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Transdifferentiation of Cultured Bovine Lens Epithelial Cells into Myofibroblast-like Cells by Serum Modulation

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An after-cataract is caused by the proliferation of residual cells over the equator of the lens. These cells subsequently migrate to the posterior lens capsule, where they undergo aberrant differentiation into fiber-like cells or transdifferentiation into fibroblast-like cells. To study the precise molecular mechanisms of transdifferentiation, an attempt was made to establish an in vitro system, in which the lens epithelial cells (LECs) of the pre-equatorial zone could be transdifferentiated into fibroblast-like cells. The required conditions for culturing the LECs were identified as consisting of four phases; intact bovine explants, explant-cultured, serum-modulated and additionally modulated LECs. The LECs of each phase were compared by examining changes in the expression of the epithelial-mesenchymal transition (EMT)-related genes and changes in cellular morphology and adhesion. The explants that were cultured in a medium containing 10% fetal bovine serum (FBS) for 2 weeks, showed changes in the expression of the EMT-related genes, although the other explant-cultured cells maintained an epithelial morphology. To introduce a transition into mesenchymal cells, the explant cultures were subcultured in a medium containing 20% FBS for six passages. These cells displayed an elongated morphology and were able to grow and migrate in a similar way to fibroblast cells. The expression of the EMT-related genes, such as, extracellular matrix proteins and integrins, was altered. This was similar to the alteration of the 3-dimensional collagen gels model previously reported. During a further process of EMT by additional serum modulation, the inhibitory effect of disintegrin on cell adhesion was gradually decreased, integrin expression was differentially regulated and α-smooth muscle actin was post-translationally modified from the point of passage number six. Overall, it can be concluded that terminal transdifferentiation accompanies changes in the cytoskeletal proteins and cell surface molecules. These are modulated in systematic patterns of post-transcriptional and post-translational regulation and patterns of gene regulation, by the synergic effects of several transforming factors contained in serum. Therefore, posterior capsular opacification may also be accompanied by this molecular mechanism.

Key Words: Cataractogenesis, after-cataract, lens, epithelium, mesenchyme, transdifferentiation, serum

INTRODUCTION

Transdifferentiation is well recognized in epithelial-mesenchymal transition (EMT). A number of recent studies have shown several growth factors and oncogenes, such as, TGF-β_1, TGF-α, acidic FGF, c-fos, v-src, v-ras, and v-mos, acting as EMT inducers.[1,2] During epithelial transdifferentiation by these inducers, the intrinsic and extrinsic organizations of cells, including the actin cytoskeleton, cell-cell adhesion molecules and several integrins, undergo changes in their structures and expression levels. EMT is a multiple process known to occur normally during embryonic development. However, it also occurs abnormally during pathologic conditions, such as fibrosis and tumorigenesis of epithelia.[3,4]

Transdifferentiation of the lens epithelial cells

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(LECs) has been demonstrated in anterior subcapsular cataracts and fibrosis types of after-cataracts.[5] The molecular mechanisms involved in such drastic changes in epithelial cells are poorly understood. Due to the general importance of EMT, several groups have tried to establish *in vitro* model systems, using well-defined cell cultures, which may be of value in examining the individual steps of such complex processes.[6-9] Examples of these model systems are demonstrated in the embryonic kidney[10] and embryonic avian lens epithelial cells suspended in 3-dimensional (3D) collagen gels.[11-13] In the embryonic avian lens epithelial cells suspended in 3D collagen gels, the cells lose polarity, develop features of an elongated morphology, synthesize type I collagen and fibronectin, and stop expressing crystallins, laminin and type IV collagen. In parallel, their receptors for the extracellular matrix (ECM) molecules are also altered, as the $\alpha 3\beta 1$ and $\alpha 5\beta 1$ integrins are more expressed by mesenchyme-like cells while $\alpha 6\beta 1$ integrin expression is decreased. However, although this model shows the well-defined EMT phenomena, it is not obvious how the EMT gene expression is regulated during the EMT process.

The aqueous humor contains a number of components that are derived mainly from the blood through the blood-aqueous barrier. The components of the aqueous humor can be altered under pathological conditions. There are higher levels of several growth factors, hormones, reactive oxygen species (ROS) and prostaglandins in the aqueous humor of eyes with cataracts.[9,14,15] LECs are in indirect contact with the aqueous humor. However, during intraocular lens (IOL) implantation, the LECs are exposed directly to the aqueous humor and blood components through the disruption of the blood-aqueous barrier, which may accelerate the EMT in the LECs.[16-18] Therefore, the transdifferentiation model by serum modulation was designed to mimic this event, which occurs during surgery.

EMT by serum modulation was attempted in the rat bladder carcinoma-derived cell line, NBT-II.[19] In this system, the NBT-II cells, when exposed to an inducing medium containing a commercial serum substitute (Ultroser G), exhibited extensive changes in their organization. These changes were accompanied by a redistribution of the desmosomal plaque proteins (desmoplakins, desmogelin, and plakoglobin) and by reorganization of the cytokeratin and actin-fodrin filament systems.

For better understanding of the molecular mechanisms regulating the EMT-induced genes during cataractogenesis, a cell culture model was designed, in which bovine LECs were modulated by changing the serum concentrations. The culturing conditions for the four phases of the LECs were identified; intact bovine explants, explant-cultured, serum-modulated and additionally modulated LECs. Examining the changes in the expression of the EMT-related genes and the changes in cellular morphology and adhesion then compared the LECs of each phase.

MATERIALS AND METHODS

Bovine lens epithelial explants and subculture

Bovine lens epithelial explants were prepared as previously described[20] and transferred to a 6-well plate in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). After 2 weeks of culture, the total cellular RNA was isolated from the explant cultures and subjected to reverse transcription-polymerase chain reaction (RT-PCR) as described in the next paragraph. For Western blot analysis, cell lysate was prepared as described below. Another set of explants were continuously subcultured in DMEM containing 20% FBS up to passage.[20]

RNA isolation and RT-PCR

Total cellular RNA was isolated by using TRIZOL reagent (Invitrogen). One (g of RNA was reverse-transcribed in a 20-μl reaction mixture by use of a kit (First strand cDNA synthesis kit; Roche, Indianapolis, IN, USA). The synthesized cDNA (0.2 to 1 μl) was amplified in a 20-μl reaction mixture by a standard PCR method. Conditions for PCR were as follows: 0.4 μM of each primer, 0.2 mM deoxynucleoside triphosphate mixture (Perkin Elmer, Foster City, CA, USA), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM

$MgCl_2$ and 1.0 U of *Taq* DNA polymerase (Perkin Elmer). Reaction mixtures were incubated in a thermal controller (Model PTC-100; MJ Research, Watertown, MA, USA) for 25 to 35 cycles (denaturation at 94℃ for 45 sec, annealing at 60℃ for 30 sec, extension at 72℃ for 1 min). The amounts of amplified products were analyzed using an image documentation system (Image Master VDS; Pharmacia Biotech, Uppsala, Sweden). DNA size markers (D-15 DNA marker; Invitrogen) were run in parallel to evaluate the predicted sizes of the amplified bands. The primer sequences specific for the genes examined and predicted product sizes, are shown in Table 1. All primer sequences, except those indicated in Table 1, were designed by using Primer 3 primer picking software through a web site run by the Center for Genome Research at the Whitehead Institute for Biomedical Research (Cambridge, MA, USA).

Proliferation and migration assay

Assays for cell proliferation were performed by the XTT process (Roche). In brief, cells were grown at a density of 1×10^4 cells/well (50% confluency), using serum free medium, in 96-well tissue culture plates (Costar, High Wycombe, Bucks, UK). After 16 hours, the medium was changed to 100 μl of phenol red free DMEM containing various concentrations of serum. After the indicated periods, cells were incubated with 50 μl of XTT reagent for 4 hours, in a humidified atmosphere (37℃, 5% CO_2) and spectrophotometrical absorbance of the samples was measured at 490 nm, using a microtiter plate reader (Bio-Tek Instruments, Winooski, VT, USA). The number of cells was determined from the linear relationship between the absorbance and the number of defined cells.

Migration assays were performed on Transwell (3-μm pore size; Becton Dickinson, Mountain View, CA, USA) as previously described,[21] with minor modifications. Cells (1×10^5) were allowed to attach and spread for 2 hours on the upper chamber in DMEM containing 1% FBS. The medium was then changed to DMEM containing 10% FBS. After 8 hours of incubation, the upper cell layer was removed with a cotton swab and cells on the underside of the Transwell were fixed, stained with toluidine blue and counted under the microscope.

Western blot and dot blot analysis

Cells were incubated on 60-mm tissue culture dishes (Costar) in serum-free medium for 24

Table 1. Conditions of Polymerase Chain Reaction and Routinely Used PCR Primers

Short name*[1]	Primer sequence	Product size (bp)	Conditions*[2]
αA-cry L	5′-ttcctgtcctccaccatca-3′	587	60℃
αA-cry R	5′-ggctactctctcaaaccctcaa-3′		
αB-cry L	5′-tccctttccactctcccag-3′	266	55℃
αB-cry R	5′-ctcaatcacatctcccagca-3′		
Col I L	5′-tccaaaggagagagcggtaa-3′	693	55℃
Col I R	5′-gaccagggagaccaaactca-3′		
Col IV L	5′-tgtaaaggagagcaaggaccac-3′	707	55℃
Col IV R	5′-agccaaccagaaactgtaggag-3′		
FN L	5′-ggtaacgaaggctccactgc-3′	735	55℃
FN R	5′-accagattcctcttatcaactg-3′		
β1-int L	5′-tcactcaaatccagccacag-3′	570	60℃
β1-int R	5′-caccaagtttcccatctcca-3′		

*[1] αA-cry, αB-cry, Col I, Col IV, FN, and β1-int represent αA-crystallin, αB-crystallin, type I collagen, type IV collagen, fibronectin, and β1-integrin, respectively.
*[2]The conditions give the annealing temperature and the number of cycles is 35.

hours. The conditioned medium was collected for dot blot analysis and ELISA as described below. Cells were washed twice with Hank's balanced salt solution (HBSS; Invitrogen) and then were lysed for 30 min at 4℃, with ice-cold lysis buffer (20 mM Tris-HCl [pH 7.5], 120 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin and 1 μM aprotinin (Sigma, St. Louis, MO, USA). After centrifugation for 20 min at 15,000 × g to remove cell debris, the supernatant was removed and stored at -70℃ before use. Protein concentrations were determined using bicinchonic acid assay (BCA; Sigma). Equal protein amounts (100 μg) were denatured in reducing Laemmli sample buffer, separated in 4 - 20% gradient SDS-polyacrylamide gel (Invitrogen) at 125V, then transferred to a nitrocellulose membrane (Hybond; Amersham, Cleveland, OH, USA) using an electroblotting apparatus (Pharmacia Biotech). For dot blot analysis, collected media were loaded onto a dot blot kit (Pierce, Rockford, IL, USA) at the rate of 10 ml/min. These electroblotted and dot blotted Membranes were blocked for 1 hour in Tween-phosphate-buffered saline (PBS-Tween) containing 5% nonfat milk powder. The membranes were then incubated at room temperature for 45 min, with primary antibody at 1 : 1,000 dilutions in PBS-Tween containing 5% nonfat milk powder. They were then washed in PBS-Tween. The primary antibodies of αA- and αB-crystallin used in this study were purchased from StressGen Biotechnologies (Victoria, BC, Canada), and those of fibronectin, laminin and α-smooth muscle actin, from Sigma. Blots were then incubated at room temperature for 45 min, with a 1 : 5,000 dilution of horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Sigma). After washing three times in PBS-Tween, the blots were detected by using chromogenic substrate solution (DAB substrate; Roche). Pre-stained molecular weight standards (SeeBlue) were purchased from Invitrogen.

Enzyme linked immunosorbent Assay (ELISA)

An ELISA was performed to measure the amount of type I collagen and several integrins. Briefly, for analysis of integrins, cultured bovine LECs were harvested with a cell scraper and homogenized in PBS, and 5 μg of total protein, as determined by a Bradford protein assay (Bio-Rad), was coated in triplicate in each well of a 96-well plastic dish. For type I collagen, 100 μl of collected medium, prepared as mentioned above, was loaded. The plates were incubated for 1 hour at 37℃, washed with PBS, blocked in PBS/3% BSA for 1 hour and then washed 3 times with ELISA wash buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Bovine-specific primary antibodies for type I collagen and integrins (Chemicon, Temecular, CA, USA) were then applied for 1 hour at room temperature, washed 3 times with ELISA wash buffer, incubated with HRP-goat anti-mouse or anti-rabbit secondary (Sigma) and detected by o-phenylenediamide (Sigma). Immediately, 50 μl of 2M H_2SO_4 was utilized to stop the reaction. Plates were read in an ELISA reader at 490 nm. To assure that measurements were conducted in the linear range, a standard curve with various amounts of cell homogenates was analyzed in parallel.

Adhesion inhibition assay

An adhesion inhibition assay was performed by a novel disintegrin, salmosin, purified from Korean snake venom.[22] Cells were adapted in serum free medium for one day and trypsinized with 0.25% trypsin-EDTA (Invitrogen). Trypsin was inactivated by soybean trypsin inhibitor (Invitrogen) and the cells were then centrifuged at 1500 rpm for 5 min and harvested. Harvested cells were plated in triplicate, at 2×10^4 cells/well, in each well of 96-well plates with various concentrations of salmosin. Following incubation for 8 hours, adhered cells were washed in PBS, fixed with 3.7% formaldehyde, stained with 0.1% crystal violet for 10 min, dried at 42℃ and read in an ELISA reader at 550 nm. Adherence was expressed as the adhesion percentage of the number of cells originally added to each well.

Statistical analysis

Values are expressed as mean ± standard deviation. Statistical analysis was performed by using SigmaPlot (Sigma), a program for Windows

98. Statistical significance was assigned at a probability value less than 0.05.

RESULTS

The bovine lens epithelial explants were primarily cultured in DMEM containing 10% FBS for 2 weeks then continuously subcultured. In this study, the cells of early-passage (EP-BLECs) were defined as those in passages 1 - 3. In comparison, the serum-modulated cells (tBLECs) were defined as those continuously subcultured in the DMEM containing 20% FBS for six passages, which changed cell features to highly elongated forms. In addition, to introduce a further transition into mesenchymal cells, the tBLECs were continuously subcultured in 20% FBS media up to 10 passages. These cells were defined as terminal transdifferentiated LECs (ttBLECs).

Transdifferentiation of bovine LECs could be identified by alterations in several markers, such as crystallins, α-SMA, ECM proteins and integrins, using bovine specific antibodies and primers. The LECs from the intact bovine lenses were compared to explant-cultured cells for 2 weeks and the EP-BLECs were compared with tBLECs. Furthermore, changes in adhesion molecule expression and the α-SMA modification of ttBLECs were examined.

Regulation of EMT-related genes in the explant cultures of bovine LECs

An early phase of transdifferentiation was analyzed by examining the mRNA levels for lens epithelial-specific and transdifferentiation-related genes, using RT-PCR (Fig. 1). The levels of type I collagen and fibronectin increased, whereas those of type IV collagen and αA-crystallin decreased dramatically. The levels of αB-crystallin and integrin β1 did not significantly change. These changes in mRNA levels suggested that the cells begin to transform into mesenchyme-like cells as they lose their lens epithelial cell characteristics. However, the morphology of the bovine LECs in the explant cultures after 2 weeks was similar to that of the epithelial cells, having a polygonal shape. To determine the α-crystallins and α-SMA protein levels, Western blot analysis was performed (Fig. 2). The concentration of αA-crystallin in the explant cultures decreased markedly, which is consistent with PT-PCR data. αB-crystallin expression was also lower, although its mRNA level was unchanged. This suggests that αB-crystallin may be regulated at a post-transcriptional level. The α-SMA expression was dramatically enhanced. Taken together, these data

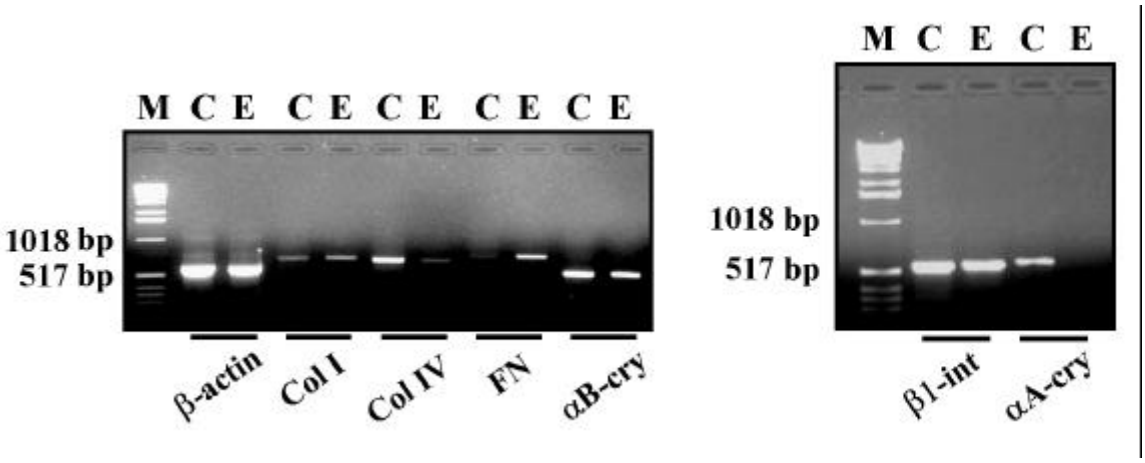

Fig. 1. Analysis of the early phase transdifferentiation, with comparison of lens-specific and transdifferentiation-related genes by RT-PCR. Bovine lens epithelial explants cultured in DMEM containing 10% FBS for two weeks (E) were compared with those in control lens epithelial cells from intact bovine lenses (C). Total RNA was isolated and subjected to RT-PCR analysis. Gene specific primers used in RT-PCR analysis were designed for type I collagen (Col I), type IV collagen (Col IV), fibronectin (FN), αA-crystallin (αA-cry), αB-crystallin (αB-cry), and β1-integrin (β1-int). M, molecular size standards. Similar data was obtained from two independent assays.

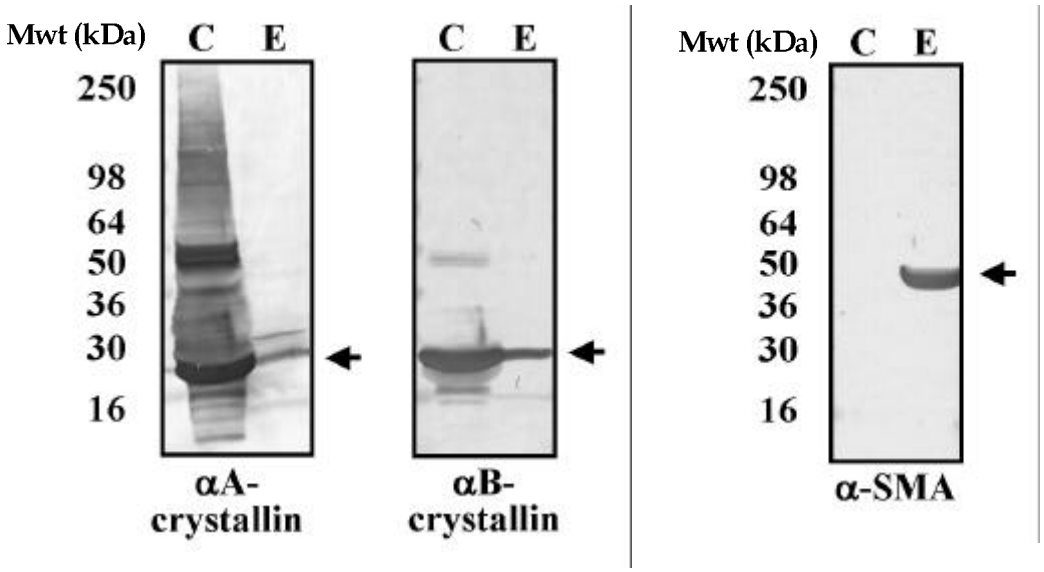

Fig. 2. Western blot analysis for α A-crystallin, α B-crystallin and α-smooth muscle actin (α-SMA), in the early phase of transdifferentiation. Cell lysates were prepared from control lens epithelial cells from intact bovine lenses (C) and bovine lens explants cultured in 10% FBS-DMEM for two weeks (E). Equal amount of proteins (150 μg for α A- and α B-crystallin; 50 μg for α-SMA) were run on SDS-PAGE (4 - 20% gradient gel, Invitrogen) and subjected to Western analysis. The arrows indicate α A-crystallin, α B-crystallin and α-SMA bands. The α-crytallins were dramatically decreased in explant-cultured LECs, whereas α-SMA was significantly induced. Data shown is from one out of two independent experiments that gave similar results. M, molecular size standards.

imply that the bovine LECs had already begun to transdifferentiate in the explant cultures.

Identification of the transdifferentiation by serum modulation

The bovine LECs of passage numbers 1 - 3 exhibited an epithelial morphology (Fig. 3A, EP-BLECs). However, the explant cultures had already undergone changes in the mRNA expression of the EMT-related genes. When these cells were continuously subcultured in the medium containing 20% FBS, th ey took on elongated features (Fig. 3A, tBLECs). The morphology of these cells was similar to fibroblasts in the front end-back end. These tBLECs proliferated in a similar way to EP-BLECs (Fig. 3B). Furthermore, these cells showed a greater migratory ability than EP-BLECs (Fig. 3C). This suggests that the multiple processes that occurred due to serum modulation, did not cause differentiation into lens fiber cells, but transdifferentiation into fibroblast-like cells.

Dot blot and ELISA analysis of the ECM proteins, revealed that the tBLECs secreted more fibronectin and collagen type I but less laminin than the EP-BLECs (Fig. 4A, B). This demonstrated that fibronectin and type I collagen were increasingly secreted during the process of explant culture and serum modulation (Fig. 1, 4).

Analysis of additional transdifferentiation by serum modulation

To investigate some further processes of transdifferentiation by serum modulation, the tBLECs were additionally subcultured in media containing 20% FBS for 10 passages and the adhesion molecule expression was analyzed by an adhesion inhibition assay and ELISA.

The adherent cells had several cell surface molecules to bind to the ECM components, mainly integrins. Some of the primary ECM protein structures contain RGD (Arg-Gly-Asp) sequences, which are recognition sites for some integrins. Salmosin, a family of disintegrins, has inhibitory effects on platelet coagulation and adherent cell adhesion. Using this inhibitory function of

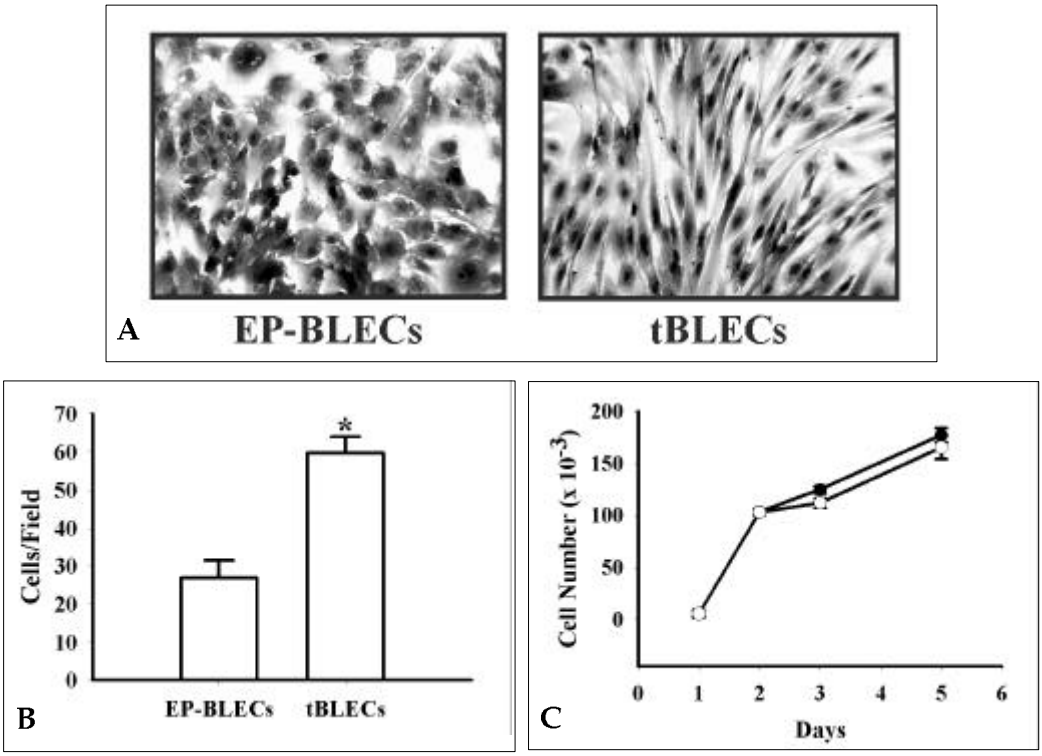

Fig. 3. Analysis of the late phase of transdifferentiation by examination of cellular morphology (A) and the rate of growth and migration (B, C). Early passage bovine LECs (EP-BLECs) were defined as, cells cultured in DMEM containing 10% FBS for 10 days after trypsinization from bovine lens explants to three passages. Transdifferentiated bovine LECs (tBLECs) were defined as, cells continuously subcultured in 20% FBS-contained DMEM for 6 passages. (A) EP-BLECs had features of epithelium, such as, a monolayer and polygonal shape. In comparison, tBLECs were elongated and preserved nuclei, unlike lens fiber cells. These cells were stained by Toluidine blue. (B) The growth rate of tBLECs was very similar to that of EP-BLECs. The number of growing cells was determined from the linear relationship between the absorbance at 490 nm and the defined cell numbers as described under Materials and Methods. (C) The migration assay was performed on the Transwell. Five random microscopic fields per well were counted and mean ± SD of triplicate determinations was shown. *Significantly different from EP-BLECs ($p<0.01$).

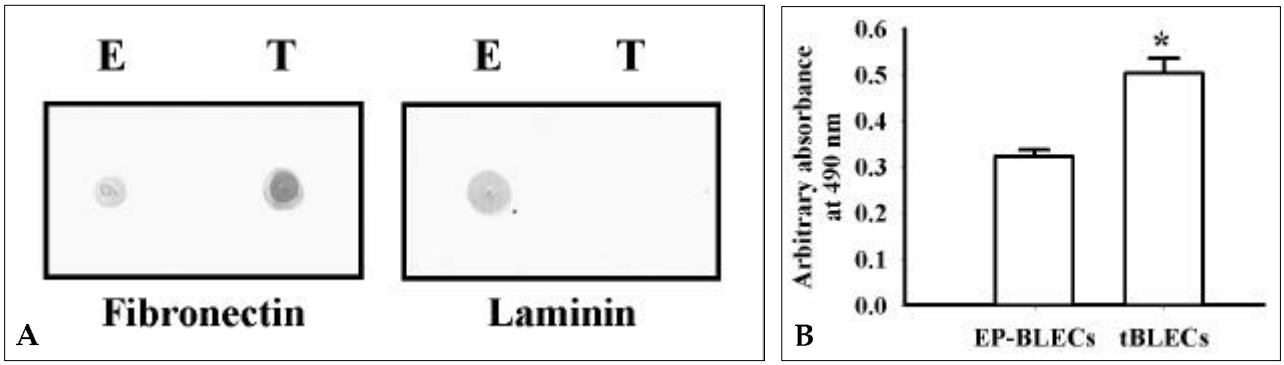

Fig. 4. Analysis for the secretion of extracellular matrix proteins. (A) Dot blot analysis for fibronectin and laminin. Conditioned media were collected and assayed as described under Materials and Methods. E and T represent EP-BLECs and tBLECs, respectively. This data showed the decrease in laminin expression in contrast to the increase in fibronectin expression, during transdifferentiation. (B) ELISA analysis for type I collagen. Conditioned media (100 μl) from EP-BLECs or tBLECs, were immobilized on ELISA plates and detected with an anti-type I collagen primary antibody and a horseradish peroxidase-conjugated anti-rabbit secondary antibody. Data is expressed as the mean concentration of type I collagen ± SD (n=3) and represent arbitrary units of type I collagen reactivity measured by absorbance at 490 nm. *Significantly different from EP-BLECs ($p<0.01$).

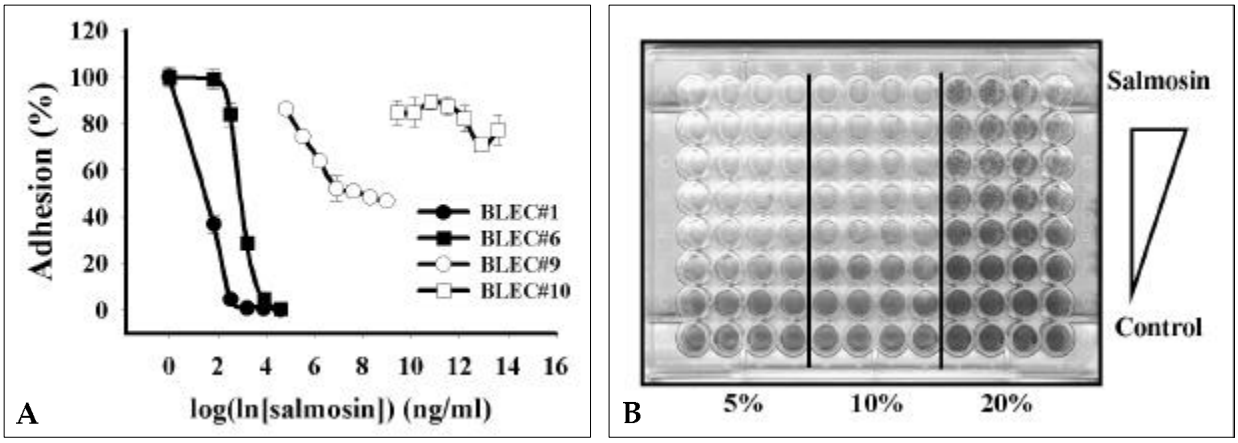

Fig. 5. Gradual alteration in cell surface molecules under the continuous subculture. Salmosin, a novel disintegrin, was reported to inhibit the adhesion of adherent cells. (A) Using salmosin, changes of adhesion inhibition in relation to subculture on 20% FBS containing DMEM, were examined. Continuous subculture made cells insensitive to salmosin. Mean ± S.E. of triplicate determinations is shown. (B) In order to verify whether insensitivity for the inhibitory effects of salmosin on adhesion was caused by serum modulation or not, examination was undertaken of the effects in bovine LECs continuously subcultured in different concentrations of serum (5%, 10%, and 20% FBS). The ranges of the concentration of used salmosin were 0 to 1600 μg/ml. Data shown is from one experiment of three.

salmosin, the changes in adhesion molecules during the process of additional transdifferentiation were examined. As the subculture proceeded, the cells became less sensitive to salmosin (Fig. 5A). In order to verify whether insensitivity to the inhibitory effects of salmosin on adhesion is caused by serum modulation or not, those effects in bovine LECs, continuously subcultured with different concentrations of serum, were investigated. At the point of passage 10, salmosin had a much lower inhibitory effect for the bovine LECs subcultured in 20% FBS, compared to the cells cultured in lower serum concentrations (Fig. 5B). It is not certain whether salmosin insensitivity may result from a decrease in the RGD-binding integrins or a relative increase in the RGD-independent adhesion molecules.

An ELISA assay was performed to confirm the changes in adhesion molecule expression during transdifferentiation by serum modulation. ELISA showed that the expression of integrins β1, α2 and α5 (fibronectin receptor) was significantly up-regulated at passage 6. Integrin α1 and α3 expressions increased markedly with additional serum modulation (Fig. 6A). The integrin β1 and α2 levels were continuously higher but integrin α5 was not additionally stimulated. The β1 integrin transcript levels were unchanged under the same conditions (data not shown), but the protein level was higher. β1 integrin may be regulated at the translational level. It was previously reported that levels of type I collagen and integrins β1, α3 and α5 were higher in avian lens epithelial cells that were suspended in 3D collagen gels.

The above data showed that integrin expression was differentially regulated. In particular, the collagen type and fibronectin receptors were dramatically up-regulated during the EMT process.

In addition, α-SMA was expressed in the EP-BLECs. Interestingly, the ttBLECs showed two unknown higher molecular proteins, immunoreactive to anti-SMA antibodies, approximately 95 kDa and 110 kDa (Fig. 6B). It is possible that these proteins may constitute covalently cross-linked forms of α-SMA or other actin-binding proteins through post-translational modification.

DISCUSSION

Transdifferentiation model by use of serum modulation

An attempt was made to establish a new EMT model system for systemically dissecting the EMT

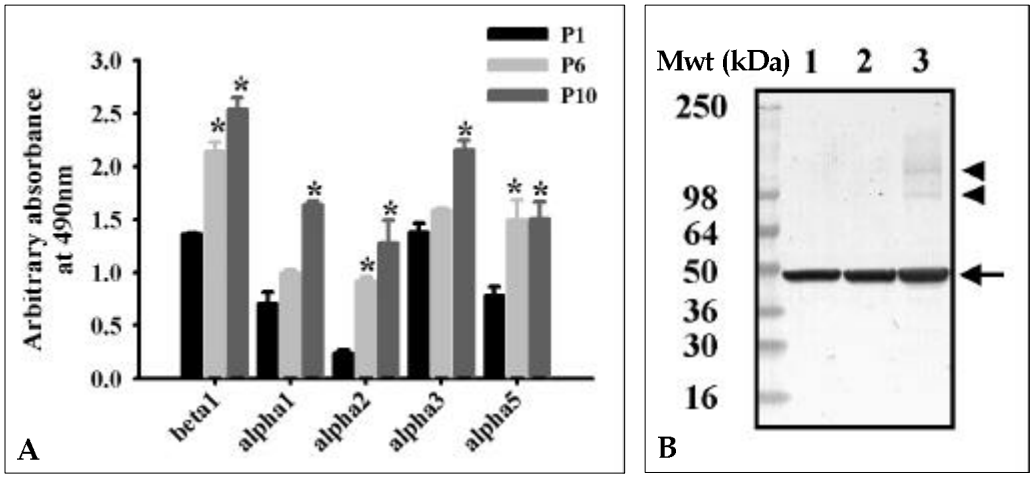

Fig. 6. ELISA analysis for integrins (A) and the modification of α-smooth muscle actin (α-SMA) (B). (A) For ELISA, protein extracts from EP-BLECs, tBLECs, and ttBLECs were immobilized on ELISA plates and detected with primary antibodies of β1-, α1-, α2-, α3- or α5-integrins and a horseradish peroxidase-conjugated anti-mouse secondary antibody. Mean ± S.E. of triplicate determinations are shown and the data represents arbitrary units measured by absorbance at 490 nm. Asterisks indicate values showing statistical significance in comparison with EP-BLECs ($p < 0.05$, analysis of variance). (B) Western analysis for α-SMA revealed no detectable changes (arrow indicates the 44-kDa SMA band). The unknown immunoreactive bands of higher molecular weight in transdifferentiated cells were observed (two upper arrow heads). Lane 1 indicates lysates of EP-BLECs subcultured in 10% FBS-DMEM at passages 1-3. Lane 2 and 3 indicates tBLECs lysate subcultured continuously in 20% FBS-DMEM at passages 6 and 10, respectively.

process in the lens, with regard to the activation or suppression of gene expression, in response to extracellular signals. Apparently, this system of serum modulation fulfilled several major EMT criteria, as described in other systems.

Initially, it was shown that the addition of increasing serum levels (containing 20% FBS) induces morphological changes in cultured bovine LECs, resembling the characteristics of myofibroblasts.

Subsequently, an alteration in ECM synthesis occurred in response to a cell change from an epithelial to fibroblastoid character, which was consistent with both the models of the suspension culture in the 3D collagen gel 13 and rat organ cultures.[9] RT-PCR and dot blot analysis revealed that the synthesis of these ECM components (mainly laminin and type IV collagen) of the normal lens capsules was halted. On the other hand type I collagen and fibronectin, which are related to mesenchymal cells, were instead up-regulated.

In the cultured explant, the α-crystallins disappeared, but α-SMA was strongly expressed. The α-Crystallins are major LEC components and play an important role in transparency as well as in stabilizing several proteins as chaperones.[23-25] The dramatic reduction in α-crystallins during EMT may result in the degradation of other proteins that preserve the transparency and characteristics of the LECs. Furthermore, α-SMA is a prominent marker for myofibroblast cells.[8,26,27] In earlier reports, α-SMA was strongly stained in the LECs of anterior subcapsular cataracts and after-cataracts. After 7 days of an explant culture of bovine LECs, α-SMA was also detected, which is similar to the results of this investigation.[28] However, for α-SMA expression, serum may be unnecessary. A recent study reported that nearly all the lens epithelial cells express α-SMA when explant-cultured on plastic, in a serum-free medium.[29]

Finally, there was also a change in the expression of several integrins, which are the receptors for the ECM proteins, as shown in the model of suspension culture in 3D collagen. The tBLECs showed stronger expression of the β1-, α2- and α5-integrins than EP-BLECs. The integrin α1 and α3 levels were slightly higher in the tBLECs at six passages. Integrins $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha3\beta1$ are known as the receptors for type I and IV collagens, which are expressed by fibroblasts and

epithelia and appear to regulate different functions.[30-32] The up-regulation of the integrins $\alpha 2$ and $\alpha 5$, probably accounts for the close relationship between the alterations of the ECM proteins and integrins.

Differential regulation in the expression of cell adhesion molecules

It appears that the cell surface molecules are altered by continuous serum modulation. This assay was evaluated by the adhesion inhibition of salmosin, a disintegrin, as a potent inhibitor of integrins capable of binding to the RGD.[22,33,34] As the cells underwent EMT, they were increasingly less affected by salmosin, suggesting that they may have increasingly expressed more RGD-independent or less RGD-dependent integrins than normal LECs. It was also confirmed that an increasing FBS concentration is essential to the ineffectiveness of salmosin. This is because at low concentrations it does not cause an alteration in adhesion (Fig. 5B). In addition, ELISA analysis showed that several integrins are differentially regulated by continuous serum modulation. The expression of integrin $\beta 1$ and $\alpha 2$ but not that of integrin $\alpha 5$, was gradually up-regulated. In addition, $\alpha 1$ and $\alpha 3$ integrin levels were slightly increased at passage 6 but dramatically up-regulated at passage 10. Overall, this study suggests that, during terminal transdifferentiation by serum modulation, the expression of the ECM proteins and their receptors, related to myofibroblast, is upregulated gradually and differentially.

Other regulations of EMT-related genes

Two unknown α-SMA bands with high molecular weights, which were not present in the cultured explants and tBLECs at passage 6, appeared in the terminally transdifferentiated cells continuously subcultured in increased serum concentrations. This indicates that α-SMA may be post-translationally modified during EMT, probably by a covalent cross-linking to other α-SMA's or with other actin cross-linking proteins, for example, smooth muscle myosin, calponin, filamin and α-actinin.[35-37]

αB-crystallin gene expression is regulated at the post-transcriptional level due to the αB-crystallin transcript level being unchanged in the explant culture, while that of the protein was dramatically reduced. A similar observation was described in a recent report describing human trabecular meshwork cells being distended and stretched with increases in intraocular pressure. Within one hour, there is a 90% decrease of cellular αB-crystallin levels. However, the αB mRNA levels remain constant.[38] In addition, the regulatory mechanisms of $\beta 1$ integrin expression may also be similar to those of αB-crystallin, due to the transcript level being unchanged (data not shown) while the protein level by ELISA analysis was higher. With the above facts taken together, it can be concluded that terminal transdifferentiation accompanies the changes in cytoskeletal proteins and cell surface molecules. These are modulated in systematic patterns of post-transcriptional regulation, post-translational regulation and a sequential pattern of gene regulation, by synergistic effects of several transforming factors contained in serum.

In addition, it was previously hypothesized that, during EMT in 3D collagen gels, there may be a sequence of gene activation.[1] A single report suggests that the expression of ED-A, a fibronectin isoform, precedes and regulates α-SMA expression by fibroblasts during granulation tissue evolution *in vivo* and after transforming growth factor-$\beta 1$ stimulation *in vitro*.[39] Therefore, it is probable there is a sequential regulation of EMT-related genes by serum modulation.

Recent studies bring into focus the initial responses of, including cross-communication among the growth factor receptors in the apical surface, integrins in the basal surface and cell-cell interaction molecules in the lateral surface, by a variety of pathogens or EMT inducers.[40-42] Transdifferentiation may have an absolute dependence on these initial responses, such as, regulation of putative master genes. Thus, further studies for transdifferentiation will be directed toward investigating the initial regulation of the putative master genes and sequential regulation of the EMT-related genes.

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