Isolation of Putative Corneal Epithelial Stem Cells from Cultured Limbal Tissue

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Purpose: To investigate methods of isolating putative corneal epithelial stem cells from cultured limbal tissue.
Methods: Three extraction techniques were compared to identify an efficient method of obtaining a large number of viable corneal epithelial stem cells from the limbus. Limbal tissues were extracted by incubation at 37℃ or 4℃ for 1 or 16 hours, respectively, with 1.2U/ml dispase/trypsin or by treatment with 0.05% trypsin and 0.01% ethyldiaminetetraacetic acid (EDTA) at 37℃ in single procedure. Collected cells were cultured on NIH/3T3-seeded plates, and colony forming efficiency (CFE) was evaluated. Fluorescence activated cell sorting (FACS) was performed with a Coulter EPICS 753 after incubation with Hoechst 33342 and propidium iodide (PI). Hoechst negative cells were obtained using gates exhibiting low Hoechst blue with a 424/44 nm BP filter. Gated cells of each fraction were re-cultured to assess the capability of colony formation.
Results: The mean numbers of viable cells obtained from treatment with dispase and trypsin was 3×10^4 cell/ml and 8.06×10^5 cell/ml at 37℃and 4℃ incubations; the number increased to 1.21×10^6 cell/ml with a trypsin/EDTA treatment ($p<0.05$). CFE was 9.67±2.13% and 6.63±2.35% in rabbit and human cells, respectively. Likewise, the Hoechst negative fraction was 3.61±0.42% and 5.21±4.91% in rabbit and human cells, respectively. The sorted Hoechst negative cells were cultured through four passages, forming small round colonies. In rabbit cells, the CFEs of Hoechst negative and positive fractions after FACS, were 12.67± 2.24% and 1.17±6.13%, respectively ($p<0.05$).
Conclusions: Putative corneal epithelial stem cells were efficiently isolated from limbal tissue using a trypsin/EDTA extraction and FACS. This technique may be very useful in tissue engineered stem cell therapy. *Korean Journal of Ophthalmology 20(1):55-61, 2006*

Key Words: Corneal epithelial stem cell, EDTA, FACS, Hoechst 33342, Trypsin

Total limbal stem cell deficiency (LSCD) is a visually devastating condition that can result from Stevens-Johnson's syndrome, ocular cicatricial pemphigoid, chemical burns, severe dry eye syndrome, repeated ocular surgeries, or chronic use of contact lens.[1,2] Autologous or allograft limbal cell transplantation has emerged as a successful surgical procedure for total LSCD, and several recent attempts have been made to transplant cultured limbal epithelial cells using various transfer mediums such as contact lens, collagen shields, and amniotic membranes.[3,4] An optimal fraction of stem cells is necessary for successful cultured limbal cell transplantations.[5,6] Thus, the added security gained by transplanting more stem cells could be achieved by optimizing culture conditions for limbal epithelial cells; isolation of stem cells from cultured limbal epithelial cells could result in both more favorable results with cultured limbal cell transplantation and a better understanding of the characteristics and the differentiation process of corneal epithelial stem cells.

Davenger and Evensen suggest that epithelial stem cells might exist in the corneal limbus,[7] and several researchers have corroborated the possibility of their existence through slow-cycling cell proliferation,[8] renewal retardation, improbable rehabilitation of corneal epithelium,[9] or unstained K3.[10] While many researchers have attempted to verify the existence of corneal epithelial stem cells in the limbus, it remains very difficult to isolate and analyze such cells, as

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quality surface markers have not yet been fully established. Meanwhile, fluorescence-activated cell sorting (FACS) based on the ability of early hematopoietic progenitors to efflux Hoeschst 33342 with an efficient efflux pump (ABCG2 transporter) has been used for isolation of hematopoietic stem cells.[11-17] Recently, putative stem cells from solid tissue, including eyes, were reported to also share the Sp phenotype;[18-22] however, the total amount of side-populated cells appears to be too small to further applications of tissue engineering, as the gated percentage is usually less than 1%. Therefore, we adopted a Hoechst negative exclusion method to efficiently isolate putative epithelial stem cells and investigated methods of extracting maximum quantities of viable basal epithelial cells from limbal tissues in order to enhance the isolation efficacy by Hoechst exclusive sorting.

The aim of this study was to compare the efficacy of three different single cell suspension extraction methods, and to investigate the colony forming abilities of isolated putative stem cells obtained from Hoechst negative exclusion sorting method.

Materials and Methods

1. Enzymatic Extraction of Epithelial Cells from the Limbal Tissues in Human and Rabbit

In accordance with the tenets of the Declaration of Helsinki and with proper informed consent, 10 human corneoscleral rims at 5 days post-harvest were obtained from the Northwest Lions Eye bank within 8 hours after penetrating keratoplasty. All the procedures conformed to the principles embodied in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Parallel lamellar dissection along the limbus (2-3 mm width) was done using a Beaver knife in right eyes following administration of muscular injections of ketamine (30 mg/kg) and xylazine (5 mg/kg) and topical anesthetic (0.5% proparacaine HCL) on 10 white rabbits weighing 2.0-3.0 kg; in all, 10 limbal tissues were obtained from rabbits. The tissues were washed 6-8 times with phosphate buffer containing 5% penicillin/streptomycin, and cut into peices (1 mm×1.5 mm×2.5 mm). Each tissue piece was treated with 1.2U/ml of dispase II at 37℃ or 4℃ for 1 h or 16 h, respectively. Subsequently, the collected sheets of epithelium were treated with 0.25% Trypsin/0.01% EDTA (Gibco. USA) at 37℃ for 2 h to generate isolated cells. In another treatment, tissues were treated with 0.05% trypsin and 0.01% ethyldiaminetetraacetic acid (EDTA) at 37℃ with gentle agitation (HB-Movcst 220, Hybaid Limited, U.K) and vortexing (Vortex Genie 2, Scientific industries, U.S.A.); suspended cells were collected 4 times every 20 minutes, and the total exposure time for suspended cell was only 20 minutes. Following collection, single cells were stained with trypan blue and counted to determine the numbers of viable cells obtained by processing. Each extraction method was repeated 5 times, and a statistical analysis was performed on a personal computer using Sigmaster ver 1.0: SPSS software (Chicago, IL, USA). A non parametric Mann-Whitney U test was performed to evaluate the statistical significance of the differences in viable cell numbers obtained by each method, and a value of $p<0.05$ was considered significant.

2. Primary Culture and Colony Forming Assay of Limbal Epithelial Cells

NIH/3T3 (ATCC, USA) were used as a feeder layer, treated with 4ug/ml mitomycin C (Sigma.USA) for 37℃ for 2 hours, and were plated at 80% confluence of an epithelial cell co-culture. Suspended cells were seeded at 1.5×10^4 cells/cm^2, and were primarily cultured with SHEM media (DMEM/F12 2:1, 10% FBS, 10 ng/ml EGF, 5 ug/ml insulin, 0.1 nM choleratoxin, 50 IU/ml penicillin-streptomycin, 0.18 mM adenine, 4 mM glutamine, hydrocortisone 0.4ug/ml, and 2 nM triiodothyronine). For the colony forming assay, primarily cultured cells were plated onto a 3T3 feeder layer at either 100, 300, or 600 cells/well in 6-well plate for 9 to 14 days. Following formation, colonies were fixed with 4% formaldehyde and stained with 1% rhodamin blue to determine colony counts.

$$\text{Colony forming efficiency (CFE, \%)} = \frac{\text{counted colony number}}{\text{initial seeding cell number}} \times 100$$

3. Fluorescence Activated Cell Sorting (FACS) of Corneal Epithelial Stem Cells

For FACS of corneal limbal epithelial stem cells, primarily cultured cells were isolated with a flow cytometer, washed with phosphate buffer, treated with 0.02% EDTA solution to remove the feeder cells, and then with 0.25% Trypsin/EDTA to harvest the cells. Suspended cells (1×10^6 cells/ml) were treated with 5ug/ml bisbenzimide H 33342 (Hoechst 33342:Sigma) at 37℃ for 30 min and re-washed with SHEM media and incubated for a 45-minute efflux period. Non-viable cells were excluded by staining with 5 ug/ml propidium iodide (Sigma) at 37℃ for 1 min. Viable cells were washed twice with SHEM media and suspended in 3 ml medium for flow cytometry (Coulter EPICS 753). Forward orthogonal scattering progress was measured with UV 100 mW (488 nm). Hoechst 33342 and propidium iodide (PI) were stimulated with UV 100 mW (351-364 nm). For Hoechst negative cells, Hoechst fluorescence was measured using a 424/44 nm BP filter, and to sort the Hoechst negative fraction as putative limbal epitheal stem cells (PLESCs), PI was estimated using a 630/22 nm BP filter. The Hoechst negative fraction was gated stepwise following sorting of the PI negative fraction. The forward scatter was proportional to cell size, and side scattering was proportional to intracellular dye granularity of Hoechst Blue. For gating of Sp cells, the

suspended cells (1×10^6 cells/ml) were incubated for a 45-minute efflux period after Hoechst treatment. Verapamil (50 uM, Sigma) was used to evaluate the Sp in which efflux activity was inhibited. To evaluate the Sp, a band pass (BP) filter of 424/44 nm and long pass filter of 675 nm were used in combination with a 640 nm long pass dichroic mirror. PI was read concomitantly using a 630/22 nm BP filter and a 610 nm short pass dichroic mirror. The fractions showing cellular disappearance after treatment with verapamil were identified as a Sp. Forward and side scatter was proportional to the intracellular dye granularity of Hoechst Red and Blue, respectively. Each FACS using Hoechst exclusion was performed in quadruplicate.

The cells in each fraction were seeded onto 12-well plates (Hoechst negative fraction) and 6-well plates (Hoechst positive fraction), with a mitomycin C-treated NIH/3T3 (ATCC) feeder layer, and were primarily cultured with SHEM media. Colony forming efficiencies were evaluated as described above in each fraction sorted by Hoechst exclusion.

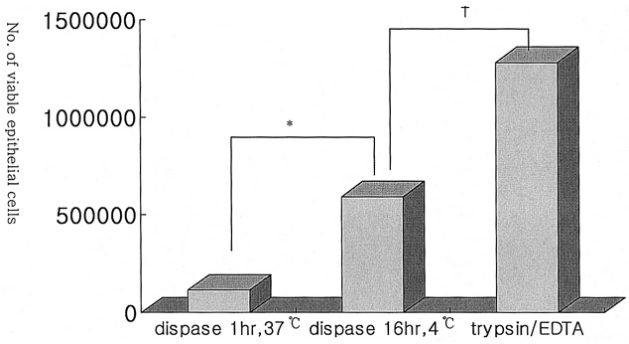

Fig. 1. Comparison of the three extraction methods used to obtain a large population of viable epithelial cells. The number of viable cells obtained every 20 minutes with 0.05% Trypsin/0.01% EDTA at 37℃ ($1.28\pm0.54\times10^6$ cell/ml) was greater than treatment with dispase II at 37℃ for 1 h ($1.15\pm1.20\times10^5$ cell/ml) or at 4℃ for 16 h ($5.93\pm4.0\times10^5$ cell/ml). *: $p<0.028$, †: $p<0.011$, Mann-Whitney U test.

Results

The number of epithelial cells collected after treatment of limbal tissue with dispase II and incubation at 37℃ for 1 h or 4℃ for 16 h was $1.15\pm1.20\times10^5$ cell/ml and $5.93\pm4.0\times10^5$ cell/ml, respectively. The latter method showed better efficacy than the former, with a statistically significant difference ($p<0.028$, Mann-Whitney U test). The number of the cells obtained with a trypsin/EDTA only treatment was $1.28\pm0.54\times10^6$ cell/ml, which was larger than the number isolated with dispase treatment at 4℃ for 16 h; this difference was statistically significant ($p<0.011$, Mann-Whitney U test, Fig. 1). Accordingly, cells were primarily cultured after isolation with a trypsin/EDTA treatment.

The limbal epithelial cells in rabbits or humans were primarily cultured on plates with mitomycin C-treated

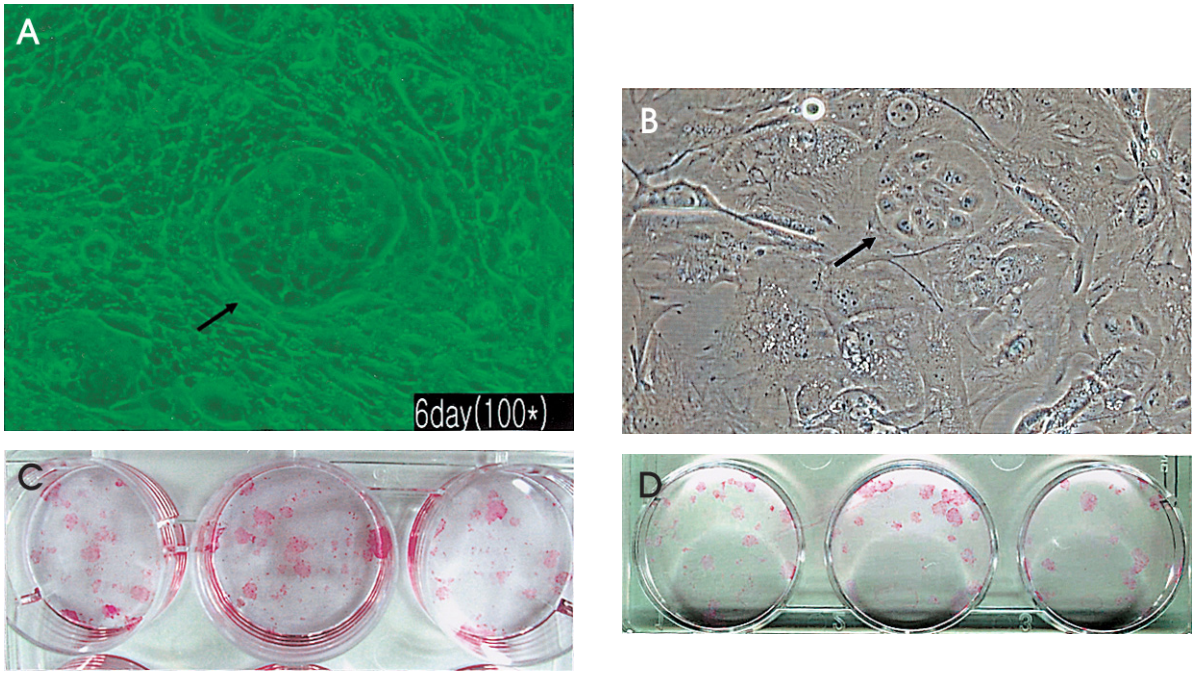

Fig. 2. Colony forming efficiencies (CFEs) of primarily cultured rabbit or human limbal epithelial cells. (A) The primarily cultured cells formed abundant colonies with well maintained round shape, exhibiting the characteristics of less cytoplasm and smaller size consistent with stem cells, as seen on phase contrast inverted microscopy. Colony of rabbit cells at 6 days (×100). (B) Colony of human cells at 6 days (×100). (C) CFEs of rabbit epithelial cells 9 days after seeding 300 cells/well on a 6-well plate was 9.67±2.13%. (D) CFEs of human epithelial cells 9 days after seeding 300 cells/well on a 6-well plate was 6.63±2.35%.

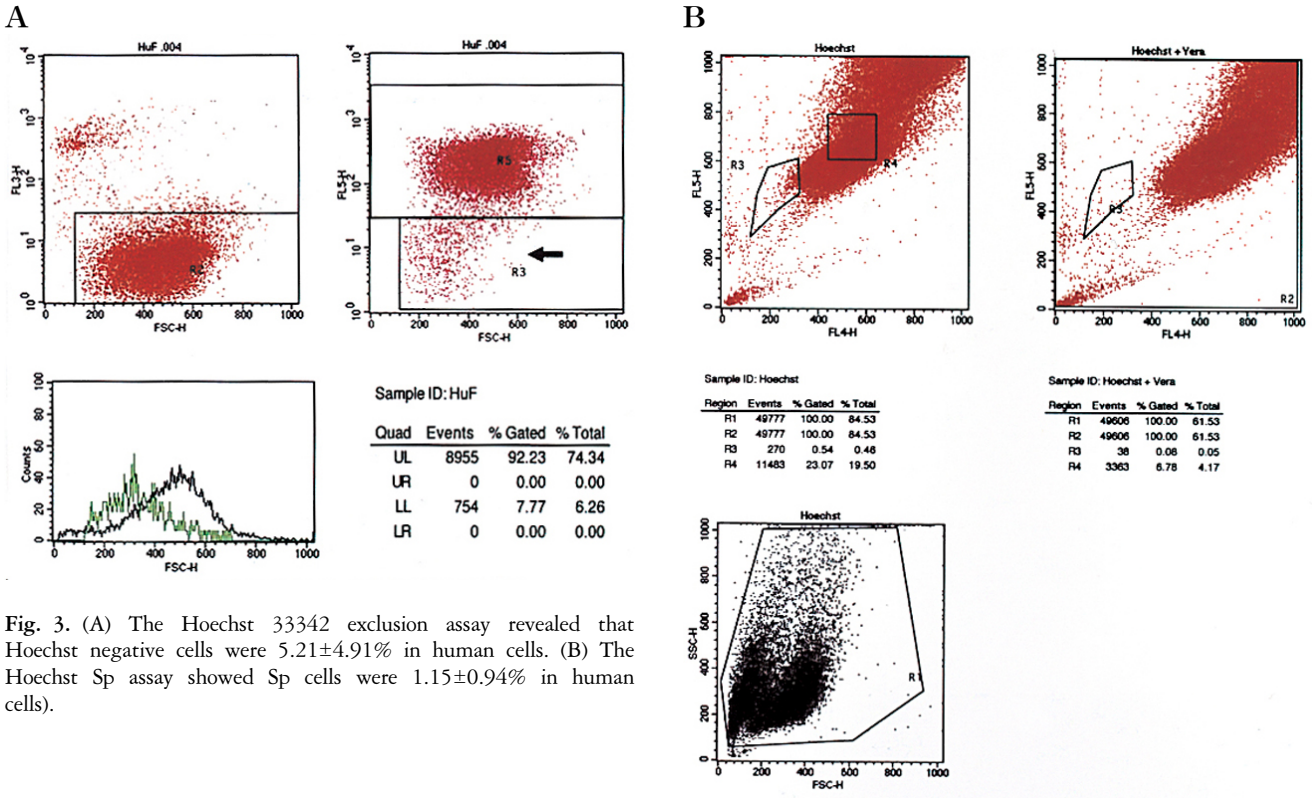

Fig. 3. (A) The Hoechst 33342 exclusion assay revealed that Hoechst negative cells were 5.21±4.91% in human cells. (B) The Hoechst Sp assay showed Sp cells were 1.15±0.94% in human cells).

NIH/3T3 feeder layers. The primarily cultured cells formed abundant colonies with well maintained round shapes, exhibiting the characteristics of decreased cytoplasm and smaller size consistent with stem cells (Fig. 2, A; B). The CFEs in rabbits and humans were 9.67±2.13% (Fig. 2C) and 6.63±2.35% (Fig. 2D), respectively. This CFE was maintained until passage 4 (P4).

The Hoechst 33342 exclusion assay revealed that Hoechst negative cells with low Hoechst blue and red were 3.61± 0.42% and 5.21±4.91% in rabbit and human, respectively (Fig. 3A). According to the Hoechst Sp assay, the mean percentage of Sp cells was 1.15±0.94% in human (Fig. 3B). Each fraction of rabbit cells isolated by Hoechst exclusion was cultured until P4 (nearly 28 days). The Hoechst negative fraction maintained the small and round morphology well, and formed colonies at high CFEs. However, while the Hoechst positive fraction formed colonies, the cells became large in size and were differentiated in colonies with low CFEs (Fig. 4A-F). The CFEs of Hoechst negative and Hoechcst positive fraction after FACS in rabbit were 12.67± 2.24% and 1.17±6.13%, respectively ($p<0.05$, Mann Whitney U test). The Hoechst positive cell fraction grew relatively rapidly and was over 85% of confluence between 6 and 7 days of culture, while the Hoechst negative cell fraction grew very slowly and displayed a similar confluence between about 9 and 12 days of culture.

Discussion

Our results suggest that corneal epithelial stem cells can be efficiently isolated by an optimal extraction method and FACS analysis. In the sampling process of cells from the tissue, it was very important to cause only minimal damage to basal cells, located in the most basal stratum, where epithelial stem cells exist. Following the verification of the usefulness of dispase by Gipson and Grill,[23] many researchers have isolated epithelial cells from the cornea by treating the cells with dispase (1.2 IU/ml) at 37℃ for 1 h.[24-26] However, metabolism enzymes, which are be easily activated at this temperature, may damage the sensitive stem cells. Therefore, we attempted to isolate epithelial cells at 4℃, expecting an inhibition of metabolism enzyme activity. Indeed, we could isolate nearly 5.2 times more limbal epithelial cells from human tissues with this method. Espana et al[27] report that the high viability of human limbal epithelial cells may be achieved at culture condition of 4℃, which is consistent with our results.

To isolate single cells from the sheets obtained using dispase, additional treatment with Trypsin-EDTA was necessary. However, this double procedure by application of dispase and Trypsin-EDTA may have caused more damage to stem cells, even in low temperatures, than would have been caused by a single step procedure. To avoid this possible damage, we simplified the extraction method by using only trypsin-EDTA; this single isolation method was used in dealing with epithelial stem cells.[28] In this method, continuous agitation and intermittent vortexing was used to

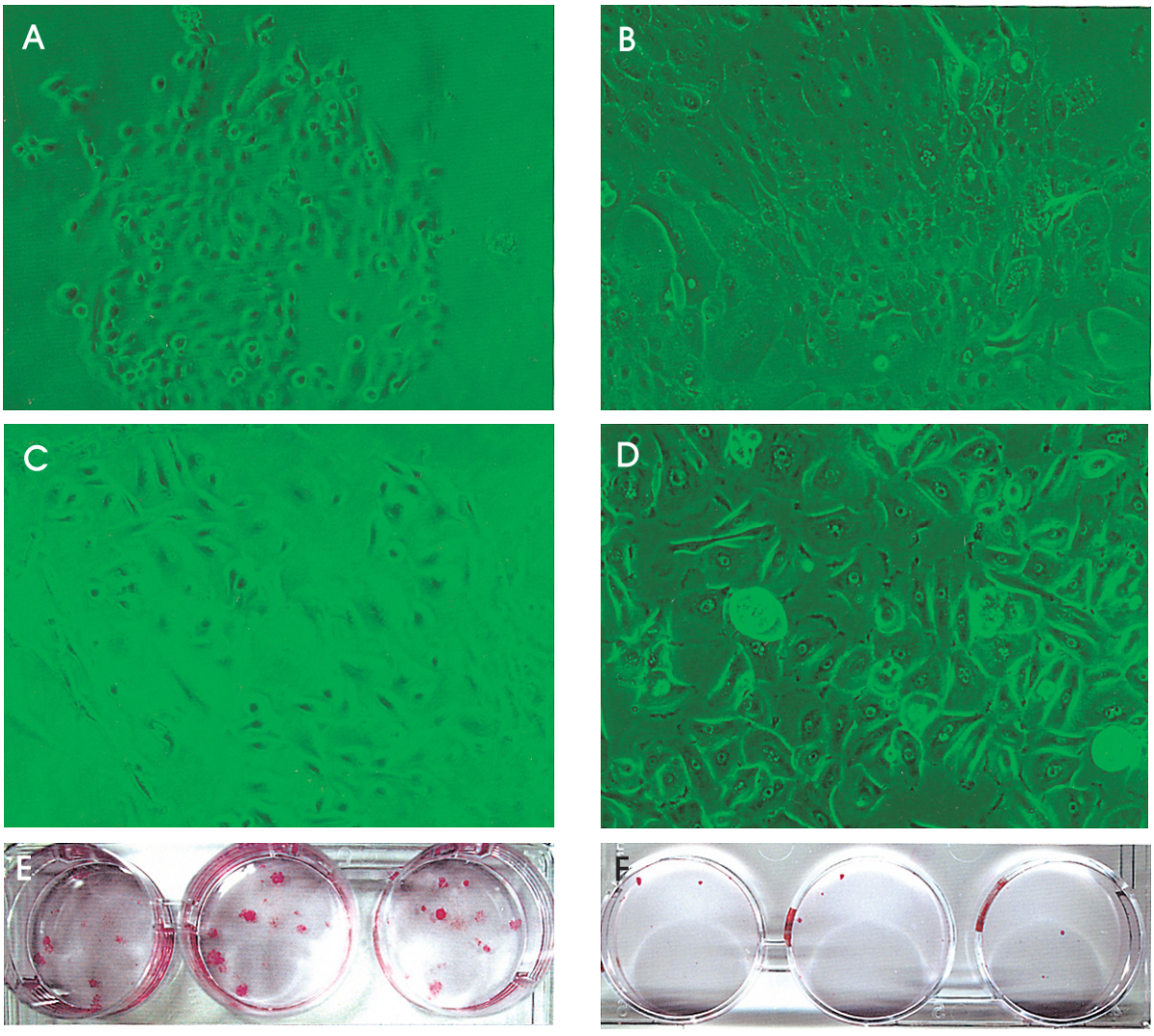

Fig. 4. Cultivation of sorted rabbit cells after Hoechst exclusion. (A) Hoechst negative cells in passage 2 cultured for 6 days. The morphology of the cells showed small and round (×100). (B) Hoechst positive cells in passage 2 cultured for 6 days(x100). The morphology of the cells revealed more hexagonal than those in Hoechst negative fraction. (C) Hoechst negative cells in passage 3 for 6 days-culture (×200). (D) Hoechst positive cells in passage 3 for 6 days-culture (×200). The cells were getting large and differentiated. The CFEs of Hoechst negative and positive fraction in 2-passage of rabbit's cells were 12.67±2.24% (E) and 1.17±6.13% (F) 9 days after seeding 100 cells/well on a 6-well plate, respectively.

isolate single cells, and suspended cells were collected at every 20 minutes to avoid further exposure to trypsin-EDTA. The yield was 2.2 times higher with a single procedure using trypsin-EDTA at 37℃ when compared to the double procedure using dispase at 4℃, 16hours and trypsin-EDTA at 37℃.

FACS with Hoechst 33342 has been attempted to separate the stem cells of neural[29] and dermal[20] tissues. These stem cells have been reported to hold an active pump[14] (i.e. ABCG2 transporter) to rapidly release toxic substances in order to maintain stem-cell properties. The ABCG2 transporter, which effluxes Hoechst dye, is preferentially expressed in stem cells, and sharply down-regulated after commitment to lineage-specific development.[14] Actually, Sp cells or stem cells isolated from the epidermal skin cells of a rat with FACS using Hoechst 33342 is 3.4%, containing over 95% of label-retaining cells, which allows for positive identification of Sp cells as stem cells.[20]

Recently, Sp fractions were also found in corneal epithelial cells,[21,22,30] however, the gated yield was usually reported as less than 1%; this finding is consistent with our results. Therefore, we adopted Hoechst exclusion based only on ability of stem cells to exclude Hoechst, and investigated CFE in gated cells after Hoechst exclusion to validate proliferation and maintenance capacities of the stem cells. In this study, the CFEs of Hoechst negative and positive fraction after FACS in rabbit were 12.67±2.24% and 1.17±6.13%, respectively ($p<0.05$). These results suggest that the method

can successfully sort cells with higher colony forming abilities. CFE was 9.67±2.13 % in rabbit before FACS. Likewise, Hoechst exclusion efficiently gated putative epithelial stem cells for further use in tissue engineering applications. Hoechst negative fractions also developed slow growing small and round shaped colonies in subcultures, suggesting that these cells may also have stem cell properties. However, the Hoechst positive cells formed rapidly growing colonies with a much higher level of differentiation. Likewise, the sizes of colonies were smaller in sorted cells than those in primary cultured cells, suggesting the possibility of some kind of damage from either UV exposure, long journey time in suspension, and possible Hoechst toxicity caused by the sorting procedure. In fact, Machalinski B et al report that stem cells damage might possibly be induced by the toxicity of Hoechst 33342 itself[24] or by the UV detector.[31] Another problem regarding evaluation of CFEs after FACS is a result of the small number of cells in Hoechst negative fractions. Specifically, we could not evaluate CFEs in Sp cells because of low cell quantities However, as far as our methods were concerned with the feasibility of Hoechst exclusion, the data is worthy of notice despite the fact that we could not directly compare the CFEs of the cells sorted by Hoechst exclusion with those of Sp cells. In fact, our CFEs in rabbit cells sorted by Hoechst exclusion were higher compared with those in Sp cells of rabbits reported by Budak et al. (2.9±1.2%)[30]

Our results also provide evidence that putative stem cells have a higher colony forming efficiencies in gated cells with Hoechst exclusion. Further investigation to confirm the identities of the stem cells isolated with Hoechst exclusion are currently underway in our laboratory.

In conclusion, the efficacy of isolation was highest with a single extraction method using trypsin/EDTA to obtain a single cell suspension of limbal epithelial cells. Hoeschst-excluding cells showed high CFEs maintaining stem cell-like morphologies, suggesting that the single method for the isolation of putative stem cells is feasible. This technique may be usefully applied in tissue engineered stem cell therapy. Additionally, our results may also assist future studies that examine the characteristics of corneal epithelial stem cells and may be critical to the development of successful corneal epithelial stem cell transplantation methods.

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