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Partial enrichment of a population of human limbal epithelial cells with putative stem cell properties based on collagen type IV adhesiveness[☆]

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Abstract

The concept that corneal epithelium stem cells reside in limbus has been recognized for more than a decade, but isolation of these stem cells has not been accomplished. This study was an initial attempt to isolate a population of human limbal epithelial cells enriched for certain putative stem cell properties based on their phenotype. Epithelial cells harvested from fresh human limbal rings and their primary cultures were allowed to adhere to collagen IV-coated dishes for 20 min and 2 hr, sequentially. The rapidly adherent cells (RAC), slowly adherent cells and non-adherent cells were evaluated for certain stem cell properties: (a) BrdU-label retention, (b) expression of basal cell (integrin $\beta 1$, p63, ABCG2) and differentiation (involucrin, keratin 12) markers, and (c) colony forming efficiency (CFE) and growth capacity on a 3T3 fibroblast feeder layer. Among unfractionated cells and the three selected populations, the RAC, accounting for about 10% of whole population, were enriched 5-fold in BrdU label-retaining cells, displayed the highest number of integrin $\beta 1$ and p63 positive and involucrin negative cells, expressed high levels of $\Delta Np63$ and ABCG2 mRNA, and lacked involucrin and K12 expression, and possessed the greatest CFE and growth capacity. These findings demonstrated for the first time that human limbal epithelial cells with stem cell properties can be partially enriched by their adhesiveness to collagen IV. The RAC population enriched for certain putative stem cell properties may prove useful in the future for transplantation to diseased and damaged corneas with limbal stem cell deficiency.

Keywords

cornea; limbus; epithelium; stem cells; integrin; adhesion; BrdU

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1. Introduction

Ocular surface diseases with corneal epithelial stem cell dysfunction, commonly known as limbal stem cell deficiency, are sight threatening and often cause blindness. There was no effective therapy for these conditions until the potential of limbal transplantation for ocular surface reconstruction was realized in the past decade. Several preliminary reports indicate that limbal epithelial cells obtained from fellow eyes of patients with limbal deficiency and cultivated on human amniotic membrane can be successfully transplanted to reconstruct damaged corneal surfaces (Koizumi et al., 2001; Pellegrini et al., 1997; Schwab et al., 2000; Tsai et al., 2000). The success of limbal transplantation has been attributed to the healing potential of the limbal stem cells that are contained within the mixture of cells transplanted in these grafts. The ability to isolate a pure population of limbal epithelial stem cells from small limbal biopsies, expand them in culture and use them for regenerating a corneal surface of normal phenotype and regenerative capacity would represent a major advance in this field.

The limbal basal epithelium contains three functionally different cell types: stem cells, transient amplifying cells, and terminally differentiated cells (Cotsarelis et al., 1989; Lehrer et al., 1998; Schermer et al., 1986). The stem cells are only a small subpopulation of these basal cells. Although the concept that corneal epithelial stem cells reside in the limbus has been recognized for more than a decade (see review articles by Dua and Azuara-Blanco, 2000; Lavker and Sun, 2000; Tseng, 1989), isolation of these stem cells has never been accomplished. This may be due to the lack of a unique marker for these stem cells, although a variety of stem cell markers have been proposed in the past decade. We have evaluated most currently proposed molecular markers related to stem cell properties to characterize a putative stem cell phenotype in human limbal epithelia. This work demonstrated that the basal cells of limbal epithelia are small primitive cells, they are p63, ABCG2 and integrin $\alpha 9$ positive, they show relatively higher expression of integrin $\beta 1$, EGFR, K19 and α -enolase, and they lack expression of nestin, E-cadherin, connexin 43, involucrin, K3 and K12 (Chen et al., 2004).

We hypothesize that this putative stem cell phenotype, especially the cell surface markers, could be used to partially enrich for a population containing putative limbal epithelial stem cells, while we recognize there is no single marker that can identify these adult stem cells to date. Integrin $\beta 1$ has been accepted as a basal cell marker associated with certain stem cell properties, and it has been used as a cell surface marker for the enrichment of putative epidermal keratinocyte stem cells (Bickenbach and Chism, 1998; Jones et al., 1995; Jones and Watt, 1993; van Rossum et al., 2002) and human prostate epithelial stem cells (Collins et al., 2001). Human skin epidermal stem cells have been enriched based on their adhesive properties to collagen IV or other extracellular matrix (Jones and Watt, 1993). For murine epidermal stem cells, approximately 10% of total basal cells and 100% of label-retaining cells could be enriched in a rapidly adherent population (Bickenbach and Chism, 1998). As an initial attempt, we adopted a previously reported method for isolating epidermal stem cells (Jones and Watt, 1993; Levy et al., 2000) to test our hypothesis that a population containing putative human limbal epithelial stem cells could be partially enriched by adhesiveness to collagen type IV.

2. Material and methods

2.1. Materials and reagents

Cell culture dishes, plates, centrifuge tubes and other plastic ware were purchased from Becton Dickinson (Lincoln Park, NJ). Nunc Lab-Tek II eight-chamber slides were from VWR International (West Chester, PA). Human type IV collagen was from BD Discovery

Labware (Bedford, MA). Dulbecco modified Eagle medium (DMEM), Ham F-12, amphotericin B, gentamicin and 0.25% trypsin/0.03% EDTA solution were from Invitrogen-GIBCO BRL (Grand Island, NY). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Dispase II and 5-bromo-2-deoxyuridine (BrdU) were from Roche Molecular Biochemicals (Indianapolis, IN). Mouse NIH 3T3 fibroblasts (ATCC CCL 92) were from American Type Culture Collection (ATCC, Rockville, MD). Mouse monoclonal antibodies (mAb) against integrin $\beta 1$, p63 and involucrin came from Lab Vision (Fremont, CA). Rabbit anti-BrdU polyclonal antibody was from Megabase Research Product (Lincoln, NE). Fluorescein Alexa Fluor 488 conjugated goat anti-mouse or anti-rabbit IgG second antibodies were from Molecular Probes (Eugene, OR). GeneAmp RNA-PCR kit was from Applied Biosystems (Foster City, CA). Heat-denatured bovine serum albumin (BSA), Mitomycin C (MMC), bovine insulin, human transferrin, sodium selenite, hydrocortisone, human EGF, cholera toxin A subunit, dimethyl sulfoxide (DMSO), Hoechst 33342, guanidium thiocyanate, DNA size marker and other reagents came from Sigma (St Louis, MO).

2.2. Corneal limbal tissues and limbal epithelial cell isolation

Fresh human corneoscleral tissues (less than 72 hr postmortem) that were not suitable for clinical use, from donors aged 19–67 years, were obtained from the Lions Eye Bank of Texas (LEBT, Houston, TX) and from the National Disease Research Interchange (NDRI, Philadelphia, PA). They were cut through the horizontal meridian, frozen and sectioned for immunostaining, whole mounted for laser scanning confocal microscopy, or prepared for explant culture.

Limbal epithelial cells, which were used for putative stem cell enrichment, were isolated from fresh limbal tissues by a previously described method (Kim et al., 2004; Tseng et al., 1996) with modification. In brief, corneoscleral tissues were rinsed with Hank's balanced solution containing $50 \mu\text{g mL}^{-1}$ gentamicin and $1.25 \mu\text{g mL}^{-1}$ amphotericin B. After carefully removing the central cornea, excess sclera, iris, corneal endothelium, conjunctiva and Tenon's capsule, the remaining limbal rims were incubated with dispase II (5 mg mL^{-1}) at 37°C for 1 hr. The limbal epithelial sheets were collected and treated with 0.25% trypsin/0.03% EDTA at 37°C for 5–10 min to isolate single cells. The isolated limbal epithelial single cells were then used for adhesion experiments.

2.3. Primary explant cultures of human limbal epithelial cells

Limbal epithelial cultures were established from limbal explants using a previously described method (Kim et al., 2004; Li et al., 2001). In brief, each limbal rim was cut into 12 equal pieces (about $2 \times 2 \text{ mm}$ size each). Two pieces with their epithelium side up were directly placed into a well of six well culture plates, and they were covered with a drop of FBS overnight. The explants were then cultured in SHEM medium, consisting of a 1:1 mixture of DMEM and Ham's F12 medium containing 5 ng mL^{-1} EGF, $5 \mu\text{g mL}^{-1}$ insulin, $5 \mu\text{g mL}^{-1}$ transferrin, 5 ng mL^{-1} sodium selenite, $0.5 \mu\text{g mL}^{-1}$ hydrocortisone, 30 ng mL^{-1} cholera toxin A, 0.5% DMSO, $50 \mu\text{g mL}^{-1}$ gentamicin, $1.25 \mu\text{g mL}^{-1}$ amphotericin B and 5% FBS, at 37°C under 5% CO_2 and 95% humidity. The medium was renewed every 2–3 days. The sub-confluent primary cultures on days 14–18 were trypsinized to prepare single cell suspensions for putative stem cell enrichment.

2.4. Adhesion of limbal epithelial cells to collagen IV

Human limbal epithelial cells isolated from fresh limbal tissues or their primary cultures were used for putative stem cell enrichment using a method reported for epidermal stem cell isolation (Jones et al., 1995; Jones and Watt, 1993) with modifications. In brief, 100 mm culture dishes were coated with human collagen IV ($20 \mu\text{g mL}^{-1}$) for 2 hr at room

temperature (RT), and then incubated with 0.5 mg mL^{-1} heat-denatured BSA in PBS at 37°C for 1 hr and rinsed with SHEM medium before use. To evaluate adhesion properties, the single-cell suspension of human limbal epithelial cells in SHEM were allowed to attach to the collagen IV coated dishes in an incubator at 37°C for different lengths of time (10, 20, 60, 120 min, and overnight). For enrichment of putative stem cells, the cells were allowed to attach to a collagen IV coated dish at 37°C for 20 min. The unattached cells within the first 20 min were then transferred to another collagen IV coated dish and allowed to adhere for an additional 100 min. The remaining unattached cells after 2 hr were collected as non-adherent cells. Unfractionated cells that were not subjected to adhesion separation were used as a control. The phenotype and properties of each group of cells were evaluated based on adult stem cell criteria. The cells were seeded on a 3T3 feeder layer to assess their growth potential. Approximately 5×10^4 cells per chamber from each group were seeded into wells of 8-chamber culture slides, incubated at 37°C overnight, and then fixed for immunofluorescent staining. The selected cell populations were also lysed in 4 M guanidium thiocyanate solution for total RNA extraction to evaluate gene expression of stem cell associated markers.

2.5. Enrichment for BrdU label-retaining cells

To evaluate the correlation between adhesion-selected populations and cell cycle, cells were labelled with BrdU and analysed by a previously reported method (Kim et al., 2004) with modification. In brief, explant cultures in 35-mm dishes that showed cellular outgrowth to 2–3 mm in diameter at day 3–5, were incubated with fresh SHEM medium containing $10 \mu\text{M}$ BrdU. After labelling with BrdU for 72 hr continually, the cultures were switched to BrdU free medium, chased for 18 days, and then used for adhesion experiment as described above. All selected populations were plated in duplicate at 5×10^4 cells per well on 8-chamber slides, fixed in cold methanol at 4°C for 10 min, and processed for immunofluorescent staining with a BrdU antibody. The BrdU labelling index was assessed by point counting through a Nikon TE200 inverted microscope using a $40\times$ objective lens and a $10\times$ subjective lens. A total of 500–951 nuclei were counted in 6–8 representative fields. This number (500 counted nuclei) was considered as a minimum requirement to obtain representative data (Goodson et al., 1998). The labelling index was expressed as the number of positively labelled nuclei/the total number of nuclei $\times 100\%$.

2.6. Immunofluorescent staining

Immunofluorescent staining was performed as a previously reported method (Chen et al., 2004; Li and Tseng, 1995; Liu et al., 2001). In brief, corneal and limbal frozen sections, primary limbal epithelial cultures or selected cell populations on 8-chamber slides were fixed with cold methanol (for cytoplasmic and nuclear protein staining) or cold 2% paraformaldehyde (for cell membrane protein staining) at 4°C for 10 min. After blocking with 5% normal goat serum in PBS for 30 min, primary monoclonal antibodies against integrin $\beta 1$ (1:200), nuclear p63 (clone 4A4, 1:1000, $1 \mu\text{g mL}^{-1}$, or involucrin (1:40, $5 \mu\text{g mL}^{-1}$) were applied and incubated for 1 hr at RT. Secondary antibodies, Alexa Fluor 488 conjugated goat anti-mouse IgG (1:300), was then applied and incubated in a dark chamber for 1 hr, followed by counterstaining with Hoechst 33342 DNA binding dye ($1 \mu\text{g mL}^{-1}$ in PBS) for 5 min. After washing with PBS, Antifade Gel/Mount (Fisher, Atlanta, GA) and a cover slip were applied. Sections were examined and photographed with an epifluorescent microscope (Eclipse 400, Nikon, Japan) with a digital camera (model DMX 1200, Nikon). The percentages of positively stained cells were calculated by a point counting method similar to BrdU labelling index.

2.7. Laser scanning confocal microscopy

For laser scanning confocal microscopy, whole fresh human corneas were fixed in fresh 2% paraformaldehyde in PBS for 10 min at RT. After fixation, they were rinsed extensively with PBS containing 0.1% Tween 20 (TPBS). The corneas were bisected, and blocked with 20% goat serum in TPBS from 2 hr or overnight to reduce nonspecific staining. The tissues were then incubated with integrin $\beta 1$ mAb (1:50) in TPBS containing 20% goat serum at 4°C overnight. Tissues without primary antibody were used as negative controls. After extensive washing with TPBS, Alexa Fluor-488 conjugated goat anti-mouse antibody (1:300) was applied for 1 hr. Tissues were rinsed and counterstained with a DNA binding dye propidium iodide ($1 \mu\text{g mL}^{-1}$ in PBS) for 5 min. After washing with PBS, corneal tissues were flattened on slides and mounted with antifade Gel/Mount (Fisher, Atlanta, GA) and cover slips. The samples were observed with a laser scanning confocal microscope (LSM 510, Zeiss with Krypton–argon and He–Ne laser, Thornwood, NY) with 488 and 543 nm excitation and emission filters, LP505 and LP560, respectively. Images were acquired with 40 $\times$ oil-immersion objectives and processed using Zeiss LSM-PC software and Adobe Photoshop 6.0.

2.8. Total RNA extraction and semi-quantitative RT-PCR

Total RNA was isolated from selected populations of human limbal epithelial cells using acid guanidium thiocyanate–phenol–chloroform extraction (Li and Tseng, 1995). The RNA was quantified by its absorption at 260 nm and stored at -80°C before use. Using a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control, the mRNA expression of different molecular markers was analysed by semi-quantitative RT-PCR (Li et al., 2001; Li and Tseng, 1995). Briefly, first-strand cDNAs were synthesized from 0.5 μg of total RNA with MuLV reverse transcriptase. PCR amplification was performed with specific primer pairs designed from published human gene sequences (Table 1) for different markers using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). Semi-quantitative RT-PCR was established by terminating reactions at intervals of 20, 24, 28, 32, 36 and 40 cycles for each primer pair to ensure that the PCR products formed were within the linear portion of the amplification curve. The fidelity of the RT-PCR products was verified by comparing their size to the expected cDNA bands and by sequencing the PCR products.

2.9. Colony forming efficiency and growth capacity

To evaluate proliferative potential of the cell populations selected by adhesion to collagen IV, a MMC treated 3T3 fibroblast feeder layer was used by a previously reported method (Kim et al., 2004; Rheinwald and Green, 1975; Tseng et al., 1996). The mouse NIH 3T3 fibroblasts, grown in DMEM containing 10% FBS to confluence, were treated with MMC (4 mg mL^{-1}) for 2 hr and then trypsinized and plated at a density of $2 \times 10^4 \text{ cells cm}^{-2}$ in 6-well plates. Each selected cell population was seeded, at least in triplicate, at $1 \times 10^3 \text{ cells cm}^{-2}$ into 6-well culture plates containing a 3T3 fibroblast feeder layer. The colony forming efficiency (CFE) was calculated as a percentage of the number of colonies at day 6 generated by the number of epithelial cells plated in a well. The growth capacity was evaluated on day 14 when cultured cells were stained with 1% rhodamine.

3. Results

3.1. Integrin $\beta 1$ expression in human corneal limbus and primary limbal explant cultures

Immunofluorescent staining of human corneal sections showed that integrin $\beta 1$ staining on the cell membranes was brighter in the basal layers than in the superficial layers of both corneal and limbal epithelia, and the highest levels of expression were observed in the basal

limbal epithelial cells (Fig. 1A and B). Laser scanning confocal microscopy using whole mounted corneas confirmed that the integrin $\beta 1$ expression was localized to the membranes of cells located in most layers of the corneal and limbal epithelia, with the brightest staining observed in the basal layer of the limbal epithelia (Fig. 1C and D). In primary human limbal epithelial cultures, the integrin $\beta 1$ antibody brightly stained the membranes of small cells, whereas weak or no staining was observed in the larger cells (Fig. 1E and F). Integrin $\beta 1$ positive cells accounted for $26.0 \pm 7.6\%$ [mean $\pm$ standard deviation ($s.d.$), $n=4$] of cells in primary limbal explant cultures.

3.2. Adhesion properties of limbal epithelial cells

The adhesion property of limbal epithelial cells was evaluated by incubating cells on collagen IV coated dishes for different lengths of time from 10, 20, 60 and 120 min to overnight. As shown in Fig. 2, summarized from six separate adhesion experiments, about 5 and 10% of epithelial cells harvested from fresh limbal tissues rapidly adhered to collagen IV coated dishes at 10 and 20 min, respectively, and the adherent cell population increased to 23 and 27% at 60 and 120 min, respectively, reaching 48% after an overnight incubation. The adherent property of primary cultured limbal epithelial cells was similar to that observed with freshly harvested limbal epithelial cells.

To evaluate their stem cell properties, limbal epithelial cells were separated into three populations based on their adhesion to collagen IV: (1) rapidly adherent cells (RAC), the cells adherent within the first 20 min, (2) slowly adherent cells (SAC), the cells adherent from 20 min to 2 hr, and (3) non-adherent cells (NAC) after 2 hr. The RAC accounted for $10.4 \pm 1.5\%$ (mean $\pm s.d.$, $n=6$) and the SAC accounted for $17.3 \pm 7.9\%$ of the whole population of limbal epithelial cells harvested from fresh limbal tissues. The percentages of RAC and SAC selected from primary cultured corneal epithelial cells accounted for $8.9 \pm 1.8\%$ and $15.8 \pm 6.8\%$ (mean $\pm s.d.$, $n=6$), respectively, slightly, but not significantly, lower than that from freshly harvested limbal epithelial cells.

3.3. Rapid adherent cells are a population enriched for BrdU label-retaining cells

The label-retaining cells (LRCs) have been widely accepted as a characteristic of epithelial stem cells. These LRCs showed putative stem cell properties, including a slow cell cycle, small size, immaturity with few organelles, and clonogenic behavior in vitro (Bickenbach, 1981; Lavker and Sun, 1982). The label retention method has then been used for 23 years to localize stem cell populations in a variety of tissues including limbal epithelia (Cotsarelis et al., 1989). We have identified about 2.3–3.73% of BrdU LRCs in human limbal epithelial cultures after chasing for 21 days (Kim et al., 2004). In the current study, about 3.15% of BrdU LRCs were detected in the limbal epithelial cultures after 72 hr labelling and chasing for 18 days. Interestingly, as shown in Fig. 3, the RAC population contained the highest number of LRCs ($16.02 \pm 2.45\%$, $P < 0.01$, $n=3$) that was enriched 5-fold from the unfractionated whole cell population ($3.15 \pm 0.55\%$). The SAC had a slightly higher number ($4.78 \pm 1.61\%$) of LRCs than all cells, but the NAC showed almost no LRCs ($0.53 \pm 0.74\%$).

3.4. Rapid adherent cells are integrin $\beta 1$ and p63 brighter and involucrin negative

To evaluate the phenotype of selected cell populations in comparison to the unfractionated whole populations, each cell population from primary cultures was seeded in 8-chamber culture slides, incubated overnight, and then fixed for immunofluorescent staining with antibodies against stem cell associated markers, integrin $\beta 1$ and nuclear transcription factor p63 (Pellegrini et al., 2001), and the differentiation marker, involucrin. As shown in Fig. 4, with Hoechst 33342 counterstaining and the unfractionated whole populations (ALL cells) as a control, the RAC population showed the highest percentage of integrin $\beta 1$ protein positive cells ($57.1 \pm 9.8\%$), significantly higher than $30.8 \pm 3.2\%$ positive cells ($P < 0.05$, $n=3$)

in the SAC group and $9.7 \pm 2.0\%$ ($P < 0.01$, $n=3$) in the NAC population. The integrin $\beta 1$ positive cells in the RAC group were enriched 2.1-fold from $26.6 \pm 3.1\%$ positive cells in ALL cells, and were 5.9 fold greater than the NAC population. The RAC population also possessed the highest percentage of p63 protein positive cells ($47.5 \pm 8.6\%$), compared with $18.8 \pm 7.1\%$ positive cells ($P < 0.05$, $n=3$) in the SAC group and $6.3 \pm 3.5\%$ ($P < 0.01$, $n=3$) in the NAC population. The p63 positive cells in the RAC group were enriched about 3.0-fold from $15.6 \pm 3.4\%$ positive cells in the ALL cells, and were 7.5-fold higher than in the NAC population. In contrast, the expression of involucrin, a differentiation marker, was negligible in the RAC population with only $1.8 \pm 0.6\%$ positive cells, significantly lower than $30.4 \pm 5.5\%$ positive cells ($P < 0.001$, $n=3$) in the SAC group and $68.6 \pm 6.1\%$ ($P < 0.001$, $n=3$) in the NAC population. The involucrin positive cells in the RAC population decreased about 23-fold from $41.8 \pm 5.6\%$ positive cells in ALL cells, and they were about 38-fold lower than in the NAC population.

3.5. Rapidly adherent cells express the highest levels of integrin $\beta 1$, p63 and ABCG2 mRNA and lowest levels of involucrin and keratin 12 mRNA

Semi-quantitative RT-PCR disclosed a differential expression pattern of stem cell associated and differentiation markers in these selected populations of limbal epithelial cells (Fig. 5). Integrin $\beta 1$ mRNA was expressed at the highest level by the RAC group although the unfractionated ALL cells and the other populations selected by adhesion to collagen IV expressed integrin $\beta 1$ abundantly (Fig. 5A). The levels of integrin $\beta 1$ expression were decreased in SAC group, and the lowest levels were seen in the NAC population. ABCG2 mRNA, a newly proposed stem cell marker (Cooray et al., 2002; Scharenberg et al., 2002; Zhou et al., 2001) was expressed at the highest level by the RAC group, while ALL cells and SAC population expressed much lower levels of ABCG2 mRNA, with undetectable expression by the NAC population (Fig. 5B). A similar expression pattern of nuclear p63 was observed in these populations. $\Delta Np63$, a truncated dominant-negative isoform of p63, is the predominant species in epithelial stem cells possessing the highest proliferative capacity (Parsa et al., 1999; Potten and Booth, 2002; Yang et al., 1999). The RAC population expressed the highest levels of $\Delta Np63$ mRNA, while the SAC and ALL cells expressed much lower levels of $\Delta Np63$ mRNA, with barely detectable $\Delta Np63$ expression by the NAC population (Fig. 5C). In contrast, the mRNAs encoding differentiation markers, keratin 12 (Fig. 5D) and involucrin (Fig. 5E) were not detected in the RAC group. They were expressed at higher levels by the SAC and at the highest levels by the NAC population. No difference in the mRNA levels of GAPDH (Fig. 5F), a housekeeping gene used as an internal control, was observed among all four populations.

3.6. Rapid adherent cells possess the greatest proliferative potential

To evaluate their growth capacity, the cells of each population selected by adhesion to collagen IV were seeded in triplicate at a density of 1×10^3 cells cm^{-2} into 6-well culture plates containing a 3T3 fibroblast feeder layer. Fig. 6A summarizes the colony forming efficiency (CFE) from four separate adhesion experiments of limbal epithelial cells. The CFE on day 6 was highest in the RAC group ($3.77 \pm 0.42\%$), significant higher than that of the ALL cells ($2.01 \pm 0.51\%$, $n=4$, $P < 0.01$), and the SAC populations ($2.45 \pm 0.61\%$, $n=4$, $P < 0.05$). The lowest CFE was seen in the NAC population ($0.91 \pm 0.32\%$, $n=4$, $P < 0.001$). The CFE in the RAC group was enriched about 1.9-fold over ALL cells, and was about 4.2-fold higher than the NAC population. Although the clonogenic ability was lower in the populations selected from the primary cultures, the pattern of CFE in their selected populations was similar to that observed with limbal epithelial cells. The RAC populations exhibit the greatest CFE at $2.10 \pm 0.40\%$ among the four cell populations, significantly higher than ALL cells ($1.02 \pm 0.16\%$, $n=4$, $P < 0.01$), the SAC ($1.30 \pm 0.25\%$, $n=4$, $P < 0.005$) and NAC populations ($0.29 \pm 0.08\%$, $n=4$, $P < 0.001$). The CFE in RAC was enriched about 2.1-fold

over ALL cells, and was 7.2-fold higher than NAC. The RAC population could grow further to become confluent in 10–14 days, while the ALL and SAC groups were slowly growing and reached confluence in 18–24 days. The NAC generated colonies failed to grow further and aborted (Fig. 6B).

4. Discussion

Adult stem cells have been observed to have a number of characteristics in morphology, phenotype and growth potential. These include: (1) slow cycling or long cell cycle time during homeostasis in vivo; (2) small size with poor differentiation and primitive cytoplasm; (3) high proliferative potential after wounding or placement in culture; and (4) the ability for self-renewal and functional tissue regeneration (see review articles by Blau et al., 2001; Cotsarelis et al., 1999; Lavker and Sun, 2000; Watt, 2000; Watt and Hogan, 2000). The cell population selected by rapid adhesion to collagen IV possesses several of these properties, including enriched slow-cycling cells identified by BrdU LRCs, less expression of differentiation markers with higher levels of molecular markers that have been identified in the basal limbal epithelium, and high proliferative capacity in culture. These preliminary results provide encouragement that it is possible to isolate a population partially enriched for putative limbal epithelial stem cell properties based on their phenotype, despite the fact that there is no single marker that can identify these stem cells today.

Adhesion to collagen type I or IV, fibronectin, laminin or other extracellular matrix has been used for isolation of putative keratinocyte stem cells (Bickenbach and Chism, 1998; Collins et al., 2001; Jones and Watt, 1993; Wan et al., 2003). Jones and Watt have successfully separated human epidermal stem cells from transit amplifying cells based on whether they were rapidly (within 20 min) or slowly adherent to the integrin $\beta 1$ ligand, type IV collagen. The rapidly adhering cells were found to have the highest proliferative potential and the lowest involucrin staining, whereas cells that adhered slowly divided only a few times before all of their progeny underwent terminal differentiation (Jones, 1996; Jones and Watt, 1993). Thus, rapidly adherent cells exhibited some stem cell properties, while the slowly adherent cells behaved like transit amplifying cells. Bickenbach and Chism (1998) found that approximately 10% of total basal cells and 100% of label-retaining cells of murine epidermal keratinocytes adhered to a substrate, collagen IV or others, in 10 min, and the rapidly adherent stem cells formed large colonies and could be used to form a structurally complete epidermis in organotypic culture. To the best of our knowledge, there have been no reported attempts to isolate limbal epithelial stem cells based on their adhesion properties of the integrins.

Like epidermis, limbal basal cells are believed to contain two types of proliferating cells: stem cells and cells with a lower capacity for self-renewal and higher probability of undergoing terminal differentiation (transit amplifying cells). Stem cells are also believed to present in limbal epithelial cultures. Although cultured limbal cells may not maintain all their in vivo characteristics after removal from their 'niche' or microenvironment, they are clonogenic and able to generate three distinct clonal forms, holoclones, meroclones and paraclones, owing to the varied self-renewal capacities (Pellegrini et al., 1999), just like previously identified in skin (Barrandon and Green, 1987). The holoclones possess the highest reproductive capacity and are believed to be founded mainly by stem cells (Pellegrini et al., 1999). Slow cycling cells identified by BrdU label-retention, a characteristic of stem cells, are also present in limbal epithelial cultures (Kim et al., 2004). We thus conducted these studies to isolate a population partially enriched for certain stem cell properties from both human limbal epithelial tissues and their primary cultures.

Our findings demonstrate that the rapidly adherent cells possess properties (Table 2) that satisfy some of the criteria of adult stem cells. Among the populations selected from limbal epithelial cells and their cultures by adhesion to collagen IV, the RAC in 20 min, accounting for about 10% of whole population, contained the highest number of BrdU label-retaining cells ($16.02 \pm 2.45\%$) that was enriched 5-fold from unfractionated primary cultures ($3.15 \pm 0.55\%$ of BrdU LRCs), displayed the highest number of integrin $\beta 1$ ($57.1 \pm 9.9\%$) and p63 protein positive ($47.5 \pm 8.6\%$) and involucrin negative stained cells, expressed the highest levels of $\Delta Np63$ and ABCG2 mRNAs and lowest levels of involucrin and K12 mRNAs, and possessed the greatest colony forming efficiency and growth capacity on a 3T3 feeder layer. Overall the RAC population exhibited a 2–3 fold of enrichment for cells with a stem cell phenotype and growth properties than the entire unfractionated cell population.

Nevertheless, the RAC population is far from pure. Our RAC populations ranged from 5% within 10 min to 10% within 20 min, which is considerably higher than estimated number of limbal stem cells. The RAC population contains only 16% slow-cycling LRCs and shows only 2-fold greater CFE than the unfractionated cells. This indicates that adhesion to collagen type IV is only one feature of limbal basal cells. Pure limbal stem cells cannot be isolated because no specific marker has been identified to date. Further selection with other cell surface markers is necessary to obtain a more enriched population.

In conclusion, our findings demonstrated for the first time that putative human limbal stem cells can be partially enriched by adhesion to collagen IV. The RAC population enriched for certain putative stem cell properties may prove useful in the future for transplantation to diseased and damaged corneas with limbal stem cell deficiency. This population could be further utilized to search for new markers to improve identification of stem cells, which would further refine the isolation methods to obtain pure stem cells in the future.

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Fig. 1. Immunofluorescent staining (A, B, E and F) and laser scanning confocal microscopy (C and D) for integrin $\beta 1$ protein expression in frozen sections of the human cornea (A) and limbus (B), in limbal supra-basal (C. Supra-L) and basal (D. Basal-L) layers of whole-mounted limbus, and in the primary cultured human limbal epithelial cells (F. Culture- $\beta 1$) with Hoechst 33342 counterstaining (E. Culture-H).

Fig. 2.

Adhesion pattern of human limbal epithelial cells, isolated from fresh limbal epithelial tissues (Limbus) and from their primary cultures (Culture), adhered to collagen type IV for different lengths of time, 10, 20, 60, 120 min and overnight (ON). The percentages of adherent cells are shown as mean $\pm$ s.d. summarized from six separate experiments.

Fig. 3.

Representative immunofluorescent staining for BrdU-label retaining cells in four populations of primary cultured limbal epithelial cells: unfractionated all cells (ALL) and three selected populations (RAC, SAC and NAC) by adhesion to collagen IV with propidium iodine (PI) counterstaining. Adhesion experiments were performed after the cell cultures labelled with 10 μm BrdU for 72 hr and chasing for 18 days.

Fig. 4. Representative immunofluorescent staining profiles for integrin $\beta 1$, p63 and involucrin in four populations of primary cultured limbal epithelial cells: unfractionated all cells (ALL) and three selected populations (RAC, SAC and NAC) by adhesion to collagen IV with Hoechst 33342 counterstaining.

Fig. 5. Representative semiquantitative RT-PCR profiles showing mRNA expression of (A) integrin β 1 (104 bp), (B) ABCG2 (379 bp), (C) Δ Np63 (440 bp), (D) keratin 12 (150 bp), (E) involucrin (121 bp) and (F) GAPDH (498 bp) by four populations of human limbal epithelial cells isolated from fresh limbal tissues: unfractionated all cells (ALL) and three selected populations (RAC, SAC and NAC) by adhesion to collagen IV. A 100 bp DNA ladder is shown in the first left lane.

Fig. 6.

(A) Colony forming efficiency (CFE) on a 3T3 fibroblast feeder layer at day 6 generated by four populations of human limbal epithelial cells isolated from fresh limbal tissues: unfractionated all cells (ALL) and three selected populations (RAC, SAC and NAC) by adhesion to collagen IV. The percentages of CFE were shown as mean $\pm$ S.D., summarized from four separate adhesion experiments. (B) Representative culture wells with 1% rhodamine staining showing growth capacity of these four populations of human limbal epithelial cells at day 14 on a 3T3 fibroblast feeder layer.

Table 1

Human primer sequences used for semi-quantitative RT-PCR

Gene	Accession	Sense primer	Antisense primer	PCR product
Integrin β 1	X07979	AGTGAA TGGGAAC AACGAGGTC	CAATTCCAGCAACCACACCA	104 bp
Δ Np63	XM_036421	CAGACTCAATT TTAGTGAG	AGCTCATGTTGGGGC/AC	440 bp
ABCG2	AY017168	AGTTCCATGGCACTGGCCATA	TCAGGTAGGCAATTGTGAGG	379 bp
K12	D78367	ACATGAAAGAAGAACCA CGAGGATG	TCTGCTCAGCGATGGTTTCA	150 bp
Involucrin	NM_005547	GGACTGCCTGAGCAAGAATGTG	TAAAGCTGCTGCTCTGGGTTT	121 bp
GAPDH	M33197	GCCAAAGGTCATCCATGACAAC	GTCCACCACCTGTTGCTGTA	498 bp

Table 2

Properties of selected populations of human limbal epithelial cells

	ALL	RAC	SAC	NAC
Adhesion time		20 min	2 hr	None in 2 hr
Population (%)	100	10.4±1.5	17.3±7.9	69.5±4.1
Label retaining cells (%)	3.15±0.55	16.02±2.45	4.78±1.61	0.53±0.74
Integrin β1+cells (%)	26.6±3.1	57.1±9.8	30.8±3.2	9.7±2.0
p63+cells (%)	15.6±3.4	47.5±8.6	18.8±7.1	6.3±3.5
Involucrin+cells (%)	41.8±5.6	1.8±0.6	30.4±5.5	68.6±6.1
Integrin β1 mRNA	++	++++	++	+
ΔNp63 mRNA	+	+++	+	±
ABCG2 mRNA	++	+++	+	±
Involucrin mRNA	+	-	+	+++
Keratin 12 mRNA	+	-	+	+++
CFE (%)	2.01±0.51	3.77±0.42	2.45±0.61	0.91±0.32
Confluent days	18-24	10-14	18-24	No confluence

ALL, the unfractionated whole cell population; RAC, the rapidly adherent cells; SAC, the slowly adherent cells; NAC, the non-adherent cells.