modulated CECs ( $3 \times 10^4$ /chamber) were seeded on four-well chamber slides. The cells were treated with inhibitors as stated above.

Cells were then washed with phosphate buffered saline (PBS) and fixed with 3% paraformaldehyde in PBS. All washes and incubations were carried out in PBS at room temperature. Cells were then permeabilized with 0.5% Triton X-100 in PBS for 5 min and blocked with 2% bovine serum albumin (BSA). The chamber slides were incubated with primary antibody (1:50 dilution) for 2 h then washed with PBS. Cells were then incubated with the biotinylated secondary antibody (Vector Laboratories, Inc., Burlingame, CA; 1:50 dilution) for 1 h followed by incubation with fluorescein conjugated to avidin (Vector; 1:100 dilution) for 30 min. Following extensive washing, the slides were examined under a Leica confocal microscope (Heidelberg, Germany). For the co-localization experiment of filamentous actin (F-actin) and  $\alpha$ -actinin, the slides were simultaneously incubated with fluorescein phalloidin (1:100 dilution) (Molecular Probes, Eugene, OR) and monoclonal anti- $\alpha$ -actinin antibody (1:100) (Sigma) for 2 h at room temperature then washed with PBS. Cells were then incubated with biotinylated anti-mouse immunoglobulins (1:100) for 1 h, followed by incubation with rhodamine conjugated to avi-

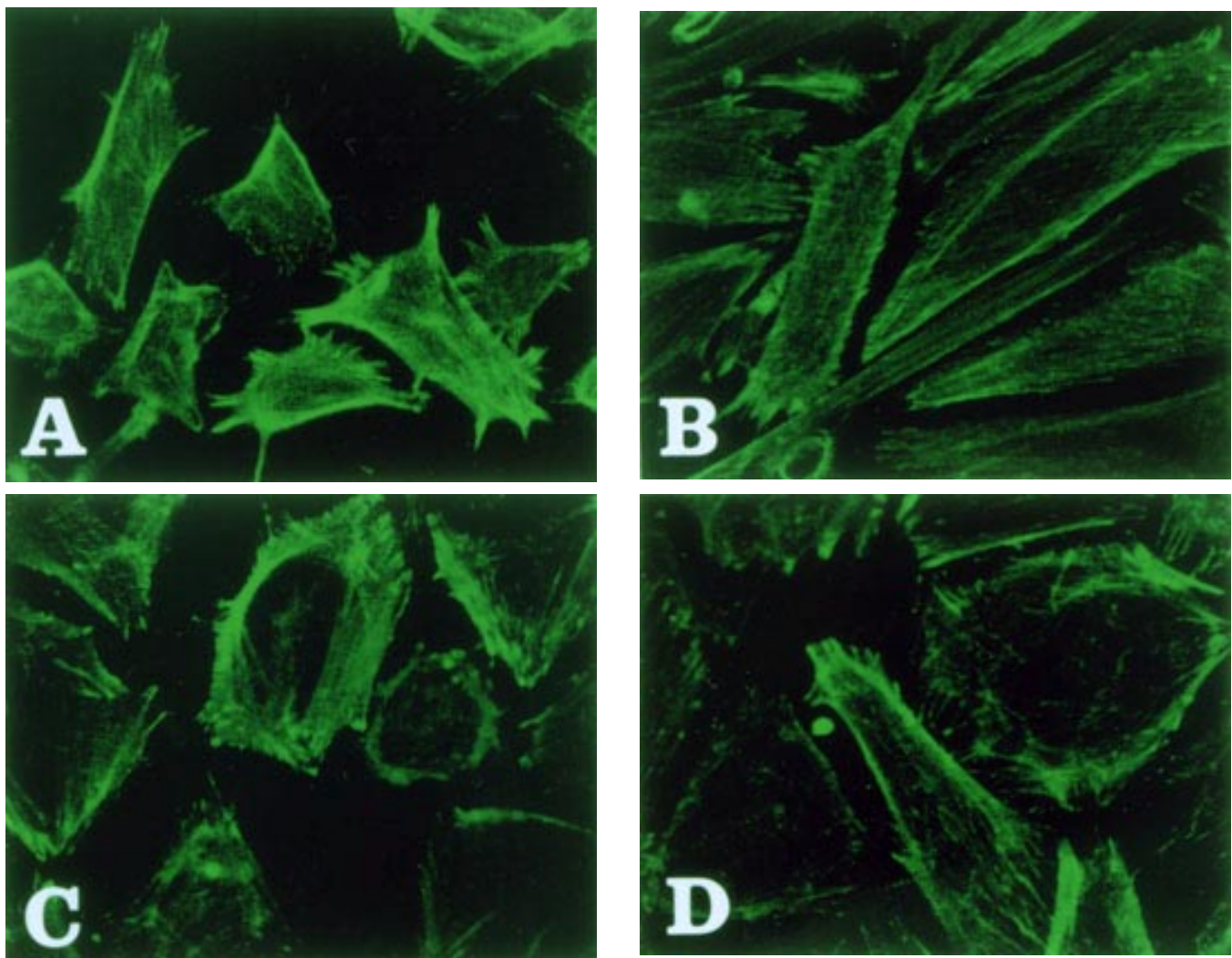


Figure 4. Immunofluorescent analysis of  $\alpha$ -actinin in CECs. Cells modulated with FGF-2 were treated with inhibitors in the presence of FGF-2 for 24 h. Cells were stained with anti  $\alpha$ -actinin antibody, processed, and analyzed on confocal microscopy as described in the text (X 400). (A) normal CECs, (B) cells treated with FGF-2, (C) cells treated with FGF-2 and LY294002 ( $20 \mu\text{M}$ ), and (D) cells treated with FGF-2 and genistein ( $10 \mu\text{M}$ ).

din (1:50) for 30 min at room temperature. Following extensive washing, the slides were examined under a Zeiss confocal microscope.

**Immunoprecipitation:** Cytoskeleton was prepared as previously described [21]. All buffers were maintained at 4°C during cytoskeleton isolation. Cells were washed with ice-cold microtubule stabilization buffer containing 0.1 M Pipes, pH 6.9, 2 M glycerol, 1 mM EGTA, and 1 mM magnesium acetate. Cells were homogenized with cold microtubule stabilization buffer containing 0.2% Triton-X-100, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were centrifuged and the pellet was dissolved in sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer followed by boiling. Protein concentration was determined using a BioRad Dc Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

**Immunoprecipitation:** Samples of a constant amount (10-20 µg) were subjected to immunoprecipitation. Five micrograms of undiluted anti-PI3-kinase antibody was added. To

this mixture, 50 µl of protein G-sepharose resin (Sigma) was added, and incubation was carried out at 4°C for 1 h. Following centrifugation at 10,000 rpm for 10 min, the resin was washed three times with PBS containing protease inhibitors (PMSF, aprotinin and EDTA). The protein was eluted from the resin by boiling in SDS-PAGE sample buffer for 5 min. After a brief spin, the supernatant was subjected to SDS-PAGE.

**SDS-Polyacrylamide Gel Electrophoresis:** The conditions of electrophoresis were as described by Laemmli [29]. Vinculin and  $\alpha$ -actinin were analyzed on a 5% gel under reduced conditions and smooth muscle  $\alpha$ -actin was analyzed on an 11% gel under non-reducing conditions using a discontinuous Tris-glycine buffer system (pH 8.3).

**Immunoblot Analysis:** Proteins separated by SDS-PAGE were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane at 0.22 ampere for 10 h in a semi-dry transfer system (Transfer buffer: 25 mM Tris, pH 8.3, 190 mM glycine, 20% MeOH). Immunoblot analysis was performed as described previously [21] using a commercial ABC Vectastain kit (Vector Laboratories). All washes and

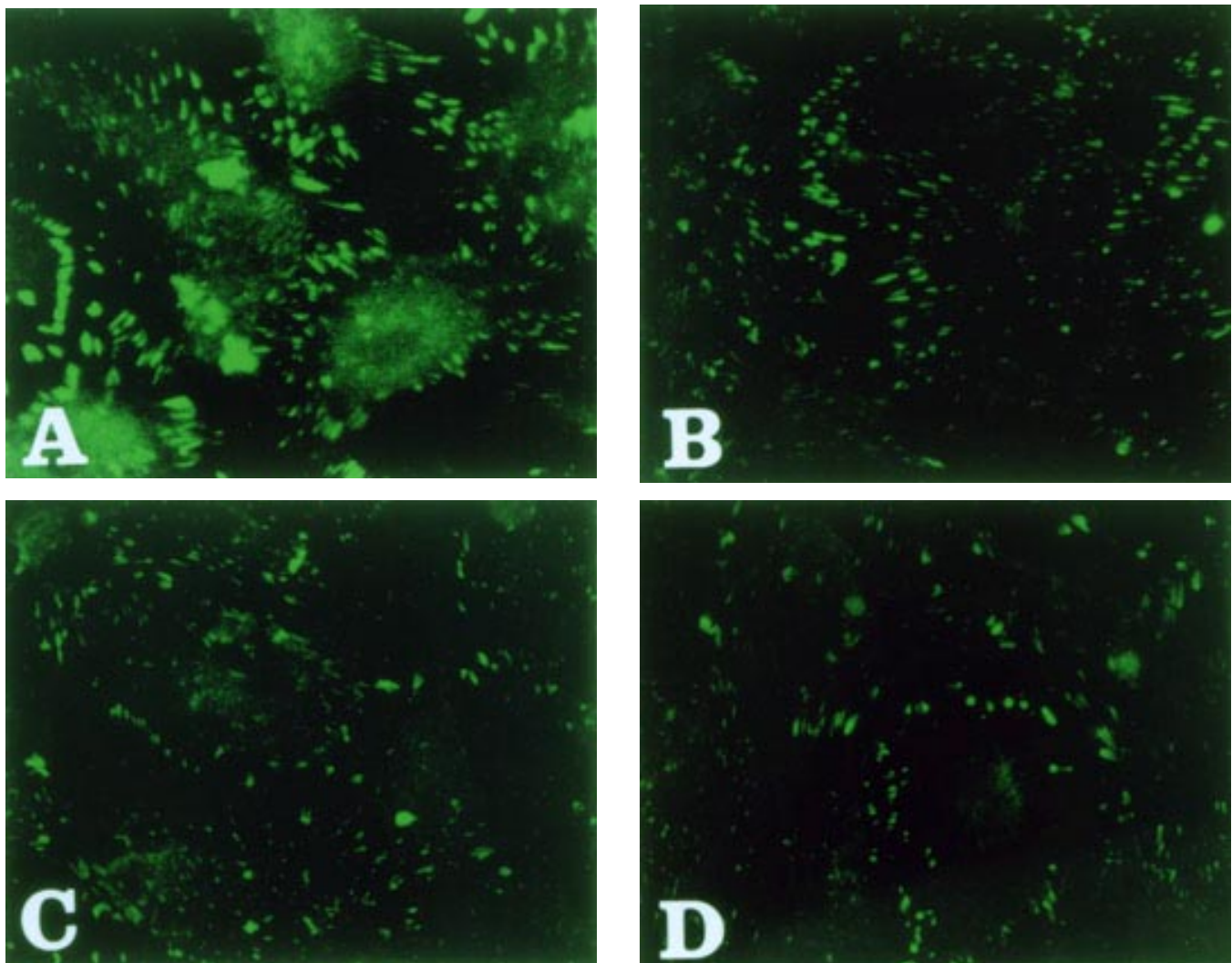


Figure 5. Immunofluorescent analysis of vinculin in CECs. Cells modulated with FGF-2 were treated with inhibitors in the presence of FGF-2 for 24 h. Cells were stained with anti-vinculin antibody, processed and analyzed on confocal microscopy as described in the text (X 400). (A) normal CECs, (B) cells treated with FGF-2, (C) cells treated with FGF-2 and LY294002 (20 µM), and (D) cells treated with FGF-2 and genistein (10 µM).

incubations were carried out at room temperature in TTBS (0.9% NaCl, 100 mM Tris-HCl, pH 7.5, 0.1% Tween 20). Briefly, the PVDF membrane was immediately placed into the blocking buffer (5% nonfat milk containing TTBS) for 1 h. The incubation with primary antibody (1:5000 dilution) was carried out for 2 h; incubation with biotinylated secondary antibody (1:2500 dilution) was carried out for 1 h; and incubation with the ABC reagent was for 30 min. The membrane was treated with enhanced chemiluminescence (ECL) reagent (Amersham Life Science, Buckinghamshire, England) for 1 min and the ECL-treated membrane was exposed further to ECL film.

## RESULTS

*Effect of Inhibitors on FGF-2 mediated Cell Shape Changes:* The involvement of PI3-kinase in the signal transduction pathway for cell shape change mediated by FGF-2 was investigated using specific antibodies (Figure 1). The confluent CECs demonstrated a polygonal monolayer, while the FGF-2-treated cells showed a loss of polygonal shape and a subsequent change to an elongated morphology. When CECs were treated simultaneously with FGF-2 and anti-FGF-2 antibody, the neutralizing antibody was able to block the modulating activity of FGF-

2. A similar reversion was shown when CECs were treated simultaneously with FGF-2 and anti-PI3-kinase antibody. On the other hand, anti-PLC- $\gamma$ 1 antibody was unable to block the modulation activity of FGF-2. This confirmed our previous finding that PLC- $\gamma$ 1 associated with vinculin and actin is not involved in the modulation of cell shape [21]. The effect of inhibitors for PI3-kinase and receptor tyrosine kinase on the fibroblastic CECs was determined (Figure 2). In the continuous presence of FGF-2, polygonal cells were completely converted to elongated fibroblastic cells. The difference in the cellular morphology of CECs treated with FGF-2 between Figure 1 and Figure 2 was caused by differences in the duration of treatment with the growth factor and when the treatment begins. When the fibroblastic CECs were treated with a low concentration of LY294002 (20  $\mu$ M) to specifically inhibit PI3-kinase, LY294002 was able to cause the cell shape to revert completely to a polygonal morphology (Figure 2C), while wortmannin (a less specific inhibitor for PI3-kinase) or genistein (an inhibitor for receptor tyrosine kinase), almost but not completely, caused the modulated cellular morphology to revert to a polygonal shape (Figure 2D). It is of interest that a high concentration of LY294002 (40  $\mu$ M) demonstrated inhibitory activity on mitogenesis as well (Figure 2F).

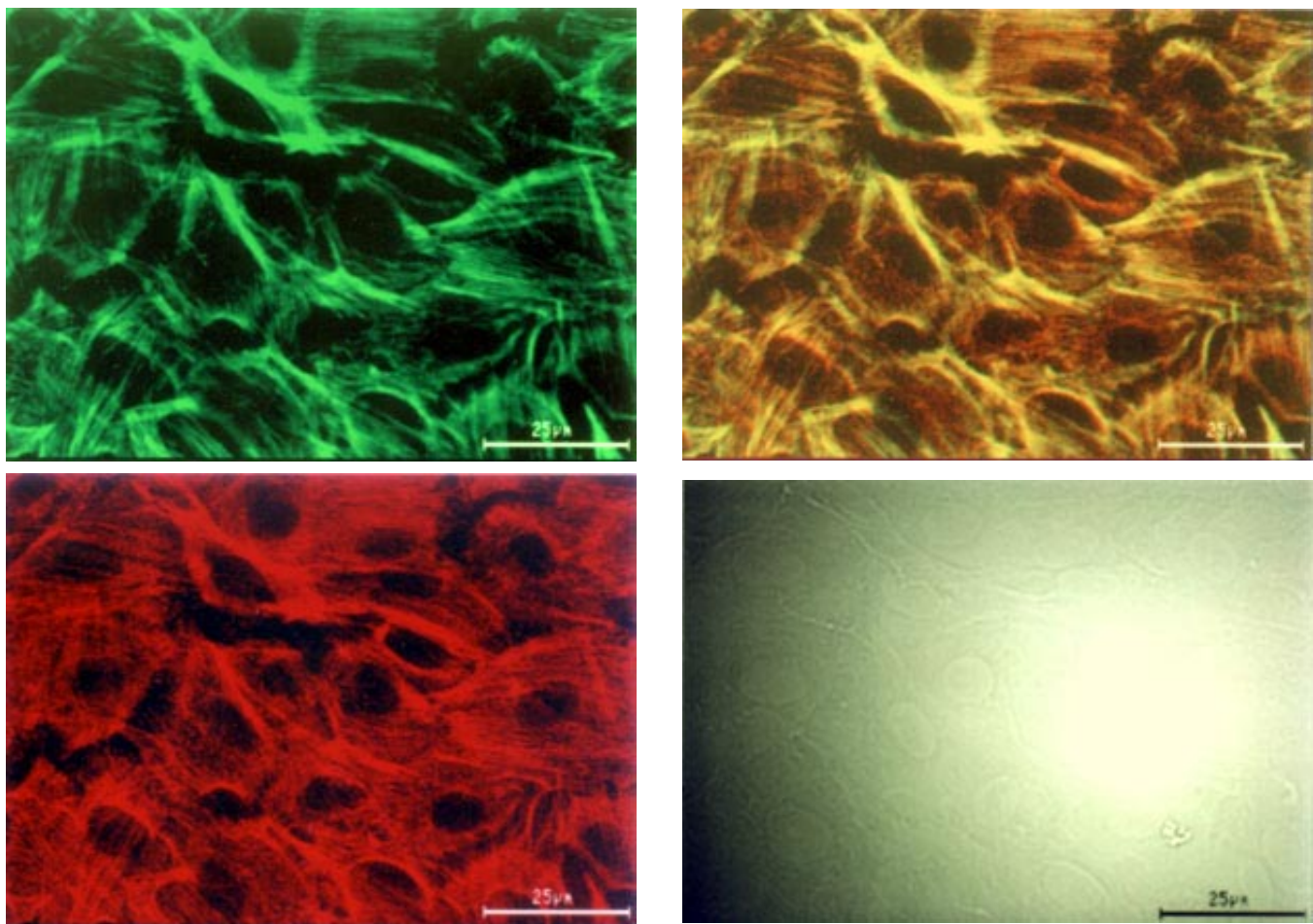


Figure 6. Colocalization of F-actin and  $\alpha$ -actinin in normal CECs. Cells were treated with Triton-X-100, BSA and double labeled for F-actin and  $\alpha$ -actinin as described in the text. Fluorescein signals (green) are F-actin positive, rhodamine signals (red) are  $\alpha$ -actinin positive, and yellow signals show the colocalization of F-actin and  $\alpha$ -actinin. The last panel is a phase-contrast image. (bar = 25  $\mu$ m).

**Actions of Inhibitors on Cytoskeleton:** To determine whether FGF-2 causes endothelial mesenchymal transformation in CECs, the expression of smooth muscle  $\alpha$ -actin, a mesenchymal marker, was determined in normal polygonal CECs, modulated fibroblastic cells, and reversed polygonal cells induced by inhibitors (Figure 3). Regardless of cell shape (polygonal or fibroblastic) or cell state (endothelial versus mesenchymal), all cells showed filamentous cytoplasmic distribution of smooth muscle  $\alpha$ -actin. The staining of smooth muscle  $\alpha$ -actin appeared to be organized into bundles, similar to stress fibers. Neither LY294002 nor genistein altered the staining profile of smooth muscle  $\alpha$ -actin, either qualitatively or quantitatively (Figure 3C and Figure 3D). When a similar analysis was performed for  $\alpha$ -actinin, an actin-binding protein, almost identical profiles to those seen with smooth muscle  $\alpha$ -actin were observed:  $\alpha$ -actinin demonstrated filamentous and punctate staining within the cells, regardless of cell shape (Figure 4). Neither FGF-2 nor inhibitors altered the staining profiles of  $\alpha$ -actinin. When the staining profiles of vinculin, another actin-binding protein, were analyzed, the characteristic punctate staining was observed in normal CECs. The cells that reverted after treatment with inhibitors, as well as the modulated fibroblastic cells, demonstrated a similar degree of punctate staining profiles (Figure 5): these cells

showed far less staining potential than the normal CECs. Nevertheless, the characteristic punctate staining potential was not altered by either FGF-2 or inhibitors (Figure 5). When filamentous actin (F-actin) and  $\alpha$ -actinin were simultaneously stained, CECs demonstrated prominent F-actin bundles and marked colocalization profiles of these proteins in normal and modulated CECs (Figure 6 and Figure 7). These cells, regardless of cell shape, showed an additional intracellular filamentous distribution of  $\alpha$ -actinin. When the fibroblastic CECs were reverted by treatment with LY294002, the induced polygonal cells demonstrated identical staining profiles to those seen in normal CECs (Figure 8):  $\alpha$ -actinin was present along the cortical actin filament with occasional intracellular stress fiber bundles and there was a marked colocalization of F-actin and  $\alpha$ -actinin.

**Association of PI3-kinase with Cytoskeleton:** Our previous study showed that PLC- $\gamma$ 1 is associated with cytoskeleton [21]; PLC- $\gamma$ 1 associated with vinculin and actin is responsible for cell proliferation mediated by FGF-2, whereas the same complex is not involved in the alteration of cellular morphology mediated by FGF-2. We, therefore, examined whether PI3-kinase also is associated with cytoskeleton proteins using immunoprecipitation with anti-PI3-kinase antibody followed by immunoblotting with anti-smooth muscle  $\alpha$ -actin antibody,

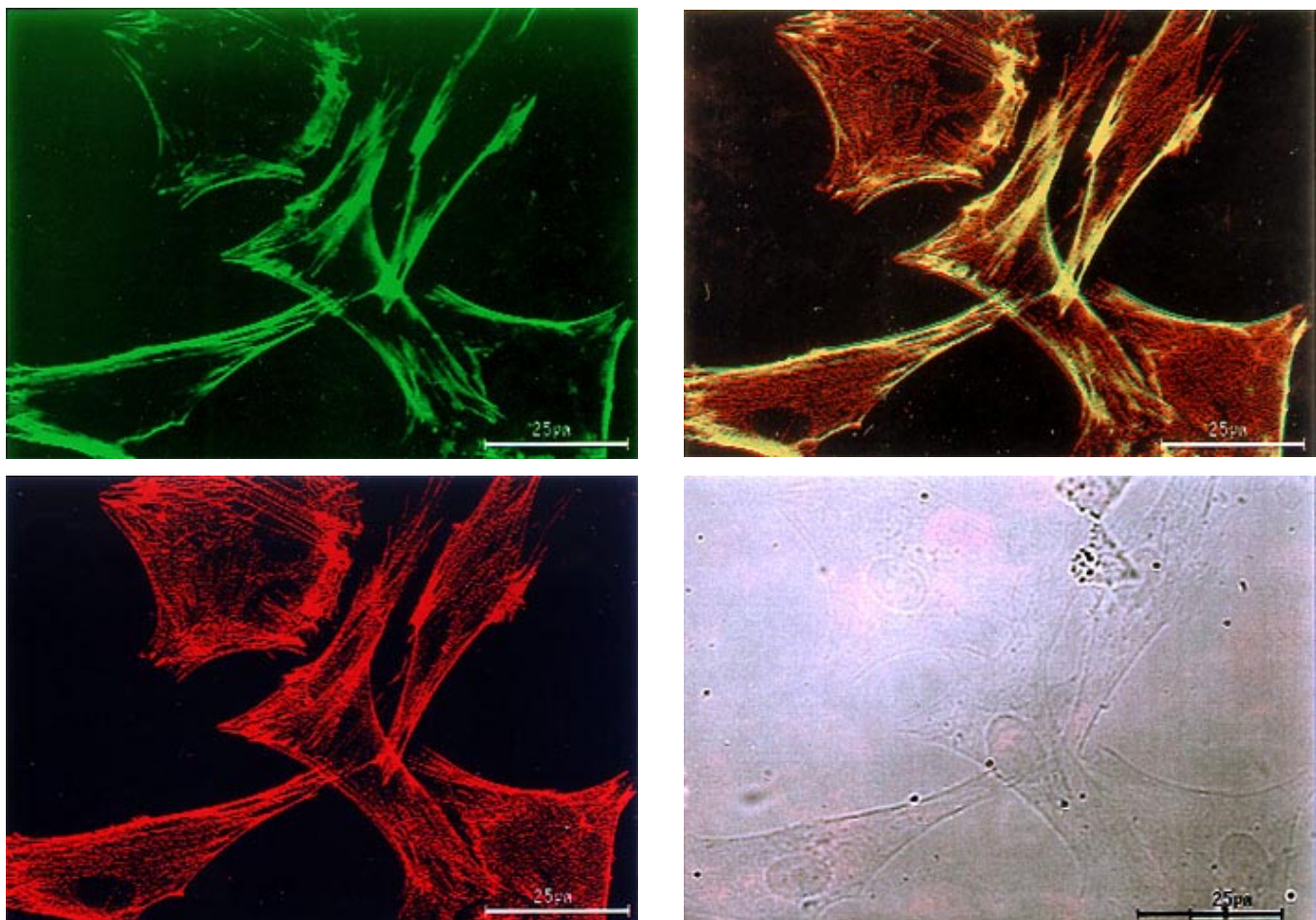


Figure 7. Colocalization of F-actin and  $\alpha$ -actinin in modulated CECs. Modulated cells were induced with treatment of FGF-2, and staining procedures were described in Figure 6 and in the text. Fluorescein signals (green) are F-actin positive, rhodamine signals (red) are  $\alpha$ -actinin positive, and yellow signals show the colocalization of F-actin and  $\alpha$ -actinin. The last panel is a phase-contrast image. (bar = 25  $\mu$ m).

anti- $\alpha$ -actinin antibody or anti-vinculin antibody (Figure 9). The immune complex contained  $\alpha$ -actinin (100 kDa), vinculin (116 kDa) and smooth muscle  $\alpha$ -actin (45 kDa), regardless of cell shape (polygonal versus fibroblastic). The amounts of  $\alpha$ -actinin and smooth muscle  $\alpha$ -actin were similar in both normal and fibroblastic CECs, whereas the level of vinculin in

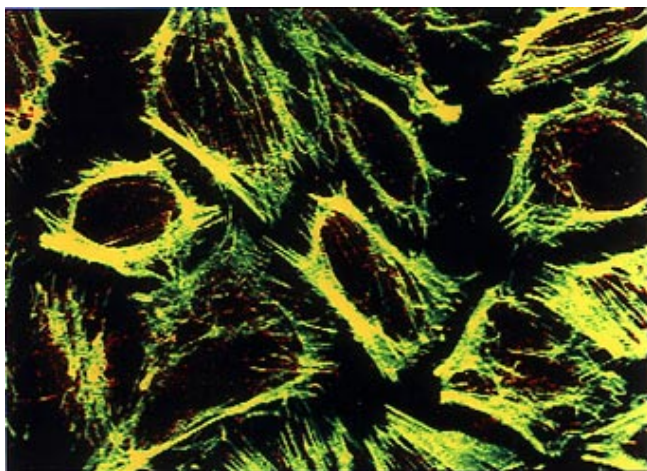
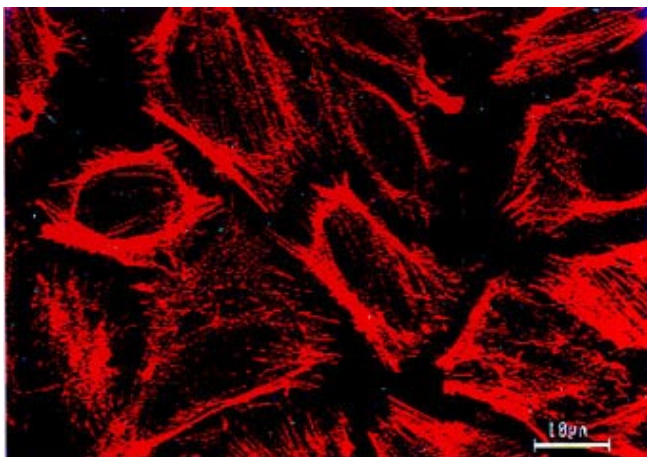
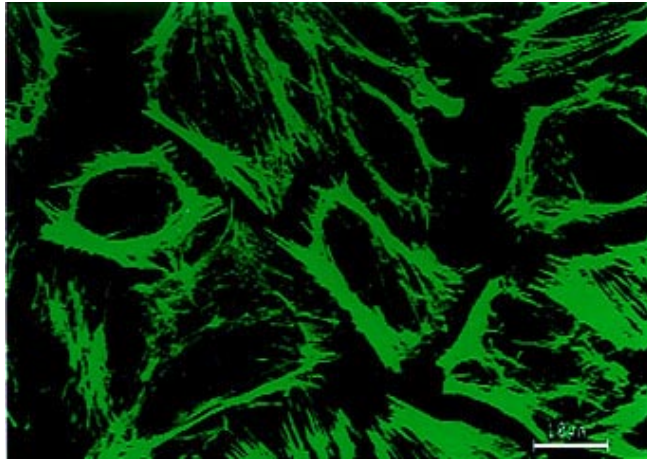


Figure 8. Colocalization of F-actin and  $\alpha$ -actinin in modulated CECs treated with LY294002. Modulated cells induced by FGF-2 were treated with LY294002 (20  $\mu$ M) and the staining procedures were described in Figure 6 and in the text. Fluorescein signals (green) are F-actin positive, rhodamine signals (red) are  $\alpha$ -actinin positive, and yellow signals show the colocalization of F-actin and  $\alpha$ -actinin. (bar = 25  $\mu$ m).

the fibroblastic CECs appeared to be much lower than that of the normal cells. These findings are in agreement with the immunofluorescent analysis.

*Effect of Inhibitors on Cell Proliferation Mediated by FGF-2:* In order to determine whether PI3-kinase is involved also in mitogenic signaling pathway mediated by FGF-2, primary CECs were treated with one of the following conditions: FGF-2, FGF-2 and genistein in concentrations ranging from 10 to 100  $\mu$ M, FGF-2 and LY294002 in concentrations ranging from 5 to 50  $\mu$ M, and wortmannin in concentrations ranging from 10 to 500 nM (Figure 10). Genistein inhibited the cell proliferation mediated by FGF-2 in a dose-dependent manner, a mild inhibitory action was observed up to 50  $\mu$ M, but there was a marked inhibition at 100  $\mu$ M. LY294002 demonstrated a moderate inhibitory action in a dose-dependent manner: approximately 60% inhibition occurred at 50  $\mu$ M. Unlike LY294002, wortmannin, a less specific PI3-kinase inhibitor, reached a maximal inhibitory activity on cell proliferation mediated by FGF-2 at low concentration of 20 nM.

### DISCUSSION

Our previous study demonstrated that in corneal endothelial cells, FGF-2 is not merely a mitogen, it is also a potent modulator of endothelial phenotypes [6,7,21]. Endothelial cells grown in the continuous presence of FGF-2 not only proliferate excessively, they also convert to fibroblast-like cells that begin to produce fibrillar collagens (types I, III and V). It is likely that several distinct signal transduction pathways contribute to the establishment of these cellular responses. To understand how these diverse actions (cell proliferation, cell shape change, collagen phenotype switch) of FGF-2 on corneal endothelial cells are relayed, we focused our attention on the earliest events, such as the direct bindings of PLC- $\gamma$ 1 and PI3-kinase to FGF receptors. We previously reported that PLC- $\gamma$ 1 is the key molecule for the mitogenic signaling pathway mediated by FGF-2 and that PLC- $\gamma$ 1 is not involved in cell shape changes mediated by FGF-2 [21]. The results presented here demonstrate that PI3-kinase is the mediator for modulation of cellular morphology of CECs. LY294002 at a

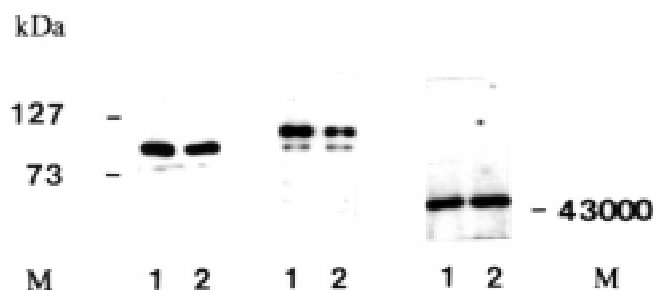


Figure 9. Association of vinculin, smooth muscle  $\alpha$ -actin and  $\alpha$ -actinin with PI3-kinase in the cytoskeleton. Cytoskeleton fractions of the first passage CECs and modulated CECs were prepared, and proteins (30  $\mu$ g/sample) associated with PI3-kinase were detected by immunoprecipitation with PI3-kinase antibody and immunoblotting with vinculin antibody, smooth muscle  $\alpha$ -actin antibody or  $\alpha$ -actinin antibody. 1, normal CECs; 2, CECs modulated with FGF-2. M, protein size marker.

concentration of 20  $\mu\text{M}$ , which is low enough to block only PI3-kinase, caused the fibroblastic morphology modulated by FGF-2 to revert to a polygonal shape. Likewise, anti-PI3-kinase antibody was able to block the activity of FGF-2 on cell shape changes. On the other hand, anti-PLC- $\gamma$ 1 antibody failed to block the modulation activity of FGF-2. These findings suggest that PI3-kinase is the key molecule in dictating cell shape mediated by FGF-2. For mitogenic activity of FGF-2, the inhibitors for PI3-kinase were able to moderately block cell proliferation. LY294002 at low concentrations showed a moderate inhibitory action on FGF-2-mediated cell proliferation. Wortmannin, a nonspecific inhibitor of the enzyme, had

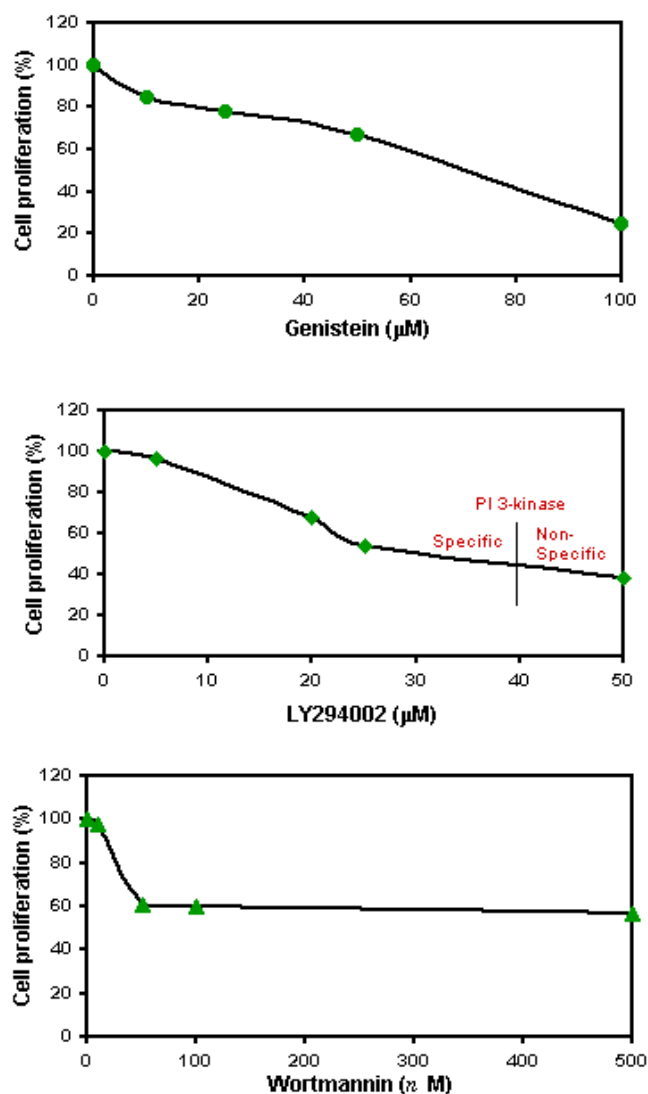


Figure 10. The effect of inhibitors on cell proliferation mediated by FGF-2 in CECs. The first passage CECs were plated into a 96-well plate and treated with one of the following conditions for 24 h when cells reached 80% confluency: FGF-2, FGF-2 and genistein in concentration ranging from 10 to 100  $\mu\text{M}$  (top panel); FGF-2 and LY294002 in concentration ranging from 5 to 50  $\mu\text{M}$  (middle panel); and wortmannin in concentration ranging from 10 to 500 nM (bottom panel). The cell proliferation assay was performed using CellTiter 96<sup>®</sup>Aqueous One Solution Cell Proliferation Assay (Promega) as described in the text. Inhibitory activity of the inhibitors were presented as percent of FGF-2 mediated cell proliferation.

a similar activity at low concentrations. These findings suggest that the initial steps of the putative mitogenic pathways initiated by association with PLC- $\gamma$ 1 and PI3-kinase are non-overlapping. It is not known whether both pathways lead to entry into S phase or whether they converge.

Our previous study also demonstrated that PLC- $\gamma$ 1 is associated with cytoskeleton, vinculin and actin [21]. Therefore, we further investigated whether PI3-kinase is associated with cytoskeleton. The immunoprecipitation followed by immunoblotting analysis demonstrated that PI3-kinase was associated with vinculin,  $\alpha$ -actinin, and smooth muscle  $\alpha$ -actin. The amount of  $\alpha$ -actinin and smooth muscle  $\alpha$ -actin associated with PI3-kinase in the fibroblastic CECs modulated with FGF-2 was similar to that of normal CECs, whereas the level of vinculin associated with PI3-kinase in the modulated cells was reduced when compared to that of normal cells. Such a finding was confirmed with the immunofluorescent staining profile of these proteins. The staining profiles of  $\alpha$ -actinin and smooth muscle  $\alpha$ -actin in the fibroblastic modulated cells were similar to those of polygonal normal cells or cells that were reversed to a polygonal shape by the inhibitors. On the other hand, the characteristic punctate staining of vinculin was less prominent in modulated CECs.

The present findings suggest that the organization and distribution of actin stress fibers (one major cytoskeleton system) is not changed as a result of the action of FGF-2 or inhibitors. These findings also confirm our previous observation that cytochalasin B does not cause the FGF-2 mediated fibroblastic morphology to revert to a polygonal shape [30], unlike colchicine, which does cause the cellular morphology to revert from fibroblastic to polygonal shape. Taken together, it is likely that what dictates cellular morphology in corneal endothelial cells is not a microfilament system but a microtubule system. Whether PI3-kinase is associated with microtubule for its action remains to be elucidated.

In summary, we conclude from the current study and our previous study that FGF-2 may use PLC- $\gamma$ 1 for mitogenic signaling and PI3-kinase for modulation of cellular morphology and mitogenic response. Further investigation focusing on the mechanism by which PI3-kinase and PLC- $\gamma$ 1 direct the initiation of cell proliferation should unravel the similarities and differences between these two pathways. Furthermore, investigations focusing on the mechanism by which PI3-kinase directs modulation of cellular morphology is expected to provide information on the specificity of the individual cellular responses.

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