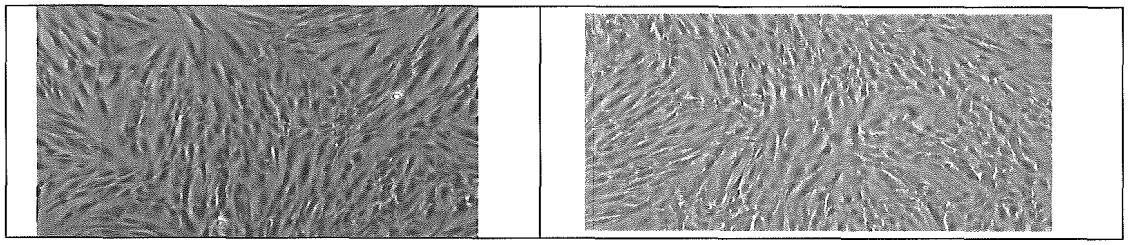
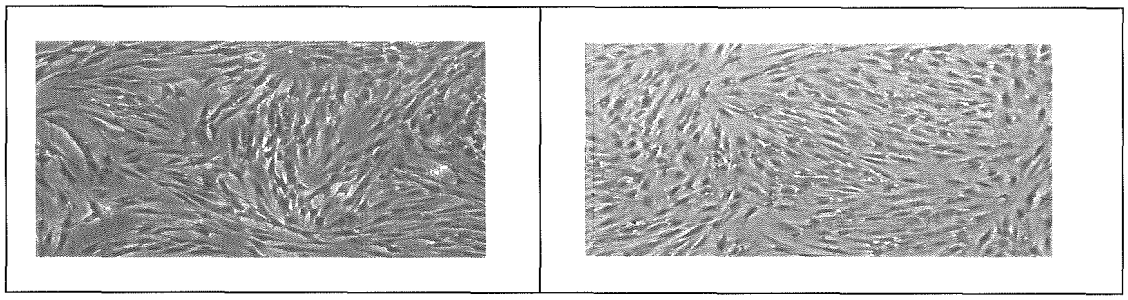
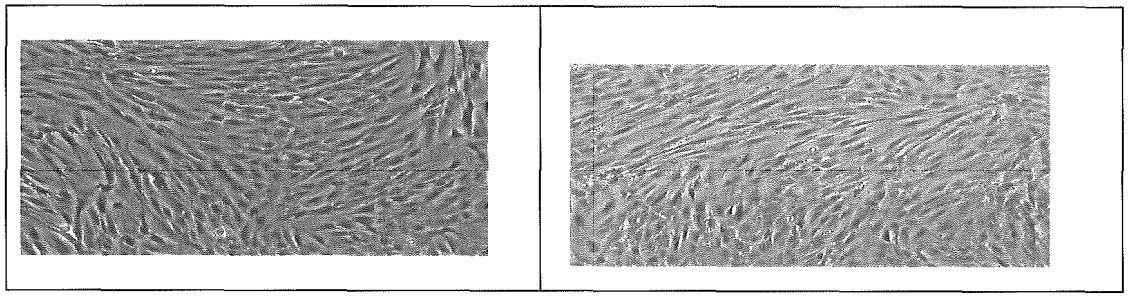
Control　2 日目　3 日目



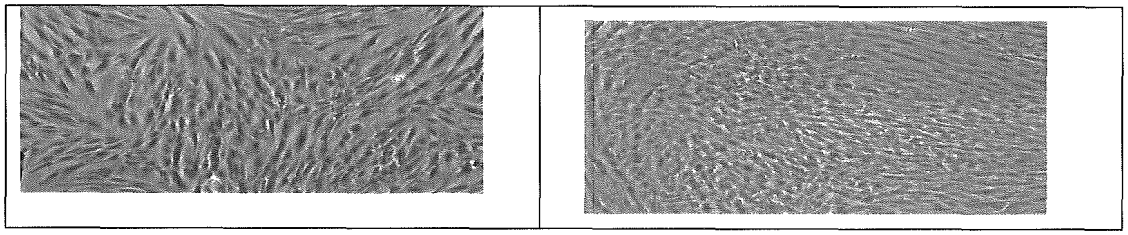
25 KGy　2 日目　3 日目



100 KGy　2 日目　3 日目



250 KGy　2 日目　4-5 日目



500 KGy　2 日目　4-5 日目

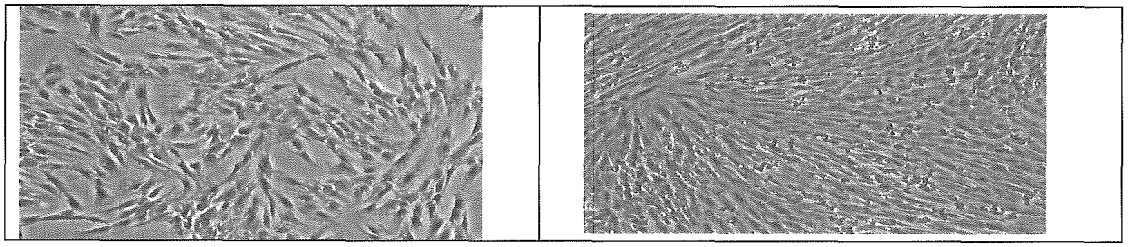
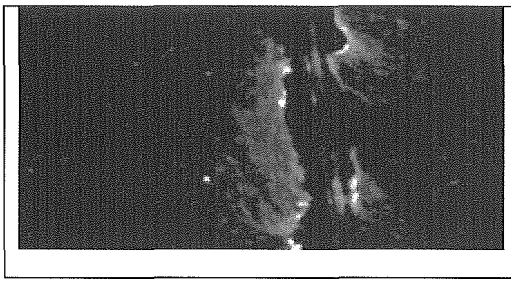
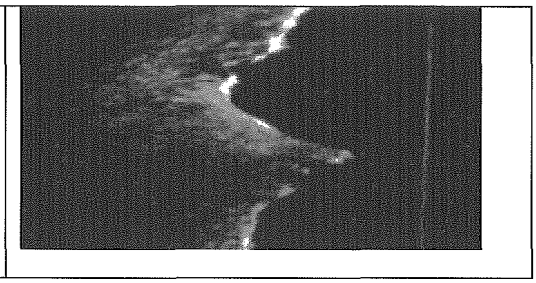


図 1 .コーティングされていないディッシュと、ポリ－N－イソプロピルアクリルアミドをコーティングしたディッシュ**(25 KGy, 100KGy, 250KGy** および **500KGy)**の正常ヒト皮膚繊維芽細胞の接着。

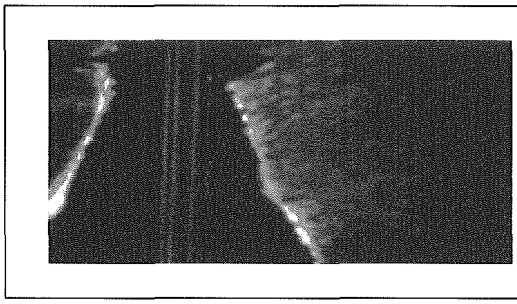
Control



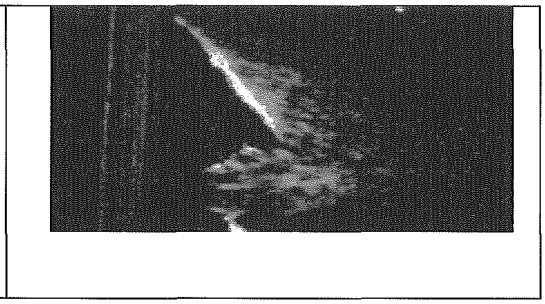
25 KGy



100 KGy



250 KGy



500 KGy

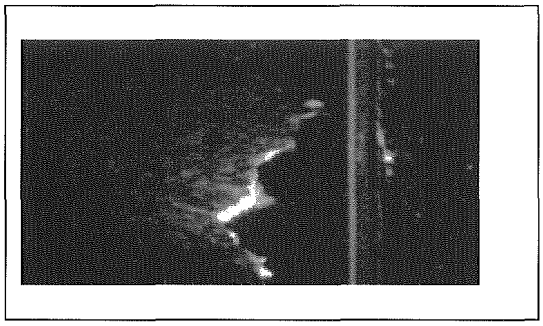


図 2. コーティングされていないディッシュと、ポリ－N－イソプロピルアクリルアミドをコーティングしたディッシュ(25 KGy, 100KGy, 250KGy および 500KGy)上の正常ヒト皮膚繊維芽細胞の SLDT 法によるギャップ結合細胞間連絡機能の測定。

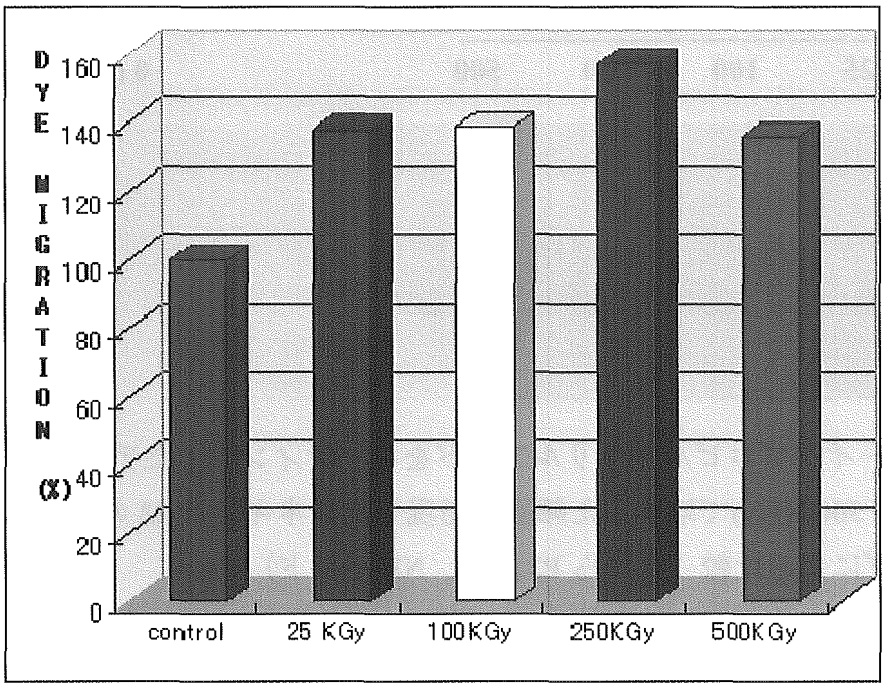


図 3. ポリーN−イソプロピルアクリルアミドをコーティングしたディッシュおよびコーティングしていないディッシュ上の正常ヒト皮膚繊維芽細胞の蛍光色素移行比率の比較。

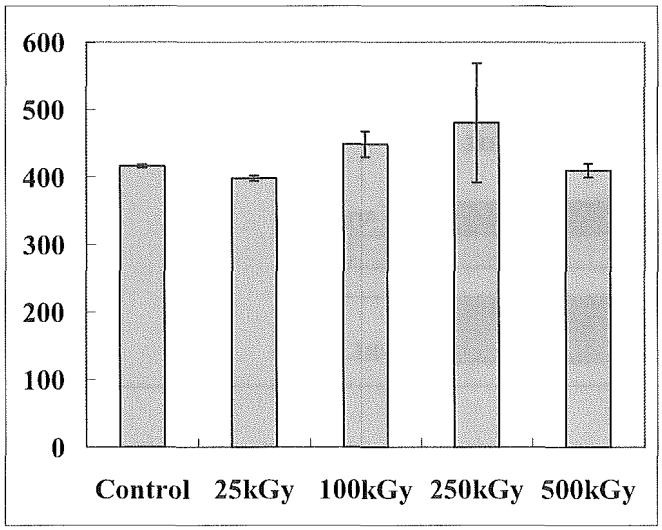


図 4. ポリーN−イソプロピルアクリルアミドをコーティングしたディッシュおよびコーティングしていないディッシュ上の正常ヒト皮膚繊維芽細胞の細胞増殖度比較。

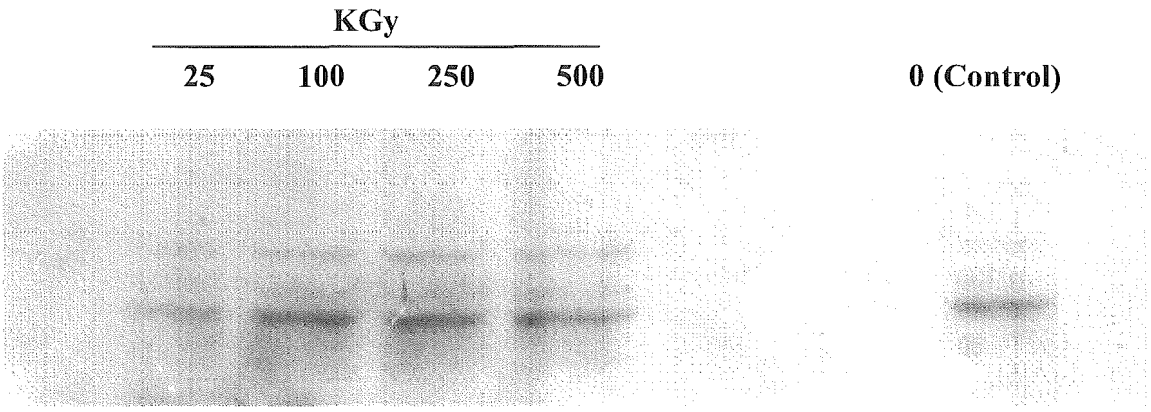


図 5. ポリ－N－イソプロピルアクリルアミドをコーティングしたディッシュ(25, 100, 250 および 500 KGy)で培養した細胞で発現したコネキシン 43 のウエスタンブロッティングによる比較（上から P2、P1、NP バンド）

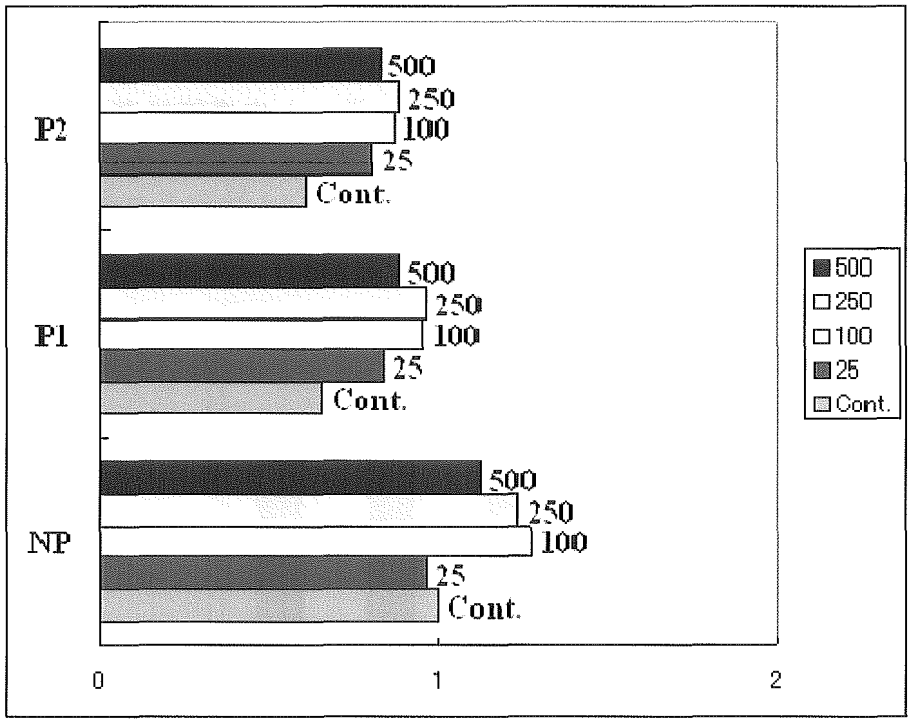


図 6　ポリ－N－イソプロピルアクリルアミドをコーティングしたディッシュ(25 KGy, 100KGy, 250KGy および 500KGy)で培養した細胞のコネキシン 43 のＮＰ、Ｐ１、Ｐ2 バンドの発現量の定量化による比較

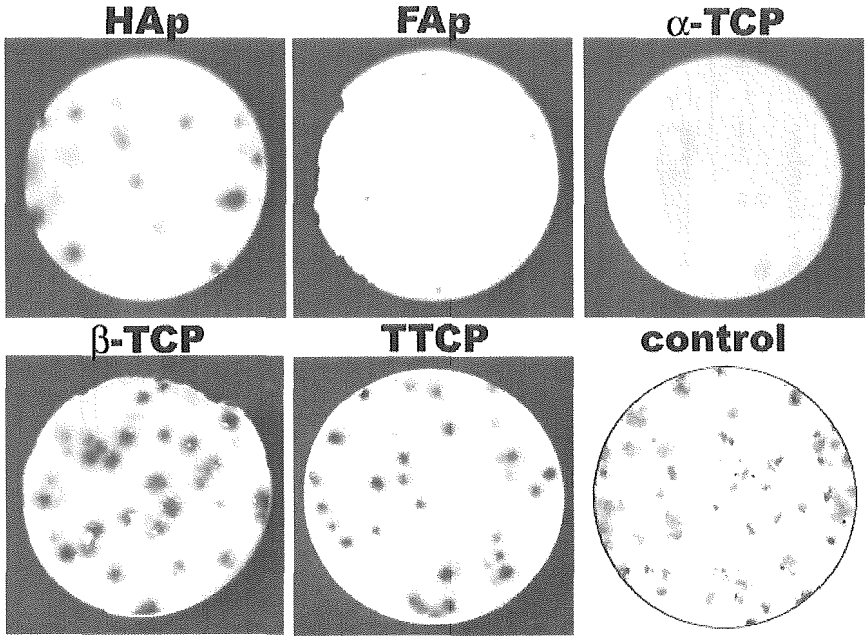


図７．種々のリン酸カルシウムバルク体上でコロニー形成.

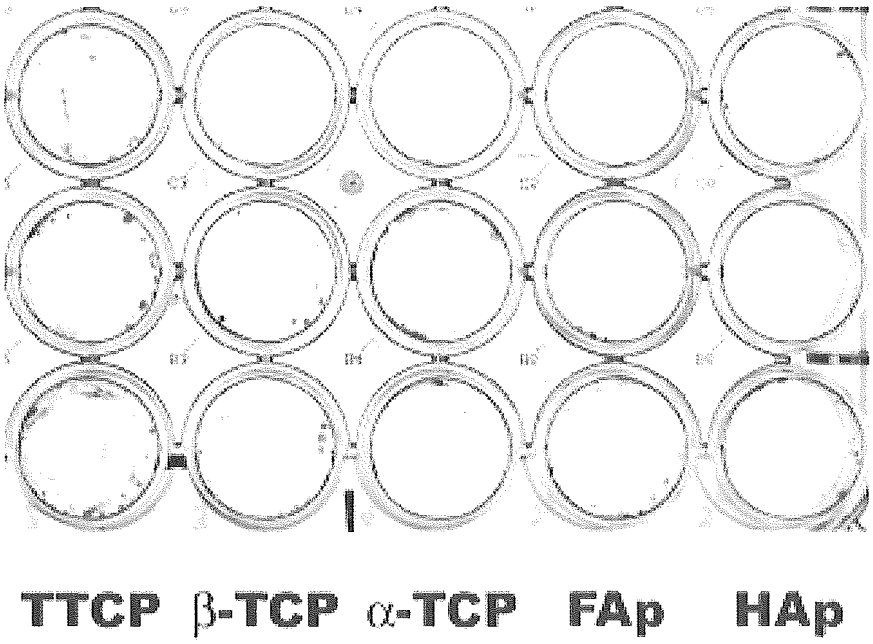


図８．リン酸カルシウムバルク体上でのコロニー試験後，これらのバルク体を取り除いた培養皿の様子.

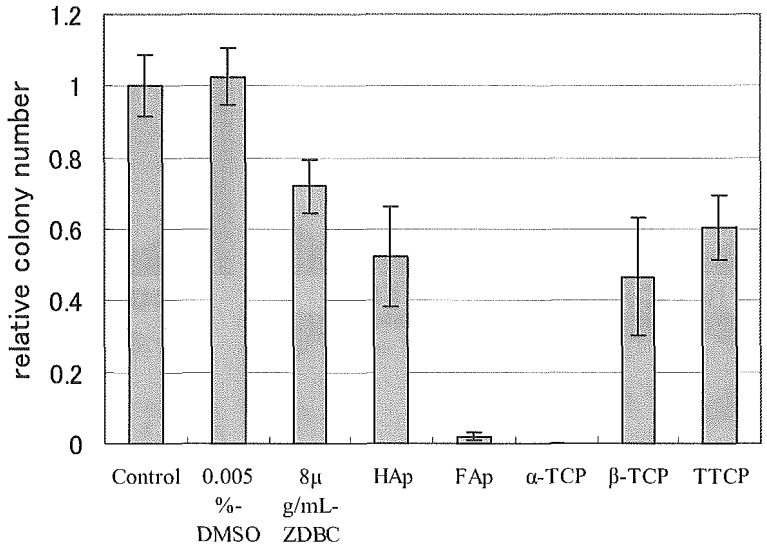


図９．種々のリン酸カルシウムバルク体上でのコロニー形成の割合.

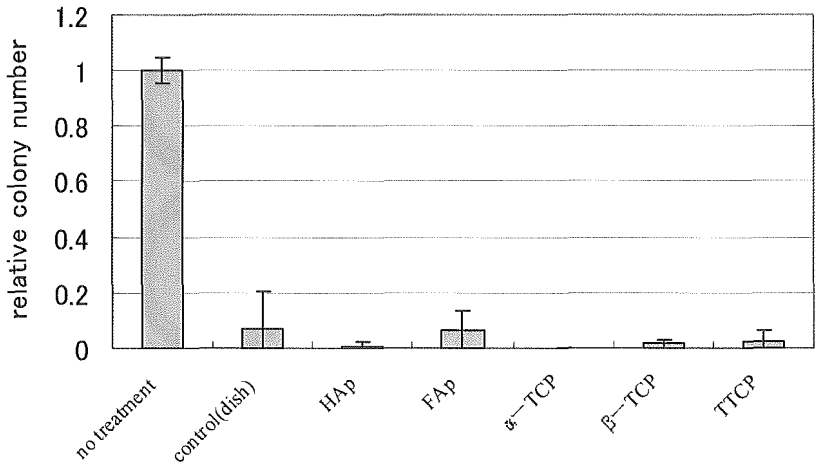


図 10. リン酸カルシウムバルク体上で 4 時間培養し，その培地を別の培養皿に移して培養した系のコロニー形成の割合.

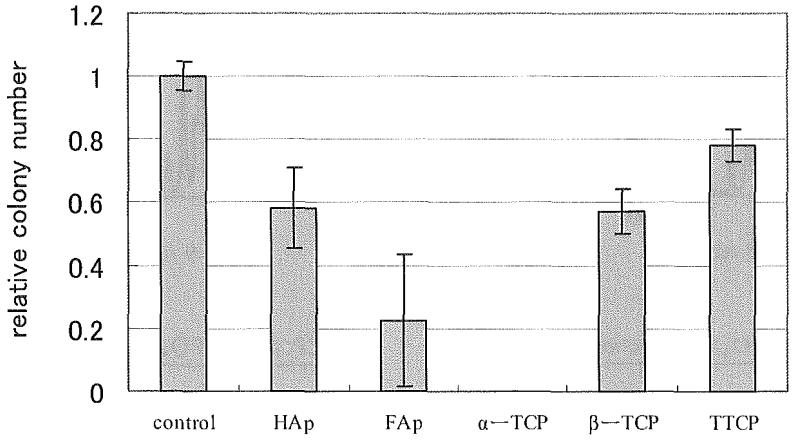


図 11. リン酸カルシウムバルク体上で 4 時間培養し，その後培地交換を行った系におけるリン酸カルシウムバルク体上のコロニー形成の割合.

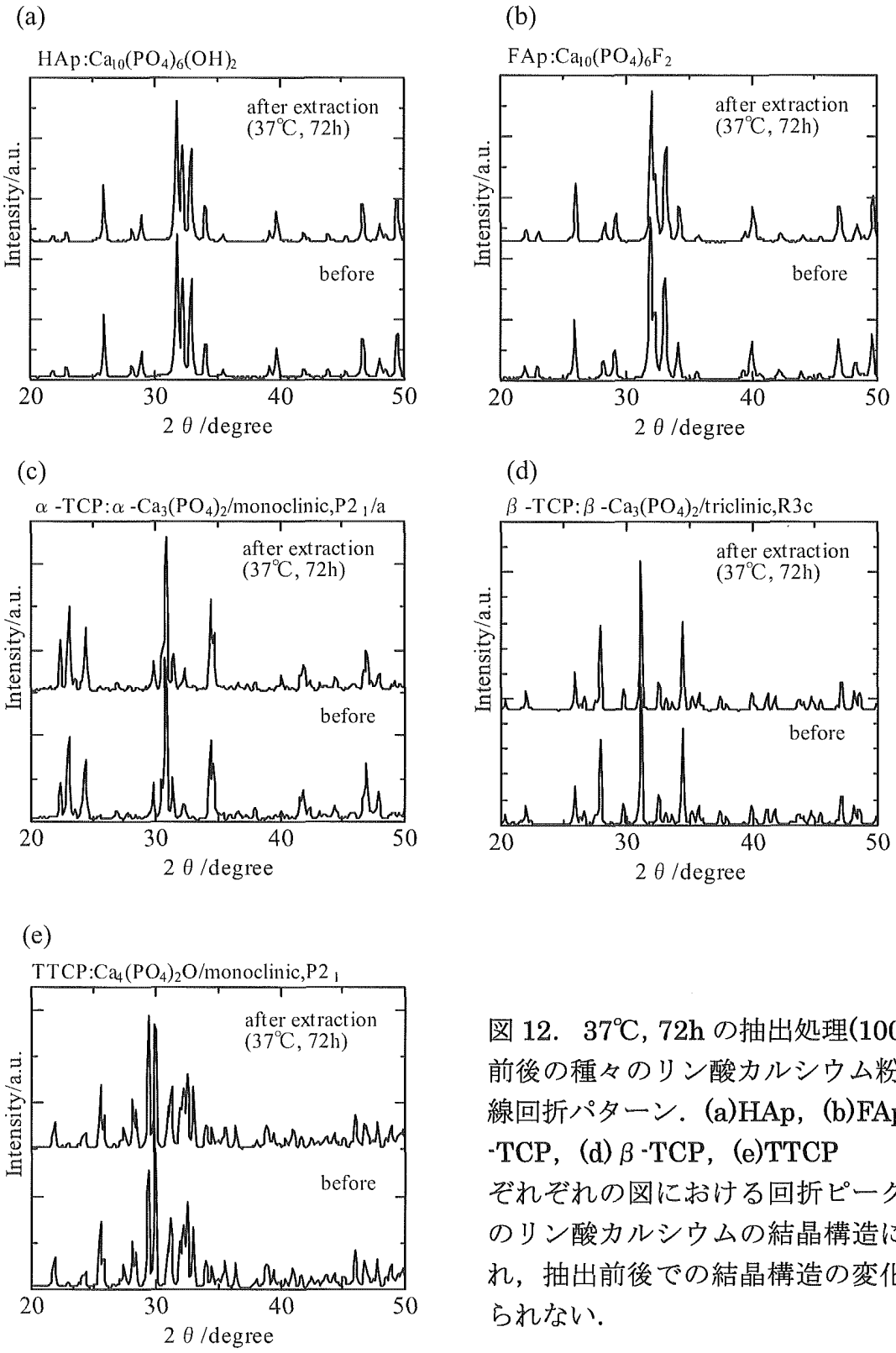


図 12. 37℃, 72h の抽出処理(100mg/ml)前後の種々のリン酸カルシウム粉末の X 線回折パターン. (a)HAp, (b)FAp, (c) α -TCP, (d) β -TCP, (e)TTCP
ぞれぞれの図における回折ピークは各々のリン酸カルシウムの結晶構造に帰属され, 抽出前後での結晶構造の変化は認められない.

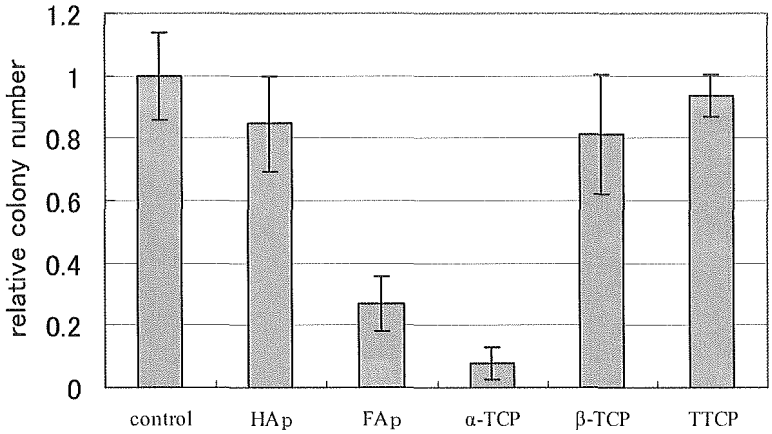


図 13. 抽出液培養した系のコロニー形成の割合

表 1. 各種リン酸カルシウムバルク体上での培養後の培地

Sample	pH
control	7.70
HAp	7.89
FAp	7.83
α-TCP	7.27
β-TCP	7.95
TTCP	8.14

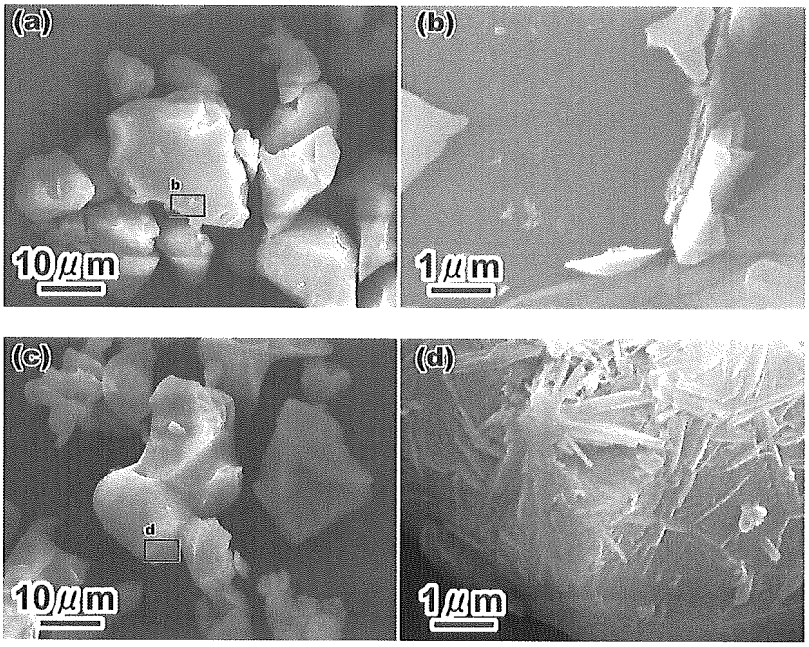


図１４．抽出処理前((a)，(b))後((c)，(d))のα-TCP の SEM 像．(b)および(d)は，それぞれ(a)，(c)中の枠内

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Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2

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Abstract Many types of organ-specific stem cells have been recently shown to exhibit a side population (SP) phenotype based on their ability to efflux Hoechst 33342 dye. Because stem cells from corneal epithelium reside in the basal layer of the limbal epithelium, the purpose of this study was to examine whether the limbal epithelium contains SP cells. The ATP-binding cassette transporter Bcrp1/ABCG2 is reported to contribute to the SP phenotype in cells from several diverse sources. Here we show data from fluorescence-activated cell sorting and real-time quantitative RT-PCR analysis showing that harvested limbal epithelial cells contain SP cells expressing ABCG2. Immunofluorescence revealed that a portion of limbal epithelial basal cells expressed ABCG2. Data indicate that ABCG2 positive limbal epithelial cells are putative corneal epithelial stem cells.

Keywords: Corneal epithelium; Limbal epithelium; Stem cell; Side population; ABCG2

1. Introduction

The cornea – the transparent outer anterior tissue layer of the eye – provides the eye with protection and refractive properties essential for vision. These functions depend, in part, on the corneal epithelium, a highly specialized cell layer comprising both basal and stratified squamous cells. Corneal epithelial stem cells reside in the basal layer of the limbus [1,2], the transitional zone between the cornea and the more peripheral bulbar conjunctiva. These cells allow the renewal of the corneal epithelium by generating transient amplifying cells that migrate, proliferate and differentiate to replace lost corneal epithelial cells [3–5]. However, because of the absence of the definite biological markers, unequivocal corneal epithelial stem cell identification remains elusive.

In 1996, Goodell et al. [6] demonstrated that mouse hematopoietic stem cells with long-term multi-lineage reconstituting ability can be isolated as side population (SP) cells based on their ability to efflux the Hoechst 33342 dye. Using dual wavelength flow cytometric analysis, SP cells were identified as a distinct population with low Hoechst 33342 blue/red fluorescence representing approximately 0.1% of total bone marrow cells [6]. SP cells have also been identified in hematopoietic compartments in a number of animals [7–10]. In addition, SP cells have been isolated from various types of adult tissue where they demonstrate stem cell activity [6,10–16]. These findings suggest that the SP phenotype represents a common feature of stem cells.

Recently, Zhou et al. [13,17] reported that the ATP-binding cassette transporter Bcrp1/ABCG2 is a molecular determinant of the SP phenotype. A number of other studies in a wide variety of organs have also indicated that the SP phenotype is largely determined by the expression of Bcrp1/ABCG2 [13,15,18–20]. More recently, Mogi et al. [21] have shown by targeted gene ablation studies in mice that serine/threonine kinase Akt signaling modulates the SP phenotype by regulating the expression of Bcrp1/ABCG2.

At present, it remains unclear if corneal epithelial stem cells might exhibit the SP phenotype. We report our recent studies on human limbal epithelium, providing evidence that these cells contain SP cell subsets expressing ABCG2, implicating possible relationships between these SP cells and limbal stem cells.

2. Materials and methods

2.1. Cell preparation

Human corneoscleral rims from USA eye bank eyes were used. Limbal tissues were obtained by using scissors, and 8.0-mm diameter central portions of corneas were obtained by trephination. Limbal tissues and central corneas were incubated separately at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Nikken Biomedical Laboratory, Kyoto, Japan) containing 2.4 units/ml dispase (Invitrogen, Carlsbad, CA) for 1 h. Epithelial cells were separated under a dissecting microscope and treated with 0.25% trypsin-1 mM EDTA solution (Invitrogen) for 15 min at 37 °C to achieve each single cell suspensions from the limbal epithelium and the corneal epithelium. Enzymatic activity was then stopped by adding an equal volume of DMEM containing 10% fetal calf serum (FCS; Morgate Biotech, Qld., Australia).

2.2. Hoechst 33342 dye exclusion assay

Separate populations of epithelial cells from the limbus and from the cornea were resuspended at 1.0×10^6 cells/ml in incubation medium

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(DMEM containing 2% FCS and 10 mM HEPES (Sigma)) and subsequently divided into two portions. After incubation in incubation medium for 90 min at 37 °C, (*R*)+-verapamil (Sigma, St. Louis, MO) or tryprostatin A (TPS-A; provided by Dr. H. Osada (RIKEN Institute)) was added to one portion (final concentration: 50 μM), after which both portions were incubated for a further 30 min at 37 °C. Antibody-mediated inhibition assay using anti-ABCG2 monoclonal antibody (5D3; eBioscience, San Diego, CA) or isotype control (mouse IgG_{2b}; eBioscience) was performed as previously described [22]. Hoechst 33342 dye (Sigma) was then added to both portions (final concentration: 3 μg/ml), and incubation continued for another 90 min at 37 °C. After the final incubation, cells were kept on ice and analyzed for Hoechst 33342 dye efflux by EPICS Altra FACS analysis (Beckman Coulter, Fullerton, CA). Prior to analysis, propidium iodide (Sigma) was added (final concentration: 2 μg/ml) to distinguish live from dead cells. Hoechst 33342 dye was excited at 350 nm using a UV laser. Fluorescence emission was detected through 450 nm BP (Hoechst blue) and 675 nm LP (Hoechst red) filters, respectively. Propidium iodide in cells was excited at 488 nm (argon ion laser) and fluorescence emission was detected through a 610 nm BP filter.

2.3. Real-time quantitative RT-PCR analysis

SP and non-SP cells were isolated from limbal epithelial cells using EPICS Altra flow cytometric sorting. Total RNA was obtained from equal numbers (2000–3000) of SP and non-SP cells according to the manufacturer's instructions (Isogen; Nippongene, Tokyo, Japan). After DNase I (Invitrogen) treatment, total cellular RNA was divided into two portions. One portion was reverse transcribed with oligo$(dT)_{12-18}$, according to the manufacturer's instructions (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen), and a 1/10 volume (2 μl) of synthesized cDNA was used as a template for PCR. The other portion was treated in the same manner except for the omission of SuperScript II reverse transcriptase (Invitrogen). The oligonucleotide primers (Invitrogen) used for ABCG2 amplification were 5′-GGTTTCCAAGCGTTCATTCAAA-3′ (forward) and 5′-TAGCCCAAAGTAAATGGCACCTA-3′ (reverse), with an expected product length of 111 bp. The TaqMan probe (Applied Biosystems, Foster City, CA) used for ABCG2 detection was 5′-CCCAGGCCTCTATAGCTCAGATCATTGTCA-3′, labeled with 6-carboxyfluorescein (FAM) at the 5′-end, and with 6-carboxytetraethylrodamine (TAMRA) at the 3′-end. Quantitative PCR was performed using ABI Prism 7900HT Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. The reaction mixture (30 μl) contained 15 μl of TaqMan Universal PCR Mastermix (Applied Biosystems), 15 pmol of forward and reverse primers, 7.5 pmol of TaqMan probe and 2 μl of the investigated sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was measured using TaqMan GAPDH Control Reagents (Applied Biosystems), according to the manufacturer's instructions. Thermocycling used 50 cycles at 95 °C for 15 s and 60 °C for 1 min with an initial cycle at 50 °C for 2 min and 95 °C for 10 min. A negative control with non-reverse transcribed total RNA as a template was included in every experiment. All assays were run in duplicate. To represent the ABCG2 mRNA expression level, we used the ABCG2 mRNA expression index as the value of the ABCG2 gene copies divided by the value of the housekeeping GAPDH gene copies.

2.4. Statistical analysis

Statistics were calculated using SigmaStat 2.0 (SPSS, Chicago, IL). To compare ABCG2 expression between SP and non-SP cells, the Mann–Whitney rank sum test was applied.

2.5. Immunofluorescence

Cryostat sections (20 μm) were cut from the limbus and from the cornea, dried for 1 h at room temperature, and fixed in acetone for 10 min also at room temperature. Immunofluorescence was performed using DakoCytomation CSA II (DakoCytomation, Kyoto, Japan). Briefly, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water for 15 min. The slides were first incubated with serum-free protein in buffer for 15 min to block non-specific binding of antibodies, then incubated at 4 °C overnight with a 1:2500 dilution of BXP-21 monoclonal antibody (MBL, Aichi, Japan). BXP-21 was diluted in tris-buffered saline containing 1% bovine serum albumin and 0.1% Tween 20. After incubation, slides were incubated for 15 min with anti-mouse immunoglobulins-HRP, followed by 15 min in fluorescyl-tyramide hydrogen peroxide in buffer. After counterstaining with propidium iodide (Sigma), slides were mounted and observed on a confocal laser scanning microscope (LSM 510 META; Carl Zeiss, Jena, Germany). For each type of tissue, identically treated negative controls were included using normal non-specific IgG_{2a} (DakoCytomation).

3. Results

3.1. SP cells are present in human limbal epithelium

In the Hoechst 33342 dye exclusion assay, a distinct population of cells with a low Hoechst 33342 blue/red fluorescence was detected in epithelial cells from the limbus (Fig. 1A; 0.20% gated cells) but not in cells from the cornea (Fig. 1B, 1D; 0.02% gated cells). Generation of this subpopulation was not seen in the presence of (*R*)+-verapamil, the specific inhibitor of Hoechst 33342 dye transport (Fig. 1C; 0.01% gated cells). These observations indicate that the limbal epithelium contains SP cells.

3.2. Effect of 5D3 and TPS-A on Hoechst 33342 dye efflux

When SP cells were incubated with an anti-ABCG2 monoclonal antibody, 5D3, recognizing an external plasma membrane epitope on living cells [13,22], prior to Hoechst 33342 dye exclusion assay, a marked decrease in SP cell dye efflux was observed (Fig. 2A). Hoechst 33342 dye efflux activity was also inhibited by a novel ABCG2 inhibitor, TPS-A (Fig. 2B), that is known not to inhibit another important ATP-binding cassette transporter, P-glycoprotein [23]. These data strongly suggest that ABCG2 is a major contributor to the SP phenotype of limbal epithelial cells.

3.3. ABCG2 mRNA expression in SP and non-SP cells

To investigate the link between the SP phenotype and ABCG2 expression, SP and non-SP cells from limbal epithelial tissues were analyzed for ABCG2 gene expression by TaqMan real-time

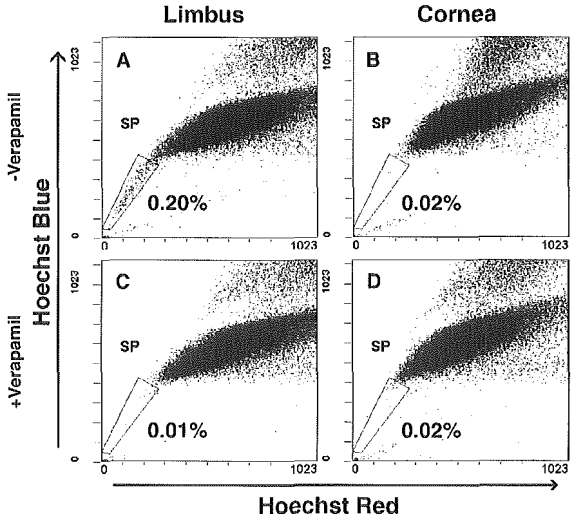

Fig. 1. Flow cytometric analysis of epithelial cells harvested from the limbus and from the cornea. Epithelial cells were isolated from eye bank limbus and cornea tissues, and analyzed for Hoechst 33342 dye efflux by FACS. (A) SP cells were detected in limbal epithelial cells after Hoechst 33342 dye staining. (B) SP cells were absent in corneal epithelial cells. (C) Dye efflux from SP cells was inhibited by verapamil. (D) Verapamil had no effect on dye efflux.

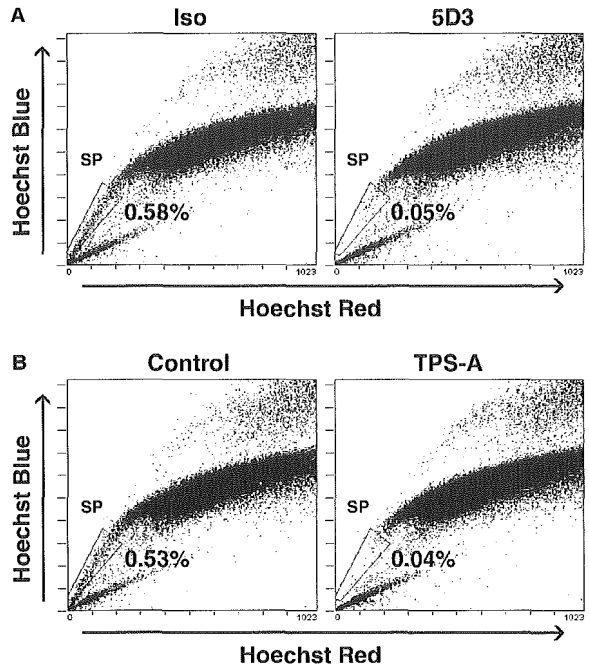

Fig. 2. Influence of ABCG2 inhibitors on the SP phenotype of limbal epithelial cells. Limbal epithelial cells were analyzed for Hoechst 33342 dye efflux in the presence of ABCG2 inhibitors. Dye efflux from SP cells was effectively inhibited by 5D3 monoclonal antibody (A) and by TPS-A (B).

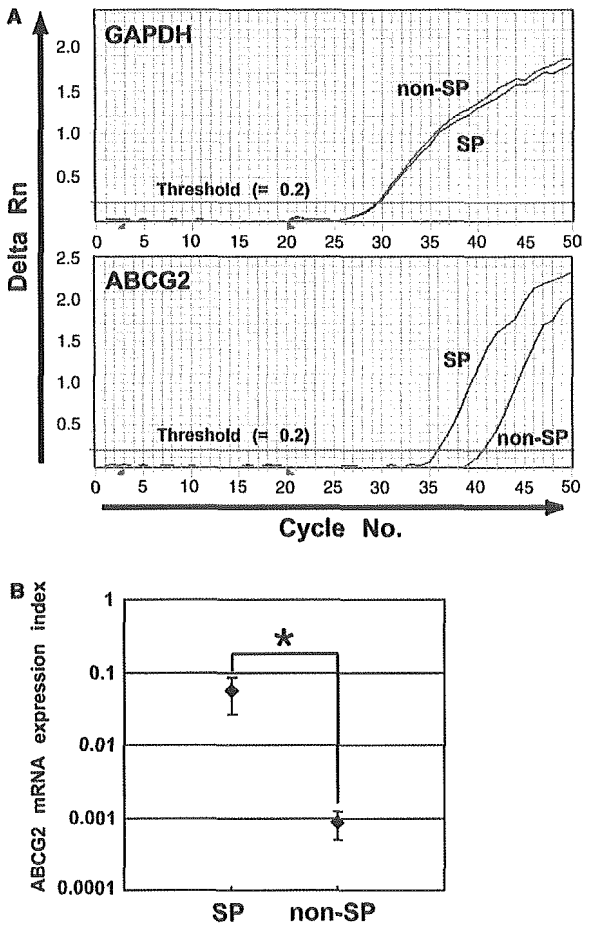

Fig. 3. Quantification of ABCG2 mRNA in SP and non-SP cells. (A) Amplification plots from real-time RT-PCR analysis. Delta Rn is plotted against cycle number. Horizontal red lines show the threshold used for calculation of ABCG2 expression indices. Real-time quantitative RT-PCR procedures were as described in Section 2. (B) ABCG2 mRNA expression indices in SP and non-SP cells. The relative expression of ABCG2 gene was normalized to that of GAPDH in each sample. Data represent the mean value from four samples. Error bars indicate the S.E. ABCG2 mRNA expression in SP cells was significantly higher than in non-SP cells ($P = 0.029$).

RT-PCR (Fig. 3A). ABCG2 mRNA expression indices were $5.61 \times 10^{-2} \pm 2.92 \times 10^{-2}$ (SP) and $8.66 \times 10^{-4} \pm 3.56 \times 10^{-4}$ (non-SP) ($n = 4$, mean ± S.E.) (Fig. 3B). Statistical analysis showed that the ABCG2 mRNA expression level in SP cells was significantly higher than in non-SP cells ($P = 0.029$).

3.4. Distribution of ABCG2-expressing cells

Our immunofluorescence data using BXP-21 monoclonal antibody specific for human ABCG2 revealed that ABCG2 is expressed in the limbal epithelial basal cells (Fig. 4A, B), but not in corneal epithelial cells (Fig. 4C). Observed ABCG2 expression in basal cells is inconsistent and discontinuous in the epithelium, and does not persist throughout the entire limbus (Fig. 4A). Fig. 4B shows that positive staining cells are present not only in the basal but also in the suprabasal cells.

4. Discussion

Corneal epithelial stem cell deficiency caused by ocular trauma or diseases causes corneal opacification and visual loss. Patients with corneal epithelial stem cell deficiency can be treated with autologous or allogenic limbal transplantation [24,25]. However, autologous limbal transplantation requires a large limbal withdrawal from the patient's eye, and allogenic limbal transplantation has a significant risk of rejection. Moreover, shortage of donor tissues is a serious current challenge. To avoid these problems, transplantations of cultivated corneal limbal epithelial cells have recently been reported [26–31]. In such tissue engineering approaches, development of new, effective methods for rapid identification and enrichment of corneal epithelial stem cells would be a considerable advantage since long-term, functional recovery of corneal epithelium is linked to graft constructs that retain viable stem cell populations [26,28,32].

In this study, we examined whether human limbal epithelium contains cells with SP phenotype, similar to stem cells in other organs [6,10–16]. Hoechst 33342 dye exclusion assays revealed that a number of SP cells are present in the limbal epithelium but not in the corneal epithelium (Fig. 1). The mean percentage of SP cells in limbal epithelial cells obtained from six independent experiments was calculated to be 0.29% (data not shown). This is a reasonable abundance value compared with that previously reported in other organs [6,10–16]. Though verapamil is generally used to identify the SP phenotype in the Hoechst 33342 dye exclusion assay, it is insufficient in specificity to demonstrate the contribution of ABCG2 to the SP phenotype of limbal epithelial cells. As ABCG2-specific inhibitors, fumitremorgin C [33], 5D3 monoclonal antibody

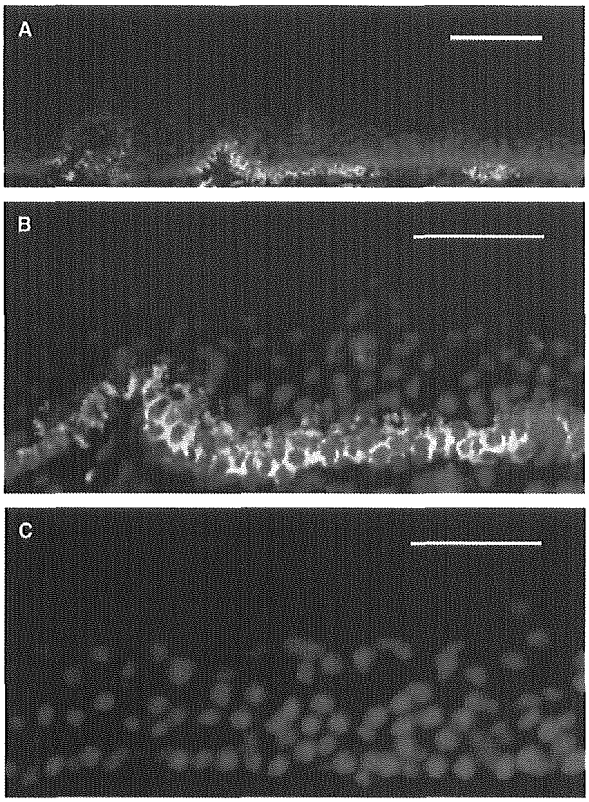

Fig. 4. Expression patterns of the ABCG2 marker using immunomarkers. Distribution of cells expressing ABCG2 protein was examined by immunofluorescence staining using the BXP-21 monoclonal antibody. (A) The basal layer of the limbal epithelium was inconsistently and discontinuously stained with anti-ABCG2 antibody (green). (B) A higher-magnification view of the limbal region showed that positively stained cells are present not only in the basal but also in the suprabasal cells. (C) ABCG2 was not expressed in the corneal epithelium. Nuclei were labeled with propidium iodide (red). Scale bar: 100 μm (A), 50 μm (B, C).

[13,22], and TPS-A [23] are reported. Our SP analysis of limbal epithelial cells using 5D3 and TPS-A showed that both 5D3 and TPS-A effectively inhibit Hoechst 33342 dye efflux from these cells (Fig. 2A, B). These data strongly suggest a correlation between SP phenotype and ABCG2 expression, but the possibility of partial contribution of other transporters to SP phenotype cannot be excluded. Real-time quantitative RT-PCR analysis showed a strong correlation between ABCG2 expression and the SP phenotype. The ABCG2 mRNA expression indices in SP and non-SP cells varied considerably between samples, but SP cells consistently expressed a significantly higher level of ABCG2 mRNA over non-SP cells (Fig. 3B). The variation observed in these experiments may be due to variable conditions of eye bank tissue samples affected by many uncontrolled factors including donor age, death-to-preservation time and tissue storage time.

Immunofluorescence work revealed that ABCG2 was inconsistently expressed within the basal layer of the limbal epithelium (Fig. 4A), but not in the corneal epithelium (Fig. 4C). These observations also indicated that ABCG2 positive cells are present not only in the basal but also in the suprabasal cells in some areas (Fig. 4B). This localization pattern for ABCG2-positive cells coincides almost identically with that for corneal epithelial stem cells demonstrated previously by several investigators [1,2,34–37]. These findings imply that ABCG2 positive cells in the limbal epithelium are putative corneal epithelial stem cells, consistent with the observation that SP cells are present in the limbal epithelium but not in the corneal epithelium (Fig. 1). Flow cytometry revealed that SP cells are only 0.20% of total limbal epithelial cells (Fig. 1A), but immunofluorescence showed that many more cells express ABCG2 (Fig. 4A, B). Because limbal epithelial basal cells have a small cytoplasmic volume, it is not clear whether ABCG2 molecules localize in the plasma membrane or in the cytoplasm (Fig. 4A, B). Since it has been reported that active ABCG2 is expressed on the surface of SP cells, and cytoplasmic ABCG2 cannot efflux Hoechst 33342 dye outside these cells [19,21,38,39], the smaller number of SP cells observed here can be explained by possible cytoplasmic localization of ABCG2. In addition, it is possible that our methods actually underestimate functional SP populations in limbal epithelial cells: cells were collected from eye bank eyes with varying death-to-preservation times and storage conditions, with the likelihood that viability, including dye efflux capability, might be reduced.

One possible biological function of ABCG2 expressed in the limbal epithelium is a protective function. Because Bcrp1/ABCG2 is known to transport a variety of toxic lipophilic compounds [40–42], ABCG2 may protect corneal epithelial stem cells from cytotoxic agents. Recently, Jonker et al. [43] found that Bcrp1/ABCG2-null mice become extremely sensitive to the dietary chlorophyll-breakdown product, pheophorbide a, resulting in severe, sometimes lethal, phototoxic lesions on light-exposed skin. This finding suggests that ABCG2 may protect corneal epithelial stem cells from solar damage. This is also consistent with the assertion that corneal epithelial stem cells are protected from solar damage by melanin pigmentation of the limbus in non-Caucasian people [2]. Another possibility is that ABCG2 may regulate the development of corneal epithelial stem cells by functioning as transporters of small hydrophobic regulatory molecules involved in proliferation, differentiation, or apoptotic pathways. However, the function of ABCG2 expressed in limbal epithelial basal cells remains a subject for further investigations.

In summary, our results suggest that the SP cell phenotype and cellular surface expression of ABCG2 may characterize putative corneal epithelial stem cells. Thus, ABCG2 may serve as a useful cell surface marker in the enrichment of viable corneal epithelial stem cells, and could be readily exploited for new tissue engineering approaches that attempt to reconstruct damaged ocular surfaces using cell transplantation methods.

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ORIGINAL ARTICLE

Corneal Reconstruction with Tissue-Engineered Cell Sheets Composed of Autologous Oral Mucosal Epithelium

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ABSTRACT

BACKGROUND

Ocular trauma or disease may lead to severe corneal opacification and, consequently, severe loss of vision as a result of complete loss of corneal epithelial stem cells. Transplantation of autologous corneal stem-cell sources is an alternative to allograft transplantation and does not require immunosuppression, but it is not possible in many cases in which bilateral disease produces total corneal stem-cell deficiency in both eyes. We studied the use of autologous oral mucosal epithelial cells as a source of cells for the reconstruction of the corneal surface.

METHODS

We harvested 3-by-3-mm specimens of oral mucosal tissue from four patients with bilateral total corneal stem-cell deficiencies. Tissue-engineered epithelial-cell sheets were fabricated ex vivo by culturing harvested cells for two weeks on temperature-responsive cell-culture surfaces with 3T3 feeder cells that had been treated with mitomycin C. After conjunctival fibrovascular tissue had been surgically removed from the ocular surface, sheets of cultured autologous cells that had been harvested with a simple reduced-temperature treatment were transplanted directly to the denuded corneal surfaces (one eye of each patient) without sutures.

RESULTS

Complete reepithelialization of the corneal surfaces occurred within one week in all four treated eyes. Corneal transparency was restored and postoperative visual acuity improved remarkably in all four eyes. During a mean follow-up period of 14 months, all corneal surfaces remained transparent. There were no complications.

CONCLUSIONS

Sutureless transplantation of carrier-free cell sheets composed of autologous oral mucosal epithelial cells may be used to reconstruct corneal surfaces and can restore vision in patients with bilateral severe disorders of the ocular surface.

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CORNEAL EPITHELIAL STEM CELLS RE-side in the basal layer of the limbus,[1,2] the transitional zone between the cornea and the bulbar conjunctiva. These cells govern renewal of the corneal epithelium[3] by generating progeny (transient amplifying cells, which are cells committed to epithelial differentiation) with limited renewal capabilities that migrate from the limbus into the basal layer of the cornea.[4]

If corneal epithelial stem cells are completely absent owing to limbal disorder from severe trauma (e.g., thermal or chemical burns) or eye diseases (e.g., the Stevens–Johnson syndrome or ocular pemphigoid), then the sources of corneal epithelial cells have been exhausted, the peripheral conjunctival epithelium invades inwardly, and the corneal surface becomes enveloped by vascularized conjunctival scar tissue, resulting in corneal opacification that leads to severe visual impairment. Such pathological characteristics are considered to represent limbal stem-cell deficiencies.[5,6]

In patients with unilateral limbal stem-cell deficiency, autologous limbal transplantation is a method of surface reconstruction of the cornea.[7] This procedure, however, requires a large limbal graft from the healthy eye (incurring a risk of causing limbal stem-cell deficiency in the healthy eye[8]), and it is not possible in patients who have bilateral lesions.[9]

Limbal-allograft transplantation can be performed in patients with unilateral or bilateral deficiencies,[10] but it requires long-term immunosuppression that involves high risks of serious eye and systemic complications including infection and liver and kidney dysfunction.[10] In patients with the Stevens–Johnson syndrome or ocular pemphigoid, graft failure is common, even with immunosuppression, owing to serious preoperative conditions such as persistent inflammation of the ocular surface, abnormal epithelial differentiation of the ocular surface, severe dry eye, and lid-related abnormalities.[11-13]

To avoid allograft rejection and improve surgical outcome, some patients with unilateral stem-cell deficiencies have had corneal epithelial grafts constructed ex vivo by the expansion of autologous limbal stem cells harvested from healthy contralateral eyes and cultivated on cell carriers such as amniotic membranes[14,15] and fibrin gel.[16] This process, however, cannot be used for bilateral total limbal stem-cell deficiencies. Therefore, we studied an alternative replacement strategy for damaged corneal epithelium involving a tissue-engineered epithelial-cell sheet comprising only the patient's own oral mucosal epithelial cells. Transplantation of autologous oral mucosal epithelial cells cultured on amniotic membranes to a rabbit corneal model has recently been reported.[17,18]

We studied a new method of transplantation involving a carrier-free cell sheet by exploiting temperature-responsive culture surfaces. By lowering the temperature, we are able to detach all the cultured cells from the surfaces as an intact transplantable cell sheet, and any carrier or scaffold is excluded from the graft.[19] We report the results of ocular-surface reconstruction in four patients with the use of cultured autologous oral mucosal epithelial cells and carrier-free tissue-replacement sheets.

METHODS

SUBJECTS

This study was approved by the institutional review board of Osaka University Medical School, in Osaka, Japan. Oral and written informed consent were obtained from all patients. Patients with bilateral total limbal stem-cell deficiency were eligible for inclusion. Exclusion criteria included glaucoma or xerophthalmia (a skinlike appearance) of the entire ocular surface. Our results are for the first four consecutive patients enrolled, each of whom had one eye grafted with a tissue-engineered epithelial-cell sheet fabricated in culture from harvested autologous oral mucosal epithelial cells in our hospital from January 2003 through March 2003 (Table 1).

All grafted eyes had been clinically diagnosed as having total limbal stem-cell deficiency with complete disappearance of the palisades of Vogt (a radial infolding at the sclerocorneal junction and a biologic marker of the location of corneal epithelial stem cells) and complete coverage by fibrovascular in-growth from 360 degrees of the limbus over the entire cornea. All patients exhibited chronic conjunctival inflammation immunologically driven by the causative diseases reported previously,[20,21] despite therapy with topical steroids. Three of the four patients (Patients 1, 3, and 4) had severe deficiency of the tear film. Lid abnormalities, including chronic blepharitis, misdirection of the eyelashes, and keratinization of the posterior lid margin, contributed to poor ocular-surface conditions and were also noted in all eyes. Patients 1 and 4 had continuous inflammation with severe tear-film and lid abnormalities and keratinization of the ocular

Table 1. **Preoperative Characteristics of Patients with Total Limbal Deficiency.**

Patient No.	Age	Sex	Diagnosis	Eye	Ocular-Surface Condition			Previous Surgery	Other Eye Diseases
					Symblepharon*	Schirmer's Test without Topical Anesthesia†	Schirmer's Test with Nasal Stimulation‡		
	yr					*mm*			
1	58	M	Stevens–Johnson syndrome (chronic phase)	Right	+	1	2	Allogeneic corneal epithelium (cultivated on amniotic membrane) transplantation in 2000	None
2	69	M	Ocular cicatricial pemphigoid	Left	+	23	26	None	None
3	77	F	Ocular cicatricial pemphigoid	Right	+	1	1	Limbal transplantation with the use of amniotic membrane in 2001	Proliferative diabetic retinopathy, branch-retinal-vein occlusion
4	75	F	Ocular cicatricial pemphigoid	Right	+	1	2	Penetrating keratoplasty in 1999	None

* The plus sign indicates that symblepharon (adhesion of one or both eyelids to the eyeball) was found at the patient's ocular surface.
† Schirmer's test without anesthesia is a commonly used clinical test of lacrimal secretion (tearing). It is performed by measuring the amount of moisture on Whatman filter paper (5 mm by 35 mm) that is placed in the margin of the lower lid for five minutes. A value of less than 5 mm indicates impaired secretion.
‡ Schirmer's test with nasal stimulation is used to measure maximal tearing and is performed by inserting a cotton swab into the nasal cavity. A value of less than 10 mm indicates decreased tearing.

surface. Three of the four patients (Patients 1, 3, and 4) had previously undergone allogeneic grafting, which had failed within one year after surgery, despite systemic and local immunosuppression with cyclosporine (trough levels of 50 to 100 ng per milliliter).

Surgical procedures for all cell-sheet autografts were performed by the same surgeon. A complete ophthalmologic examination included measurement of best corrected visual acuity, slit-lamp biomicroscopy, tonometry, and indirect ophthalmoscopy and was performed in all patients every two to four weeks during the follow-up period, starting two weeks after transplantation. The assessments of visual outcomes were carried out by investigators who were not involved in performing the procedures and were not informed about which eye underwent transplantation or whether the assessment was preoperative or postoperative.

CULTURE AND FABRICATION OF AUTOLOGOUS ORAL MUCOSAL EPITHELIAL-CELL SHEETS

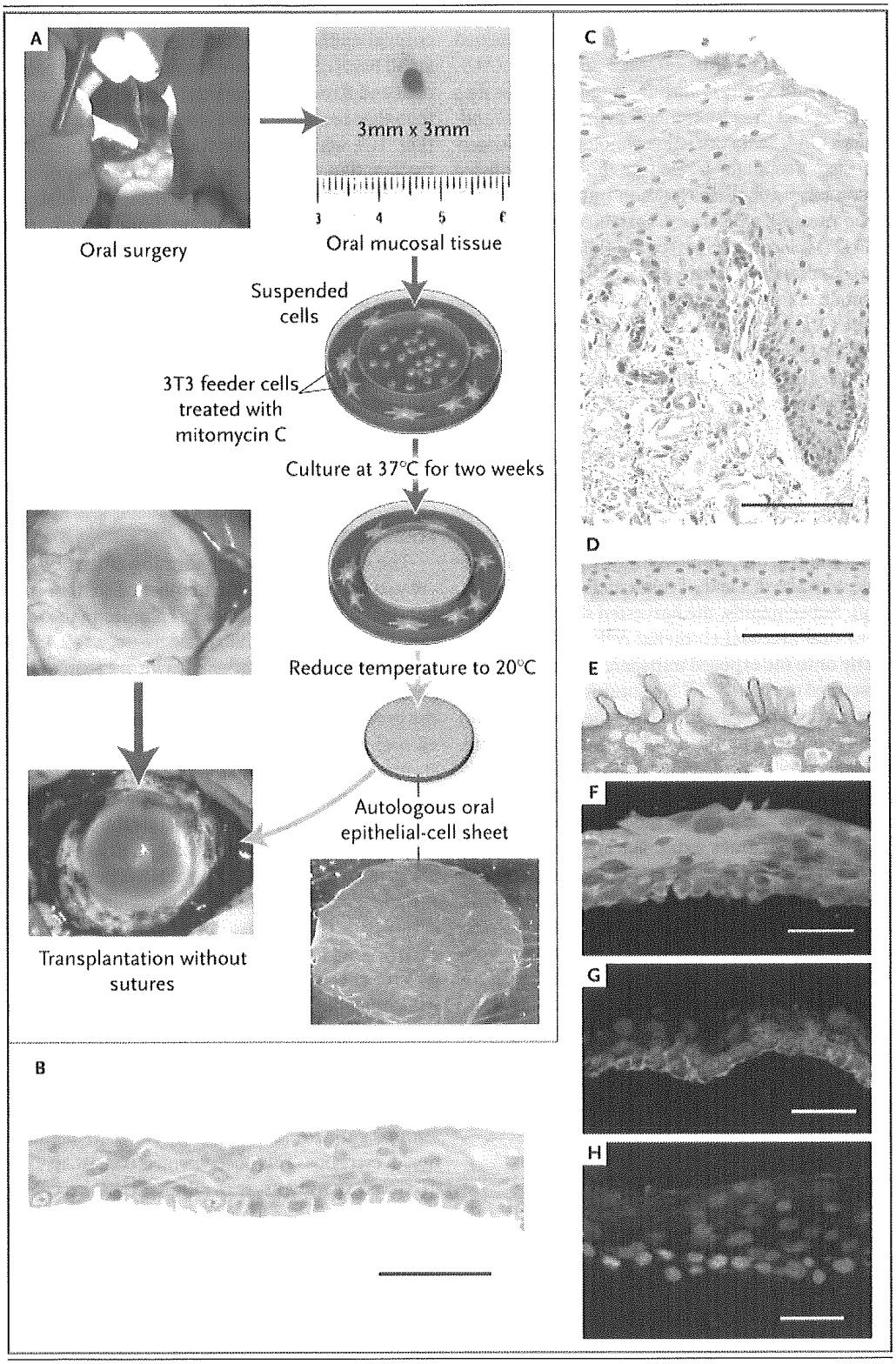
After each patient's oral cavity was sterilized with topical povidone-iodine, a 3-by-3-mm specimen of oral mucosal tissue was surgically excised from the interior buccal mucosal epithelium while the patient was under local anesthesia with xylocaine (Fig. 1A). Oral mucosal epithelial cells were collected by removing all epithelial layers after treatment with dispase II (3 mg per milliliter, Roche), at 37°C for one hour. Collected materials were placed in trypsin and EDTA for 15 minutes to form single-cell suspensions. Temperature-responsive cell-culture inserts (CellSeed) were prepared with the use of commercial cell-culture inserts (Falcon, Becton Dickinson) according to specific procedures described previously.[22] The temperature-responsive polymer poly(*N*-isopropylacrylamide), which reversibly alters its hydration properties with temperature, is chemically immobilized in thin films on cell-culture surfaces, facilitating cell adhesion and growth in normal culture conditions at 37°C. Reducing the temperature of the culture below 30°C causes this surface to hydrate and swell rapidly, prompting complete detachment of adherent cells without the use of typical proteolytic enzymes or treatment with EDTA. Confluent cell cultures on these surfaces can be conveniently harvested as a single, unsupported contiguous cell sheet, retaining cell-to-cell junctions as well as deposited extracellular matrix on the basal surface of the sheet.[23] Enzyme-free harvest permits the cell sheets to be readily manipulated, transferred, layered, or fabricated, because they adhere rapidly to other surfaces, such as traditional culture plastics,[22] other cell sheets, and tissues in vivo.[19]

To prepare lethally treated feeder layers, subconfluent NIH 3T3 cells were incubated with 16 μg of mitomycin C per milliliter for two hours at 37°C and then trypsinized and seeded onto tissue-culture wells (35-mm diameter, Becton Dickinson) at a density of 2×10^4 cells per square centimeter. Oral epithelial cells were separated from these feeder-layer cells during culture with temperature-responsive cell-culture inserts. We confirmed that multilayered cell sheets were fabricated only in the presence of 3T3 cells in the culture system. After culture in vitro for 14 days, epithelial-cell sheets (23.4 mm in diameter) were harvested by reducing the temperature to 20°C.

For colony-forming assays, treatment with trypsin and EDTA was used to isolate single cells from oral mucosal epithelium. Cells were counted, seeded onto culture dishes (35-mm diameter, Becton Dickinson), and cultured with feeder layers treated with mitomycin C. After cultivation for 10 to 12 days, dishes were fixed and stained with rhodamine B. Colony formation in the entire dish was screened under a dissecting microscope.

Figure 1 (facing page). **Transplantation of Autologous Tissue-Engineered Epithelial-Cell Sheets Fabricated from Oral Mucosal Epithelium.**

Panel A shows the removal of oral mucosal tissue (3 by 3 mm) from patient's cheek. Isolated epithelial cells are seeded onto temperature-responsive cell-culture inserts. After two weeks at 37°C, these cells grow to form multilayered sheets of epithelial cells. The viable cell sheet is harvested with intact cell-to-cell junctions and extracellular matrix in a transplantable form simply by reducing the temperature of the culture to 20°C for 30 minutes. The cell sheet is then transplanted directly to the diseased eye without sutures. In Panel B (the scale bar represents 50 μm), harvested cell sheets have three to five cell layers and do not resemble the original oral mucosa as shown in Panel C (the scale bar represents 100 μm) as closely as they resemble normal corneal epithelium as shown in Panel D (the scale bar represents 100 μm). Panel E shows a transmission electron micrograph of developed microvilli on the apical surface of the cell sheet. Specimens of human tissue-engineered epithelial-cell sheets harvested by reducing the temperature of the culture are immunostained green with anti–keratin 3 antibodies (Panel F), anti–β_1 integrin antibodies (Panel G), and anti-p63 antibodies (Panel H). The nuclei in Panels F, G, and H are shown in red. The scale bars represent 50 μm in Panels F, G, and H. The specimens in Panels B, C, and D are stained with hematoxylin and eosin.


A
Oral surgery
3mm x 3mm
Oral mucosal tissue
Suspended cells
3T3 feeder cells treated with mitomycin C
Culture at 37°C for two weeks
Reduce temperature to 20°C
Autologous oral epithelial-cell sheet
Transplantation without sutures
B
C
D
E
F
G
H