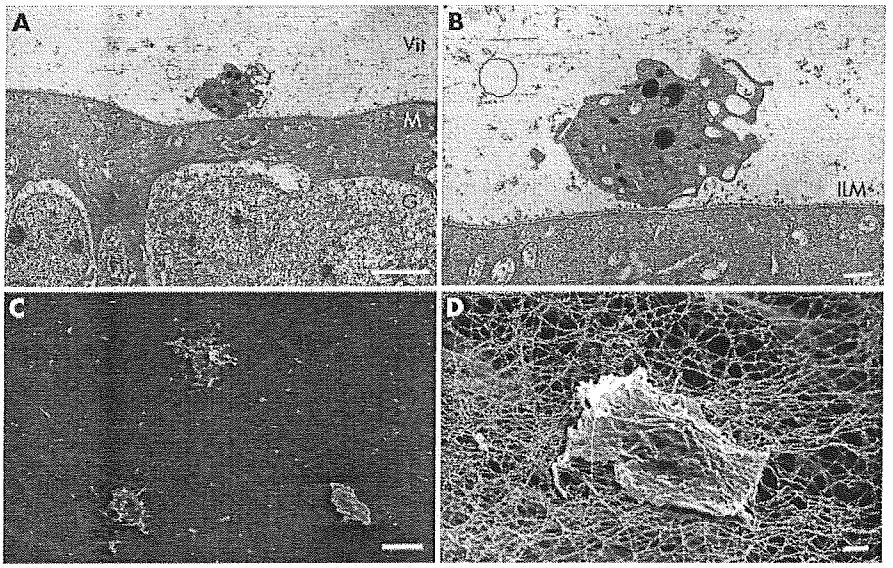

Figure 1 The morphological characteristics and the distribution of hyalocytes. TEM micrograph of a hyalocyte was distributed in the vitreous cavity close to the retina (A). Higher magnification showed the hyalocyte was completely free and close to the inner limiting membrane (ILM) of the retina (B). SEM micrograph showed three of the free hyalocytes were distributed randomly in the vitreous cortex very close to the retina, which lies in the background (C). A higher magnification view showed the cell was entangled in a collagen fibril network in the vitreous cortex and a few protuberances are observed at the cell surface (D). (Original magnification A, ×2600, bar 5 μm; B, ×6000, bar 1 μm; C, ×1100, bar 10 μm; D, ×4300, bar = 1 μm).

thickness by argon plasma coater (Eiko). Next, the retina was studied using a Jem 840 scanning electron microscope (Jeol).

Immunohistochemistry

For immunohistochemical examinations, the rat eyes were enucleated and fixed in 4% paraformaldehyde, embedded in paraffin. Anti-ED1, ED2 antibodies (Serotec, Oxford, UK), and control IgG were used at a 1:100 dilution. Anti-ED1 antibody recognises monocyte macrophage, and anti-ED2 recognises tissue macrophages in rat.[18] Propidium iodide (PI) was used for nuclear staining.

Cy5 labelled secondary antibody (Zymed Laboratories, San Francisco, CA, USA) was used at a dilution of 1:200 for 20 minutes, and then observed by fluorescence microscopy (Olympus, Tokyo).

Preparation of cell suspensions and chimeric mice

To characterise the infiltrating hyalocytes, we produced chimeric GFP mice, by a previously described method.[19] Briefly, the WT B6 mice were lethally irradiated with x ray of 9 Gy; these eyes were protected with lead shields to prevent radiation retinopathy. Then they were intravenously injected via a caudal vein with 0.2 ml of PBS containing EGFP transgenic mice BM cells ($6\times10^6-1\times10^7$). Successful BM transplantation was confirmed by the identification of GFP^+ cells in the blood 2 weeks after irradiation and then used for experiments.

In vivo cellular tracking using fluorescence microscopy

To directly observe the donor derived GFP^+ cells, the peripheral blood cells in chimeric mice were obtained and suspended in 2 μg/ml PI for identification of the nuclei in cells, they were then observed by fluorescence microscopy (Olympus, Tokyo). The eyes from WT mice were used as control eyes.

Flow cytometry analysis (FACS)

The spleen cells of the chimeric mice were obtained and determined by flow cytometry using EPICS XL (Becman Coulter, Mannheim, Germany). GFP fluorescence was measured at the same excitation/emission wavelength as FITC.

Histological analysis

The chimeric mice were sacrificed at 0, 4, 6, 7, and 12 months after BM transplantation. The eyes were enucleated, futher fixed in 2% paraformaldehyde for 3–4 hours and dehydrated in acetone for 5 minutes at 4°C, embedded in Technovit 8100 (Heraeus Kulzer, Werheim, Germany) on crushed ice and cut to 3 μm thick serial sections. The samples were observed by fluorescence microscopy (Olympus, Tokyo).

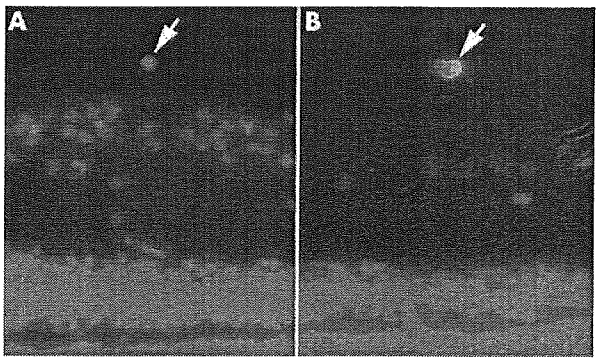

Figure 2 Immunophenotypic analysis of hyalocytes characterisation. The normal adult rat hyalocytes were double immunostained with ED1 and PI, ED2 and PI, respectively at 6 months post-transplantation. ED1 antibody, bearing the characteristic phenotype of monocyte derived macrophages, did not react with hyalocytes (PI^+, $ED1^-$) (A, arrowhead). The ED2 expression was typically associated with hyalocytes (PI^+, $ED2^+$) (B, arrowhead). (Original magnification ×400).

Statistical analysis

The number of cells per 1 mm area in 10 histological sections for each eye was measured and six eyes were analysed using analysis software (MacScope, Mitani, Fukui, Japan). The results were expressed as the means (SD). Student's *t* test was used to calculate the probability by comparing data between the groups and $p<0.05$ was considered to be significant.

RESULT

Hyalocytes

Hyalocytes were examined in rat vitreous cavity by TEM and SEM. Ultrastructural characterisation of the rat hyalocytes was performed using the electron microscope (fig 1).

Figure 1A illustrated a TEM micrograph of a hyalocyte distributed in the vitreous cavity close to the retina. The cell

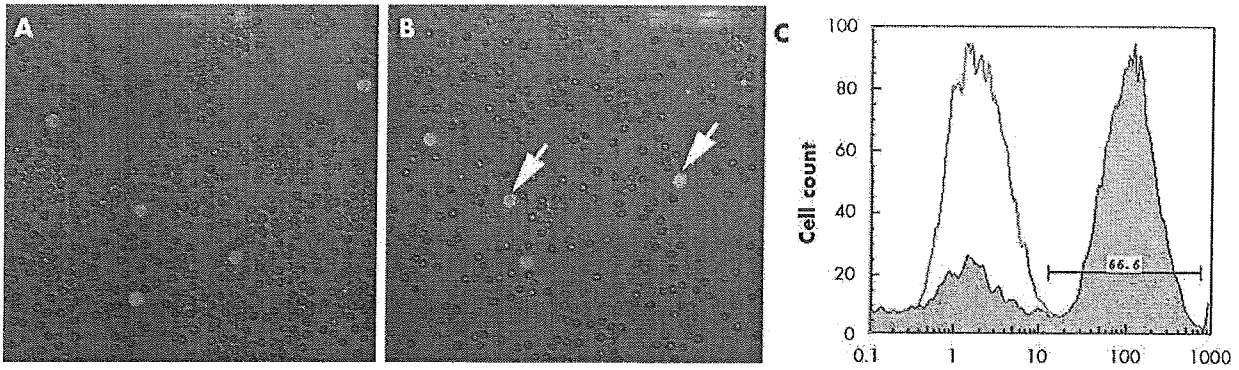

Figure 3 Fluorescein microscope analyses of peripheral leucocytes in chimeric mice. Four weeks after reconstitution, the peripheral blood leucocytes of normal C57BL/6 as a control (A). In the chimeric mice, some of the peripheral leucocytes expressed both PI^+ and GFP^+ (yellow) (B). (Original magnification ×100). Flow cytometric analyses of splenocyte chimerisms of radiation BM chimeras (C).

surface was irregular with numerous slender processes. Higher magnification of the micrograph (fig 1B) clearly showed the hyalocyte was a completely free cell separated from the inner limiting membrane (ILM) of the retina. It was surrounded by collagen fibrils in the vitreous cortex. Numerous electron dense heterogeneous contents were observed inside the hyalocyte. The cytoplasmic granules showed the morphological characteristics of lysosomes: a single membrane, a halo, and a dense homogeneous matrix.

The examination of the hyalocytes with the SEM has previously been described in the rabbit.[20] Here, we examined them in the rat. The hyalocytes revealed their complex and warty surface. In a survey view of the posterior part of the eyeball, free hyalocytes were found distributed randomly on the vitreous cortex of the retinal ILM (fig 1C). A high magnification view of the hyalocyte showed the cell was entangled in a collagen fibril network in the vitreous cortex (fig 1D). The same feature of hyalocytes was also observed in mice (data not shown).

Immunophenotypic analysis of hyalocytes expressing ED2

Immunohistochemical examinations were performed to analyse and characterise the rat vitreous hyalocytes. The adult rat hyalocytes were double immunostained with ED1 and PI, ED2 and PI, respectively. ED1 recognises an antigen in monocytes and in most macrophages, free and fixed.[21] ED2 recognises membrane and cytoplasmic antigens of tissue macrophages, discriminating between distinct subpopulations of macrophages.[21] The immunophenotypic analysis demonstrated that most of the hyalocytes were ED2 positive (90%), and few hyalocytes were ED1 positive (15%). Only 5% of hyalocyte showed both ED1 and ED2 positive staining. Antibody directed against ED1, bearing the characteristic phenotype of monocyte derived macrophages, did not react with hyalocytes (fig 2A). Antibody directed against ED2 was typically associated with hyalocytes, and illustrated that vitreous hyalocytes have the characteristic phenotype of tissue macrophages (fig 2B).

GFP^+ cells detected in BM chimeras

Four weeks after reconstitution, the peripheral blood cells and the splenocytes from the radiation BM chimeric mice were examined for GFP expression. The green light of the native GFP or fusion proteins can be detected by fluorescence microscopy or flow cytometry in living cells without fixation.[15] As a control, the peripheral leucocytes of the normal C57BL/6 mouse observed PI^+ by a fluorescein microscope (fig 3A). In contrast, in the radiation BM chimeric mice, some of the peripheral leucocytes expressed both PI^+ and GFP^+ (fig 3B). Moreover, we also obtained the chimeric mice splenocytes and analysed them by flow cytometry. After 4 weeks of BM transplatation, approximately 66% of splenocytes from chimeras contained a high density of GFP^+ (fig 3C).

Hyalocytes regenerated and totally replaced within 7 months

To further elucidate the origin and regeneration of hyalocytes, we also examined the chimeric mice at 0, 4, 6, 7, and 12 months after BM transplatation. As determined microscopically, 0 day after BM transplantation, the hyalocytes contained only PI but not GFP in vitreous cavity (fig 4A). Virtual results were observed in naive mouse vitreous (data not shown). Six months after BM transplantation, the donor derived (GFP^+) hyalocytes were readily observed in the chimeric vitreous cavity (fig 4B). Moreover, the GFP^+ hyalocytes were distributed in the vitreous cavity close to the retina as described above (fig 1). These results suggested that hyalocytes are derived from BM. By evaluating the average ratios of GFP^+ cells in total PI^+ cells in vitreous cavity, we examined the turnover period of the hyalocytes. The hyalocytes were replaced gradually after BM cells transplantation. Four months after BM transplantation, more than 60% of hyalocytes were replaced by the BM derived GFP^+ cells. Approximately 90% of total vitreous hyalocytes were GFP^+ in the chimeric mice 7 months after BM transplantation (fig 4C). These cells have a similar character and distribution to the naive hyalocytes by TEM and SEM (data not shown). Taken together, these results indicate that the rodent hyalocytes were of BM origin and regenerate gradually even under the physiological conditions.

DISCUSSION

We demonstrated for the first time that rodent hyalocytes were derived from BM cells. Hyalocytes were found in the vitreous cavity and most of the hyalocytes had the morphological characteristics of macrophage that expressed ED2, but not ED1, confirming their identification as tissue macrophages. The same immunophenotypical characterisation was also observed in murine hyalocytes by F4/80 (data not shown). By using the chimeric mice, we revealed that the BM derived hyalocytes (GFP^+/PI^+) were turned over gradually, and replaced almost all of the hyalocytes in 6 months. These results suggest that BM derived cells might differentiate into hyalocytes and turn over even under normal physiological conditions. The large number of BM derived cells lining in the vitreous cavity suggests that they make a contribution to maintaining the vitreous.

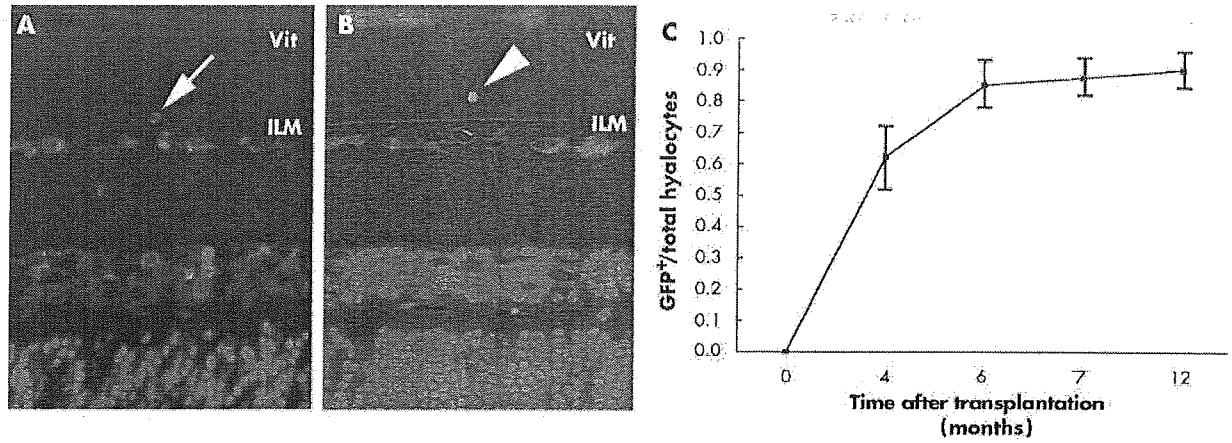

Figure 4 The kinetics of the hyalocytes. Chimeric mice were used to analyse the hyalocytes' origin and regeneration at 0, 4, 6, 7, and 12 months after BM transplantation by fluorescent microscopy. The PI^+ nuclei associated GFP^- cells, indicating recipient derived hyalocyte, were seen in the mouse right after BM transplantation (A). The donor derived (GFP^+) hyalocytes were observed 6 months after BM transplantation (B). The time dependent ratio of GFP^+ hyalocytes in the chimeric mice (C) (original magnification ×400).

We also indicated that BM derived GFP^+ cells (GFP^+/PI^+) occurred and turned over the residual host hyalocytes in the vitreous cavity with time. The kinetics of both resident and infiltrating macrophages have been thoroughly investigated in brain by GFP chimeric models.[22] The turnover of resident macrophages, however, was not revealed adequately. Several studies revealed that the BM derived cells infiltrated into the central nerve system (CNS) across the blood-brain barrier in normal condition.[23 24] Although infiltrating/GFP^+ macrophages were physiologically (without any injury or inflammation) detected in the brain of the chimeric mouse, the proportion of GFP^+ macrophages was small.[22 25] Even under the pathological coditions including demyelation and ischaemic injury, resident/GFP^- macrophages were dominant compared with GFP^+ macrophages for up to 4 weeks.[26] In contrast, we revealed that 90% of resident macrophages in vitreous cavity were physiologically replaced by BM derived macrophages for up to 7 months. Our data suggested that levels of resident macrophages might not been maintained by their proliferation but by being produced by BM in physiological situations. This discrepancy between brains and eyes remains unclear. The observation period might be one of the reasons for the discrepancy between replacement rates of tissue macrophages (CNS and retinas). In these studies, many GFP^+ cells existed around blood vessels in the CNS.[22–26] Hyalocytes examined in this study exist directly on the ILM near the vascular rich region. The location might be another possible reason of this discrepancy. Further studies should be done to check whether tissue specific mechanisms, which make circulating macrophages infiltrate and reside in the tissue, exist.

Chimeric mice stably reconstituted with BM cells represent a good model for analysis of the mechanism of BM cell infiltration in the eye. However, in preparing chimeric mice, irradiation of the recipient mice is necessary to kill their own BM before transplantation; this might induce an inflammatory response. This irradiation causes gliosis by activation of astrocytes and microglia in the brain.[22 23] We used a lead cup to prevent the eyes being damaged; we did not detect any histological changes such as gliosis and acute inflammatory responses in all retinas examined.

Although our data cannot definitively conclude a role for hyalocytes in the vitreous cavity, the inhibitory effects of vitreous hyalocytes on endothelial cell and RPE proliferation have been recently demonstrated.[10 11] Further investigations are needed to delineate the functional characterisation of hyalocytes. Increased knowledge of the origin and character of hyalocytes should improve our understanding of various vitreoretinal pathologies, such as epiretinal membrane formation, proliferative vitreoretinopathy, and diabetic retinopathy, and lead to improved methods of prevention; furthermore, this will be also an important step towards optimising regenerative therapies.

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SHORT COMMUNICATION

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Gene expression and immunolocalisation of a calcium-activated chloride channel during the stratification of cultivated and developing corneal epithelium

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Abstract The spatial and temporal localisation of a calcium-activated chloride channel (CLCA) and its mRNA was investigated, during the in vivo and in vitro development of stratified epithelia, by fluorescence immunohistochemistry and quantitative polymerase chain reaction in embryonic chicken corneas and the expansion of excised human corneal stem cells on amniotic membrane. Single-layered human epithelial cultures on amniotic membrane and early day embryonic chicken corneas expressed relatively little human CLCA2 or its chicken homologue. However, as the epithelium in both models matured and the number of cell-layers increased, the gene expression level and protein staining intensity increased, primarily within the basal cells of both the cultured and embryonic tissues. These results demonstrate that human CLCA2 protein and mRNA expression are elevated during epithelial stratification, suggesting that this protein plays a role in the growth of multi-layered corneal epithelia during both natural development and tissue cultivation.

Keywords Calcium-activated chloride channel · Corneal epithelium · Development · Immunohistochemistry · Real-time polymerase chain reaction · Chick

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Introduction

Calcium-activated chloride channels (CLCA) are members of the anion conductance family of proteins. The first distinct CLCA protein was purified from apical membranes of bovine trachea (Ran and Benos 1991) and the cloning and characterisation of the first human member (CLCA1) followed soon afterwards (Gruber et al. 1998). Several members of the CLCA gene family have now been cloned and analysed from several different mammalian species, including bovine, pig, mouse and human (Cunningham et al. 1995; Gandhi et al. 1998; Gruber et al. 1998; Gaspar et al. 2000).

The cloning of a second human homologue, CLCA2, from a human lung cDNA library has subsequently revealed additional expression in the human trachea and mammary gland (Gruber et al. 1999). The same homologue has since been found to play a key role in the adhesion of tumour cells to lung endothelia (Abdel-Ghany et al. 2001) and in the tumorigenicity of human breast cancer (Gruber and Pauli 1999; Li et al. 2004) by virtue of its specific integrin-β4-binding domain (Abdel-Ghany et al. 2003). More recently, the distribution of CLCA2 in normal human corneal epithelia has been investigated by immunogold labelling and electron microscopy and found to be predominantly expressed along the cytosolic basal aspect of the basal epithelia in close apposition to hemidesmosomes (Connon et al. 2004). Moreover, human CLCA2 is also significantly expressed in tissues with stratified epithelia and, within these tissues, has been shown to be colocalised with integrin β4 by double immunofluorescence staining (Connon et al. 2005). The human CLCA2 molecule has

been revealed to be both an ion channel and a cell adhesion molecule suggesting an intriguing multifunctional capability. However, its function within normal tissue, such as the cornea, has yet to be fully understood.

The surface of the cornea is composed of a smooth continuous stratified epithelium and serves as the frontal barrier to the whole eyeball and maintains key optical features, such as refraction and transparency. To preserve these functions the corneal epithelium is in a constant state of cell renewal, which is in part dependent upon the integrity of basement membrane, cell-cell and cell-matrix interactions. The consistency of the corneal basement membrane is also an important factor during wound healing (Fujikawa et al. 1984; Power et al. 1995; Suzuki et al. 2000) and defects to basal cell adhesion are thought to be responsible for corneal dystrophies, such as Thiel-Behnke and Reis-Bucklers' dystrophies (Ridgway et al. 2000). Previously, human CLCA2 has been shown to be the most abundant chloride channel in the corneal epithelium, implying that it has an important role in the maintenance of corneal transparency (Itoh et al. 2000). The present study has been conducted to evaluate changes in human CLCA2 expression between developing corneal epithelium undergoing stratification and the previously reported expression in quiescent corneal epithelium (Itoh et al. 2000; Connon et al. 2004). Expression of human CLCA2 in cultivated corneal epithelia and of a chick homologue of human CLCA2 in embryonic corneal epithelia has been examined. Our results show increased basal cell expression with improved stratification by using the techniques of quantitative polymerase chain reaction (PCR) and immunofluorescence staining.

Materials and methods

Embryonic chicken corneas

The use of animals in this experiment was carried out in accordance with European Commission Directive 86/609/EEC. Fertile chicken eggs were collected from a commercial hatchery (Hy-line UK, Warwickshire, UK) at days 12, 14, 16 and 18 of incubation, as confirmed by using Hamilton-Hamburger staging (Hamburger and Hamilton 1951; Hamburger 1992). Ten corneas were excised at each time-point and immediately processed for immunohistochemistry.

Cultivation of human primary cells by using corneal stem cells

The cultivation of primary human corneal epithelia (pHCLE) grown on denuded human amniotic membrane (HAM) closely followed the method described previously (Koizumi et al. 2000). Briefly, six limbal explants (the limbus forms the outer rim of the cornea and possibly contains its stem cells) of approximately 5 mm×3 mm were dissected from each donor cornea and placed epithelium-side-down onto HAM spread on 24-mm diameter polycarbonate membrane culture inserts (Transwell, Corning, New York). The corneal epithelial cells were co-cultured with mitomycin-C-inactivated 3T3 fibroblasts in a Dulbecco's modified Eagles medium and Ham's F-12 media (DMEM/F12; 1:1 mixture) containing 10% fetal calf serum (FCS), 5 mg/ml insulin, 0.1 nM cholera toxin, 10 ng/ml epidermal growth factor (EGF) and 50 IU/ml penicillin-streptomycin. Cultures were incubated for 2 weeks in submerged conditions followed by 1 week in "air-lifting" conditions in which the upper surface of the cells was exposed to the air. The medium was changed every 2 days. All incubations were carried out at 37°C in a 5% CO_2–95% air incubator.

RNA isolation, reverse transcription and real-time PCR analysis

Isolation of mRNA from pHCLE grown on HAM was achieved by scraping the cells off the HAM after its careful removal from the culture insert. Total RNA was isolated, according to the manufacturer's instructions (TRIzol Reagent; Invitrogen, Carlsbad, Calif.) and its integrity was confirmed to be intact by 1% agarose gel electrophoresis (data not shown).

The RNA was reverse-transcribed to single-stranded cDNA by using random hexamer-primed reverse transcriptase (Superscript II Rnase H-; Invitrogen). Reverse transcription (RT) PCR amplification was preformed with *Taq* DNA polymerase (Platinum Taq; Invitrogen) with the following gene-specific primers: human CLCA2, sense 5′-GATGGGAGT ACAGCTTCAAGA-3′ and antisense 5′-T TTTCCCTCTTTTCCACACC-3′; β-actin (as positive control), sense 5′-GGACTTCGAGCAAGAGATGG -3′ and antisense 5′-ATCTGCTGGAAGGTGGACAG-3′. Optimised PCR conditions were as follows: 3 cycles from 94°C to either 70°C, 68°C, 66°C, 64°C or 62°C for 30s each followed by 3 cycles from 94°C to 60°C to 72°C and then from 94°C to 58°C to 72°C for 30 s each and then 25 cycles from 94°C to 55°C to 72°C for 30 s each, finishing with 6 min at 72°C in 0.2-ml thin-walled tubes by using a Biometra T-gradient Thermoblock. Negative control reactions were obtained by performing the PCR under identical conditions except with reverse transcription reaction mixtures in which the SuperScriptII RNase H^- was replaced by an equal volume of deionised water.

Real-time PCR was undertaken with the cDNA products obtained as described above and probes against human CLCA2 and β-actin (Applied Biosystems, Calif., USA) by using a thermal profile of 50°C for 2 min, 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Real-time PCR measurements were detected by an ABI Prism 7000 (Applied Biosystems). For each of the tissues,

real-time PCR values against human CLCA2 were normalised against the corresponding values for the housekeeping gene, β-actin. Real-time PCR was repeated at least four times for each sample and the results were averaged.

Immunohistochemistry

The embryonic chicken corneas from developmental days 12, 14, 16 and 18 and pHCLE after 1, 2 and 3 weeks cultivation were embedded in Tissue Tek (Miles, Ind., USA), snap-frozen and sectioned (7 μm thick), the sections being stored at −80°C until used. The cryosections were collected on silanised glass slides and fixed in Zamboni fixative at 4°C for 10 min followed by several washes in 0.01 M phosphate-buffered saline (PBS). Non-specific antibody-binding sites were blocked by incubation of the sections for 30 min with 0.01 M PBS containing 1% bovine serum albumin, after which the sections were incubated with anti-CLCA2 antibody (×10,000 dilution) overnight at 4°C, followed by washes (3×5 min) in 0.01 M PBS.

The preparation and characterisation of the human CLCA2 polyclonal antibody has been described previously (Connon et al. 2004). Antibody cross-reactivity regularly occurs between species and the human CLCA2 genomic sequence has been identified within a recently predicted chicken CLCA gene (GI:50751359), inferring successful cross-reactivity of the human CLCA2 antibody with the chicken homologue.

The sections were incubated at room temperature for 1 h with anti-rabbit IgG antibody (×1000 dilution) conjugated to Alexa Fluor 488 (Molecular Probes, Ore., USA). After being washed with PBS, the sections were mounted in medium containing PI (Vector, Calif., USA) and examined by fluorescent microscopy (Leica, TCS SP2). Negative controls were performed by replacing the primary antibody with rabbit immune serum at the same concentration.

Results

In vitro expression of human CLCA2 in pHCLE

The qualitative level of human CLCA2 mRNA in pHCLE was investigated by RT-PCR. The pHCLE expressed human CLCA2 mRNA after 1, 2 and 3 weeks in culture (Fig. 1a). Agarose gel electrophoresis analysis demonstrated a single distinct band of the expected size for human CLCA2 and a band for β-actin from pHCLE, establishing the integrity of the RNA isolated from the cultured cells. No band was observed in the absence of reverse transcriptase in control reactions.

A quantitative analysis of human CLCA2 mRNA expression in cultured corneal epithelium was undertaken by real-time PCR. The pHCLE in culture, displayed a significant increase in human CLCA2 expression between 1 and 2 weeks, followed by a smaller, less significant increase between 2 and 3 weeks (Student *t*-test, $P=<0.03$ and 0.08 respectively; Fig. 1b).

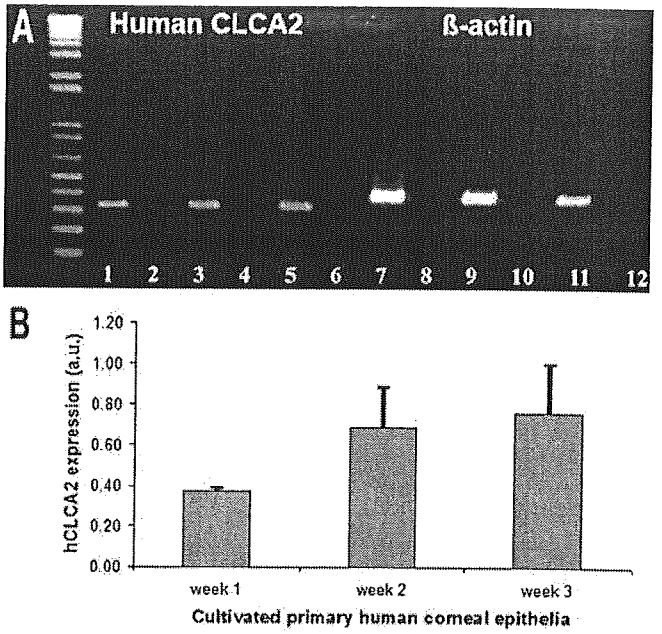

Fig. 1 Reverse transcription (RT) and real-time polymerase chain reaction (PCR) results for human CLCA2 expression in cultivated primary human corneal epithelia (pHCLE). **a** RT-PCR demonstrated qualitative human CLCA2 expression in pHCLE. PCR product bands were detected after 1 week (*lane 1*), 2 weeks (*lane 3*) and 3 weeks (*lane 5*) of culture. Corresponding product bands for β-actin were also detected (*lanes 7, 9, 11*, respectively). No PCR products were detected in the control lanes for human CLCA2 (*lanes 2, 4, 6*) or β-actin (*lanes 8, 10, 12*). **b** Real-time PCR analysis of cultivated pHCLE demonstrates a quantitative marked increase in the level of CLCA2 mRNA expression between 1-week and 2-week cultures. The final amount of human CLCA2 at 3 weeks is approximately twice that at 1 week (*error bars* standard deviation, *a.u.* arbitrary units).

Immunolocalisation of human CLCA2 in pHCLE

We investigated the in vitro expression of human CLCA2 protein by immunohistochemistry of cultivated pHCLE. Examination of the phenotype and distribution pattern of cells expressing human CLCA2 protein during the cultivation of stratified corneal epithelium on denuded HAM was carried out after 1 week, 2 weeks and 3 weeks in culture. The expression of human CLCA2 protein was not detected after a 1-week culture in which the cells were primarily distributed as a monolayer across the HAM (Fig. 2a). After 2 weeks, human CLCA2 was detected sporadically, displaying a membrane distribution pattern and being primarily associated with layered epithelial cells (Fig. 2b). Following "air-lifting", the 3-week culture was more stratified in appearance and human CLCA2 expression was located almost exclusively along the basal aspect of the basal epithelial cells (Fig. 2c). Denuded HAM and denuded HAM after removal of pHCLE following a 3-week culture period both stained negatively for CLCA2 (data not shown).

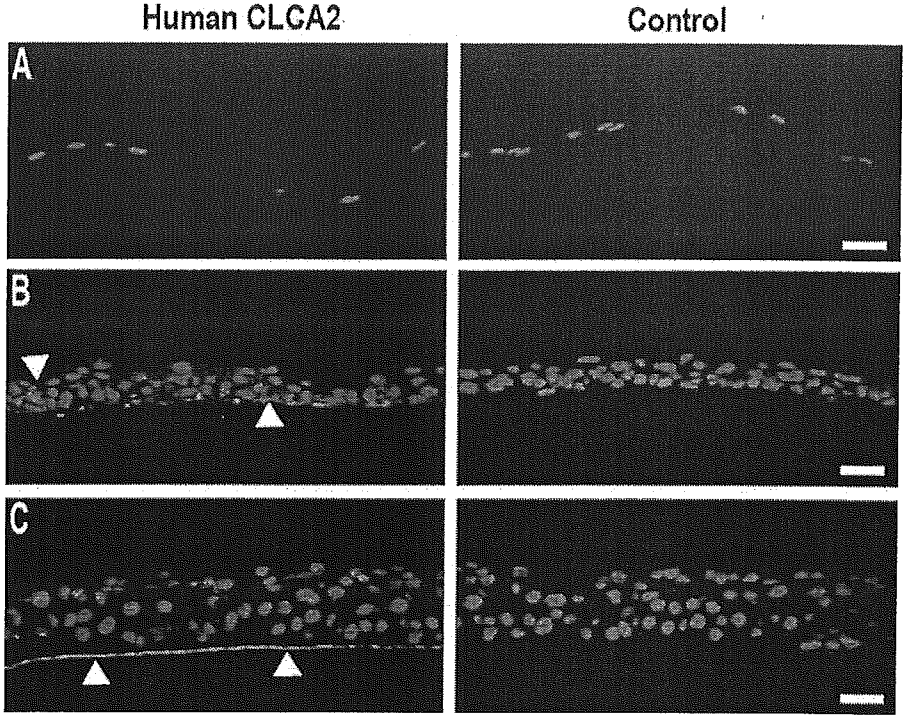

Fig. 2 Immunofluorescent labelling of anti-human CLCA2 antibody (*green*) in cultivated human corneal epithelial cells (*red*). **a** An undetectable level of human CLCA2 was noted within the 1-week monolayer. **b** After 2 weeks, the cultured epithelia had become stratified and human CLCA2 protein displayed a membrane staining pattern (*arrowheads*). **c** By 3 weeks, the cultivated epithelia were highly stratified and human CLCA2 expression had changed from a general membrane staining pattern to one that was predominantly expressed along the basal aspect of the basal epithelia (*arrowheads*). *Bars* 40 μm

Epithelial stratification and expression of a chicken homologue of human CLCA2 in the developing embryonic chicken cornea

To investigate the role of human CLCA2 during epithelial stratification further, the embryonic chicken cornea was employed as an in vivo model system. Most current knowledge about corneal development has been provided by work on the embryonic chicken by virtue of its morphological similarities to the human eye and the relative ease with which the tissue is acquired. Immunolocalisation of the chicken homologue of human CLCA2 protein was examined at days 12, 14, 16 and 18 of chicken corneal development. At developmental day 12, expression was not detected within the epithelium (Fig. 3a). Patchy expression was detected at day 14 along the basal aspect of the basal epithelial cells (Fig. 3b). By day 16, protein expression of the chicken homologue of human CLCA2 was less patchy along the basal epithelium (Fig. 3c) and, finally at day 18, was continuously expressed along the basal aspect of the basal epithelium (Fig. 3d). The epithelial thickness and stratification of the developing chicken cornea are known to increase gradually with developmental age (Hamburger and Hamilton 1951; Hamburger 1992). This increase in epithelial thickness correlated closely with the stronger staining patterns of the chicken homologue of human CLCA2 observed in the basal epithelial cells of the developing chicken cornea. Thus, these results were wholly consistent with those from the cultured human corneal epithelia in which human CLCA2 mRNA and protein expression markedly increased between monolayer and stratified conditions.

Discussion

In this study, we demonstrate, for the first time, a significant difference in the relative levels of human CLCA2 mRNA and protein expression during the stratification of corneal epithelium. First, we investigated human CLCA2 mRNA and protein expression during the progression from monolayered to multi-layered corneal epithelia by using a primary human corneal cell line containing putative corneal stem cells. Gene expression and protein levels increased markedly between the 1-week and 2-week cultures, the time at which the cultured cells started to form multiple layers. Thus, an increase in human CLCA2 expression concomitant with the formation of multiple corneal epithelial cell layers in vitro was indicated. To investigate the potential association between human CLCA2 and epithelial stratification further, the chicken homologue of human CLCA2 was immunolocalised to the secondary cornea of the embryonic chicken. As the embryonic epithelium became naturally stratified over time, the level of this homologue increased correspondingly, similar to the human in vitro results. This result provided strong evidence for the suitability of the in vitro cell culture method to reproduce in vivo developmental effects in epithelial stratification and indicated that the measured increase in human CLCA2 expression was not an artefact of tissue culture.

The preferential expression of human CLCA2 in progressively more multi-layered corneal epithelia implies a role for this gene in epithelial development and/or stratification via presently unidentified means. Previous studies into the function of human CLCA2 have suggested that it is involved with cell attachment and growth retardation.

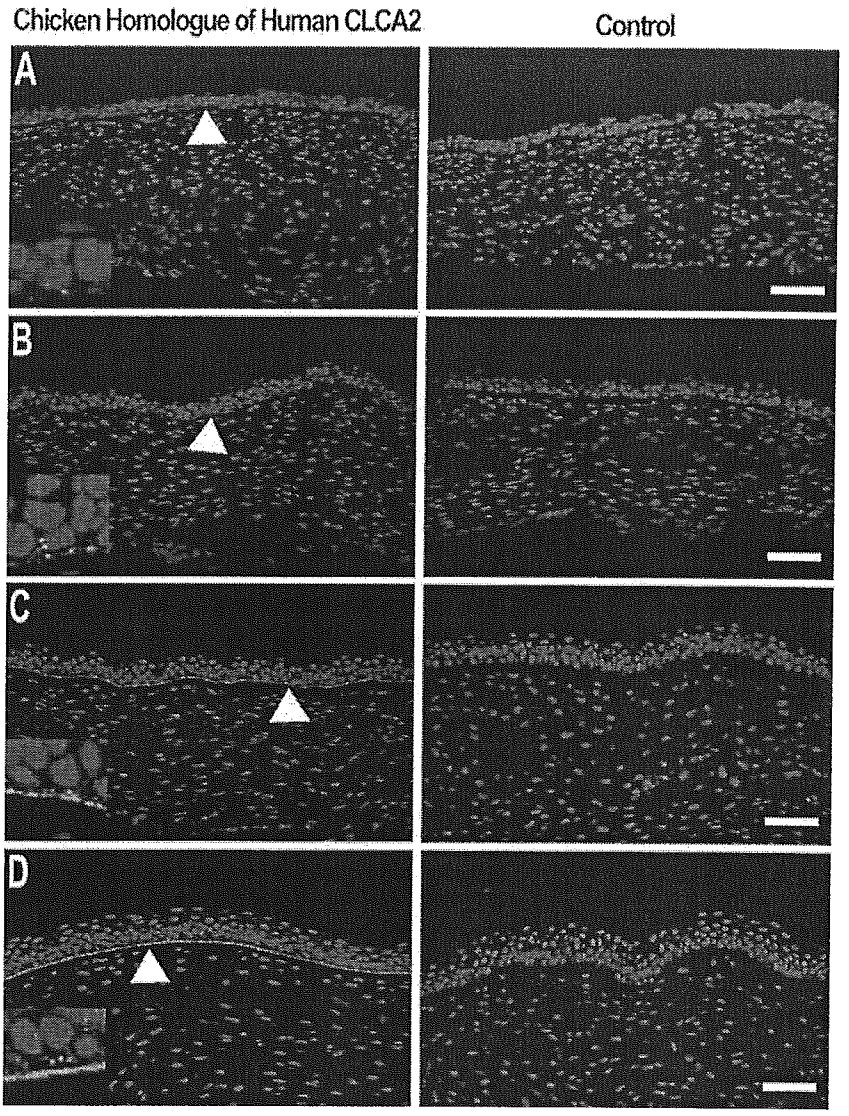

Fig. 3 Immunofluorescent labelling of the chicken homologue of human CLCA2 (*green*) in embryonic chicken corneal epithelial cells (*red*). **a** At developmental day 12, the corneal epithelium was 2–3 cells thick and the protein was undetectable. **b** By developmental day 14, the epithelium was approximately three cells thick and the chicken homologue of human CLCA2 was detected in small amounts along the basal epithelia. **c** On day 16, the epithelia had continued to stratify becoming four cells thick and the expression of the chicken homologue of human CLCA2 had also increased in the basal epithelia. **d** At developmental day 18, the embryonic chicken corneal epithelium was well-stratified (approximately 5 cells thick) resembling that of the mature chicken (data not shown). Furthermore, the staining pattern of chicken homologue of human CLCA2 was continuous and more intense along the basal aspect of the basal epithelia. *Inserts* ×5 (*arrowheads* mark their original positions). *Bars* 100 μm

Within the basal epithelial cells of normal stratified tissues, human CLCA2 has been co-localised to integrin β4 and hemidesmosomes within the cornea (Connon et al. 2004, 2005), both integrin β4 and hemidesmosomes being important for cell-matrix adhesion (Trinkaus-Randall and Gipson 1984; Stepp et al. 1990; Dowling et al. 1996). Moreover, investigation of human CLCA2 as a potential tumour suppressor has revealed its ligation to integrin β4 via a short semi-conserved motif, its loss of expression in metastatic cells and, when subsequently induced, a corresponding loss of proliferation (Gruber and Pauli 1999; Abdel-Ghany et al. 2001, 2003; Li et al. 2004). The lack of human CLCA2 expression in multiplying cells has also been noted in this study, as evidenced by its paucity in cultivated proliferating corneal monolayers. More recently, similar observations have been made during a study of the mouse orthologue, mCLCA5, which is also highly expressed in the eye (Evans et al. 2004) and whose expression in normal cells correlates with fully differentiated tissue undergoing slow or arrested growth (Beckley et al. 2004).

Human CLCA2 involvement with cell attachment suggests that its observed increase in basal epithelia during stratification can be explained by a corresponding rise in mechanical stress at the points of basal-cell-matrix adhesion, assuming a monolayered epithelium experiences lower shearing forces than its multi-layered counterpart. Interestingly, in normal and developing tissue, the basal epithelium is thought to be an area of cell proliferation; however, this region is now also associated with the expression of human CLCA2 (present results). The ability of human CLCA2 to retard cell growth, as has been shown in tumorous tissues, might also occur in quiescent or developing tissue, although its presence in an area of cell proliferation creates a possible dilemma. A potential, though speculative, explanation for this physiological conundrum could be that, during tissue growth, the expression of human CLCA2 stabilises proliferating cells by increasing their adhesion to the basement membrane, possibly via ligation to integrin β4 and/or the formation of hemidesmosomes. This, in turn, would lead to adhesion-generated

signalling resulting in differentiation and finally stratification of the tissue.

Previous studies into the function of human CLCA2 in normal tissues have concentrated on quiescent tissue, describing both the type of tissue in which it is expressed and corresponding cellular location (Gruber et al. 1999; Itoh et al. 2000; Connon et al. 2004, 2005). This investigation significantly builds upon these earlier findings by correlating human CLCA2 expression with the actual development of multi-layered tissues and highlights the importance of cell-matrix adhesion during this process. Moreover, a continued study of expression of CLCA2 in the developing corneal epithelium may ultimately lead to a better understanding of its molecular mechanisms and underlying function, potentially resulting in therapeutic treatments for corneal wound healing and disease.

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角膜再生医療

Regenerative Therapy for Cornea

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➡Key Words:
Ocular surface, stem cell, cornea, oral mucosa, substrate, amniotic membrane

Abstract

再生医学・再生医療とは，胚性あるいは体性幹細胞などを利用し生体組織を再生あるいは各種臓器機能の代替を行おうとする新しい医学研究分野である。近年，再生医学研究の波は眼科領域にも波及し，眼表面再建における角膜上皮再生の役割に関心が寄せられている。幹細胞を*in vitro*で培養し*in vivo*へ移植する培養上皮移植術は，細胞移植という新しいコンセプトを打ち立てたことになる。そのストラテジーとしては，細胞ソースの選択や組織幹細胞，基質，増殖因子などに対する知見を集積し，生体外で細胞を正常に分化・増殖させ，組織構築を図る必要がある。本稿では，実用化の始まった再生医療として，現段階での角膜再生へのアプローチの現状と課題を紹介する。

はじめに

ポストゲノム時代の21世紀に入り，人体の臓器や組織を再生しようとする再生医学，再生医療の分野はさまざまな領域で関心を集め，精力的に研究が進められている。ES細胞などの胚性幹細胞や骨髄幹細胞などの体性幹細胞等を用いて生体外(*in vitro*)で臓器，組織を再生させ移植する治療法は，従来の移植医療にかわる新しい細胞治療といえる。眼科領域，特に角膜における再生医学研究の歴史は古く，その技術開発および臨床応用への進展は，他の臨床医学領域に比べ発展し，その一部はすでに実用化がはじまっている。

1．角膜の構造

ヒトが外界から得る情報の大部分は五感の一つである視覚がつかさどっている。その中で角膜は，前眼部の1/6の面積を占める透光体組織であり，高い透明性とバリアー機能を持ち，光を屈折させて眼内に導くとともに，外界の刺激から眼球を物理的，生物学的に防御する役割を担っている。角膜の組織構造は表層から，角膜上皮層，角膜実質層，角膜内皮層の3層の細胞層に分けることができる(図1)。その最表層にある角膜上皮層は表面外胚葉由来の重層扁平上皮であり，周辺の輪部上皮や結膜上皮と一体になって眼表面を構成している。角膜実質層は，角膜全体の厚みの90%以上を占め，角膜の基本骨格を構成している。血管は存在せず，コラーゲン線維束が規則正しく配列した透明組織である。角膜内皮層は角膜実質層と同じく神経堤由来の組織であり，角膜の最後面に位置する単層の細胞層で，ポンプおよびバリアー機能により角膜内の水分含有率を適切に調節し，角膜の透明性を維持している。これらの3層構造のいずれかに病変が生じると，角膜は透明性を失い，視力低下の原因となる。従来の角膜移植は，角膜の全層を置換する全層角膜移植術が行われてきた。しかしな

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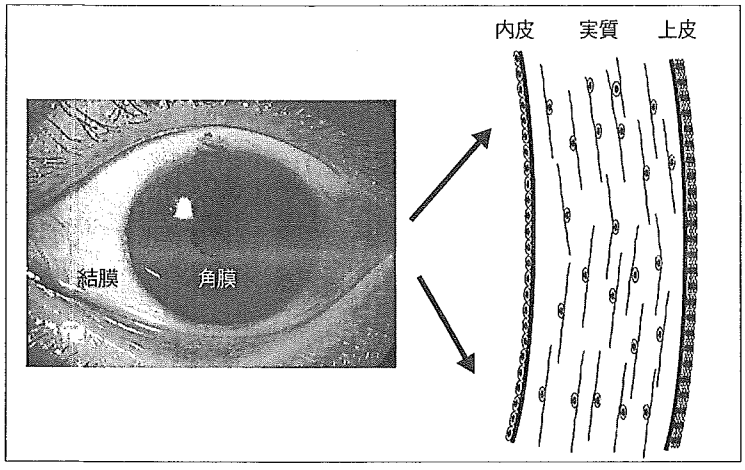

図1　ヒト角膜の模式図
眼表面は，角膜および結膜の2つの細胞群より構成されている。角膜はその表層から①角膜上皮，②角膜実質，③角膜内皮の3つの細胞層からなる。

がら，近年の再生医療技術の飛躍的な発展により，今日では障害をうけた部位のみを移植する角膜移植法が開発されている。すなわち，上皮が傷害されているケースは上皮のみを移植する，また内皮が傷害されているケースは内皮のみを移植すると言った角膜パーツ移植のコンセプトである。

■2．角膜上皮の再生

角膜上皮疾患の中で，種々の治療に抵抗性を示す疾患としてはStevens-Johnson症候群，眼類天疱瘡，熱化学外傷などの難治性眼表面疾患があげられる。難治性眼表面疾患では，角膜上皮幹細胞が広範囲に障害されて枯渇し，正常な角膜上皮を供給することができず，周辺の結膜上皮が炎症，瘢痕などを伴って角膜表面を覆い，著しい視力障害をきたす。これらの疾患に対する基本的な治療戦略としては，正常な角膜上皮細胞（幹細胞を含む）で角膜を再建する必要性がある。

1）*in vitro*から*in vivo*へ －培養角膜上皮移植術の開発

米国と異なり我が国では慢性的な角膜のドナー不足の問題があり，再生医療学的見地からは必要とする角膜移植片が少量であれば理想的である。そこで難治性眼表面疾患に対する眼表面再建術として近年注目を集めているのが培養角膜上皮移植術である。必要とする細胞を少量採取して生体外(*in vitro*)で培養上皮シートを作成し，*in vivo*へ移植するというコンセプトは，これまでの角膜移植の長い歴史の中で細胞移植(cellular surgery)の分野を確立したといえる（図2）。その先駆けとなったのは，1997年Pellegriniらによる*Lancet*誌での臨床報告である。この発表以降，培養角膜上皮移植に関する基礎および臨床研究が世界的に精力的に行われた。これまでその培養基質には，生体材料由来の羊膜[1,2]，滅菌処理された凍結乾燥羊膜[3]，生体吸収性高分子のフィブリン[4]，また温度応答性培養皿を用いた手法などが報告されている[5]。いずれの培養角膜上皮シートに関しても，臨床応用後の長期における比較試験による手術成績の評価，及び疾患や症例に応じた術式の選択が必要であると思われるが，現在世界的に他施設での有効性のコンセンサスが得られている基質としては羊膜があげられる。

2）アロからオートへ －培養口腔粘膜上皮移植術の開発

難治性眼表面疾患の大部分が両眼性であるため，その治療法としてはアロ培養角膜上皮移植が行われてきた。しかしアロ移植ゆえに術後多量の免疫抑制剤を長期にわたり使用する必要があり，拒絶反応や感染症が術後成績に大きく影響を与えているのが現状であった。これらの疾患に対しては可能であれば拒絶反応の危険性のない自己（オート）移植の開発が理想的である。以上の観点から，眼表面以外の自己粘膜上皮の中で，角膜型ケラチン3を保持する口腔粘膜上皮が着目され，羊膜を基質

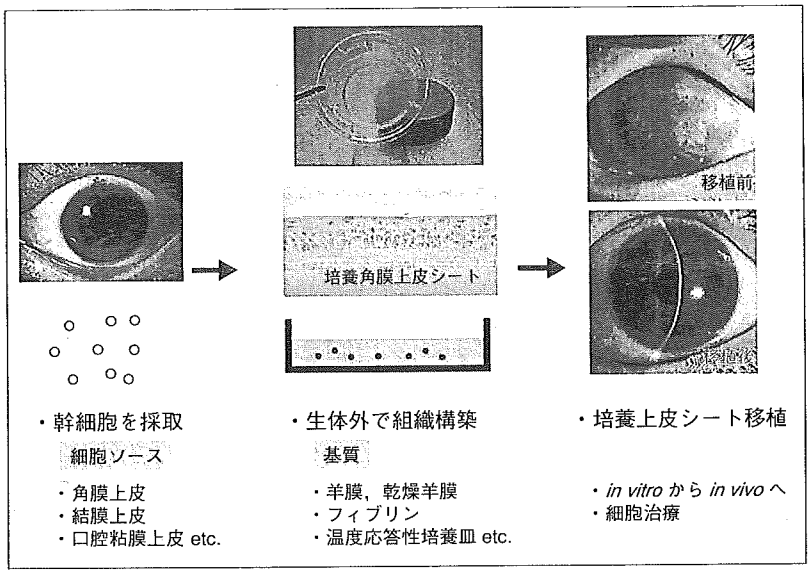

図2　角膜再建に対するコンセプト
増殖能力の高い幹細胞等を細胞ソースとし，適切な基質を用い組織工学の技術を応用して*in vitro*で生体に近い組織構造をもった培養上皮シートを作成し，*in vivo*へ細胞移植を行う。

に用いた自己の培養口腔粘膜上皮シートによる眼表面再建術が開発された。家兎での動物実験レベルでその有効性が確認され[6]，2002年より，急性期および慢性期の難治性眼表面疾患に対するヒトへの臨床応用が開始された（図3）[7]。また培養の基質には羊膜のほかに，温度応答性培養皿を用いた手法が報告され，慢性期のStevens Johnson症候群，眼類天疱瘡の臨床試用においても上皮修復が可能であることが報告された[8]。但しいずれの手術法でも臨床経過の中で周辺からの血管新生を生じる症例が多いのが現状である。今後，培養口腔粘膜上皮シートの眼表面での生着性・細胞動態等を長期にわたり注意深く経過観察し，本手術法の適応，治療効果などを検証していく必要がある。

■3．角膜実質の再生

角膜実質に関して，現在臨床応用可能な再生医療学的アプローチは少ない。実質再生のための素材開発として，いわゆる，細胞成分を含まない生体適合性を高めた人工角膜の開発の歴史は50年以上にも遡る。現在使用されている合成素材としてはPMMA (polymethyl methacrylate)などを用いたプロトタイプが開発されているが，術後眼内炎，感染などの合併症を併発する確率が高く，また学術的な報告も少ないため，その有効性について論じ難い面がある。このような合併症を克服するため，人工角膜の組織適合性を高める目的で，自己の歯根部を利用した人工角膜(osteoodonto-keratoprosthesis)も開発されている。しかし，この方法では二度にわたる技術的難易度の高い手術が必要であるため，一般に普及するには至っていない。一方，角膜実質細胞をコラーゲン，コンドロイチン硫酸，ゼラチンなどの細胞外マトリックス中で培養して再生しようとする試みも行われている。しかし，臨床応用に向けて，移植に耐え得るだけの力学的強度や透明性の保持，血管新生や神経再生の点など，越えるべきハードルは依然高い。

■4．角膜内皮の再生

角膜内皮細胞はヒトの生体内では増殖せず，創傷時には周辺の細胞が拡大して創傷治癒される。よって，角膜内皮細胞は障害されるとともにその細胞数は減少し，内皮機能不全による水疱性角膜症をきたす。この水疱性角膜症は現在の角膜移植適応疾患の第一位であるため，角膜内皮の再生は非常に重要な研究課題である。近年，生体内では増殖しないとされた内皮細胞は，適切な細胞外基質と増殖因子を用いることによって生体外で増殖可能であることがわかってきた。臨床応用をふま

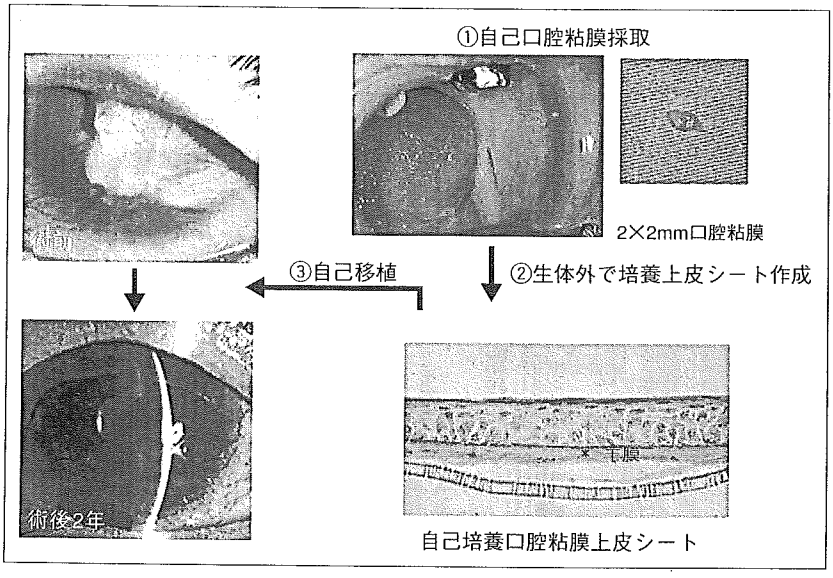

図3　自己培養口腔粘膜上皮移植術の臨床応用例
①厳密な口腔内管理後，培養に必要な少量(2×2mm)の口腔粘膜を採取。②羊膜上で培養口腔粘膜上皮シートを作成。培養口腔粘膜上皮シートは5-6層の重層粘膜上皮を形成し，*in vivo*の角膜上皮層と類似した組織像を示す。③術前，角膜は瘢痕を伴う結膜侵入により高度の視力障害が認められるが，自己培養口腔粘膜上皮移植後2年，周辺からの血管侵入を認めるものの，眼表面は上皮欠損なく安定している。

えた現状の解決すべき点としては，いかにして培養角膜内皮細胞の細胞密度を保つか，またどのようにして*in vitro*で作成した角膜内皮細胞シートを*in vivo*に持ち込むかといった点である。現段階で報告されている培養基質としては，羊膜[9]，コラーゲンシート[10]などがあげられ，臨床応用へ向け本格的なこのラインの研究がすすめられている。

■　おわりに

近年の再生医療研究の進歩は，角膜移植，特に角膜上皮再建の歴史に大きな革新をもたらした。*in vitro*から*in vivo*への細胞移植治療は，傷害されている部位のみを再建する角膜移植法へのパラダイムシフトを起こした。現行の課題としては，各種の培養上皮移植の臨床成績を詳細に検討する必要性がある。また恒久的な再建を目標とした場合，移植シートにどの程度幹細胞が存在するかなどの組織幹細胞に関する知見を集積する必要性がある。さらに今後このラインの再生医療を行う上で，本術式の安全面と倫理面の問題にも気を配る必要性がある。特に培養過程で使用する血清やfeeder cellの問題など，科学的根拠に基づく再生医療を開発していくのが我々の責務である。

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角膜再生医療の現状

Regenerative therapy for cornea

Keywords
眼表面，角膜上皮
結膜上皮
口腔粘膜上皮
羊膜
幹細胞

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Summary

Cornea is an avascular, transparent structure consisting primarily of 3 layers, the corneal epithelium, stroma, and endothelium. In cases of corneal epithelial stem cell deficiency, corneal epithelial replacement using a tissue engineering technique holds much promise for ocular surface reconstruction. Autologous cultivated corneal epithelial stem cell sheets are the safest and most reliable epithelial sheets we can use for such treatment, however, they are not useful for treating bilaterally affected ocular surface disorders. In order to treat these bilateral cases, we must choose either an allogeneic cultivated corneal epithelial sheet or an autologous cultivated oral mucosal epithelial sheet. In the case of the former, the threat of immunological reaction must be dealt with. Therefore, it is imperative that we have a basic understanding of the immunological aspects of ocular surface reconstruction using allogeneic tissues. In the case of the latter, a basic understanding of ocular surface epithelial biology is required as the sheet is not exactly the same as corneal epithelium. Studies on corneal stroma and corneal endothelium regeneration are currentry in progress all over the world.

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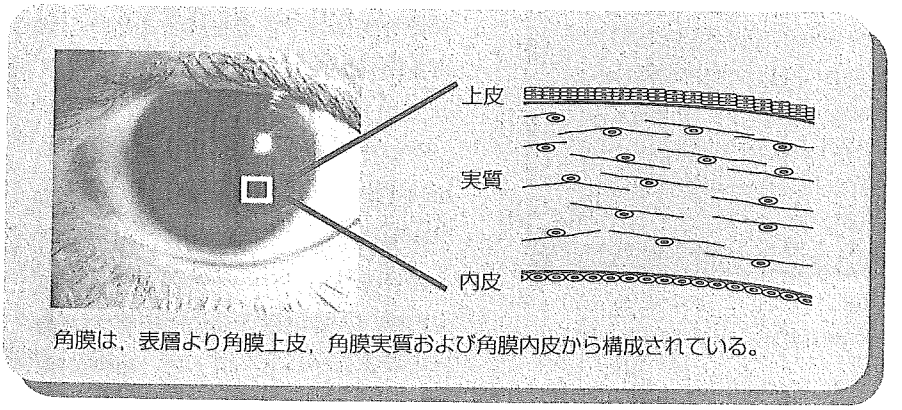
はじめに

“再生”とは，失われた生体の一部が再び作られることである。科学的に述べれば，“生体で失われた細胞，組織が分裂能力を有した特殊な細胞（幹細胞，前駆細胞など）の分化，増殖により修復され，元の状態へと回復する現象”ともいうことができる。わが国では，提供者（ドナー）の絶対的不足という現状もあり，必要な細胞，組織，臓器を生体外で大量生産するといった再生医学的研究に大きな関心が寄せられている。そして，バイオテクノロジーの飛躍的な進歩に支えられた近年の再生医療・再生医学研究の発展には目を見張るものがあり，特に眼科領域における再生医療の技術開発および臨床応用への進展は，他の臨床医学領域に比べ進んでいるといえる。眼科領域における研究対象としては，前眼部領域（角膜疾患）と後眼部領域（網膜変性

症，視神経疾患）に大きく分けることができる。本稿では，我々の研究グループが精力的に関与している角膜の再生医療・再生医学の現状と課題について述べる。

眼と角膜の構造

我々人類は，さまざまな日常生活の局面で，視・聴・嗅・味・触覚の五感を体感し，生命活動を営んでいる。その中で，ヒトが外界から得る情報の大部分は視覚から得ていると考えられており，視覚は極めて重要な機能を担っているといえる。視覚をつかさどる角膜は，直径約12mm，厚さ約500μm，前眼部の1/6の面積を占める透光体組織であり，高い透明性とバリアー機能をもち，眼球を外傷や病原菌の侵入など外界の刺激から，物理的，生理的に保護する役割を果たしている。角膜組織は，上皮，実質，内皮の3つの細胞層からなり，各細胞層が相互間でネットワーク機能を果たし，角膜の透明性・恒常性維持を担っている（図1）。角膜上皮は表面外胚葉由来の非角化重層扁平上皮で，周辺の角膜輪部上皮や結膜上皮と一体になって眼表面を構成している。角膜実質は，角膜全体の厚みの約90％以上を占めるいわば骨格部分であり，主として角膜実質細胞と細胞外マトリックスであるコラーゲンやプロテオグリカンから構成され，高い透明性を維持している。角膜内皮は角膜実質と同じく神経堤由来の細胞であり，角膜の最後面に位置する1層の細胞層で，ポンプ機能により角膜内の水分含有率を適切に調節している。角膜のいずれかの細胞層に不可逆性の変化により混濁が生じた場合，根本的な再建策としては角膜移植術が必要となる。従来は角膜の全層を置換する全層角膜移植術がスタンダードであったのに対し，近年では障害を受けた部位のみを移植する，侵襲の少ない角膜パーツ移植術が考案・開発されている。



角膜は，表層より角膜上皮，角膜実質および角膜内皮から構成されている。

図1　角膜の構造

ocular surface（眼表面）と幹細胞

1977年，Thoftにより，角膜，結膜およびその境界部位の角膜輪部などの表面外胚葉由来の粘膜上皮を包括的にまとめて“ocular surface（眼表面）”としてとらえていこうという概念が提唱された。それ以降，角膜上皮幹細胞を含むocular surfaceに関するさまざまな細胞生物学的知見が明らかとなった。一般的に幹細胞の定義としては，①長期増殖能，②自己複製能をもち，③自身とは異なる前駆細胞や分化した細胞を作る能力をもつことなどがあげられる。角膜上皮幹細胞に関しては，①角膜上皮に特異的な細胞骨格蛋白ケラチン3/12陰性の細胞群が角膜輪部基底層に存在する[1]，②この細胞がslow-cycling cellであり[2]，③*in vitro*の条件でも高い増殖能を保持する[3]，といったさまざまな状況証拠から，角膜輪部の基底層に存在すると考えられている（図2）。角膜輪部には，茶色の色素沈着を伴う放射状の柵状構造［palisade of Vogt（POV）］が認められる。臨床的に，このPOVの消失はすなわち，角膜上皮幹細胞の消失を示唆する重要な所見である[4]。角膜上皮幹細胞は，定常状態では非常にゆっくりとした細胞周期で角膜上皮を供給している。そして創傷治癒など，必要時にすみやかに分裂増殖し，角膜上皮基底細胞（transient amplifying cell：TA cell）を供給する。角膜輪部で増殖した角膜上皮幹細胞は，一部は幹細胞と

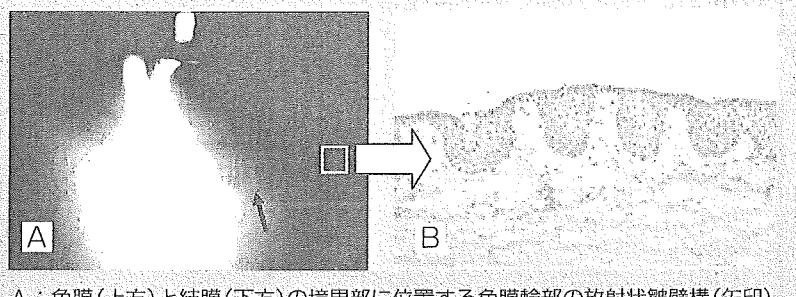
してそのまま輪部にとどまり，残り（TA cell）は角膜周辺部から角膜中心部に向かって分化・増殖する。この細胞動態は，XYZ理論として提唱され，X：角膜上皮基底細胞の分裂，Y：角膜周辺部から角膜中央部への移動，Z：角膜上皮表層細胞の脱落とし，定常状態ではX＋Y＝Zとして，角膜上皮細胞の供給と脱落の均衡が保たれている（図3）[5]。角膜上皮幹細胞が広範囲に喪失した場合，正常な角膜上皮細胞を供給することが不可能となり，周辺の結膜上皮が炎症，瘢痕，血管新生などを伴って角膜表面を覆い，不可逆性の視力障害をきたす。この角膜上皮幹細胞の異常をきたす疾患は“難治性眼表面疾患”と呼ばれ，その病態と治療法の開発にさまざまな研究者が挑んできた。

角膜上皮の再生医療

難治性眼表面疾患には，Stevens-Johnson症候群，眼類天疱瘡，熱化学外傷などがあげられる。これらの疾患に対する基本的な治療戦略としては，正常な角膜上皮細胞（幹細胞を含む）で角膜を再建する必要性がある。その中には，大きく分けて，①*in vivo*の組織をそのまま移植して再建する角膜上皮形成術[6][7]や角膜輪部移植術[8]，②*in vitro*（生体外）で適切な組織幹細胞を用いて培養上皮シートを作製し，移植しようとする培養上皮移植術の二つに分けられる（図4）。前者は，移植片からの再生上皮による角膜再建であり，広い意味で第一世代の角膜上皮の再生医療と位置付けることができる。一方本稿では，近年開発された第二世代の角膜上皮の再生医療である，培養上皮移植を中心に解説する。

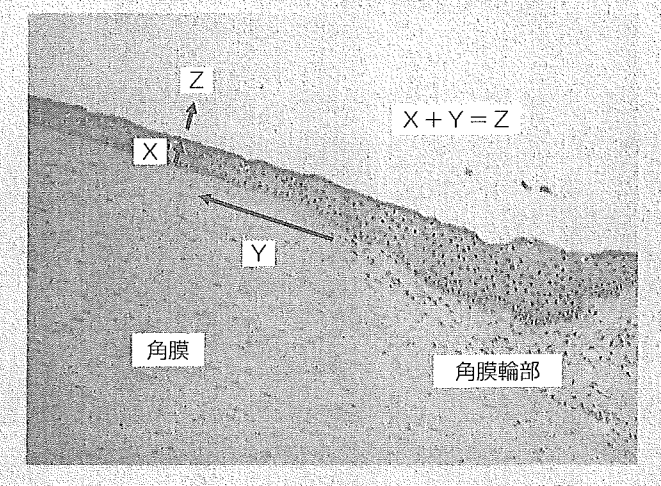
1）培養角膜上皮移植術

米国と異なり，わが国では移植片が慢性的に不足しており，近年の再生医療学的見地からは組織片を少量採取して*in vitro*で培養して増殖させ，*in*



A：角膜（上方）と結膜（下方）の境界部に位置する角膜輪部の放射状皺壁構（矢印）に角膜上皮幹細胞が存在すると考えられている。
B：角膜輪部の組織像（HE染色）。乳頭状の構造を示し，その基底細胞の一部が角膜上皮幹細胞であると推測される。角膜中央部の上皮にはこのような構造はみられず，4～5層の重層扁平上皮を示す。

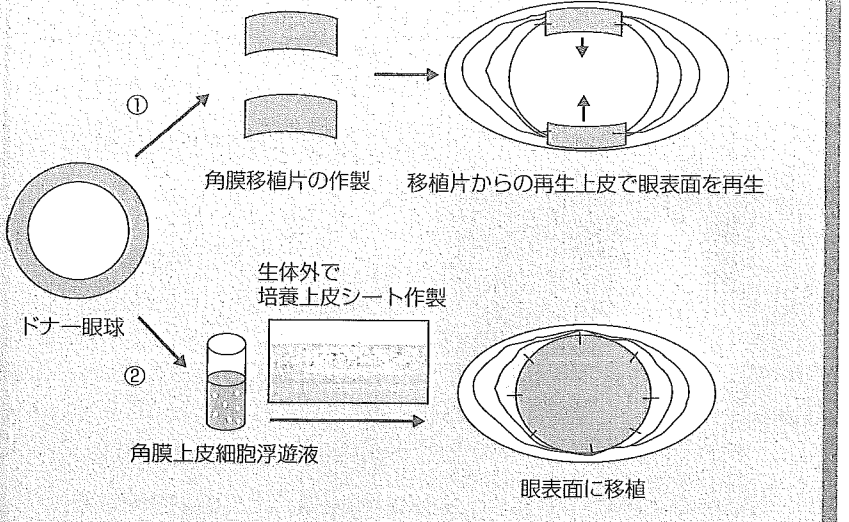
図2　角膜輪部の構造（⇒巻頭Color Gravure参照）



角膜輪部の基底部に存在する角膜上皮幹細胞は，角膜中央部へ求心性の動きをして角膜上皮を供給し，その恒常性を保っている。

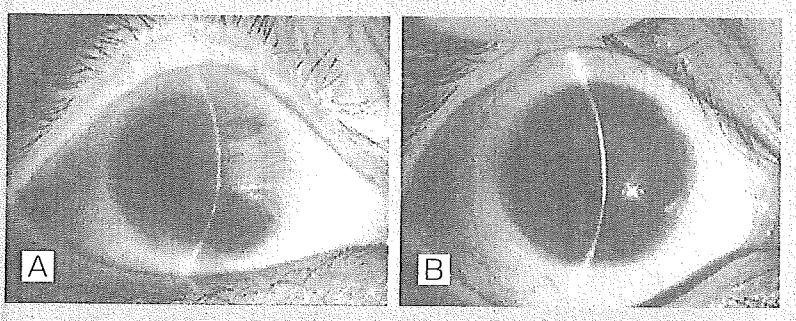
図3　XYZ理論の模式

vivo へ移植する手法が理想的である。1997年，イタリアのPellegriniらにより培養上皮移植の臨床例がはじめて報告されて以降[9]，この分野の治療法の開発が精力的に行われた。*in vitro* の条件で再生組織を作製するためには，幹細胞の性質を保持し，かつ細胞が分化・増殖しやすい環境を整える必要がある。特に，難治性眼表面疾患では，上皮の再建のみならず，細胞外マトリックスを含めた基質の正常化が必須であると考えられる。現在，その応用が報告されている基質としては，生体材料の羊膜[10)-12)]や滅菌凍結乾燥羊膜[13)]，生体吸収性高分子のフィブリン[14)]，また基質を用いない培養方法として温度感受性培養皿などがあげられる[15)]。その中で，現在世界的に他施設でのコンセンサスが得られている基質としては羊膜があげられる。



①*in vivo* expansion法。ドナー眼球より角膜移植片を作製し，眼表面へ直接移植する。移植片からの再生上皮で，眼表面を再建する。
②*ex vivo* expansion法。ドナー眼球より少量の細胞を採取し，生体外で培養して上皮シートを作製し，眼表面へ移植する。移植直後から眼表面は上皮で再建することが可能である。

図4　角膜上皮再生に対するストラテジーの模式図



A：術前，角膜は瘢痕化と血管侵入を伴った結膜侵入を認め，視力障害をきたしている。
B：術後1年，角膜はアロの培養角膜上皮シートで欠損なく再建されている。

図5　培養角膜上皮幹細胞移植術の臨床応用例（慢性期Stevens-Johnson症候群）
（⇒巻頭Color Gravure参照）

治療手法としては，片眼性疾患の場合は，健眼より輪部組織を少量採取して培養角膜上皮シートを作製し，患眼に移植する自己培養角膜上皮移植術が，術後の拒絶反応などもなく理想的である。これまで多施設での臨床応用例が報告されている[9) 10) 16) 17)]。両眼性疾患では，自己輪部組織は利用できないため，ドナー眼球による他家（アロ）培養角膜上皮移植術を選択することとなる[11) 12)]。台湾，米国，インド，日本をはじめ，世界の多施設で臨床応用が報告されており，組織採取や培養方法から移植，術後管理に至るまで安定した術式となっている（図5）。特に羊膜を基質に用いた本術式は，従来外科的手術が禁忌と考えられてきた急性期の症例に対して，培養上皮シートによる速やかな眼表面の上皮化により，炎症，瘢痕を抑制し，眼表面再建が可能

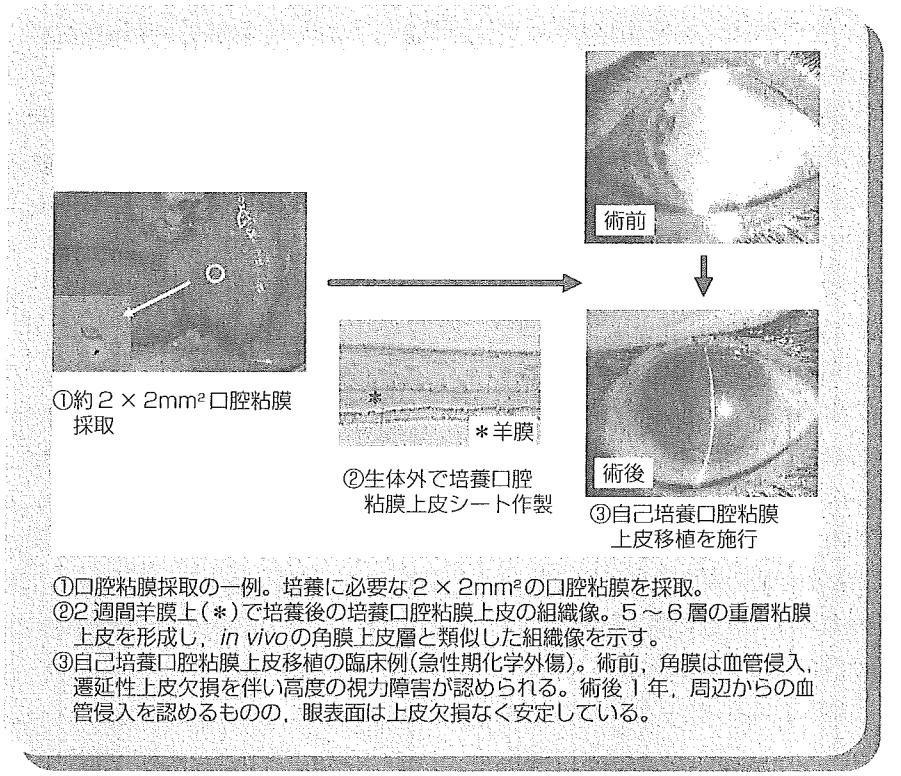
であることや，術後混濁が生じた場合に，シートによる再移植が安全で容易に行えることなどの利点があげられる[18)19)]。

2）培養口腔粘膜上皮移植

難治性眼表面疾患に対する外科的再建法としては，これまで主に前述の輪部移植や海外ドナー角膜を用いたアロ培養角膜上皮移植術が行われてきた。しかし，いずれもアロ移植ゆえに術後多量の免疫抑制剤を長期にわたり使用する必要があり，拒絶反応や感染症が術後成績に大きく影響を与えているのが現状であった。難治性眼表面疾患の大部分が両眼性であるため，自己の角膜組織は利用することはできないが，恒久的再建を目標にするのであれば，拒絶反応の危険性のない自家（オート）移植の開発が理想的である。以上の観点から，眼表面以外の自己粘膜上皮の中で，口腔粘膜上皮に着目し，自己の培養口腔粘膜上皮シートを用いた眼表面再建術が開発された。我々の施設では，家兎での動物実験レベルでその有効性を確認し[20)-22)]，2002年より，急性期および慢性期の難治性眼表面疾患に対するヒトへの臨床応用を開始した（図6）[23)24)]。また羊膜のほかに，温度感受性シートを用いた本手術法で，慢性期のStevens-Johnson症候群，眼類天疱瘡の臨床試用においても上皮修復が可能であることが報告された[25)]。ただし，いずれの手術法でも臨床経過の中で周辺からの血管新生を生じる症例が多く，拒絶反応は回避できるが，培養上皮シートの生物学的性状の違いや疾患による差異など，検討すべき課題は多い。我々の施設での長期観察例では，術後30ヵ月経過してもなお，眼表面は培養口腔粘膜上皮シートで再建されていることから，少なくともこの手術法により，異所性粘膜である口腔粘膜上皮幹細胞が眼表面で生着可能であることがわかった。今後，培養口腔粘膜上皮シートの眼表面での生着性・細胞動態などを長期にわたり注意深く経過観察し，本手術法の適応，治療効果などを検討していく必要がある。

角膜実質，角膜内皮の再生医療

角膜上皮とは異なり，角膜実質再生に対する臨床応用可能な再生医療学的アプローチは少なく，いまだに研究段階を逸脱できていない[26)]。角膜実質の材料開発としては，細胞成分を含まない生体適合性を高めた人工角膜が開発されている。現在使用されている素材としては，PMMA（polymethyl methacrylate）やePTFE（polytetrafluoroethylene）などのプラスチック系合成ポリマーを用いたプロトタイプが開発されているが，術後眼内炎，感染，



①口腔粘膜採取の一例。培養に必要な2×2mm²の口腔粘膜を採取。
②2週間羊膜上（＊）で培養後の培養口腔粘膜上皮の組織像。5～6層の重層粘膜上皮を形成し，*in vivo*の角膜上皮層と類似した組織像を示す。
③自己培養口腔粘膜上皮移植の臨床例（急性期化学外傷）。術前，角膜は血管侵入，遷延性上皮欠損を伴い高度の視力障害が認められる。術後1年，周辺からの血管侵入を認めるものの，眼表面は上皮欠損なく安定している。

図6　自己培養口腔粘膜上皮移植の模式図（➔巻頭Color Gravure参照）

脱落などの合併症を併発する確率が高く，その適応には議論の余地が残る。最近では，合成ポリマーと天然ポリマーを組み合わせて生体適合性を高める試みも施行されており，このラインの研究は発展していくものと思われる。また，このような合併症を克服し，人工角膜の組織適合性を高める目的で，自己の歯根部を利用した人工角膜移植法（osteoodonto-keratoprosthesis）が開発され，国内でも臨床応用されている。本術式では劇的な視力改善が得られるが，二度にわたる技術的難易度の高い手術が必要であるため，一般に普及するには至っていない。一方，角膜実質細胞をコラーゲン，ゼラチン，コンドロイチン硫酸などの細胞外マトリックス中で培養して再生しようとする試みも行われている。しかし，臨床応用に向けて，その力学的強度や透明性の保持，血管や神経再生の課題など，移植可能な素材を確立するために越えるべきハードルは依然高い。

ヒトの角膜内皮細胞は生体内では増殖せず，内皮細胞が傷害を受けた場合，周辺の細胞が拡大する創傷治癒過程によって補われる。よって，角膜内皮細胞は傷害されるとともにその細胞数は減少し，最終的には内皮機能不全による水疱性角膜症をきたす。この水疱性角膜症はわが国の現在の角膜移植適応疾患の第一位であるため，角膜内皮の再生の潜在的需要は非常に高い。従来，これらの疾患に対して，全層角膜移植が施行されてきたが，その手術成績は他の角膜疾患に比べて決して満足できるものではなかった。近年，生体内では増殖しないとされた内皮細胞は，適切な培養環境（細胞外基質と増殖因子）を用いることによって生体外で増殖可能であることがわかってきた。角膜パーツ移植のコンセプトのもと，培養角膜内皮移植の臨床応用が現実味を帯びつつある。現段階での課題としては，いかにして角膜内皮細胞培養法を安定化させ，培養角膜内皮細胞の細胞密度を高く保つか，またどのようにして侵襲なく *in vitro* で作製した角膜内皮細胞シートを *in vivo* に持ち込むかといった点である。これまで報告されている培養基質としては，羊膜[27]，コラーゲンシート[28]，温度応答性培養皿などがあげられ，臨床応用へ向け本格的なこのラインの研究がすすめられている。また，神経幹細胞などで用いられているスフィア法による角膜内皮細胞の培養過程での工夫や，その幹細胞に関する知見も散見されはじめている。

今後の展望と課題

近年の再生医療・再生医学研究の進歩は，長い角膜移植の歴史にも大きな転機をもたらした。*in vitro* から *in vivo* への組織幹細胞を用いた細胞移植治療は，従来型の全層角膜移植から，傷害されている部位のみを置換する角膜パーツ移植法へのパラダイムシフトを起こした。しかし，角膜の再生と一口に言っても，生体内で比較的単純な組織であるにもかかわらず，ようやくその上皮の再生に手が届きはじめているのが現状であり，再生医療の可能性と限界をよくよく見定めなければならない。現在の角膜再生の課題としては，特に培養上皮移植の長期における臨床成績の検討を詳細に行うとともに，培養上皮シートの生物学的特徴を分析する必要性がある。それには，角膜上皮や口腔粘膜上皮などの組織幹細胞やその環境（ニッチ）の役割に関する知見を集積すると同時に，その幹細胞を保存し，マスターセルとして利用する研究も必要になると思われる。また本術式の安全面と倫理面に対する配慮も必要であり，培養過程で使用する血清やフィーダー細胞の問題など，科学的根拠に基づく医療を実現させるのが我々医療にたずさわる者の責務である。

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