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Effects of Transforming Growth Factor Beta on Cytoskeleton Structure and Extracellular Matrix in Mv1Lu Mink Epithelial Cells

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Abstract: Previous studies have shown that transforming growth factor beta (TGF-β) is a potent regulator of cell growth and differentiation. To study the effects of TGF-β on cell morphology and cytoskeleton reorganization, we conducted a survey using Mv1Lu mink lung epithelial cells with antibodies to cytoskeletal proteins and an extracellular matrix protein. While the untreated cells showed a cuboidal shape of typical epithelia, the Mv1Lu cells displayed a drastic shape change in the presence of TGF-β. This alteration was most prominent when near-confluent cells were treated with TGF-β. Since the morphology alteration is known to be accompanied by the reorganization of cytoskeletal proteins in other cell types, we investigated the intracellular distribution of the three major cytoskeletal structures: microfilaments, microtubules, and intermediate filaments. In the microfilament system, TGF-β induced new stress fiber formation, which was caused primarily by the polymerization of cytoplasmic G-actin. However, TGF-β appeared not to induce any significant changes in microtubular structures and vimentin filaments as determined by indirect fluorescence microscopy. Finally we confirmed the rapid accumulation of fibronectin by immunoblot analysis and chased the protein locations by immunofluorescence microscopy.

Key words: cytoskeleton, extracellular matrix, Mv1Lu cells, stress fiber, TGF-β.

TGF-β is a potent regulator of cell growth and differentiation, and exerts a variety of biological activities depending on the target cell type (Roberts and Sporn, 1991; Derynck, 1994). TGF-β is secreted in a latent form comprised of a homodimer of 105 kDa of which the C-terminal 112 amino acids of each chain forms the mature active 25-kDa cytokine. The remaining N-terminal 75-kDa dimer has been designated the latency-associated peptide (LAP). The active portion of the molecule is cleaved from the LAP during or shortly after secretion of the complex from the cell (Miyazono *et al.*, 1991). Following interaction with receptors, TGF-β has the ability to stimulate or inhibit cell proliferation. The anti-proliferative activity is well studied in epithelial cells, whereas the growth stimulatory effects occurs mainly in cells of mesenchymal origin such as fibroblasts.

TGF-β can induce anchorage-dependent and anchorage-independent growth of fibroblast in some cases, but it inhibits the growth of normal as well as tumor-derived cells in other cases. However, TGF-β is a strong inhibitor of adipogenic and myogenic differentiation under conditions in which it does not alter the proliferation of preadipocytes or myoblast. TGF-β also has been reported to inhibit mitogen-induced lymphocyte and thymocyte proliferation, down regulate receptor and cytokine expression, and inhibit generation of CTL and LAK cells. In addition, TGF-β has been found to induce the expression of CD8 on thymocytes suggesting that it has a differentiative function in addition to its anti-proliferative activity. TGF-β also increases the expression of several extracellular matrix proteins and integrin receptors for extracellular matrix components, and inhibits the proteolytic degradation of this matrix (Edward *et al.*, 1987; Roberts *et al.*, 1988). How TGF-β exerts its variety of activities at the receptor level is at present largely unknown. Chemical cross-linking of radiolabeled TGF-β to cell surface TGF-β-binding proteins has revealed the existence of up to nine different receptors (Massague, 1992). Among these, the types I, II, and III TGF-β receptors are most frequently detected on the surface of many different cell. The type III receptor is a cell surface proteoglycan that presumably does not have any signaling activity (Lopez-Casillas

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et al., 1991; Wang *et al.*, 1991).

The mink lung epithelial cell line Mv1Lu provides the opportunity to study the cellular responses to TGF-β. The proliferation of Mv1Lu cells is potently inhibited by TGF-β (Associan *et al.*, 1984; Tucker *et al.*, 1984). The phenotypic responses of Mv1Lu cells to TGF-β is characterized by a flat, very enlarged cell morphology and accumulation of extracellular matrix fibronectin (Like and Massague, 1986). These morphological changes probably result from the rea rrangement of cellular cytoskeletons and extracellular matrix components. Although Mv1Lu cells are one of the cell types used for the bioassay of TGF-β, few studies have been conducted on the identification of cellular components or factors leading to the morphological changes of the cell type. In this report, we studied the effects of TGF-β on actin filaments, microtubules, and intermediate filaments, which are the three major types of cytoskeletal components involved in cell shape and morphology, by immunofluorescence microscopy and Western blotting analysis.

Materials and Methods

Cell culture

Mink epithelial cells (Mv1Lu, CCL64) were grown in DMEM with 10% fetal bovine serum (Hyclone, Logan, USA) and maintained at 37℃ in a humidified atmosphere of 7% carbon dioxide. The cells were cultured for various periods of time in the presence or absence of human TGF-β (type-1, R & D System Inc., Minneapolis, USA). Since concentration of TGF-β ranging from 1 to 10 ng/ml gave similar results, we treated the cells with TGF-β at a concentration of 2 ng/ml throughout the experiments.

Immunofluorescence microscopy

Cells for immunofluorescence were seeded onto 18 mm diameter glass coverslips in a 12-well plate, cultured for 1 day and then treated for 2 days with TGF-β, unless otherwise indicated. Untreated control cells were cultured for the same length of time as treated cells, and all samples were processed together. Cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 15 min, washed with PBS, and permeabilized with cold methanol for 1 min or PBS with 0.5% Triton-X 100 for 5 min. The cells were blocked with 10% fetal calf serum in PBS for 20 min and washed with PBS twice. The cells were then treated with primary antibodies for 2 h, washed in PBS and reacted with fluorescein-conjugated goat anti-mouse or rabbit antibodies (Jackson ImmunoResearch, West Grove, USA). After washes, the coverslips were mounted on a microscope slide using a 90% solution of glycerol in PBS. Photography was carried out using an Olympus epifluorescence microscope and Kodak T-max ASA 400 (Kodak, Rochester, USA). All primary antibodies were obtained from Sigma Chemical Co. (St. Louis, USA). Anti-actin antibody was developed in rabbit using actin from chicken (cat. A2668). Anti-vimentin (V5255), anti-β-tubulin (T4026), and anti-fibronectin (F7378) were mouse monoclonal antibodies.

Preparation of Triton-X insoluble cytoskeletons

Cellular fractions enriched with cytoskeletons were prepared as follows. Mv1Lu cells either treated with TGF-β or not were grown in 100 mm culture dishes. The cells were pelleted by centrifugation in 15-ml tubes and lysed for 10 min at room temperature at 10^6 cells/ml in 0.6 M KCl, 1% Triton-X 100, 10 mM $MgCl_2$, 1 mM PMSF, 100 μg/ml leupeptin, 10 μg/ml aprotinin. DNase was added to a final concentration of 25 μg/ml and incubated for 10 min at room temperature. Lysates were centrifuged at top speed for 10 min in a microcentrifuge. The pellets were suspended in PBS with the protease inhibitors, centrifuged for 10 min, and resuspended in PBS with 0.5% SDS.

Western blot analysis

For immunoblotting, proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) were electrophoretically transferred to nitrocellulose membrane (Towbin *et al.*, 1979), and the membrane was rinsed briefly in distilled water and air-dried. The blots were blocked with 2% non-fat dry milk in PBS for 1 h. After rinsing with PBS, the blot was incubated with primary antibodies for 1 h and washed 3 times in PBS containing 0.3% Tween 20 at 5-min intervals. The blots were treated with alkaline phosphatase (AP)-conjugated goat secondary antibody (Jackson ImmunoResearch, West Glove, USA) for 1 h and washed 3 times. Following the final rinse for 5 min with an AP buffer (100 mM Tris-HCl and 5 mM $MgCl_2$, pH 9.5), color reaction was started by incubating the blots in AP buffer containing nitro blue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP). [For 10 ml of solution, 60 μl of NBT (50 mg/ml in 70% dimethylformamide) and 30 μl of BCIP (50 mg/ml in 100% dimethylformamide) were added to the 10 ml of AP buffer]. When the color reaction had reached the desired intensity, the reaction was stopped by rinsing the membranes with several changes of distilled water. The blots were photographed while still moist.

Results

Responses of Mv1Lu cells to TGF-β

The proliferation and morphology effects of TGF-β on the Mv1Lu mink epithelial cells were studied. As

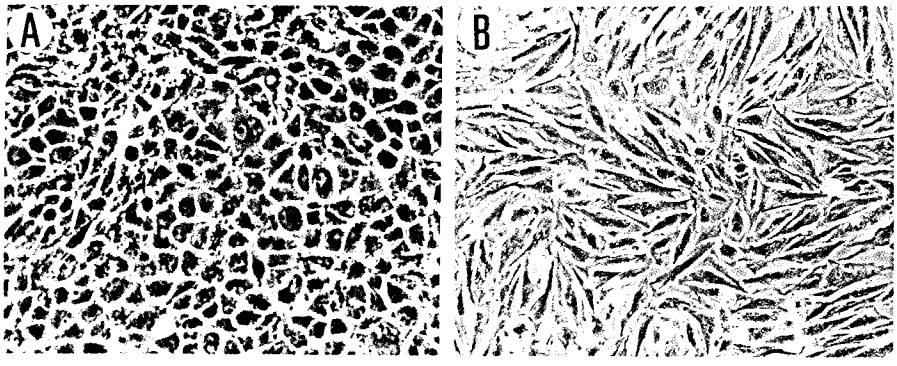

Fig. 1. Effect of TGF-β on morphology of Mv1Lu mink lung epithelial cells. Mv1Lu cells were seeded onto 18-mm round coverslips in a 12-well plate and grown for 1~2 days until cells became almost confluent. Then, the cells were grown for 2 days in a medium containing 10% fetal bovine serum alone (A) or with 2 ng/ml TGF-β (B). The TGF-β treated cells became flattened and spindle-shaped, whereas untreated cells showed a typical cuboidal shape of epithelial cells. (magnification ×400).

described previously by others (Like and Massague, 1986), the Mv1Lu cells did not grow in the presence of TGF-β, remaining at the same density at which they were seeded. Since TGF-β has also been shown to inhibit the growth of a number of cell types in monolayer cultures, we tested the effect of TGF-β on cell proliferation of other cell types including NIH 3T3, Cos-1, Cos-7, Vero, Vero 76, Swiss 3T3, HeLa, SP2-o myeloma, human neuroblastoma, human glioma, and human Jurkat T cells. Among the cell types examined, only Swiss 3T3 cells showed a notable growth inhibition and the other cells seemed to be unresponsive to TGF-β at the maximum concentration of 10 ng/ml and even for longer culture periods. However, Swiss 3T3 cells showed no or weak cross-reactivity with the antibodies used in this study. Thus, we used Mv1Lu cells for immunofluorescence microscopy.

Cultures of Mv1Lu cells grown for 2 days in the presence of 2 ng/ml displayed a dramatic alteration in morphology under a phase-contrast microscope. They became elongated and spindle-shaped, forming a pattern not seen in control cells, whereas the untreated Mv1Lu cells revealed cuboidal appearance characteristic of epithelial cells (Fig. 1A & B). The effect on cell shape became apparent within 24 h after addition of TGF-β. This alteration was most prominent when near-confluent cells were treated with TGF-β for 2 days. However, the cells seeded at low density and treated with TGF-β for 2 days did not show the shape change as prominently as the near-confluent cells, though earlier studies indicated the alteration observed at low cell density (Like and Massague, 1986).

Reorganization of actin filaments

The cytoskeleton of eukaryotic cells is composed of the different filamentous networks: actin microfilament, microtubule, and intermediate filament. Since the actin filaments are known to play a key role in cell shape, cell attachment, and spreading on the substrate, we investigated the structural changes of the cytoskeleton in conjunction with cell morphology by immunofluorescence microscopy. Cells were fixed, permeabilized, and treated with an anti-actin antibody. In the cultures of Mv1Lu cells grown in the presence of TGF-β for 2 days, formation of new stress fibers was observed (Fig. 2). A period of culture more than 2 days in the presence of TGF-β did not increase the contents of stress fibers. On the contrary, fewer numbers of stress fibers were detected in control cells. For unknown reasons, the antibody also recognized nuclei in Mv1Lu cells.

To determine whether the stress fiber formation in TGF-β treated cells resulted from the change in the amount of actin such as from new actin protein synthesis or increased polymerization of G-actin into F-actin, we compared the amount of G- and F-actins in TGF-β treated and untreated cells. From the gel analysis, TGF-β treatment appears to increase the total actin pool slightly (Fig. 3). For the estimation of F-actin content, half of the cells were extracted with a buffer containing 0.5% triton-X 100. The amount of Triton-X insoluble filamentous actin was quite different between TGF-β treated and control cells. As in the case of immunofluorescence staining, TGF-β treated cells had more F-actin content than untreated cells. The result indicates that the new stress fiber formation was contributed primarily by the polymerization of cytoplasmic G-actin.

Microtubules and intermediate filaments

Next we investigated whether there are any structural changes induced by TGF-β treatments in two other cytoskeletons, microtubules and intermediate filament. To localize microtubules, the cells were probed with an anti-β-tubulin antibody. As shown in Fig. 2B & b,

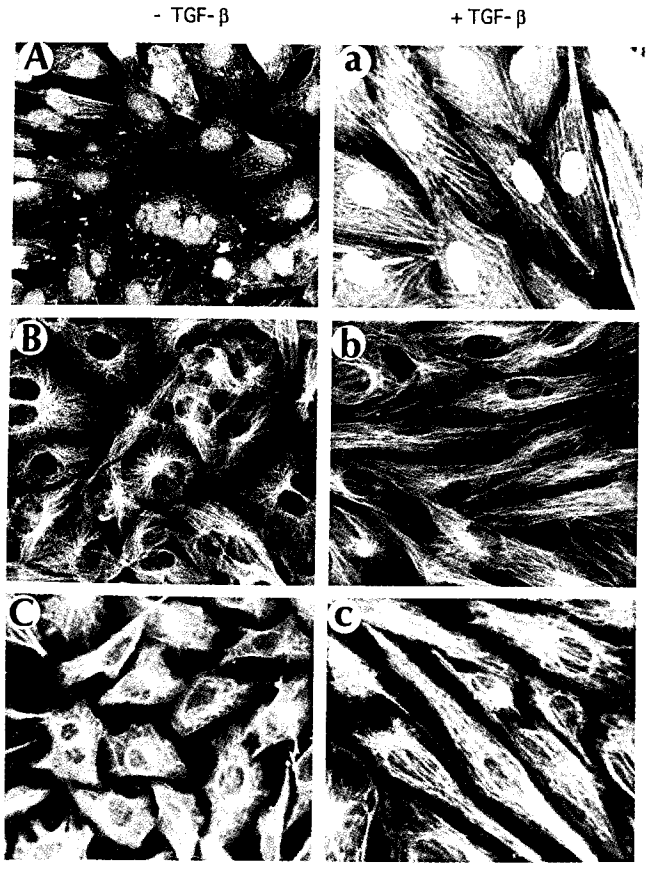

Fig. 2. Effects of TGF-β on cytoskeletal structures of Mv1Lu cells. The Mv1Lu cells treated with TGF-β (a, b, c) or not (A, B, C) were processed for immunofluorescence microscopy with anti-actin (A, a), anti-β-tubulin (B, b), and anti-vimentin antibodies. (Magnification ×600).

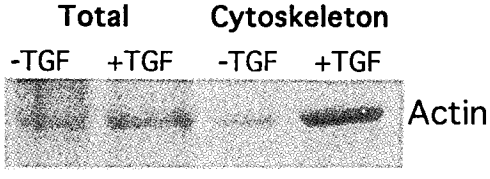

Fig. 3. TGF-β induces polymerization of G-actin to F-actin. The Mv1Lu cells treated with TGF-β (+TGF) or not (−TGF) were grown for 2 days and harvested by scrapping with a rubber policeman. The cells were homogenized with a probe sonicator (total) or extracted with a buffer containing 0.5% Triton-X 100 to enrich F-actin (cytoskeleton). The same amount of proteins were separated by SDS-PAGE and stained with Coomassie blue. TGF-β treatment appears to increase a total actin pool slightly and polymerization of G-actin into F-actin.

the cells showed well-developed microtubules. In TGF-β treated cells, however, significant changes in microtubular structures were not detected by fluorescence microscopy; for instance, fluorescence intensity or rearrangement of microtubule organizing center, except the elongated microtubule structures. Since it has been reported that vimentin is differentially expressed by cytokines, we chose vimentin among the intermediate filament proteins (Hornbeck, 1993). Likewise, we observed that immunostaining patterns of vimentin filament were not affected by TGF-β treatment in Mv1Lu cells (Fig. 2C & c). These changes seemed to result from the cellular morphology alteration, from cuboidal shape to spindle shape.

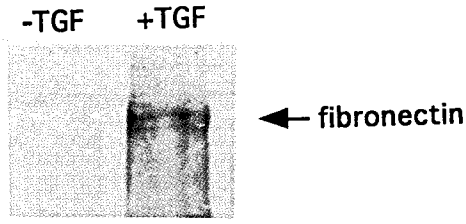

Fig. 4. Immunoblot analysis of TGF-β treated Mv1Lu cells. The total proteins from TGF-β treated cells were separated by SDS-PAGE and immunoblotted with an anti-fibronectin antibody.

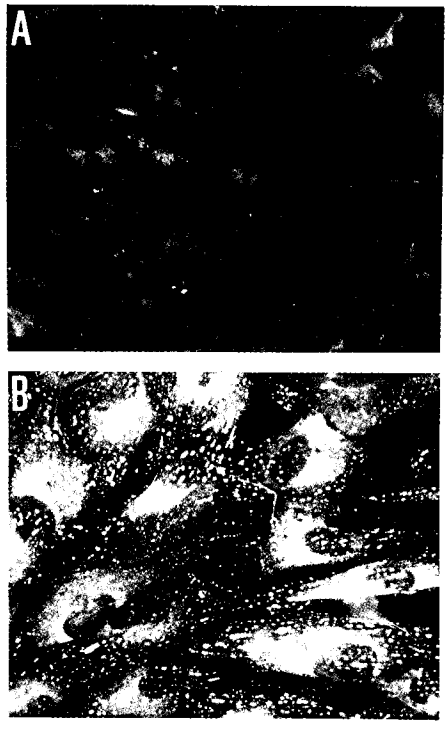

Fig. 5. Localization of fibronectin in Mv1Lu cells by immunofluorescence microscopy. The Mv1Lu cells treated with TGF-β (B) or not (A) were fixed and reacted with anti-fibronectin antibody. Major site of fibronectin accumulation appeared to be endoplasmic reticulum, suggesting that the protein was still being synthesized for 2 days of TGF treatment in Mv1Lu cells. (magnification ×600).

Fibronectin accumulation

Since TGF-β was known to increase the expression of extracellular matrix proteins, we compared the amount of fibronectin, which is one of the extracellular matrix protein, between TGF-treated and untreated cells. As shown by immoblotting, the Mv1Lu cells dis-

played an extensive accumulation of fibronectin (Fig. 4). To localize the accumulation site and confirm the immunoblotting experiment, we chased the protein by immunofluorescence microscopy, and found that fibronectin seemed to be located mainly in an ER-like structure. As shown Fig. 5, the staining pattern of fibronectin was similar to that of a typical ER. It suggests that fibronectin was still being synthesized for 2 days of TGF-β treatment and accumulated extracellularly thereafter.

Discussion

One of characteristic responses of Mv1Lu cells to TGF-β is a flat and very enlarged cell morphology (Like and Massague, 1986). The morphological change was accompanied by the rearrangement of cellular cytoskeletons and increased synthesis of extracellular matrix components. Eukaryotic cells contain three major classes of cytoskeletal fibers: 6 nm actin filament, 25 nm microtubules, and 10 nm intermediate filaments. While the Mv1Lu cell is one of the cell types for bioassay of TGF-β, few studies have been conducted on the cytoskeletons of the cells. In this report, we investigated the effects of TGF-β on the cytoskeletal organizations including actin filaments, microtubules, and intermediate filaments. Among the cytoskeletons, actin filament rearrangement was found to be a prominent change detected by immunofluorescence and Western blot analysis.

Cell morphology change and rearrangement of actin microfilament into stress fiber by TGF-β treatment have been shown in a number of cell types. In renal proximal tubule cell TGF-β provoke a dramatic rearrangement of actin microfilament (Humes *et al.*, 1993). Endothelial cell morphology is altered by TGF-β treatment, followed by the modification of actin microfilament staining pattern, in which dense peripheral band staining disappeared and staining intensity of cytoplasmic stress fiber increased (Coomber, 1991). Treatment of mouse osteoblastic cells with TGF-β induced rapid cell spreading and increased actin stress fibers (Lomri and marie, 1990). When other cytokines such as epidermal growth factor (EGF) and TGF-β were treated together, it was reported that TGF-β augmented the effects of EGF. In the presence of TGF, the cells became flattened and actin stress fibers were well developed compared to those cultured in its absence. In Mv1Lu cells, cell shape change is accompanied by the rearrangement of actin filament, and this stress fiber formation seems to be primarily contributed by the polymerization of G-actin into F-actin. In addition, a slight increase in de novo synthesis of actin was detected with TGF-β treatment.

Microtubules, like actin filaments, are universal components of all the eukaryotic cells and play one of the most important roles during cell division, by pulling chromosomes to each pole (Dustin, 1984). One of the effects exerted by TGF-β is growth arrest or inhibition in susceptible cells, such as Mv1Lu cell (Like and Massague, 1986). Thus, we suspected that there might be structural changes of microtubules in Mv1Lu cells in response to TGF-β treatment. However, no significant change in microtubular structures was detected in TGF-β treated cells by fluorescence microscopy, except the elongated microtubule structures. Intermediate filaments are composed of distinct intermediate filament proteins that are coded by a multigene family, the members of which are regulated developmentally in a tissue specific fashions (Bershadsky and Vasiliev, 1988). Particularly, among the intermediate filaments, vimentin is differentially expressed by cytokines, IL2 and IL4 in immune cells (Hornbeck, 1993). Like β-tubulin staining, it seemed to be that immunostaining patterns of vimentin filament was not affected by TGF-β treatment in Mv1Lu cells. However, we can not exclude the possibility that there might be some modifications of vimentin molecules such as phosphorylation. With the expectation that the accumulation site of fibronectin had some relationship with cytoskeletons, particularly with actin filament, we immunostained Mv1Lu cells with an anti-fibronectin antibody. However, the immunostaining pattern of fibronectin was quite different from that of stress fibers.

Regardless of cell types, it seems to be that cell morphology alteration by the TGF-β treatment accompanies rearrangement of actin microfilaments. Most cell types so far studied showed new formation of stress fibers after TGF-β treatment. Thus, actin microfilament plays a pivotal role in cell shape change. The signal transduction pathways leading from receptor activation to actin remodeling are still unclear, but recently some investigations have been undertaken (Nobes and Hall, 1994; Ridney, 1994). The signals that trigger these different cytoskeletal patterns act through diverse receptors, but now it appears that the pathway leading from these receptors converge on one or more members of the Rho family of small GTP-binding proteins (Ridney and Hall, 1994; Nobes and Hall, 1995). The family members include Rho and Rac, and Cdc42 which are recently joined. However, the cells and cytokines used in the studies have been limited to particular cell types or cytokines, for instance, EGF. Thus, few studies regarding signal transduction and F-actin remodeling of TGF-β have been reported. In this respect, Mv1Lu cells provide an excellent system to study the linkage. Signal transduction between TGF-β receptor and F-actin for-

mation could involve a Rho-dependent signaling pathway, because of all of the known cells use the pathway. The possibility can be tested by the inhibition of Rho protein with C3 transferase in a future study.

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