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# Downregulated Expression of Integrin $\alpha$6 by Transforming Growth Factor-$\beta_1$ on Lens Epithelial Cells *in Vitro*

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**Integrins represent the main cell surface receptors that mediate cell–matrix and cell–cell interactions. They play critical roles in adhesion, migration, morphogenesis, and the differentiation of several cell types. Previous studies have demonstrated that members of the fibroblast growth factor (FGF)-2, transforming growth factor (TGF)-$\beta_1$, and insulin growth factor (IGF)-1 play important roles in lens biology. In particularly, TGF-$\beta_1$ appears to play a key role in extracellular matrix production, cell proliferation, and cell differentiation of lens epithelial cells. In this study we investigated the effects of FGF-2, TGF-$\beta_1$, and IGF-1 on the modulation of integrin receptors using lens epithelial cell lines (HLE B-3 and $\alpha$TN-4) and lens explants. We found that the expression of integrin $\alpha$6 is downregulated by TGF-$\beta_1$, but is not responsive to FGF-2 or IGF-1. The promoter activity of the integrin $\alpha$6 gene decreased upon TGF-$\beta_1$ treatment in a transient transfection assay, and flow cytometric analysis demonstrated the reduced expression of integrin $\alpha$6 by TGF-$\beta_1$, whereas significant changes were not observed in the level of integrin $\alpha$6 after the addition of FGF-2. These findings suggest that the reduced expression of integrin $\alpha$6 caused by TGF-$\beta_1$ might play a role in the activation of the cell cycle genes required during the fiber differentiation of the lens.** © 2001 Academic Press

***Key Words:*** **integrin; transforming growth factor-$\beta$1 (TGF-$\beta_1$); promoter; gene regulation; differentiation.**

The lens is composed of two major cell types: epithelial cells, which are primarily located at the anterior of the lens, and fiber cells, which are located at the cortical and posterior region (1, 2). Previous studies have shown that the lens epithelial cells (LECs) differentiate into lens fiber cells, and that this is followed by the degeneration of their nuclei and organelles as development proceeds. On the other hand, LECs become transdifferentiated into myofibroblast or mesenchyme-like cells during the formation of anterior subcapsular cataracts and after-cataracts (2–7). These processes require cell–cell or cell–extracellular matrix contacts, which can be modulated by cell adhesion molecules. Among these superfamilies of cell adhesion molecules, the integrins are considered to be one of the important as mediators of cell–cell and cell–ECM interactions (8–11).

Integrins are heterodimeric molecules that consist of noncovalently bound $\alpha$ and $\beta$ subunits. Each integrin has intracellular, transmembrane, and extracellular domains and transduces both internal signals and signals from the extracellular matrix (10, 12, 13). Integrins also participate in several important physiologic and pathologic processes, such as leukocyte trafficking (14) and inflammatory-immunologic cellular responses (15). It is known integrin expression affects a wide variety of developmental and cellular processes, including the regulation of gene expression, and cell adhesion, migration and morphology (16, 17). Condic and Letourneau (18) demonstrated that integrin regulation maintains neuronal growth-cone motility over a broad range of ligand concentrations, allowing axons to invade different tissues during development and regeneration. In contrast to situations, in which integrin regulation may be part of a larger change in cell state, Condic and Letourneau demonstrated the regulation of integrin in terminally differentiated neurons under conditions that do not appreciably affect cell morphology.

Significant progress has been made during the past several years to promote the significance of integrins in

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the lens cell differentiation and cataractogenesis processes. LECs of human cataracts express $\beta_1$, $\beta_2$, $\alpha_2$, and $\alpha_5$ integrins in specimens removed during surgery (19). Moreover, $\beta_1$ integrin, ICAM-1, and CD44 are all involved in LEC attachment and growth on collagen and laminin *in vitro* (20). In particular, integrin $\alpha$6 has been shown to mediate several significant functions: (i) it is known as a key mediator in lens differentiation during the developmental process (21); (ii) Georges-Labouesse *et al*. showed that integrin $\alpha$6 null ($-/-$) mice die at birth with severe skin blistering and defects in the cerebral cortex and retina (22). Interestingly, synergistic integrin $\alpha$3 and integrin $\alpha$6 null mice show distinctive developmental defects in lens, namely, a severe disorganization of the cortex and a disorganization of the retinal ganglion cells and nerve fiber layers of the retina.

The $\alpha$6 integrin subunit dimerizes with either the $\beta$ 1 or the $\beta$4 subunit to form receptors for various laminin isoforms (12, 23). Two splice variants of the cytoplasmic domains of $\alpha$6, $\alpha$6A and $\alpha$6B, have been identified. The cytoplasmic domains of $\alpha$6A and $\alpha$6B are encoded by separate exons and present entirely different sequences, and with the exception of the GFFKR motif, are present in all a subunits. Although the existence of alternatively spliced forms of integrin subunits has been known for some time, their functional significance remains to be elucidated.

Growth factors and cytokines modulate proliferation, differentiation, fibrosis, and apoptosis in several cell systems. Specifically, insulin-like growth factor (IGF)-1, transforming growth factor-$\beta$ (TGF-$\beta$), and basic fibroblast growth factor (FGF-2) have been implicated in the regulation of normal and pathological conditions of the lens (24–27). The ocular lens has recently been shown to be a target of TGF-$\beta$. Previous studies have shown that TGF-$\beta$ can disturb the normal lens architecture and induce changes in LECs that are analogous to those found in forms of human subcapsular cataract (28, 29).

The influence of TGF-$\beta$ on LECs includes the control of proliferation (both inhibitory and stimulatory), extracellular matrix production and modification, and the regulation of terminal differentiation (30). After TGF-$\beta$ treatment, passaged cells, primary explants and organ cultured lenses express $\alpha$-smooth muscle actin, another indicator of cataractogenic changes. Also, epithelial cells in primary explants and organ cultured lenses secret extracellular matrix components like laminin, fibronectin, type I collagen, and heparan sulfate proteoglycan (31). Whereas passaged cells become mitotically inhibited when exposed to TGF-$\beta$, the accumulation of large plaques of cells beneath the capsule of TGF-$\beta$ exposed organ cultured lenses indicates that some limited form of cell proliferation occurs (29). In primary cultures of chick LECs, Richiert and Ireland showed that TGF-$\beta$ promotes cell proliferation concomitant with the increased secretion and deposition of ECM proteins, particularly fibronectin (32). We hypothesize that TGF-$\beta$-mediated actions in the lens must be tightly regulated in order to avoid proliferative responses due to the inappropriate production of both normal and abnormal lens capsule ECM components.

In addition, TGF-$\beta$ has been implicated in the transdifferentiation and fibrosis of LECs. We have previously reported that TGF-$\beta_1$ enhances extracellular matrix production, such as, fibronectin, type I collagen, and the $\alpha$-SMA protein of lens epithelial explants (7). Recent transgenic studies using the dominant negative-TGF-$\beta$ receptor approach have shown that TGF-$\beta$ plays an important role in the normal maturation and survival of lens fiber cells (33). Nishi *et al*. assessed the effect of cytokines on the mitosis and collagen synthesis of the LECs of human cataracts (34). While IL-1 and FGF-2 increased mitosis and collagen synthesis significantly, TGF-$\beta_2$ decreased mitosis significantly, but also significantly increased collagen synthesis (34). These experiments support the role of TGF-$\beta$ as a potential regulator of lens differentiation and cataractogenesis.

Although the correlation between the expression and function of integrins in LECs has been previously studied, no report has been published to date, which demonstrates that exogenous stimuli modulate the expression of integrins in LECs or its possible correlation with cell–matrix interactions. Therefore, the present study was undertaken to investigate the modulation of integrin receptors in LECs, particularly, of those induced by members of several important growth factors.

## MATERIALS AND METHODS

*Cell culture and TGF-$\beta_1$ treatment.* Human lens epithelial cell line (HLE B-3) was kindly provided by Dr. Usha Andley and maintained as described previously (35). The murine lens epithelial cell line, $\alpha$TN4, kindly provided by Dr. Paul Russell (NEI, NIH, Bethesda, MD), was grown in Dulbecco's modified Eagle medium (DMEM; GibcoBRL, Grand Island, NY) supplemented with 10% fetal bovine serum(FBS; GibcoBRL), 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin as described previously (36). The cultures were treated with 5 ng/ml of human recombinant TGF-$\beta_1$, 75 ng/ml of IGF-1, and 50 ng/ml FGF-2 in serum-free minimum essential medium (MEM) (Sigma, St. Louis, MO) and Dulbecco's Minimum Essential Medium (DMEM; Gibco BRL) after 24 h of 1% serum-adaptation period. At the indicated time points, the cells were harvested for the following experiments.

*Total RNA Preparation and RT-PCR.* Total cellular RNA was isolated by using TRIzol reagent (Gibco BRL). One microgram of RNA was reversed-transcribed by using a cDNA synthesis kit (Boehringer Mannheim, Indianapolis, IN). Then, the cDNA was amplified in a 20 $\mu$l reaction mixture by PCR with the following condition: 0.4 $\mu$M each primer, 0.2mM deoxynucleoside triphosphate mixture (Perkin–Elmer. Corp., Foster City, CA), 50 mM KCl, 10mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, and 1 unit *Taq* polymerase (Perkin–Elmer). Reaction mixtures were incubated in a thermal controller (Model PTC-100, MJ Research Watertown, MA) for 35 cycles of denaturation for 45 s at 95°C, annealing for 1 min at 58°C, and extension for 45 s at 72°C. The amounts of amplified products were analyzed using an image documentation sys-

tem (ImageMaster VDS, Pharmacia Biotech Inc., Uppsala, Sweden). The primer sequences specific for the genes examined and the predicted sizes for human $\alpha$6 integrin isoform cDNA were 5′-CTAACG-GAGTCTCACAACTC-3′ and 5′-ACTCTGAAATCAGTCCTCAG-3′, yielding an amplification product of 844 and 714 bp and mouse $\alpha$6 integrin isoform cDNA were 5′-GACTCTCAACTGCAGCGTCA-3′ and 5′-CTCTCGCTCTTCTTTCCG-3′, yielding an amplification product of 550 and 420 bp. Primers for human integrin $\alpha$6 cDNA were 5′-GTACAGTTGTTGGCGAGCA-3′ and 5′-TGCATCAGAAGTAAGCCT-CTC-3′, yielding an amplification product of 785 bp and for human $\alpha$5 cDNA were 5′-ACTCAACTGCACCACCAATC-3′ and 5′-CCATC-CATGAAGAGGGTATG-3′, yielding an amplification product of 785 bp. Primers for human $\alpha$V cDNA were 5′-GCTGGTCTTCGTTT-CAGTGT-3′ and 5′-GAGCAACTTCCACAACCCA-3′, yielding an amplification product of 550 bp and for human $\beta$1 cDNA were 5′-AACCTTCAGTGGAAAGCCA-3′ and 5′-TGAATACACAAAGGCCA-ACA-3′, yielding an amplification product of 621 bp. Primers for human laminin cDNA were 5′-ACCAGGCAAACCAATGAATC-3′ and 5′-TCCTCCAAGACCTCCAACA-3′, yielding an amplification product of 807 bp and for mouse laminin cDNA were 5′-CCTGGAAACT-GGATTTTGGA-3′ and 5′-GGTCTGCCATTGATGAGTGA-3′, yielding an amplification product of 303 bp. $\beta$-actin was amplified as an internal control, which primers for human cDNA were 5′-ATCATGT-TTGAGACCTTCAACACC-3′ and 5′-CATGGTGGTGCCGCCAGA-CAG-3′ and for mouse cDNA were 5′-AGGCCAACCGCGAGAA-GATGACC-3′ and 5′-GAAGTCCAGGGCGACGTAGCAC-3′, yielding an amplification product of 552 and 250 bp.

*Northern blot analysis.* Total RNA (30 $\mu$g) was used on each lane of 1% formaldehyde/agarose gel and transferred to a nitrocellulose membrane (Optitran BA-S; Schleicher & Schuell, Dassel, Germany) using TurboBlotter transfer system (Schleicher & Schuell, Dassel, Germany). The membrane was fixed by UV cross-linking method ($200 \times 100$ $\mu$J/cm$^2$) (Stratalinker; Stratagene, La Jolla, CA). Human integrin $\alpha$6 cDNA clone was generously provided by Dr. Arnoud Sonnenberg (The Netherlands Cancer Inst., Amsterdam, The Netherlands). cDNA probe (25 ng) for integrin $\alpha$6 and $\beta$-actin were labeled with 50 $\mu$Ci of [$\alpha$-$^{32}$P]dCTP (Amersham) with random priming method (Random primed DNA labeling kit; Boehringer Mannheim). Blots were prehybridized at 42°C for 3 h in 50% formamide, 5× SSPE, 10× Denhardt's solution, and 0.5% SDS, and then hybridized with a specific activity of $1 \times 10^9$ cpm/$\mu$g [$\alpha$-$^{32}$P]dCTP-labeled probes at 42°C consecutively for 16 h. Blots were then washed with 0.2× SSC and 0.1% SDS for 1 h at 65°C and exposed to X-ray film. Equal loading was assessed by hybridization with human $\beta$-actin probe.

*Construct of integrin $\alpha_6$ promoter/luciferase reporter.* To analyze the TGF-$\beta_1$ activity on integrin $\alpha$6 mRNA transcription by using promoter-reporter assay, we created a plasmid containing integrin $\alpha_6$ promoter with luciferase structural gene. We conducted PCR to obtain human integrin $\alpha_6$ gene promoter region by using the primers which comes from study by Nishida *et al.* (37), forward (5′-CAGGAGCCTTCATGCCACCTACACA-3′) and reverse (5′-GGC-GACTTACTCGGGCAACAAGAG-3′). The 830 base pair PCR products were cloned into the pGEM-T Easy Vector according to manufacturer's protocol (Promega, Madison, WI). The sequence of cloned promoter region was verified by automatic sequencing service provided by Genome Center, Korea Research Institute of Biotechnology & Bioscience, Taejon. The inserted promoter was excised from the pGEM-T Easy vector and subcloned into pGL3-Basic (Promega, Madison, WI), that has a luciferase reporter gene.

*Transient transfection assays.* Ten micrograms of integrin $\alpha_6$ plasmid DNA and one microgram of pCMV-$\beta$ plasmid DNA (Clontech, Palo Alto, CA) with 2× HeBS buffer and $CaCl_2$ were incubated for 20 min at room temperature to form calcium phosphate–DNA precipitate. 70–80% confluent HLE B-3 cells on 6-well plates were used for transient transfection assays. One hour prior to transfection, the cells were fed with complete media. Three hundred microliter of calcium phosphate–DNA complex precipitates were overlaid to the cells and incubated for 4 h. Then the cells were treated with TGF-$\beta_1$. Twenty-four hours after TGF-$\beta_1$ treatment, the cells were harvested and the luciferase activity was measured as following.

*Luciferase and $\beta$-galactosidase activity assays.* The cell pellet was lysed with 250 $\mu$l of lysis buffer (200 mM sodium phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 100 mM $\beta$-mercaptoethanol, and 1.33 mg/ml ONPG), and centrifuged at 10,000*g* for 10 min at 4°C to remove cell debris. Cell lysate was mixed with 100 $\mu$l of luciferase substrate buffer (Promega, Madison, WI) and luciferase activity was measured immediately (Turner-20/20 luminometer, Sunnyvale, CA). To measure $\beta$-galactosidase activity, 50 $\mu$l of 2× $\beta$-galactosidase buffer (200 mM sodium phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 100 mM $\beta$-mercaptoethanol, 1.33 mg/ml ONPG) was added to 50 $\mu$l of cell lysate. Following incubation at 37°C for 30 min, 1 M sodium carbonate was added. Then, the absorbance at 420 nm was measured with a spectrophotometer (SEPCTRAmax 250 Microplate Spectrophotometer, Molecular Devices Corps., Sunnyvale, CA). The promoter/reporter luciferase activity was normalized by $\beta$-galactosidase activity.

*Fluorescence-activated cell sorting (FACS) analysis.* HLE B-3 cells, treated with TGF-$\beta_1$ and FGF-2 for 72 h, were washed twice with phosphate-buffered saline (PBS), detached with 1 mM EDTA/1 mM EGTA, and resuspended in PBS. The cells were incubated with 40 min at 4°C with anti-integrin $\alpha_6$ (Immunotech, Miami, FL), anti-integrin $\alpha_5$ (Upstate Biotechnology, Lake Placid, NY), and anti-integrin $\beta$ 1 (Biosource, Camarillo, CA). Immunoglobulin IgG was used as a negative control. After washing twice with PBS containing 0.5% BSA, the cells were incubated with affinity purified fluorescein isothiocyanate-conjugated goat anti-rat F(ab′) fragments (1:500 dilution) and sheep anti-mouse (1:200 dilution) for 40 min. Following washing with PBS for two times, the cells were fixed with 2% paraformaldehyde in PBS. Integrin expression was analyzed using FACScan software (Becton–Dickinson, Mountain View, CA).

*Culture of mouse lens epithelial explants.* All animal procedures conformed to Institutional Guidelines and the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. Eyes were removed from 8-week-old Balb/c mice under sterile conditions, placed in medium, that is M199 containing bovine serum albumin and antibiotics, and preincubated at 37°C in 5% $CO_2$/air. Lenses were removed and incubated in 2 ml for 1 h. Epithelia were peeled away from fibers and pinned out with the cellular surface uppermost in cultured dishes containing 1 ml medium. The whole epithelium was used, unless otherwise specified, and each dish contained 9 or 10 explants. Approximately 3 h after preparation of explants, medium was replaced and TGF-$\beta_1$ were added to five final concentrations of 10 ng/ml.

## RESULTS

### *Differential Modulation of Integrin mRNAs by Different Growth Factors in LEC Lines*

To demonstrate the effects of several growth factors on the expression of integrin mRNAs for $\alpha$5, $\alpha$6, and $\alpha$V, total cellular RNA was isolated for RT-PCR analysis from human lens epithelial B-3 cells (HLE B-3) and murine lens epithelial cells ($\alpha$TN4). After incubation with or without IGF-1 (75 ng/ml), TGF-$\beta$1 (5 ng/ml), or FGF-2 (50 ng/ml) for 24 h, the expression of integrin mRNAs were detected with a nested set of specific oligonucleotide primers for $\alpha$5, $\alpha$6, and $\alpha$V. First, to investigate the possible structural differences within the cytoplasmic domain of the $\alpha$6 subunits expressed by LECs, the distribution of the $\alpha$6 isoforms

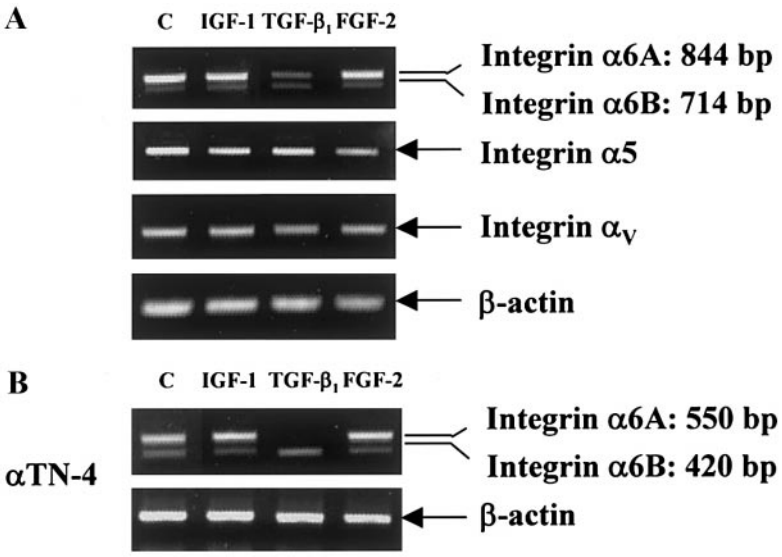


**FIG. 1.** Expression and modulation of integrin $\alpha 5$, $\alpha 6$, and $\alpha V$ mRNA expression by IGF-1, TGF-$\beta_1$, and FGF-2. (A) Human lens epithelial B-3 cells cultured in the absence (lane 1) or in the presence of IGF-1 (75 ng/ml), TGF-$\beta_1$ (5 ng/ml) and FGF-2 (50 ng/ml) for 24 h (lanes 2–4). Total cellular RNA was isolated and RT-PCR was conducted as described under Materials and Methods. $\alpha 6$ mRNA isoforms were detected by RT-PCR with a nested set of oliogonucleotide primers. With these primers, the A and B isoforms rise to products of about 844 (550) and 714 (420) bp, respectively. Both $\alpha 6$A and $\alpha 6$B isoform bands are visible in HLE B-3 and $\alpha$TN-4 cell lines and mouse explants. TGF-$\beta_1$-treated cells display the reduced amount of $\alpha 6$A and no noticeable change of $\alpha 6$B isoform. These results, together with the cultured cell data (data not shown), indicate that the expression of the $\alpha 6$ isoforms is cell-type specific. (B) The murine lens epithelial cell line, $\alpha$TN4, cultured in the absence (lane 1) or in the presence of IGF-1 (75 ng/ml), TGF-$\beta_1$ (5 ng/ml), and FGF-2 (50 ng/ml) for 24 h (lanes 2–4). Total cellular RNA was isolated and RT-PCR was conducted as described under Materials and Methods.

was identified in cultured human (HLE B-3) and mouse ($\alpha$TN-4) lens epithelial cell lines. The LECs tested contained both $\alpha 6$A and $\alpha 6$B mRNA at ratios characteristic of the individual cell lines. In contrast, immortalized mouse embryonic fibroblasts (NIH-3T3) expressed exclusively $\alpha 6$A, as previously published (12) (data not shown). A nested set of primers, derived from the human $\alpha 6$ sequence (38), were used to ensure the specificity of the reaction. Figure 1A shows the RT-PCR fragments amplified from the $\alpha 6$A and $\alpha 6$B mRNAs, which corresponded to the size expected, 844 bp and 714 bp, respectively.

Using oligonucleotide primers flanking the 3′ end of the integrin $\alpha 6$ coding region, as previously published (12), we obtained two products of 550 and 420 base pairs from $\alpha$TN-4 cells and mouse explants (Fig. 1B). For convenience, we refer to the published form of $\alpha 6$ as $\alpha 6$A.

To determine whether the expression of integrins could be modulated upon the administration of several growth factors to LECs, HLE B-3 and $\alpha$TN-4 cells were allowed to respond over a period of 24 h in the absence of serum. The morphology of the TGF-$\beta_1$ treated LECs was not dramatically different from that of un-treated cells. Unexpectedly, PCRs of cDNA from TGF-$\beta_1$ treated cells only downregulated the upper fragment corresponding to the $\alpha 6$A cytoplasmic sequence. However, similar amplification of the cDNA from the TGF-$\beta_1$ treated NIH 3T3 cells produced two distinct fragments, which were shown by nucleotide sequencing to be the $\alpha 6$A and $\alpha 6$B isoforms, respectively (data not shown).

As shown in Fig. 1A, the level of $\alpha 6$A integrin mRNA was decreased by TGF-$\beta_1$ treatment, while no significant changes occurred in $\alpha 5$ and $\alpha V$ integrin mRNAs. Moreover, IGF-1 slightly reduced the expression of integrin $\alpha 6$ mRNA in mouse lens epithelial cells, but no significant alteration in HLE B-3 cells (Fig. 1B). In contrast, FGF-2 treatment altered neither integrin $\alpha 6$ expression in HLE B-3 cells nor in the murine lens epithelial cell ($\alpha$TN-4) line.

### *TGF-$\beta_1$ Downregulated the Expression of Integrin $\alpha 6$ and Laminin mRNA in Cultured LECs*

To investigate the mechanism by which TGF-$\beta_1$ leads to the reduced expression of integrin $\alpha 6$ mRNA, HLE B-3 cells were incubated with TGF-$\beta_1$ at various concentrations from 1 to 20 ng/ml, which have been previously shown to be relevant concentrations for ECM matrix synthesis (31). As shown in Fig. 2A, RT-PCR analysis demonstrated the decreased expression of integrin $\alpha 6$ mRNA and laminin mRNA in a dose dependent manner. In contrast, TGF-$\beta_1$ treatment did not alter $\beta 1$ integrin mRNA levels. Northern blot also demonstrated a significantly reduced expression of integrin $\alpha_6$ mRNA in response to TGF-$\beta_1$. Blots were

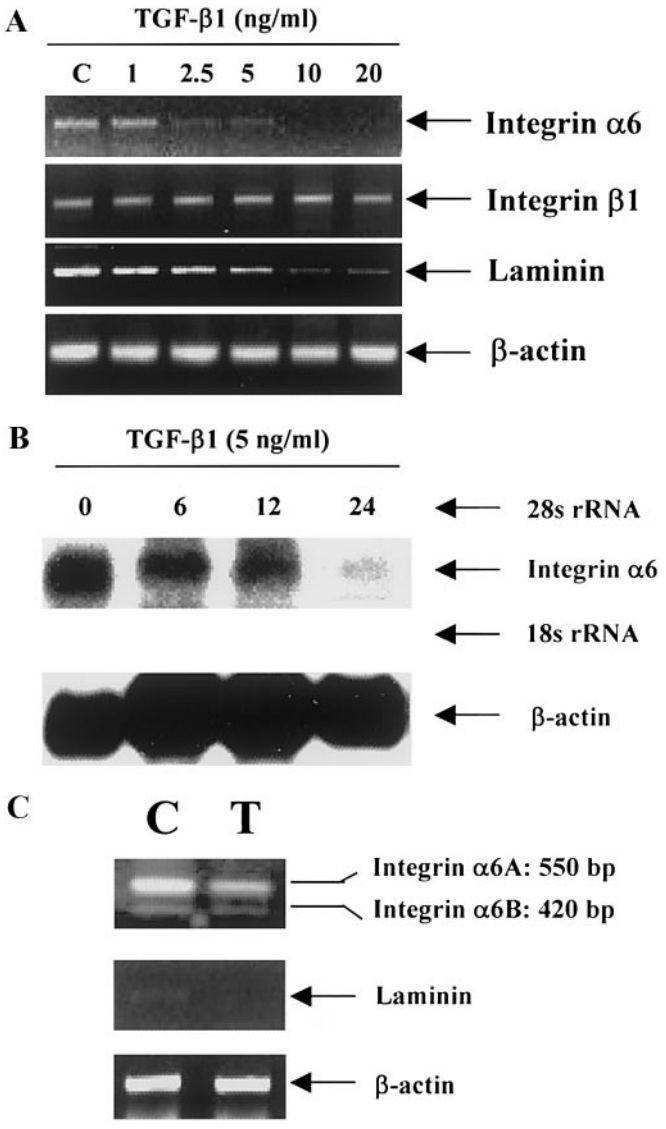


**FIG. 2.** Effect of TGF-$\beta_1$ on integrin $\alpha$6, $\beta$1, and laminin mRNA levels in cultured lens epithelial cells. (A) The expression of integrin $\alpha$6, $\beta$1, and laminin mRNA in HLE B-3 cells cultured in the absence of (lane 1) or in the presence of 1, 2.5, 5, 10, 20 ng/ml TGF-$\beta_1$ for 24 h (lanes 2–6). (B) Nothern blot analysis of integrin $\alpha$6 mRNA in TGF-$\beta_1$-treated HLE B-3 cells. HLE B-3 cells (80–90% confluent) growing in serum free medium were treated with 5 ng/ml TGF-$\beta_1$ for 0–24 h and total RNA was isolated at indicated time points, and subjected to Northern blot analysis. (C) Lens epithelial explants were cultured for 24 h with 10 ng/ml TGF-$\beta_1$. Primers specific for mouse integrin $\alpha$6 isoforms were derived from GenBank sequences. The position corresponding to the $\alpha$6 (550 and 420 bp) and $\beta$-actin amplification products is shown; C, control cells; T, cells cultured with TGF-$\beta_1$.

stripped and reprobed for $\beta$-actin as a loading control (Fig. 2B).

To investigate whether TGF-$\beta_1$ can alter the integrin $\alpha$6 receptors of explanted LECs, lens epithelial explants from P9 mouse were cultured with or without TGF-$\beta_1$. None of the controls or TGF-$\beta_1$ treated explants showed any of the cataractous changes (i.e., spindle-shaped cells) associated with TGF-$\beta_1$ exposure. However, Fig. 2C illustrates that TGF-$\beta_1$ markedly reduced the expressions of $\alpha$6A integrin and laminin mRNA levels in mouse lens epithelial explants within 24 h. In contrast, there was no apparent alteration of integrin $\alpha$6 upon exposure to FGF-2 (data not shown).

### *TGF-$\beta_1$ Reduced the Promoter Activity of Integrin $\alpha$6 in Human Lens Epithelial B-3 Cell Line*

The integrin $\alpha$6 promoter region was amplified using promoter specific primers by PCR and cloned into pGL3-basic, as described under Materials and Methods (37, 39). The DNA sequence of human integrin $\alpha_6$ promoter, which is termed pI$\alpha$6, was found to be 100% identical to the sequences reported in GenBank (AF078694). The 830 bp integrin $\alpha_6$ upstream fragment (pI$\alpha$6) is proved to be transcriptionally active showing that the promoter we cloned has a promoter activity, and that it is able to recruit the various transcription factors involved in the gene transcription of integrin $\alpha$6. pI$\alpha$6 had 54 times the promoter activity of the negative control (pGL3-basic). Figure 3 shows that TGF-$\beta_1$ mediated approximately a 52% reduction in the promoter activity of human lens epithelial B-3 cells over a period of 24 h, which is consistent with the results of RT-PCR and Northern blot analysis. In contrast, there was no apparent alteration of integrin $\alpha$6 in the promoter activity upon exposure to FGF-2.

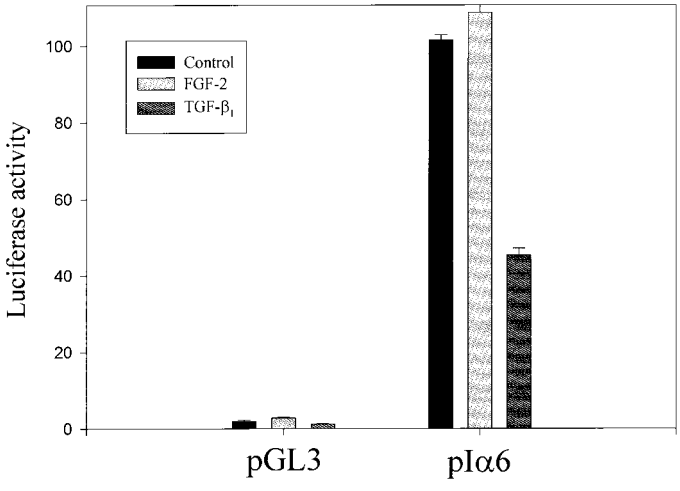


**FIG. 3.** Effect of TGF-$\beta_1$ and FGF-2 on the promoter–reporter constructs for $\alpha$6 integrin. Effect of TGF-$\beta_1$ on human integrin $\alpha$6 promoter transcriptional activity was determined in transient transfection experiments of HLE B-3 cells. $3 \times 10^5$ cells were transfected with 10 $\mu$g of promoter–reporter plasmid and 1 $\mu$g of pCMV $\beta$-galactosidase DNA as an internal control. Luciferase activity was measured as relative light units and normalized by $\beta$-galactosidase activity. Induction of promoter transactivation was calculated by comparing luciferase activity in the presence or absence of TGF-$\beta_1$ (5 ng/ml) and FGF-2 (50 ng/ml). The experiments were repeated three times, and the representative result is shown. Error bar is the mean of the three replicates $\pm$ SD.

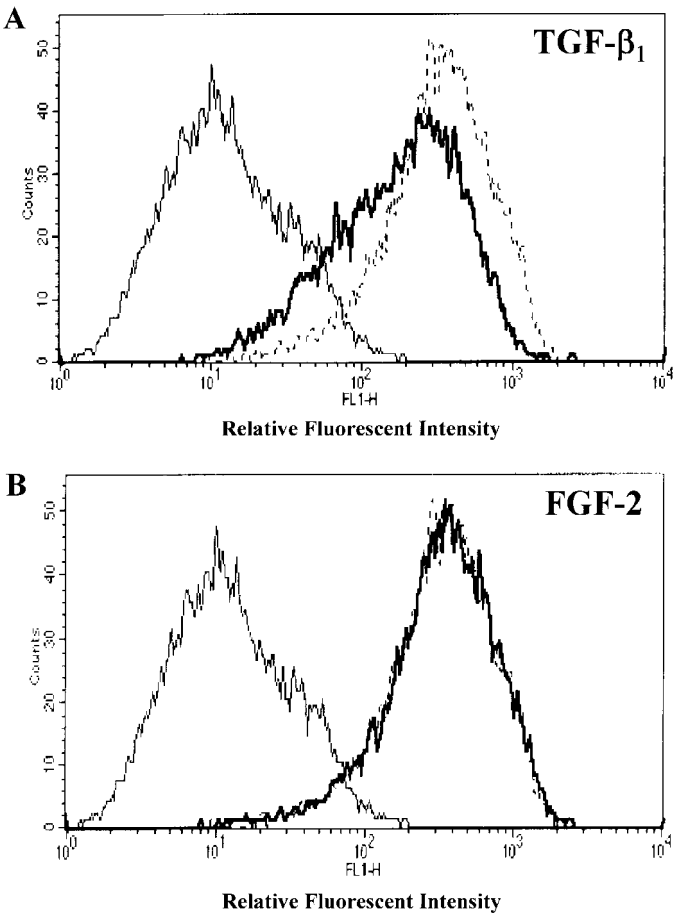


**FIG. 4.** Histograms of flow cytometric analysis for the $\alpha$6 subunit with TGF-$\beta_1$ and FGF-2 in the human lens epithelial B-3 cell line. Control (dotted lines) or TGF-$\beta_1$ treated (solid lines) and FGF-2 treated (solid line) HLE B-3 cells were cultured in serum-free MEM with TGF-$\beta_1$ (5 ng/ml) and FGF-2 (50 ng/ml). After 3 days later, cells were labeled by primary and secondary antibodies and then assayed by FACS as described under Materials and Methods. The y axis represents the cell number and the x axis represents the relative fluorescent intensity. Cell number is plotted as a function of relative fluorescence intensity on a log scale. One representative of three independent experiments performed is shown.

### *Expression of the α6 Subunit in the TGF-β1 Treated B-3 Human Lens Epithelial Cells*

To correlate mRNA changes with subsequent changes in cell surface expression, HLE B-3 cells were cultured in TGF-$\beta_1$ (10 ng/ml) and FGF-2 (100 ng/ml) treated serum-free medium, and cell surface $\alpha_6$ integrin expression was analyzed by indirect immunofluorescence. FACS analysis was also carried out on a parallel set of cultures. After treatment with TGF-$\beta_1$, a marked reduction in the expression of $\alpha$6 was observed TGF-$\beta_1$, whereas FGF-2 appeared to have no effect on the regulation of integrin $\alpha$6 (Figs. 4A and 4B). Constitutive $\alpha$6 integrin subunit expression was demonstrated on unstimulated HLE B-3 cells as shown by the dotted line in Fig. 4. A markedly reduction in the surface expression of integrin $\alpha$6 was also observed after TGF-$\beta_1$ treatment for 72 h. The geometric mean was shifted from 313.13 (no treatment; dotted line) to 165.64 (TGF-$\beta_1$ treatment; solid line) and to 312.89 (FGF-2 treatment; solid line) with the base value of 12.38 (negative control). In contrast, less pronounced changes were observed in $\beta$1 integrin (data not shown). The overall changes observed among the integrin subunits are shown in Fig. 4, results are from one of three independent experiments.

## DISCUSSION

In this paper, we report that TGF-$\beta_1$ downregulates the expression of integrin $\alpha$6 in cultured LECs. In an attempt to demonstrate that TGF-$\beta_1$ induces the downregulation of integrin $\alpha$6 at the transcriptional level, transient transfection assays were performed. TGF-$\beta_1$ was found to repress luciferase activity, whereas pI$\alpha$6 did not respond significantly to FGF-2 treatment. Changes in integrin $\alpha$6 expression induced by TGF-$\beta_1$ revealed the existence of an unique mechanism of integrin $\alpha$6 regulation, since the expressions of the other integrin complexes of LECs, namely $\alpha$5 and $\alpha$v, were unaltered. Although we did not detect a change in $\alpha$3 integrin mRNA on treating HLE B-3 cells with TGF-$\beta_1$ (data not shown), we do not know whether TGF-$\beta_1$ modulates the expressions of other laminin receptors in these cells. Alternatively, the decrease in laminin mRNA observed may be due to the remodeling of the extracellular matrix components by metalloproteinases (our unpublished data). Such changes may result in different functional responses in LECs.

It is also interesting to note that in the case of the integrin $\beta_1$ class, the changes in expression occur via an alteration of the $\alpha$ subunits, while the synthesis of the $\beta_1$ subunit remains only slightly changed. Treatment with IGF-1 and FGF-2 did not change the expression of $\alpha$6 integrin in HLE B-3, though a slight alteration was found upon IGF-1 treatment in mouse lens epithelial cells. Thus, TGF-$\beta$1 is the only cytokine among those tested which affects the integrin $\alpha$6 expression of LECs. However, this does not account for the presence of other receptors that can be modulated by other growth factors and cytokines. The regulation of cell-surface receptor levels by TGF-$\beta_1$ would allow LECs to respond rapidly to different conditions, without requiring alterations in the overall synthesis of other receptors. The regulation of integrins by TGF-$\beta_1$ may be exclusive to LECs, when they are challenged by changing amounts and composition of ECM in different tissues they encounter.

The downregulation of integrin $\alpha$6 that we have reported here may have implications for several LEC functions. Walker and Menko (21) reported that during the lens fiber cell maturation process, $\alpha$6 integrin is dramatically downregulated as the cells undergo ter-

minal differentiation involving the loss of nuclei in the nuclear fiber zone. They speculated that this is of specific significance, because the loss of $\alpha$6 in this cell population, and the concomitant dissociation of LECs from the matrix capsule, could trigger the loss of their organelles and nuclei, an event which is pivotal to the lens becoming a clear crystalline structure. Recently, De Iongh *et al.* reported that TGF-$\beta$ signaling is important during the terminal lens differentiation process (22). This study has also confirmed that the competency of LECs to respond to TGF-$\beta_1$ can be modulated by integrin receptors.

The observation from the current study concerning the downregulation of integrin $\alpha$6 by TGF-$\beta_1$ is entirely consistent with a role for integrin $\alpha$6 during the terminal differentiation process. Our current data, led us to speculate that the downregulation of integrin $\alpha$6 may be mimicked *in vitro* by the exposure of cultured LECs to TGF-$\beta_1$. However, the isoform switches between the $\alpha$6B and $\alpha$6A were not observed, which we attribute in part to the fact that the cultured LECs we used had a predominance of the $\alpha$6A isoform. This study also emphasizes the importance of considering TGF-$\beta_1$ in the lens differentiation process in addition to its potential role in cataractogenesis. Wride and Sander (3) reported that the addition of TNF-$\alpha$ to LEC cultures enhanced nuclear degeneration in explanted LECs, and further suggested that TNF-$\alpha$ could play a role in lens fiber cell denucleation during embryonic development. At present, it remains unclear what events or signaling transduction pathways are active in the terminal differentiation of LECs. Although the subsequent downstream pathways are not completely understood, the interactions of T$\beta$RI with several cytoplasmic proteins have been described- the most important of these being the Smad family of proteins (40, 41). In addition, signaling via T$\beta$RII has also been shown to occur and has been associated with TGF-$\beta$ mediated growth arrest (42). Recent studies suggested that this receptor is involved in effecting cell cycle arrest by associating with cyclin B, a cytoplasmic protein involved in activation of Cdc2 kinase and the entry of cells into the G2/M phase of the cell cycle. Bachelder *et al.* demonstrated the integrin $\alpha$6 associated survival signaling pathways by tumor suppressor p53 (43).

However, the specificity of lens cell responses to TGF-$\beta_1$ via the regulation of $\alpha$6 integrin remains to be established, since changes in linkage of integrin $\alpha$6 to the cytoskeleton correlated with the activation of other receptors, enabling it to participate in intracellular signaling events and creating the opportunity for $\alpha$6 integrin to participate in lens cell migration (44). However, the exact mechanism by which cells move within the ECM are still the subjects of investigation (37, 39, 45–50). Recently, Torimura *et al.* suggested the participation of integrin $\alpha_6\beta_1$ in the metastasis and invasion of hepatoma cells, by demonstrating the promoted attachment of hepatoma cells to laminin (51). Although the physiological functions of integrin $\alpha$6 have not been thoroughly investigated in LECs, its possible involvement in the adhesion and migration of LECs cannot be underestimated.

In view of the significance of the integrin $\alpha$6 gene, relatively few analytical studies have been undertaken upon the integrin $\alpha$6 subunit promoter (37, 39). The 830 bp integrin $\alpha$6 promoter we cloned accommodated the putative binding sites for various transcription factors, such as, SP1, AP2, GRE/PRE, and CREB, and these elements are postulated to be responsible for the modulation of the integrin $\alpha$6 gene expression observed during cell differentiation, development, and embryogenesis.

The mechanism by which TGF-$\beta_1$ downregulates the expression of $\alpha$6 is currently under investigation. In conclusion, we show for the first time that TGF-$\beta_1$ downregulates the expression of integrin $\alpha$6 on lens epithelial cell *in vitro.*

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