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Integrin β1-mediated signaling is involved in transforming growth factor-β2-promoted migration in human lens epithelial cells

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Purpose: Transforming growth factor-beta 2 (TGF-β2) is a potent growth inhibitor and apoptosis inducer. However, typically TGF-β2 loses its growth-inhibitory and apoptosis-inducing effects but stimulates the migratory capacity of epithelial cells. In this study, we investigate the possible involvement of integrin and integrin-mediated signaling in TGF-β2-promoted migration and adhesion in human lens epithelial cells.

Methods: A human lens epithelial cell line (HLE B-3) was treated with 100 pg/ml TGF-β2 for 6 h, 12 h, and 24 h. In vitro wound healing assay and cell adhesion assay were performed to detect the effect of TGF-β2 on HLEC adhesion and migration. Integrin β1 expression changes in HLECs during the treatment of TGF-β2 were detected in protein levels and mRNA levels using confocal microscopy, flow cytometric analysis, and real time quantitative reverse transcription polymerase chain reaction (RT-PCR). Focal adhesion kinase (FAK) activity was examined by FAK phosphorylation and total tyrosine phosphorylation during treatment with TGF-β2 in HLEC. Production of endogenous TGF-β2 was measured by ELISA assay.

Results: In this study, we found that TGF-β2 significantly stimulated cell adhesion and migration in HLECs. By immunofluorescence staining and western blotting, we observed that TGF-β2 markedly enhanced the expression of integrin β1 and the Tyr-phosphorylation of focal adhesion kinase (FAK). Real time quantitative RT-PCR also showed the mRNA level of integrin β1 was upregulated. Neutralizing anti-integrin β1 monoclonal antibody significantly ($p<0.05$) inhibited TGF-β2-promoted HLEC adhesion and migration.

Conclusions: TGF-β2 promoted HLEC adhesion and migration in vitro. Integrin β1 and integrin-mediated signaling are necessary for TGF-β2-promoted adhesion and migration in human lens epithelial cells.

Posterior capsular opacification (PCO) is a common complication of cataract surgery and occurs when residual lens epithelial cells (LEC) undergo proliferation and transdifferentiation [1-3]. This process involves LEC migration and cell-extracellular matrix (ECM) adhesion [4-6]. It has been demonstrated that transforming growth factor-beta (TGF-β), a multi-functional polypeptide, is involved in various ocular diseases including pathological changes [7,8]. For human lens epithelial cells (HLEC), TGF-β is a potent growth inhibitor and apoptosis inducer. However, TGF-β commonly loses its growth-inhibitory and apoptosis-inducing effects and paradoxically stimulates the metastatic capacity of many epithelial cell lines [9,10]. However, currently little is known about TGF-β-promoted cell migration and adhesion in HLEC or its mechanisms.

The cell-extracellular matrix interactions that lead to signal transduction are mediated mainly through integrins, which are intracellularly linked to cytoskeleton components [11] and other protein molecules [12-14]. Integrins are heterodimeric transmembrane glycoproteins consisting of one α- and one β-subunit that are noncovalently linked. They bind to capsular ECM proteins such as collagen type IV and laminin as well as to fibronectin (Fn), which is present only in the embryonic lens capsule [15-17]. Integrin binding to extracellular matrix protein or integrin crosslinking increases tyrosine phosphorylation of focal adhesion kinase (FAK). FAK is a tyrosine kinase considered a central molecule in integrin-mediated signaling and it is involved in cellular motility and protection against apoptosis. As integrins are cell surface adhesion molecules involved in epithelial cell attachment and are receptors for ECM components, they may have a significant role in the adhesion and migration of LEC across the lens capsule leading to posterior capsule opacification.

Here, we have investigated the role for TGF-β2 in HLECs and found that TGF-β2 significantly enhances cell migration and adhesion. The present studies were designed to investigate how TGF-β2 promoted cell migration and adhesion in vitro, focusing particularly on integrin β1 expression and activation of FAK.

METHODS

Cell culture and treatment: The human lens epithelial cell line HLE B-3 was purchased from ATCC and maintained as described previously [18]. In short, the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY). The cells were grown to confluence at 37 °C in a humidified atmosphere containing 5% CO_2, washed with Ca^{2+}/Mg^{2+}-free PBS (PBS-CMF), and dissociated with trypsin-

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EDTA solution (0.05% trypsin-0.02% ethylenediamine tetra acetic acid [EDTA] solution; Gibco). The cell cultures were plated in a 100 mm culture dish, allowed to reach 85-90% confluence, and supplemented with recombinant human TGF-β2 (R&D Systems, Inc., Oxon, UK) in serum-free medium. TGF-β2 was reconstituted in PBS containing 2 mg/ml bovine serum albumin and stored at -20 °C before use. The medium was changed every 24 h and the appropriate agents were added to the fresh medium. The treated cells were compared with control cultures that were incubated under identical conditions but in the absence of TGF-β2 in the medium. Cells were used at about the sixth to tenth passage for all experiments.

Confocal microscopy and flow cytometric analysis of integrin β1 expression: Cells were cultured on coverslips that were kept in a T6-well plate for 16-20 h before treatment. After treatment with or without 100 pg/ml TGF-β2 for 12 h and 24 h, the cells were washed with isotonic PBS (pH 7.4) then fixed in 4% paraformaldehyde solution in PBS for five min at 4 °C. The coverslips were washed three times with PBS, and nonspecific binding sites were blocked in PBS containing 10% normal goat serum and 0.3% Triton X-100 for 1 h. The cells were incubated with mouse anti-human monoclonal integrin β1 (556047; BD Biosciences San Jose, CA) in PBS (containing 0.3% Triton X-100 and 10% normal goat serum) for 90 min at 4 °C and washed three times with 0.3% Triton X-100 in PBS. The cells were then incubated with goat anti-mouse IgG-FITC (sc-2010; Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 0.3% Triton X-100 and 10% normal goat serum for 45 min at 4 °C in the dark and washed three times with 0.3% Triton X-100 in PBS. The samples were stored in the dark at 4 °C until examination by confocal microscopy. The nuclei were stained with 0.25% propidium iodide (PI; Sigma Chemical Company, St. Louis, MO) for 10 min before being examined under a Fluoview FV1000 confocal microscope (Olympus, Tokyo, Japan). The relative amount of the β1 subunit was analyzed on a FACScan (Becton-Dickinson & Co., Mountain View, CA). For each sample, data from 10,000 cells were collected. Cells used as control were incubated with secondary antibody alone to show the background fluorescence.

Western blotting of phosphotyrosine and focal adhesion kinase: The polyclonal antibody against p-FAK (Tyr 576)-R and monoclonal antibody against FAK were obtained from Santa Cruz Biotechnology, Inc. After different time periods, the cells were lysed in ice-cold modified radioimmunoprecipitation (RIPA) buffer (10 mmol/l Tris-HCl, 150 mmol/l NaCl, 1.0% NP40, 0.25% deoxycholate, 0.1 mmol/l PMSF, 0.1 mmol/l TLCK, 0.1 mmol/l iodoacetamide, 10 μg/ml leupeptin, and 10 μg/ml aprotinin; pH 7.4) at 4 °C for 30 min. Cell lysates were centrifuged at 13,000x g for 10 min to remove insoluble material, and the supernatant was transferred to a fresh tube. Protein samples were boiled and protein assays were performed with the Bradford assay (Bio-Rad Laboratories, Hercules, CA) [19]. Twenty micrograms of the supernatant of each protein sample were separated by 12% SDS-PAGE and subsequently blotted onto polyvinylidene difluoride (PVDF) membranes (100 V for 1 h), and the membranes were blocked in incubation buffer (10 mmol/l Tris-HCl, 50 mmol/l NaCl, 0.05% Tween 20, pH 7.5 containing 5% BSA) at room temperature for 1 h while rocking. Membranes were incubated with anti-P-Tyr576 or anti-FAK mAb in an incubation buffer containing 5% BSA overnight at 4 °C while rocking, and, after washing three times, they were incubated with anti-mouse specificanti-IgG-alkaline phosphatase for 2 h at room temperature then visualized by enhanced chemiluminescent detection methods.

RNA isolation and real time quantitative reverse transcriptation polymerase chain reaction: Total cellular RNA was extracted from $2x10^7$ HLECs, using TRIzol reagent then digested with DNase I (Life Technologies, Rockville, MD). Purified RNA was resuspended in 20 μl of RNAse-free water. Three microliters RNA was then used as a template for cDNA synthesis in the presence of 1 μl of M-MLV reverse transcriptase (200 U; Promega, Madison, WI), 4 μl of First Strand buffer (5X; Promega), oligd(T) 2.5 μmol/l, 1 μl of dNTP (10 mM; Roche, Lewes, UK), and 5 μl of RNAse-free water. After incubation for 60 min at 42 °C, the reverse transcriptase was inactivated at 72 °C for 10 min. cDNAs were amplified by real-time PCR, using the Applied Biosystems FTC 2000 sequence detection system. The reaction mixture (40 μl) consisted of 4 μl cDNA, 35.1 μl SYBR Green PCR mix (Biocore, Hangzhou, China), 0.5 μl of 5 U Taq DNA polymerase, and 0.3 μl primer (20 pmol/μl each primer; Invitrogen, Life Technologies, Carlsbad, CA). The cDNA was denatured by heating to 94 °C for three min. The template was amplified using 40 cycles with each cycle containing an incubation step of 94 °for 20 s, an annealing step of 55 °for 20 s, and an extension step of 72 °C for 30 s. The primer set for integrin was 5'-GCC TTA CAT TAG CAC AAC ACC-3' and 5'-CAT CTC CAG CAA AGT GAA AC-3'; for GAPDH was 5'-GTC GGT GTG AAC GGA TTT-3' and 5'-ACT CCA CGA CGT ACT CAG C-3'. A cycle threshold (C_T) value was recorded for each sample. PCR reactions were set up in triplicates and the mean of the three C_Ts was calculated. A $2^{-\Delta\Delta CT}$ method was applied to the raw C_T values to find a relative gene expression [20].

Migration assays: Cells were seeded in Falcon T6-well culture dishes at a density of $3x10^5$ cells per well and 24 h later (or 80% confluence), "scratch-wounds" were created by scraping the confluent cell monolayer with a sterile pipette tip to make an approximately 1.0 mm gap. After careful washing to remove detached cells, the cells were cultured in serum-free medium and were cultured with or without the anti-integrin β1 antibody (0.5 μg/ml) before the treatment with 100 pg/ml TGF-β2. The cultures were incubated for a further 24 h. Migration was quantified by counting cell numbers that had advanced into the cell-free space from a number of randomly chosen 1.0 mm segments of the initial wound border. Matched pair marked wound regions were photographed again at different time points. Each point represents the mean±SD from at least four wounds in every experiment. Photographs were taken of the wounded area at 12 and 24 h using an inverted Olympus microscope.

Cell adhesion assay: Ninety-six well plates (Nunc, Roskilde, Denmark) were coated at 37 °C for 1 h with 100 μl

(5 mg/ml) fibronectin (Fn) and 100 mg/ml poly-lysine in PBS. The plates were washed twice with PBS and blocked with 100 µl 1% BSA for 1 h at 37 °C. Wells were washed twice with PBS and stored at 4 °C before use. Cells were collected and resuspended in complete DMEM without serum. A total of 10,000 cells in 100 µl were added into each substrate-coated well, and the plates were incubated for 30 min at 37 °C in 5% CO_2. Unattached cells were gently washed away with PBS. The attached cells were fixed with 4% formaldehyde, stained with 0.5% crystal violet overnight, destained with distilled

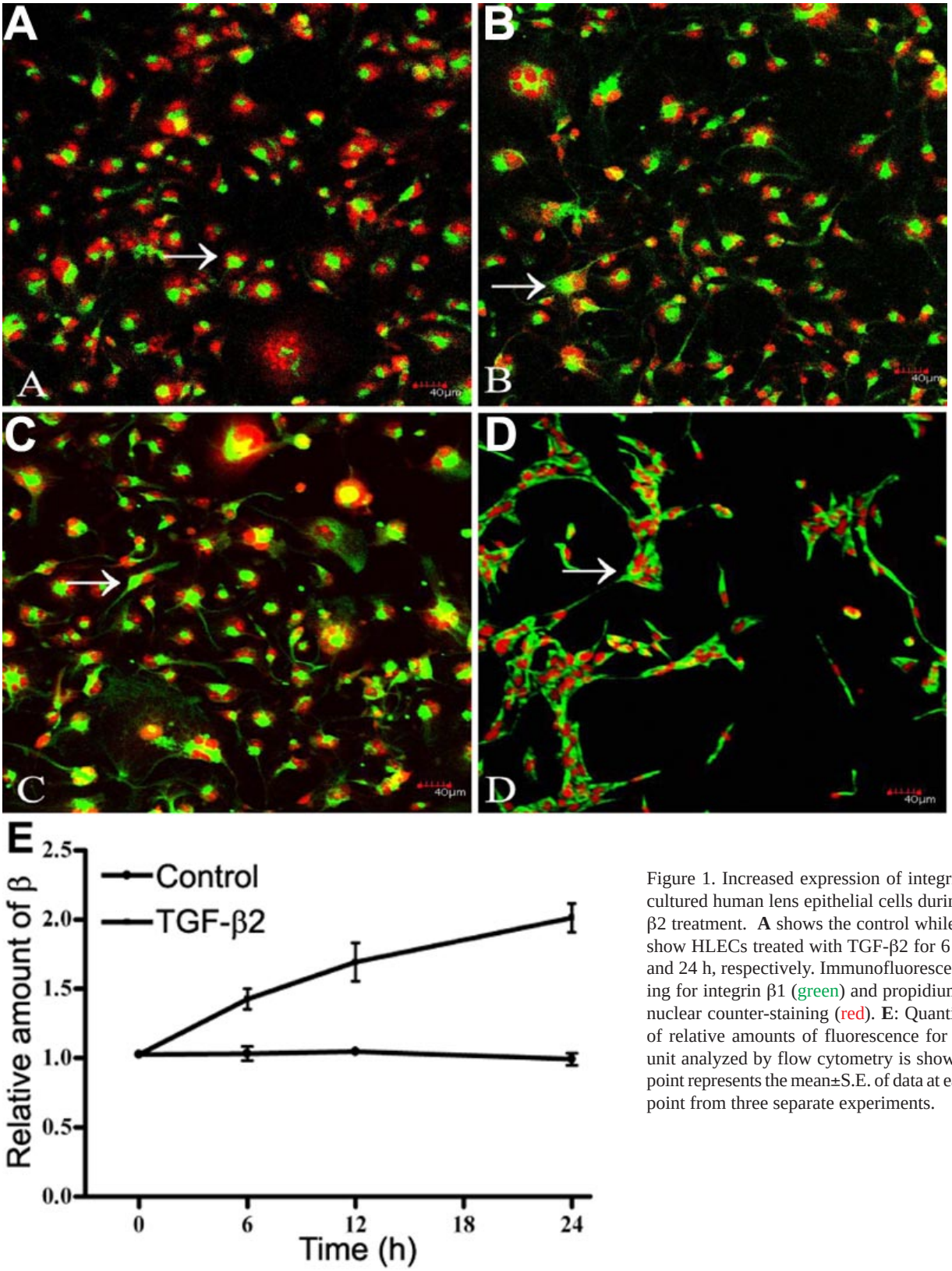

Figure 1. Increased expression of integrin β1 in cultured human lens epithelial cells during TGF-β2 treatment. **A** shows the control while **B** to **D** show HLECs treated with TGF-β2 for 6 h, 12 h, and 24 h, respectively. Immunofluorescent staining for integrin β1 (green) and propidium iodide nuclear counter-staining (red). **E**: Quantification of relative amounts of fluorescence for β1 subunit analyzed by flow cytometry is shown. Each point represents the mean±S.E. of data at each time point from three separate experiments.

water, solubilized in 36% acetic acid, and quantified by a microtiter plate reader.

Statistics: Data are expressed as the mean±S.E. from a minimum of three independent experiments unless otherwise indicated. Statistical analysis was performed with Student's t-test using SAS statistical analysis software with a $p < 0.05$ as the criterion of significance.

RESULTS

TGF-β2 stimulates the expression of integrin β1 and Tyr-phosphorylation of focal adhesion kinase in human lens epithelial cells: Cell migration through the ECM is generally mediated by integrins on the cell membrane. Studies in various systems indicate that TGF-β modifies integrin expression and thus alters cell adhesion and migration [21,22]. To investigate the effects of TGF-β on lens integrins, HLEC were treated with 100 pg/ml TGF-β2 for 6 h, 12 h, and 24 h. Cells were analyzed for their expression of integrin β1 on the cell surface by confocal microscopy and flow cytometric analysis. We found that TGF-β2 increased integrin β1 subunits in a time-dependent manner. β1 subunits were increased approximately 2.0 fold to that of the control 24 h after TGF-β2 treatment (Figure 1).

FAK activity was examined by FAK phosphorylation and total tyrosine phosphorylation during treatment with TGF-β2 in HLEC. The lysates of cells were analyzed with antibodies to FAK followed by western blotting with an antibody specific for Tyr 576-phosphorylated FAK. After cells were incubated with TGF-β2 for 6 h, 12 h, and 24 h, the expression of FAK was apparently unaffected while the tyrosine phosphorylation of FAK was increased to 3.75 fold, 4.82 fold, and 5.64 fold at 6 h, 12 h, and 24 h, respectively in contrast to that of the control (Figure 2). This suggests that TGF-β2 increases FAK phosphorylation and leaves the amount of FAK unaltered.

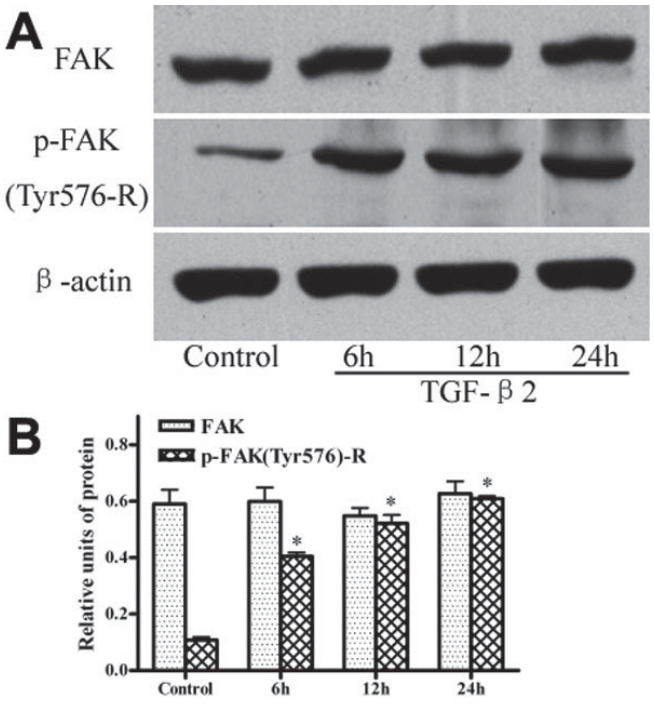

Figure 2. TGF-β2 stimulates Tyr-phosphorylation of FAK in human lens epithelial cells. **A**: Immunoblots for total FAK, phospho-Y576 FAK, and β-actin of HLEC cultured for 6 h, 12 h, and 24 h with TGF-β2. **B**: Quantification of immunoblots shows a significant ($p<0.05$) increases in FAK phosphorylation on Y576 in HLEC cultured for 6 h, 12 h, and 24 h with TGF-β2. Data show the mean±S.E. of three experiments and one representative example is shown.

Effect of TGF-β2 on mRNA levels of integrin β1: Real time RT-PCR was carried out to determine whether TGF-β2 stimulates gene expression of integrin β1 in HLECs. After serum starvation, HLECs were treated with 100 pg/ml TGF-β2 for 6 h, 12 h, and 24 h. The results (Table 1) show that treating cells with TGF-β2 for the indicated times gradually upregulated β1 subunit expression with a 2.6 fold increase after 24 h.

Effects of blocking integrin β1 on cell migration and adhesion on fibronectin promoted by TGF-β2: Integrins are cell surface adhesion molecules involved in epithelial cell attachment and receptors for ECM components. Since TGF-β2 enhanced the expression of integrin β1, we wanted to determine whether TGF-β2 could enhance cell adhesion onto extracellular matrix Fn. As shown in Figure 3, cell adhesion onto Fn increased 1.49 fold at 12 h and 1.67 fold at 24 h with a concentration of 100 pg/ml TGF-β2. However, when the cells were treated with integrin β1 antibody, adhesion cells only increased 1.28 fold at 12 h and 1.3 fold at 24 h. These results indicated that blocking integrin β1 decreased cell adhesion enhanced by TGF-β2.

Adhesion plays an important role during the process of migration. We therefore performed an in vitro wound healing assay to evaluate the effect of TGF-β2 on the migration of HLECs and found that cell migration was promoted by TGF-β2 treatment for different time periods. As shown in Figure 4, cell migration increased 10.2 fold at 12 h and 23.1 fold at 24 h with a concentration of 100 pg/ml TGF-β2. However, when the cells were treated with integrin β1 antibody, cell migration only increased 2.5 fold at 12 h and 10 fold at 24 h. These results indicated that integrin β1 blocking induced a significant decrease in the cell migration promoted by TGF-β2.

DISCUSSION

Cell adhesion molecules, especially integrins, are signal transducers within the cell as well as receptors for the extracellular matrix. Integrin ligation to matrix components induces a conformational change in the cytoplasmic tail of the integrin, which allows it to bind to the actin cytoskeleton and signal transduction molecules [23,24]. This allows integrins to act as bidirectional signal transducers [25]. Zhang and colleagues [26] found integrins β1, β2, α2, α3, and α5 in 70%, 65%, 75%, 70%, and 80% of human cataract lens epithelial cells, respectively.

Integrin expression has been investigated in LEC lines from several species in vitro. Lim and colleagues [27] demonstrated the presence of integrin α3, α5, α6, and αv in the

human LEC line HLE-B3 and the murine LEC line αTN4. The integrin β1 subunit is reported to play an important role in the adhesion and invasion of many cells [28,29]. The regulatory effect of TGF-β on integrins has long been a subject of investigation. TGF-β modulates several integrins (α1, α2, α3, α5, and β1) on many types of cells (chondrocyte, breast cancer cell, fibroblast, osteoblast, colon carcinoma cells, etc.) [30,31]. In this study, we also found that TGF-β2 significantly upregulates integrin β1 expression at protein level in vitro (Figure 1) and at the mRNA level. These results confirm that TGF-β is able to initiate cellular and molecular changes in lens epithelial cells that are associated with PCO development [32].

For human lens epithelial cells, TGF-β is a potent growth inhibitor and apoptosis inducer. However, TGF-β usually loses its growth-inhibitory and apoptosis-inducing effects and instead stimulates the metastatic capacity of many epithelial cell lines [33]. We hypothesized that TGF-β2 might modify those aspects of cell migration, positions, and development that are guided by adhesion to ECM by modulating the expression of integrin β1. We first investigated cell adhesion following TGF-β2 treatment in HLECs, and the results showed that cell adhesion onto Fn was increased (Figure 3). This effect may be secondary to TGF-β2-induced overexpression of cell surface integrin β1 since anti-integrin-β1-blocking antibody significantly inhibited this effect. We performed an in vitro wound healing assay to evaluate the effect of TGF-β2 on the migration of HLECs and found that cell migration was promoted by TGF-β2 treatment (Figure 4). Cell migration relies upon forces generated by the cell and the regulation of the adhesive strength between the integrin and cell substratum interface [34].

These observations provide compelling evidence that integrin β1 plays a vital role during TGF-β2-induced adhesion and migration of HLECs in vitro. We based this conclusion on the following observations: (1) TGF-β2 specifically stimulated integrin β1 expression in cultured HLEC so that the stimulated cells acquire a migratory phenotype; and (2) anti-integrin-β1-blocking antibody inhibited this acquired motile activity.

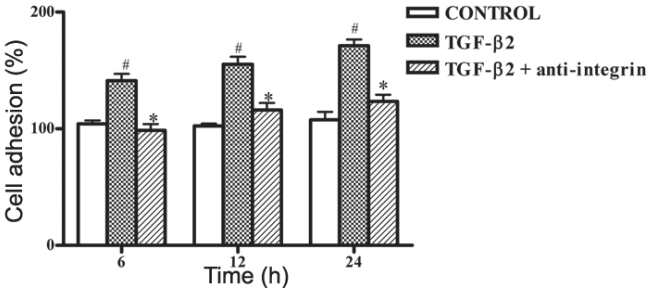

Figure 3. TGF-β2 enhances cell adhesion onto fibronectin. HLEC were treated with 100 pg/ml TGF-β2 for 6 h, 12 h, and 24 h with or without addition of anti-integrin β1 antibody and cell adhesion was assayed as described in the Methods. Data was normalized to the control values and each data point represents the mean±S.E. of three experiments. Statistically, significant differences are indicated by the sharp (hash mark; $p<0.05$) compared with the control group and by the asterisk ($p<0.05$), compared with TGF-β2 group.

Table 1. Relative quantitation using the comparative C_T method A

Time	Integrin β1 Average C_T	GAPDH Average C_T	ΔC_T Integrin β1 - GAPDH	$\Delta\Delta C_T$ ΔC_T - ΔC_T Control	$2^{-\Delta\Delta C_T}$ Relative to Control
Control	27.76 ± 0.03	22.01 ± 0.02	5.75 ± 0.02	0 ± 0.02	1.0 (0.99-1.01)
6h	30.56 ± 0.10	24.81 ± 0.06	5.01 ± 0.08	-0.72 ± 0.08	1.65 (1.56-1.74)
12h	31.02 ± 0.06	26.37 ± 0.03	4.65 ± 0.04	-1.1 ± 0.04	2.14 (2.08-2.20)
24h	31.15 ± 0.02	26.80 ± 0.03	4.35 ± 0.03	-1.4 ± 0.03	2.64 (2.58-2.69)

The ΔC_T value is determined by subtracting the average GAPDH C_T value from the average integrin β1 C_T value. The calculation of $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value. This is subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value. The range given for integrin β1 relative to the control is determined by evaluating the expression: $2^{-\Delta\Delta C_T}$ with "$\Delta\Delta C_T$ plus s" and "$\Delta\Delta C_T$ minus s", where "s" equals the standard deviation of the $\Delta\Delta C_T$ value.

FAK is an important downstream molecule of integrin signaling whose tyrosine phosphorylation and activation is triggered by the ligation and clustering of the integrin. The phosphorylation of FAK can be detected in attached cells [34,35]. We therefore decided to examine the phosphorylation status of FAK, which has been shown to be important for mediating the biological effects of integrin β1. In response to integrin engagement, FAK is phosphorylated at tyrosine residue 576 (Tyr-576) in its kinase domain[36]. Thus, we examined the phosphorylation status of this tyrosine residue in the FAK molecule. TGF-β2 increased the expression of FAK phosphorylation at Tyr 576 without affecting the total FAK expression in the cells (Figure 2).

The observations that TGF-β2 upregulated integrin β1 expression and enhanced cell adhesion and migration support the hypothesis that TGF-β2 may regulate HLEC behavior and

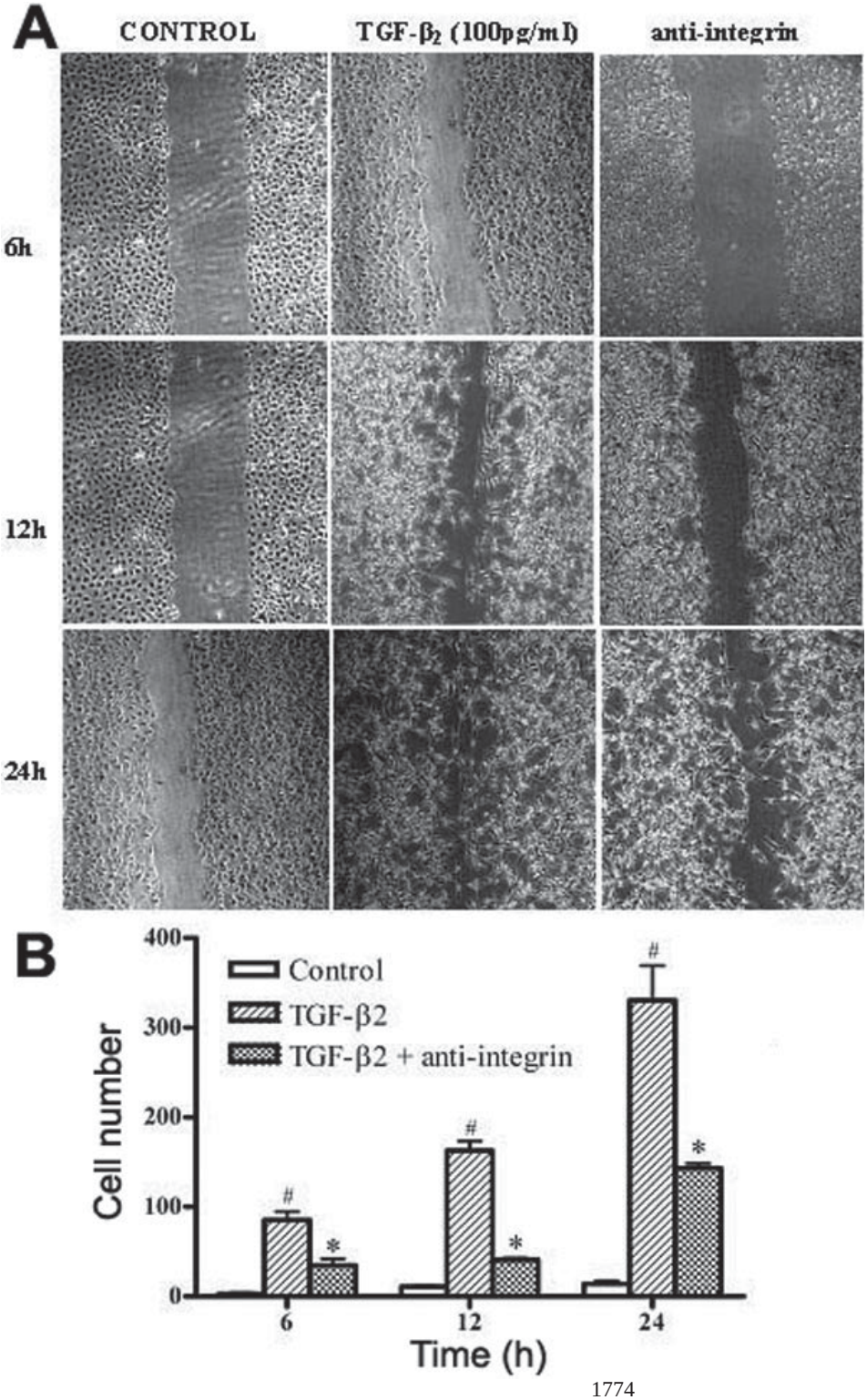

Figure 4. Inhibition of integrin β1 inhibits TGF-β2-induced migration. **A**: In vitro "scratch" wounds of confluent cell monolayer is cultured with or without 100 pg/ml TGF-β2 and with or without anti-integrin β1 antibody for 6 h, 12 h, or 24 h. **B**: Cell migration rate was measured by counting cell numbers that advanced into the cell-free space. Each data point represents mean (cells/mm^2)±S.E. of three experiments. Statistically, significant differences are indicated by the sharp (hash mark; $p<0.05$) compared to the control group and the asterisk ($p<0.05$) compared with the TGF-β2 group.

PCO formation by integrin-mediated signaling in HLEC. The mechanisms regulating integrin-stimulated cell migration are very complex, and the activation of tyrosine kinases plays an important role in these events [34]. In cultured cells, FAK overexpression can increase Fn-stimulated cell motility and this activity depends upon the integrity of the FAK autophosphorylation site [37,38]. We postulate that increased integrin expression at the cell surface and increased cell adhesion enhances integrin clustering, which stimulates FAK activation and promotes cell migration. Consistent with this, our results show that TGF-β2 increased tyrosine phosphorylation of FAK in HLE-B3 cells. This suggests that increased phosphorylation of FAK is at least part of the signaling that takes place during TGF-β2-induced cell migration.

In conclusion, the results presented in this paper strongly suggest that TGF-β2 promotes HLEC adhesion and migration in HLEC in vitro, which are associated with PCO development. Integrin β1 and integrin mediated-signaling play a critical role in this process.

ACKNOWLEDGEMENTS

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