博士論文

論文題目 Development of corneal epithelial cell sheets using limbal stem cells for corneal regenerative therapy in dogs

(犬の角膜再生医療を目指した輪部幹細胞由来角膜上皮シートの開発)

氏名　南　垠列

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Laboratory of Veterinary Surgery
Department of Veterinary Medical Sciences
Graduate School of Agricultural and Life Sciences
The University of Tokyo

東京大学大学院農学生命科学研究科
獣医学専攻博士課程 獣医外科学研究科室

平成 22 年度入学

ナン　ウンリョル
南　　垠 列
Nam　Eun Ryel

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Chapter 1

General introduction

Section 1. Structure of cornea and turnover of corneal surface

Cornea is a transparent avascular tissue at the front of the eye, and transmits, and focuses light to retina to enable visual perception. Three cellular layers composed the cornea: an epithelium, a stroma, which comprises 90 % of the corneal volume with collagen fibrils, and a single layer of endothelium. The corneal epithelium is composed of a single basal layer, two to three middle layers of polygonal cells, and two to three surface layers of nonkeratinized squamous cells. These cells express K3, which is cornea-specific keratin and considered as a marker of differentiated corneal epithelial cells. The transparency of the corneal epithelium is maintained by the regularly controlled differentiation of corneal epithelial cell lineage.

Limbus is a narrow transitional zone between cornea and conjunctiva which contains a reservoir of corneal epithelial stem cells (1, 2). The stem cells are involved within limbal epithelial crypts, 'Palisades of Vogt'. which provide shelter from trauma deep into the stroma, nutrients from adjacent limbal vessels, and barriers from ultraviolet radiation through the intermingled pigmented melanocytes (3). The stem cells are smaller than basal cells of the central cornea, and have the highest self-renewing capacity and slow cell cycling.

Stem cells in the limbal basal layer support the turnover of corneal surface (4). Stem cells in limbus contribute to and continuously replenish the corneal epithelium through centripetal migration from peripheral cornea. This continuous renewal of epithelial cells keeps the cornea transparent by preventing invasion of cells and blood vessels from conjunctiva into cornea (5).

Transient amplifying cells (TACs), which are rather differentiated daughter cells, are derived from stem cell pool in limbus, and migrate through the epithelium with high proliferative capacity and rapid cell cycling (6, 7). Young TACs maintain

stemness constantly proliferate in the basal layer of the peripheral cornea, and keep the centripetal migration of the corneal epithelial cells from the limbus (8). Terminally differentiated cells are produced after multiple rounds of replication of the TACs, and compose the most numerous of the cell population in the corneal epithelium. They have no proliferation capacity, and desquamate from the central corneal surface (9).

Several proteins have been used as corneal epithelial stem/progenitor cell markers. In those markers, p63 and ATP-binding cassette transporter group2 protein (ABCG2) have been used widely for the study of corneal epithelial regeneration in human. Pellegrini et al. have demonstrated that p63, one of the intranuclear transcription factors, which is found in epidermal development, can be used to a stem/progenitor cell marker of corneal epithelial lineage (10, 11). In these studies, one particular isoform, DNp63, in the basal epithelium of limbus was not found in human central cornea epithelium with immunohistochemistry. DNp63 is an important transcription factor in the proliferation, and the differentiation of the epithelium (12, 13). ABCG2, which commonly indicates side population cells that have stem cell characteristics (14), has been found in the basal limbal epithelial cells by Watanabe et al (15, 16). Budak et al. showed the expression of ABCG2 in isolated human and rabbit limbal epithelial cells which have proliferative and colony forming capacity during cultivation and differentiation (17).

Section 2. Treatment of ocular surface defects in small animals

Corneal injury caused by trauma, infection, or diseases such as keratoconjunctivitis sicca or trichoma are very common in dogs. Depending on underlying cause or depth of injury, medical or surgical treatments are required. In case the injury is severe, aggressive surgical techniques such as nictitating membrane flap or conjunctival autograft are commonly applied to provide vascularization or restoration of defected corneal tissue (18, 19). However, these surgical methods just protect cornea from further damages to the defected area, and often fail to support adequate healing of corneal surface by blood supply (20). In addition, vascular and conjunctival tissue may remain after restoration of cornea and obscure vision depending on the ulcer size, depth, and position (21). Inadequate healing of epithelial injuries can lead to corneal opacity, ulcers, perforations, or even vision loss.

Corneal transplantation is an essential rescue technique for the treatment of severe corneal damage in human to restore the corneal transparency. Although the corneal transplantation has been reported, the number of canine cases is very limited due to insufficient resources of cornea because eye bank like as in human is poorly equipped. Additionally, it was reported to be very difficult to control graft rejections or failure resulting in corneal severe vascularization or edema in dogs (22).

Section 3. The cultivated corneal epithelial cell sheet

3.1 History

In humans, over 10 million people worldwide are suffering from corneal blindness by traumas, infections, inflammations, and congenital defects. These reasons of corneal disorders deplete stem cells in limbus, leading to visual impairment that resulted from vascularization, and fibrosis of corneal surface (23, 24). Although corneal transplantation has been performed widely as the effective treatment for those disorders in human cases, shortage of suitable donors, and failures due to immune rejections are also major limitation of this surgical procedure (25, 26).

In 1997, Pellegrini et al. reported a successful cultivation and transplantation of corneal epithelial cell sheet from small limbus tissue of patient's own healthy eye in a patient, who had unilateral stem cell deficiency. In this study, transparency, and visual acuity of the patients was improved without neovascularization (27). However the mechanism of regeneration in the corneal surface by the transplanted epithelial sheet is still unclear, many successful transplantations using with the corneal epithelial sheets cultivated from limbal corneal stem cells have been reported (28-31). These reports have noted that the progenitor or immature cells with abundant proliferative property such as young TACs maintained in the sheet are essential to encourage long-term graft survival, facilitate reepithelization, and transparency by preventing the invasion of conjunctival epithelial cells. Rama et al. (32) suggested that amount of cells with strong expression of p63, which indicated high potential of self-renewal and proliferation were significantly associated with successful transplantation.

As the corneal epithelial cells can be expanded *ex vivo* easily, small amount of tissue is needed to cultivate the enough size of corneal epithelial cell sheet for the

treatment of damaged cornea, and the autologous transplantation is possible if the contralatral eye of the patient is intact. Therefore, the transplantation of cultivated corneal cell is promising treatment to conquer the shortage of donor of corneal transplantation and to prevent immunological rejection (27).

3.2 Scaffolds

Various culture methods have been employed to develop corneal epithelial cell sheet using several kind of scaffold (28-30, 33). Scaffold acts as substrate for adhesion and supports proliferation of the corneal epithelial cells. And restoration stem/progenitor cells is necessary for regeneration of the ocular surface with less immunogenicity (34). Amniotic membrane is one of the most popular scaffolds for cultivation of the corneal epithelial cell sheet. Corneal epithelial cell sheet cultivated on the amniotic membrane was reported to induce successful ocular surface regeneration after transplantation (33, 35, 36). Type I collagen gel has received attention as a scaffold to cultivate corneal epithelial cell sheet because type I collagen is a major constituent of the corneal stroma. However, these materials need enzymes such as trypsin and dispase to separate the corneal epithelial cell sheet from the substrate. This process damages the cultivated cell sheet. To overcome this problem, temperature-responsive culture dish was developed harvest the cell sheet without chemical substances (37).

3.2.1 Amniotic membrane

An amniotic membrane, which consists of an avascular stromal matrix and a thick basement membrane, is the most inner layer of the placenta. The amniotic membrane has been used as transplant material for severe ocular surface damage (38-40). An amniotic membrane is believed to suppress inflammation by secretion of anti-inflammatory cytokines such as interleukin-10 and inhibin, and anti-inflammatory protease inhibitors such as α1 anti-trypsin inhibitor and inter-α-trypsin inhibitor (41). Moreover, the stromal matrix of amniotic membrane may suppress of TGF-β signaling that are responsible for scar formation (42, 43).

Tseng et al. found that denuded amniotic membrane, which was eliminated amnion epithelium, promoted migration, proliferation, and differentiation of limbal cells (44). Following further investigations revealed that the denuded amniotic membrane preserves progenitor cells, and has been considered as a suitable carrier for the cultivated corneal epithelial tissue (45-48). The corneal epithelial cell sheet cultivated on amniotic membrane has developed and transplanted to reconstruct ocular surface of patients with limbal stem cell deficiency. The results of the transplantation showed improvement of visual acuity, facilitated epithelialization, hindered neovascularization and inflammation (33, 35, 36). However, the use of an amniotic membrane may give rise to some problems such as general versatility and infections. The amniotic membrane can be available only when caesarean section is performed and preservation facilities are needed. In addition, strict donor screening should be conducted to avoid the risk of transmittable viral agents (49).

3.2.2 Collagen

Animal body consists a viriety of collagens. Biomaterial structure of the collagen may affect the cell attachment, growth, and differentiation. Type I collagen is easy to obtain due to abundant amount in body and high biocompatible with low immunogenicity (50, 51). These characteristics of type I collagen allow application to regenerative therapy of the ocular surface as well as heart muscles and nerves by tissue engineering (52, 53). And type I collagen has been demonstrated to retain stem cell reservoir and promote proliferation of the corneal epithelial cells (54). Atelocollagen which is treated with proteolytic enzymes to remove the terminal telopeptides (51) has low antigenicity and immunogenicity compared to native collagen (8). Several researches have reported that the transplantation of type I atelocollagen gel to the vocal cords (55), cartilage (56), and cornea (57), showed low or no rejections in dogs. Therefore, atelocollagen is also a promising scaffold of the

corneal epithelial cell sheet. However, collagen gel is produced by crosslinking methods, which make the gel stable and increase resistance to enzymatic degradation (58), and needs enzymes such as glutaraldehyde, which is a toxic agent.

3.2.3 Temperature-responsive culture dish

The temperature-responsive culture dish developed by Okano and his colleagues, and is composed of temperature-responsive poly(N-*isopropyl acrylamide*). The temperature-responsive culture dish enables cell attachment and proliferation at 37°C, and releases the cultivated cells from the bottom of the dish when the temperature is lowered to below 20°C (59). Compared to harvest methods using chemicals such as trypsin and dispase II, which damage cell interactions and the extracellular matrix, the cultivated tissue can be harvested maintaining an intact structure of extracellular matrix (37). A corneal epithelial cell sheet cultivated on a temperature-responsive culture dish has been reported to attach to the exposed host corneal stroma spontaneously, and removed abnormal conjunctival tissue by the extracellular matrix contained in the cell sheet.

Section 4. Purpose of this study

Corneal injury is common disease in dogs. However, in the case of large corneal defects, a conjunctival flap or graft may not be able to recover corneal transparency because large grafts may leave opaque areas and blood vessels on the cornea, even if the grafted tissue is removed after corneal epithelial regeneration. Therefore, the transplantation of corneal epithelial cell sheet is expected to be a clinically valuable treatment, and may overcome limitations of the standard treatment for severe corneal damage in dogs. However, development and transplantation of the cultivated corneal epithelial cell sheet in dogs has been hardly reported.

Therefore, the purpose of this study was to develop and evaluate methods of cultivation of corneal epithelial cell sheet using with corneal epithelial stem cells derived from limbus in dogs. Scaffold carrier materials including canine amniotic membrane and atelocollagen gel were employed and temperature-responsive culture dish was additionally applied to select an optimal method to cultivate promising corneal epithelial cell sheet in which corneal stem/progenitor cells were highly preserved in chapter 2. And in chapter 3, culture methods including air exposure and degradation of the collagen gel were investigated to develop the most suitable culture method for transplantation in clinical settings. Finally, corneal epithelial cell sheet cultivated by most suitable methods in chapter 2 and 3 was transplanted into a canine corneal defect model to demonstrate safety and efficacy of the transplantation of in chapter 4.

Chapter 2

Comparison of the canine corneal epithelial cell sheets cultivated from limbal stem cells on canine amniotic membrane, atelocollagen gel, and temperature-responsive culture dish

Introduction

Recently, experimental transplantation of corneal epithelial cell sheets cultivated from limbal stem cells has yielded encouraging results in a rabbit model (60). This technique has been applied for the reconstruction of severe corneal defects caused by serious trauma, chemical burns, and genetic disease in humans, as it provides corneal healing and rapid reepithelialization with fewer adverse effects (e.g., corneal and conjunctival inflammation, and corneal opacity) than conventional approaches such as the Boston Keratoprosthesis (61).

In dogs, nictitating membrane flap or conjunctival autograft are often applied to treat several corneal injury (62). However, these treatment methods are not sufficient to restore corneal transparency depending on the depth and size of the injury because they often allow invasion of vascular and conjunctival tissue during wound healing (21). Therefore, transplantation of the cultivated corneal epithelial cell sheet including stem cells is expected to be a promising treatment to recover corneal transparency in dogs.

To cultivate a corneal epithelial cell sheet, corneal epithelial stem cells are typically cultured on a substrate or materials such as amniotic membrane (45-48), collagen gel (57), or temperature-responsive culture dish (37) to allow cells to adhere and proliferate easily and to maintain stem/progenitor phenotype of cultured epithelial cells. When assuming practical transplantation, the cultivated sheet should be better to have a similar histological morphology to the native corneal epithelium and enough stiffness to endure surgical manipulation and suture. Air exposure of cell sheet is usually performed during cultivation to acquire those characteristics when the amniotic membrane or the collagen gel is used as a scaffold. On the other hand, the temperature-responsive culture dish, which is developed to detach the cell sheet only by dropping in temperature, is not suitable for air exposure method. However, this

relatively new culture method can preserve basal membrane of the sheet without chemical digestion and have been reported to be effective to transplant into exposed corneal stroma (63). Therefore, the most suitable choice of the substrates or materials to cultivate the corneal epithelial cell sheet is important for the future clinical application of the cultivated cell sheet.

Although several reports have evaluated the corneal cell proliferation and preservation of progenitor cells on collagen substrate or amniotic membranes in human (64, 65), there is no report comparing directly the corneal epithelial sheets cultivated on these materials in dogs. The purpose of the study in this chapter was to evaluate the cultivated epithelial cell sheet on an amniotic membrane, atelocollagen gel and a temperature-responsive culture dish to compare the structure of the sheets by histopathologically and the characteristics of cells involved in the sheets immunohistopathologically and to determine the most suitable material to cultivate canine corneal epithelial cell sheet for promising transplantation in clinical application.

Materials and Methods

The animal experiments in this chapter were approved by the Animal Care Committee of the Graduate School of Agricultural and Life Science at the University of Tokyo.

Preparation of amniotic membrane

Amniotic membranes obtained from a dog (a 5-year-old Labrador Retriever) that had undergone Caesarean section were used. The obtained amniotic membrane was rinsed three times with phosphate-buffered saline (PBS) containing gentamicin according to the procedures described for preparing a human amniotic membrane for the same purpose (66). After cutting the amniotic membrane to approximately 3.5 × 3.5 cm to fit a six-well cell culture insert, the membrane was immersed in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin with 0.5 M dimethyl sulfoxide (DMSO; Wako, Osaka, Japan) for 5 minutes, then with 1.0 M DMSO for 5 minutes, and again with 1.5 M DMSO for 5 minutes. The samples were then stored at -80°C in 1.5 M DMSO until use. After thawing, the amniotic membrane was incubated with 1.2 U dispase II (neutral protease, grade II; Roche, Mannheim, Germany) at 37°C for 10 minutes and then the amniotic epithelial cells were removed from the amniotic stroma by gentle scrubbing with a cell scraper. After the separation of the epithelial cells, the amniotic membrane was fixed on a six-well cell culture insert (transparent polyethylene terephthalate membrane, 0.4 μm pore size, BD Biosciences, Bedford, MA, USA) with the basement membrane side up, using 6-0 nonabsorbable suture materials to inhibit shrinkage of the membrane.

Preparation of collagen gel

The collagen gel was prepared with 0.1 M NaOH solution, 10 × phosphate buffered saline (PBS), and a porcine dermis type I collagen solution of atelocollagen (3 mg/ml in 0.02 M acetic acid; Nippi, Tokyo, Japan) at a 1: 1: 8 ratios by volume(67). The solution was supplemented with 0.1% glutaraldehyde (Wako, Tokyo, Japan) for crosslinking and gently mixed at 4°C. After that, 120 µl of the blended reagent was spread onto the surface of the culture insert in the six-well plate and stored at 37°C for two hours. Then, the collagen gel was washed with PBS twice and immersed in sterilized PBS overnight at 37°C to wash out remaining glutaraldehyde before use.

Preparation of 3T3 cells

3T3 fibroblast cells (Japanese Collection of Research Bioresources, Tokyo, Japan) were used as feeder cells that promote proliferation of cells and retain of stem/progenitor cells to cultivate corneal epithelial cell sheet. Confluent 3T3 fibroblasts were incubated with 4 µg/ml of mitomycin C (Wako, Tokyo, Japan) for two hours at 37°C under 5% CO_2, and then were trypsinized. Single cells of 3T3 fibroblasts were replaced onto six-well cell culture dishes or a temperature-responsive culture dish at a density of 3.3×10^4 cells/cm^2.

Isolation and culture of limbal epithelial cells

Limbal epithelial tissues were collected from the intact eyes of eight healthy beagle dogs which had been humanly euthanized after used for other research purposes. Tissue collection was performed within two hours after euthanasia. Then, the obtained corneoscleral rim was exposed to 2.5 U dispase II in DMEM/Ham's F12 nutrient mixture (3:1; Invitrogen) containing 10 mg/ml penicillin, 25 µg/ml streptomycin sulfate, and 0.5 µg/ml amphotericin B (Invitrogen) for two hours at 37°C. Two hours later, the limbal epithelium were gathered from the corneoscleral rim under observation by light microscopy and trypsinized for 15 minutes to collect single

cells. The isolated single cells were cultured in DMEM/Ham's F12 mixture, supplemented with 10% FBS, 10 ng/mL human recombinant epidermal growth factor (EGF), 10 mg/ml human recombinant insulin, 10 mg/ml penicillin, 25 μg/ml streptomycin sulfate, and 0.5 μg/ml amphotericin B (Invitrogen) on a type I collagen coated plastic dish at 37°C under 95% humidity and 5% CO_2. The culture medium was changed every other day until the cells reached 100% confluency. After confluency, cells were harvested from the culture dish and seeded on canine amniotic membrane, collagen gel in the six-well culture insert or temperature-sensitive culture dish at a density of approximately 3.5×10^4 cells/cm^2 in the presence of 3T3 feeder cells. The culture was continued until the cells reached 100% confluency with changing the medium every other day. Then, confluent epithelial layers on the amniotic membrane and the collagen gel were exposed to air for 5 days to promote differentiation and stratification of the epithelial cells by removing the medium in the cell culture inserts and lowering the level of the medium in the six-well plate, After 5 days of air exposure, the confluent epithelial sheets on these substrates were harvested by digestion with 1.25 U/ml of Dipase II (Fig. 2-1-A). The cell culture in the temperature-responsive culture dish was continued for 14 days and the confluent epithelial sheet was harvested by dropping in temperature from 37°C to 20°C (Fig. 2-1-B).

Histological examination and immunofluorescence

Cultivated cell sheets, and normal canine ocular surface including cornea and limbus were embedded in OCT compound, frozen in liquid hexane, and stored at −80°C until use. Then, 4-μm and 7-μm frozen sections were prepared using a cryomicrotome for hematoxylin and eosin (H&E) staining and immunofluorescence staining, respectively. For immunofluorescence, after washing the sections in PBS for 5 minutes three times, they were blocked by incubation with 10% normal goat

serum for one hour at room temperature. Anti-rabbit Ki-67 (1: 100; Abcam, Cambridge, UK), anti-mouse keratin 3 (K3 1:50; Progen, San Francisco, CA, USA), anti-mouse ABCG2 (1:100; Medical & Biological Laboratories Co. Ltd., Nagoya, Japan), and anti-mouse p63 (1:50; Abcam) were applied to the prepared sections, which were incubated overnight at 4°C. Then, the sections were rinsed in PBS for five minutes, treated with Cy3-conjugated goat anti-mouse IgG antibody (Zymed, San Francisco, CA, USA) or Cy3-conjugated goat anti-rabbit IgG antibody (Zymed) for 45 minutes, and washed in PBS three times. The sections were coverslipped using an anti-fading mounting medium, 4'-6-Diamidino-2-phenylindole (DAPI)-containing VECTA SHIELD® (Vector Laboratories, Inc., Burlingame, CA, USA). After mounting the coverslips, fluorescence was detected by laser confocal microscopy (Olympus, Tokyo, Japan).

Real-time quantitative RT-PCR

To investigate the expression of ABCG2 and p63 mRNA, total RNA was isolated from the cultivated sheets on the amniotic membrane, on the collagen gel, and on the temperature-responsive culture dish using the RNeasy mini kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol. The genomic DNA was eliminated from RNA extractions with DNase I (QIAGEN). Spectrophotometrically quantified RNA (5 μg) was precipitated using Ethachinmate (Wako), and the cDNA was synthesized with reverse transcriptase (Superscript III, Invitrogen) as per the manufacturer's protocol and stored at -20°C until use. A real-time quantitative PCR was performed by real-time monitoring of the increase in the fluorescene of SYBR Green dye (Thunderbird SYBR qPCR Mix, Toyobo, Osaka, Japan) with the Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA). The primers specific for dogs were designed using the software Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) with sequence data from the NCBI

database as described in Table 1. The PCR conditions were as follows: 1 cycle at 50°C for 2 min and 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min. To evaluate the specificity of the amplified products in each experiment, a melting-curve analysis immediately performed after the amplification protocol. The expression levels of mRNA quantities across different samples were normalized to the mRNA quantity of the endogenous control gene glyceraldehye-3-phosphate dehydrogenase (GAPDH). Each experiment was performed in triplicate.

Statistical analysis

The mean values and standard deviations of mRNA expression of ABCG2 and p63 in cultivated corneal epithelial sheets were calculated and the statistical analysis was performed using Excel (Microsoft, WA, USA). One-way ANOVA followed by the Tukey-Kramer test was used to determine the statistical differences between the expression levels of each gene in cell sheets cultivated on different materials. Significance was accepted at $p < 0.05$.

Results

Single cells isolated from limbal epithelial tissue adhered to the amniotic membrane and the collagen gel within 24 hours and formed colonies within 3 days. The cells on the amniotic membrane and the collagen gel reached 100% confluency and formed monolayers after 5 and 7 days, respectively. The stratification of monolayers on each substrate was observed under a light microscope after exposed to the air by lowering the level of the culture medium to the base of the insert. On the other hand, the single cells adhered to the temperature-responsive culture dish within two days and colonized after 5 days and reached 100% confulency after 14 days.

H&E staining revealed that the cell sheets cultivated on the amniotic membrane and on the collagen gel formed six to eight layers (Fig. 2-2-A) and four to five layers (Fig. 2-2-B), respectively. Both cultivated cell sheets consisted of a basal layer, two to five layers of ovoid cells containing some vacuoles, and one or two layers of flat cells. The basal layers of cultivated cell sheets adhered to both substrates. By contrast, the cell sheet cultivated on the temperature-responsive culture dish had only two or three layers including basal layer (Fig. 2-2-C). Normal corneal epithelium and limbus composed of five to seven (Fig. 2-2-D) and three to eight cell layers (Fig. 2-2-E), respectively.

In the immunofluorescence staining, the basal cells in the sheets on the amniotic membrane dish were weakly positive for Ki-67 (Fig. 2-3-A). On the other hand, a large numbers of cells strongly positive for the proliferation marker Ki-67 were detected in the basal layer of the cultivated epithelial sheets on the collagen gel (Fig. 2-3-B), and the expression of Ki-67 was rare in the basal cells on the temperature-responsive dish (Fig. 2-3-C). The corneal epithelium-specific marker K3 was observed in all layers of the cell sheets cultivated on the amniotic membrane (Fig. 2-4-A) and the temperature-responsive culture dish (Fig. 2-4-C). In contrast,

expression of K3 was observed in the suprabasal layer of the cell sheet cultivated on the collagen gel (Fig. 2-4-B) and was not detected in the basal layer. In all cultivated epithelial cell sheets, the expression of ABCG2 was only observed in the basal layer (Fig. 2-5-A, B, C). The expression of p63, another stem/progenitor epithelial cell marker, was observed in the basal layer of the cell sheet cultivated on the amniotic membrane (Fig. 2-6-A) and the temperature-responsive culture dish (Fig. 2-6-C). However, the cultivated sheet on the collagen gel contained more cells positive for p63 in two to three layers including the basal layer (Fig. 2-6-B).

In normal canine cornea and limbus, positive expression of Ki-67 was occasionally observed in the basal cells (Fig. 2-3-D, E). K3 was observed in all layers of the central corneal epithelium (Fig. 2-4-D) and the limbus except for the basal layer (Fig. 2-4-E). ABCG2 was only expressed in the limbal basal layer (Fig. 2-5-D, E), while p63 was expressed in the basal layer of both limbus (Fig. 2-6-D) and central corneal epithelium (Fig. 2-6-E) and strongly positive cells were crowded around limbal basal area.

The real-time quantitative RT-PCR revealed that the mRNA expression of ABCG2 in the sheet cultivated on the amniotic membrane was 9.9 and 7.2 times greater than that in the sheet cultivated on the collagen gel and the temperature-responsive culture dish, respectively. The sheet cultivated on the amniotic membrane showed significantly higher expression of ABCG2 compared to the sheet cultivated with other methods (Fig. 2-7-A). However, the expression level of p63 in the sheet on the collagen gel was 2.8 and 3.2 times greater and significantly higher than that in the sheets cultivated on the amniotic membrane and the temperature-responsive culture dish, respectively (Fig. 2-7-B).

Discussion

In this study, the cells derived from limbus adhered and proliferated on the amniotic membrane, collagen gel and the temperature-responsive culture dish, and the cell sheets were successfully cultivated. However, the cell activities and the phenotypes of cultivated sheets were different depending on the culture methods. The cells cultured on the collagen gel showed the highest ability of colony formation and proliferation. Moreover, greater expression of Ki-67, which is associated with cell proliferation (68) was obviously detected in the basal cells on the collagen gel. The cells on the amniotic membrane reached confluency faster than the cells on the temperature-responsive culture dish and showed weak Ki-67 positive expression in the basal layer. The amniotic membrane and collagen gel are similar in composition to corneal stroma which include extracellular matrixes including type I collagen and coordinate adhesion, proliferation, migration, and function of the cells (69, 70).

On the contrary, temperature-responsive culture dish supported lesser in adhesion and proliferation of the corneal epithelial cells than the other two substrates.

In this study, I used glutaraldehyde is widely used to increase the mechanical strength of gel by cross-linking (71). However, glutaraldehyde is also known to be a cytotoxic chemical which may cause irritation of eyes, the respiratory tract, and skin in humans (72). It was reported that glutaraldehyde at over 1.0% concentration causes corneal injury, but it does not affect cells at concentrations of less than 0.1% (73). In this study, 0.1% glutaraldehyde was used in an attempt to limit the cytotoxicity of the gel while increasing its stiffness. In addition, we submerged the collagen gel in PBS overnight to eliminate the remaining glutaraldehyde that could not crosslink to the collagen. No apparent apoptosis or diminution by cytotoxic effects of the glutaraldehyde was shown in the cultivated corneal epithelium. Therefore, it

was supposed that canine corneal cells are safe from the cytotoxic effects of glutaraldehyde at a concentration of 0.1%.

By H&E staining, stratified layers on the collagen gel and amniotic membrane and one or two layers on the temperature-responsive culture dish appeared. Although the number of cell layers of the cultivated sheet on the collagen gel was fewer than the layers on the amniotic membrane, the stratified tissue on the collagen gel composed of four to five layers and was morphologically most similar to a normal corneal epithelium. While cells cultivated on the temperature-responsive culture dish reached over confluent, the cells rarely formed such stratified structures. Air exposure method requires lowering the medium levels in the plate, cells should be cultured in appropriate apparatus like as a culture insert used in this study. Therefore, the cultivated cells on the temperature-responsive culture dish were not applied air exposure method to promote stratification of the tissue. The cultivated cells on the two biosubstrates in cell culture inserts were exposed to a liquid-and-air interface and resulted in the difference in the number of cell layers compared to the cultivated sheet on temperature-responsive culture dish. Although the detail mechanism of the air exposure cultivation method remains unclear, it has been revealed that air exposure promotes differentiation and stratification of the corneal epithelial cells (74, 75).

I used the corneal epithelium-specific marker K3 antibody to determine whether the cultivated layers certainly composed of corneal epithelial cells. Positive expression of K3 has been observed in the all layer of the native canine corneal epithelium except for limbal basal cells. The basal layer of the sheet on the collagen gel also did not express K3, whereas all layers of the sheet on the amniotic membrane and the temperature-responsive culture dish showed positive expression. Therefore, the tissues cultivated on the three substrates are considered to the

corneal epithelium. Moreover, no K3 expression in the basal layer of the cell sheet on the collagen gel indicated that more undifferentiated cells were maintained in the basal layer of the sheet on the collagen gel compared to that on amniotic membrane and temperature-responsive culture dish.

In dogs, the expression of ABCG2 is locally observed in basal layers of normal limbus where corneal stem cells are believed to locate. In contrast, the expression of p63 is widely detected throughout entire cornea, however, strongly positive cells are mainly crowded around limbal basal area. Therefore, both antibodies could be used as stem/progenitor cell markers in dogs. The basal layer of the sheet cultivated on all materials faintly expressed positive reactions for ABCG2. On the contrary, the expression of p63 was clearly detected in the sheet cultivated on collagen gel and the positive expression of p63 appeared in two or three layers including basal layer and the expression of p63 was limited in the basal layer of the sheet cultivated on amniotic membrane and the temperature-responsive culture dish. Positive expressions of the stem/progenitor cell markers suggested that the basal layer of the cultivated corneal epithelial sheet on all the carriers included more or less corneal stem/progenitor cells.

Real-time RT-PCR analysis revealed that the cells cultivated on the amniotic membrane showed the highest expression of ABCG2. On the other hand, the cultivated sheet on collagen gel showed the highest value of p63 gene expression and the lowest value was detected in the sheet on the amniotic membrane. Even though the technique of exposure to air could proliferate terminal differentiation of corneal epithelial cells, cultivated sheets on canine amniotic membrane and collagen gel successfully retained corneal stem/progenitor cells compared to the sheet cultivated on temperature-responsive culture dish. Higher expression of p63 is detected not only in the stem cells but also in the young TACs (76). Therefore more

cells strongly positive for p63 and higher expression of p63 gene in the sheet on collagen gel suggested that the collagen gel offered extracellular matrix for the cells and could promote differentiation from stem to progenitor cells like as young TACs with preservation undifferentiated cells. The fact that K3 expression was not detected in the basal layer of the sheet on collagen gel could also support the presence of absolutely immature corneal stem/progenitor cells.

The mechanism of regeneration of the corneal surface with the transplanted epithelial sheet is still unclear. However, much successful transplantation of cultivated corneal epithelial sheets has been reported in human ophthalmology (28-31). These researchers have noted that the inclusion of the progenitor cells or the proliferative corneal epithelial cells such as the young TACs in the sheet are essential to encourage long-term graft survival, facilitate reepithelization, and maintain transparency by preventing the invasion of conjunctival epithelial cells. Rama et al. suggested that a significant amount of p63-bright cells that have abilities of self- renewal and proliferation were associated with successful transplantation (32). Therefore, the cells cultivated on collagen gel presented similar structures to the normal corneal epithelium and was likely to have the most abundant stem/progenitor cells among the three types of sheets grown on the different materials.

Additionally, the safety of substrate itself could be an important issue when considering the application in clinical cases. Amniotic membrane is one of most popular carrier material of the corneal epithelial cells. However, the use of an amniotic membrane may give rise to some problems such as infections after transplantation of the cell sheet. And strict donor screening should be conducted to avoid the risk of transmittable viral agents (49). On the contrary, the corneal epithelial cell sheet cultivated on the collagen gel can be transplanted to cornea without the substrate because the collagen gel may be easily degraded by collagenase.

Therefore, the collagen gel could be safer and more promising substrate than the amniotic membrane for clinical application.

In this study, corneal epithelial cell sheet cultivated on collagen gel showed similar morphology to normal corneal epithelium and was thought to retain more stem/progenitor cells than the sheets grown on the other two materials, the amniotic membrane and the temperature-responsive culture dish. Therefore, collagen gel may be the most suitable material among the three to promise the effective transplantation to canine corneal injury in clinical cases. Further study is necessary to investigate the actual efficacy of corneal surface reconstruction after transplantation in dogs.

Table. 2-1 Specific primers designed for canine gene GAPDH, ABCG2, and p63.

Gene	Accession No.		Sequence
GADPH	NM_001003142	F	TGACACCCACTCTTCCACCTTC
		R	CGGTTGCTGTAGCCAAATTCA
ABCG2	NM_001048021	F	CAGGGCTGTTGGTAAATCTCA
		R	TACTGCAAAGCCGCATAACC
P63	XM_545249	F	CGGAAGGCGGATGAGGACAGCATCA
		R	AAAAGGGCGCTTCGTACCGTCACCG

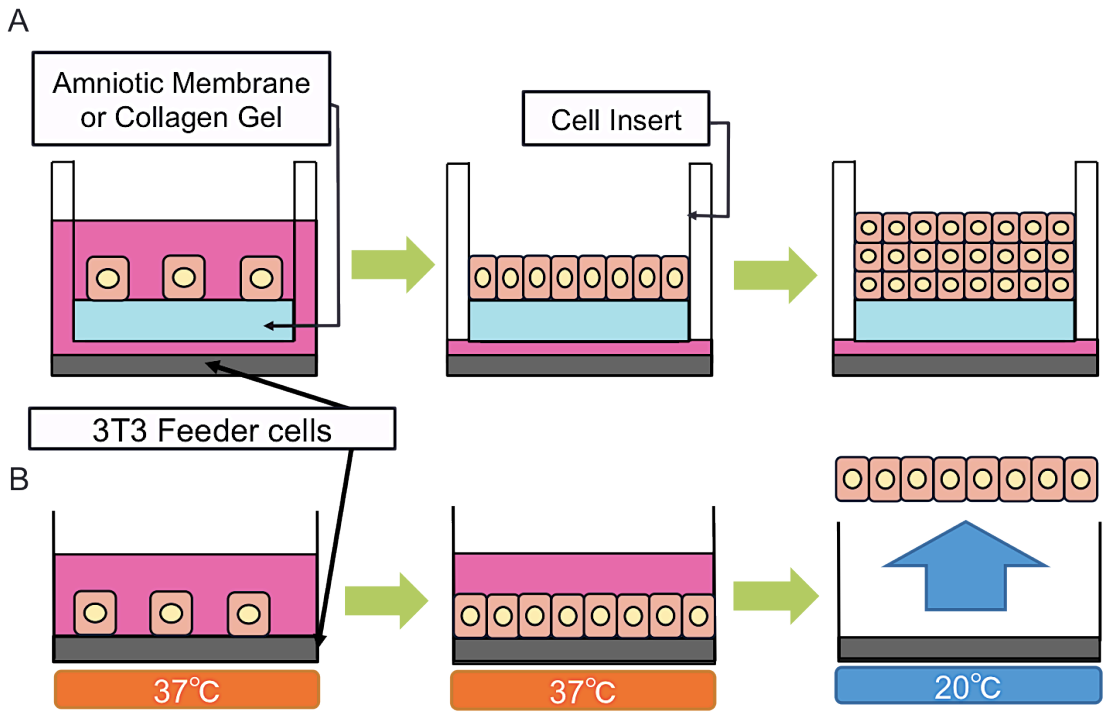

Fig. 2-1 Preparation of the cell sheet cultivated on the amniotic membrane, collagen gel and the temperature-responsive culture dish.

The corneal epithelial cells were seeded and cultivated on the canine amniotic membranes or the collagen gel in the six-well culture insert. The confluent epithelial layers on these substrates were exposed to air for 5 days by removing the medium in the cell culture inserts and lowering the level of the medium in the six-well plate. After 5 days, the confluent epithelial sheets on the amniotic membrane and the collagen gel were harvested by digestion with dispase II (A). The cells seeded on the temperature-responsive culture dish cultivated at 37°C for 14 days. After 14 days, the confluent epithelial sheet was harvested by dropping in temperature from 37°C to 20°C (B).

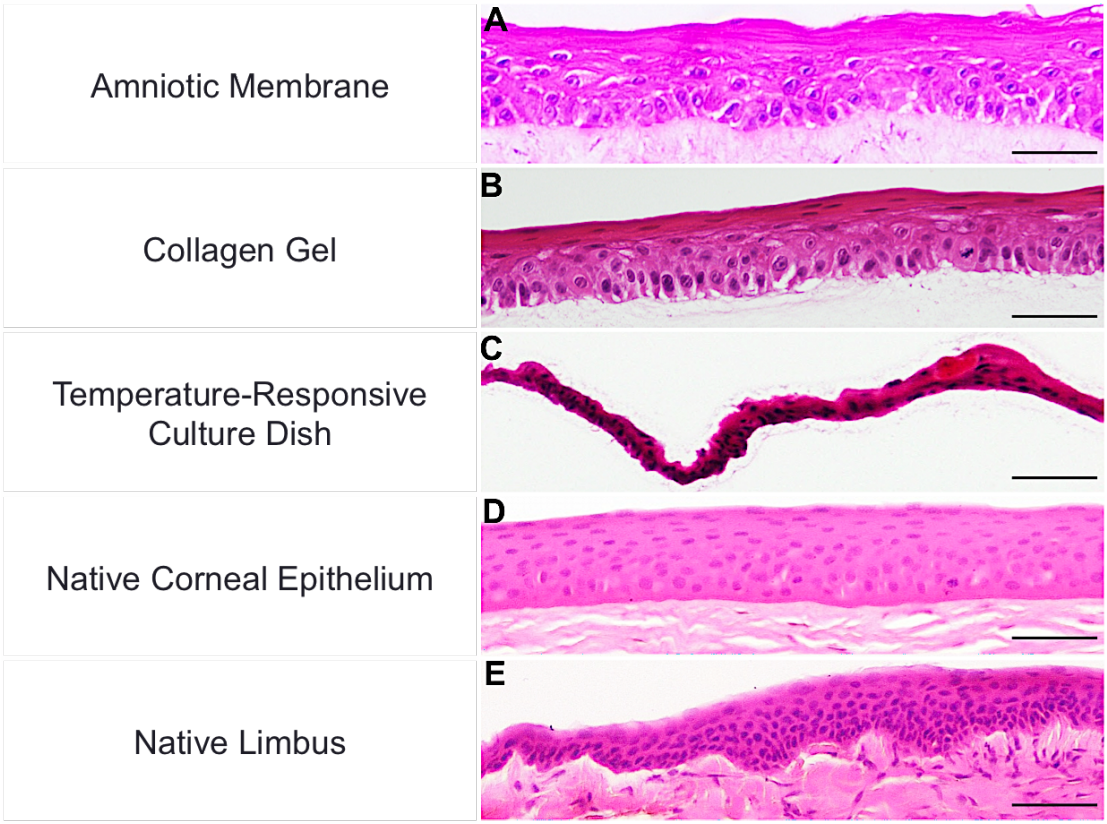

Fig. 2-2 H&E staining of the cultivated corneal epithelial cell sheets and canine normal cornea.

The cell sheets cultivated on the amniotic membrane and on the collagen gel formed six to eight layers (A) and four to five layers (B), respectively. By contrast, the cell sheet cultivated on the temperature-responsive culture dish had only two or three layers including basal layer (C). Normal canine corneal epithelium and limbus composed of five to seven (D) and three to eight cell layers (E), respectively. Bar scale: 50 μm.

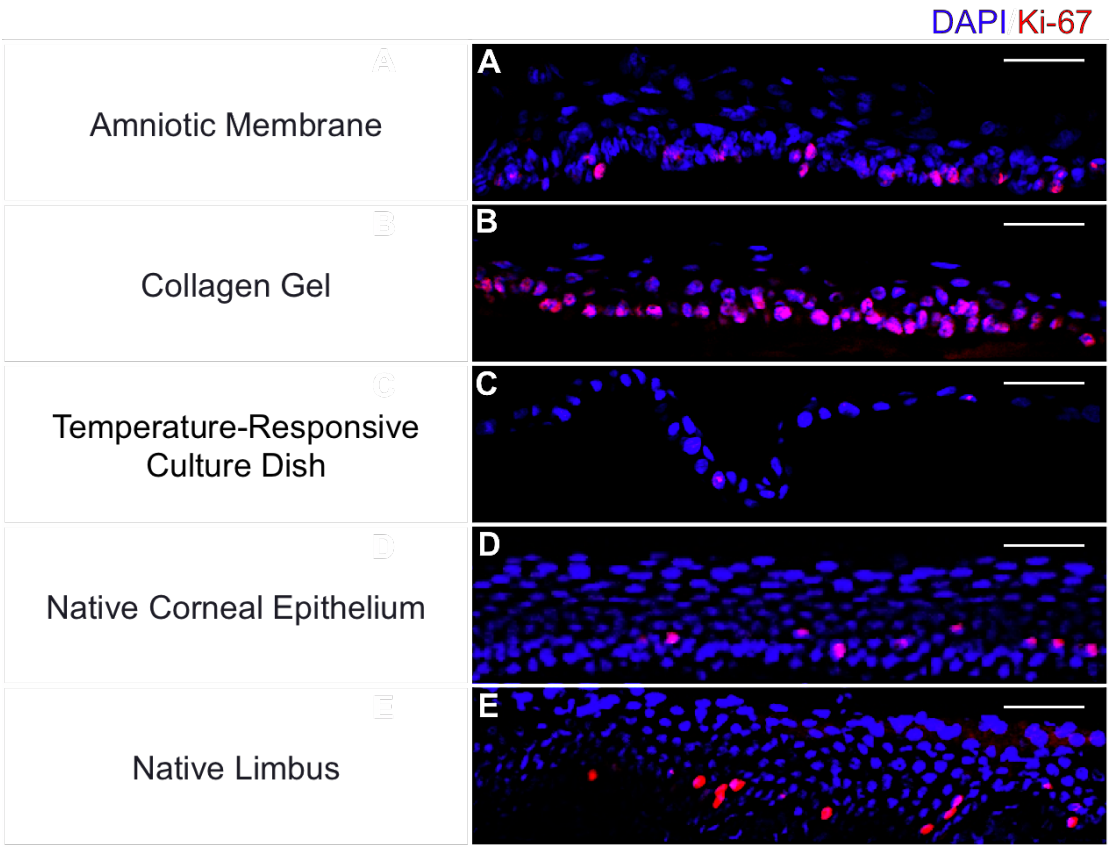

Fig. 2-3 Immunohistochemistry for Ki-67 in the cultivated corneal epithelial cell sheets and normal canine cornea.

The basal cells in the sheets on the amniotic membrane dish were weakly positive for Ki-67 (A). On the other hand, a large numbers of cells strongly positive for the proliferation marker Ki-67 were detected in the basal layer of the cultivated epithelial sheets on the collagen gel (B), and the expression of Ki-67 was rare in the basal cells on the temperature-responsive dish (C). In the normal canine cornea and limbus, positive expression of Ki-67 was observed occasionally in the basal layer (D, E). Bar scale: 50 μm.

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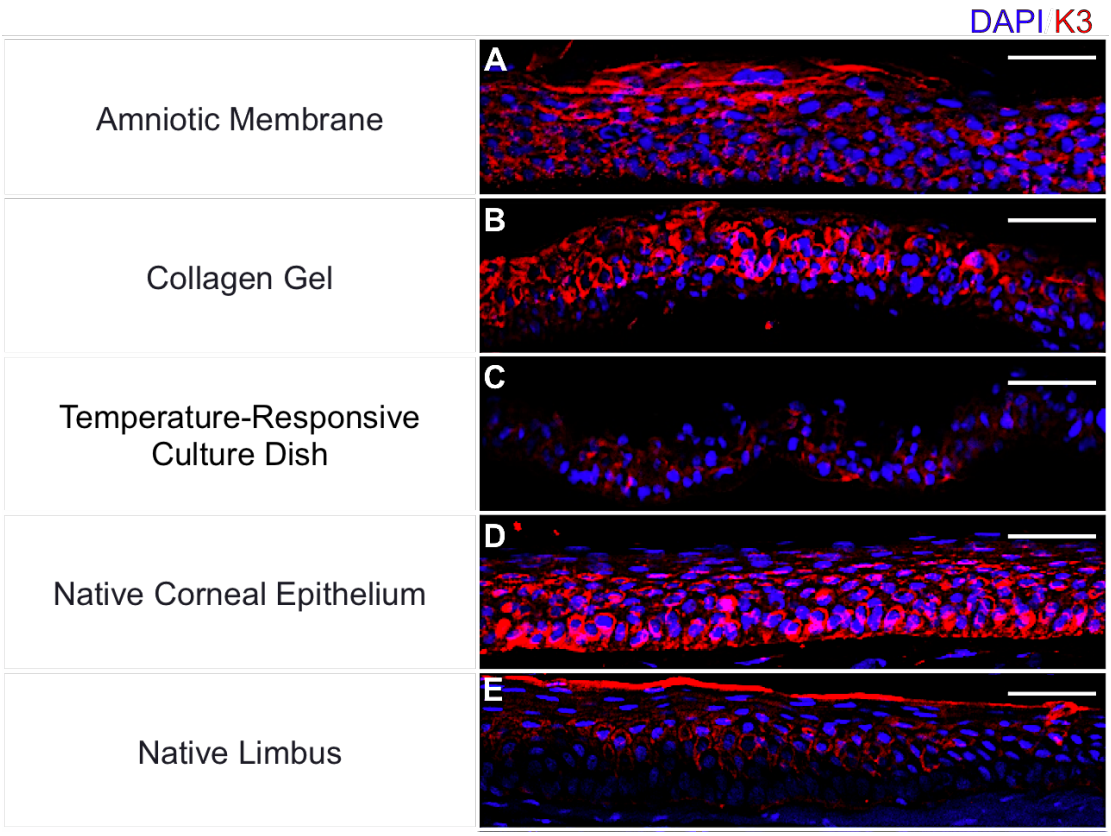

Fig. 2-4 Immunohistochemistry for K3 in the cultivated corneal epithelial cell sheets and normal canine cornea.

K3 was observed in all layers of the cell sheets cultivated on the amniotic membrane (A) and the temperature-responsive culture dish (C). In contrast, expression of K3 was observed in the suprabasal layer of the cell sheet cultivated on the collagen gel and was not detected in the basal layer (B). In the normal canine cornea, K3 was observed in all layers of the central corneal epithelium (D) and limbus except for the limbal basal layers (E). Bar scale: 50 μm.

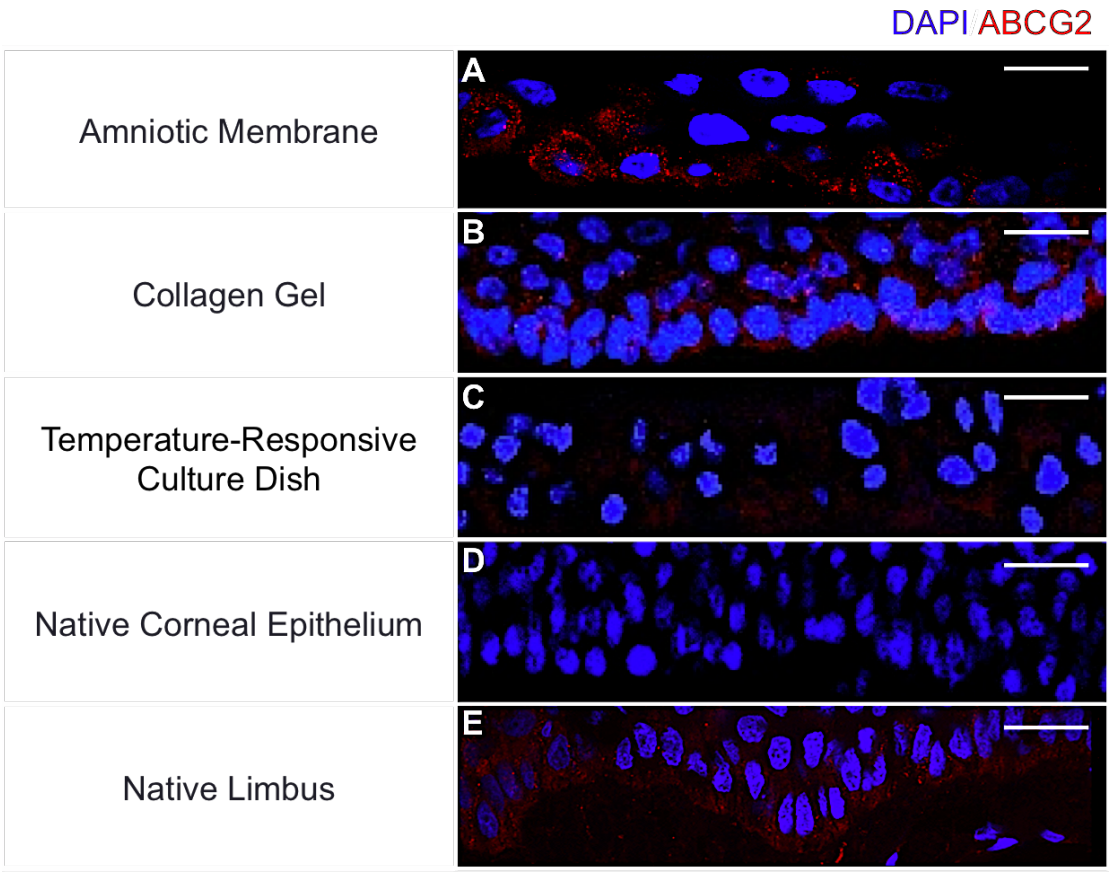

Fig. 2-5 Immunohistochemistry for ABCG2 in the cultivated corneal epithelial cell sheets and normal canine cornea.

In all cultivated epithelial cell sheets the expression of ABCG2 was only observed in the basal layer (A, B, C). In normal canine cornea, ABCG2 were only observed in the limbal basal layer (D, E). Bar scale: 25 μm.

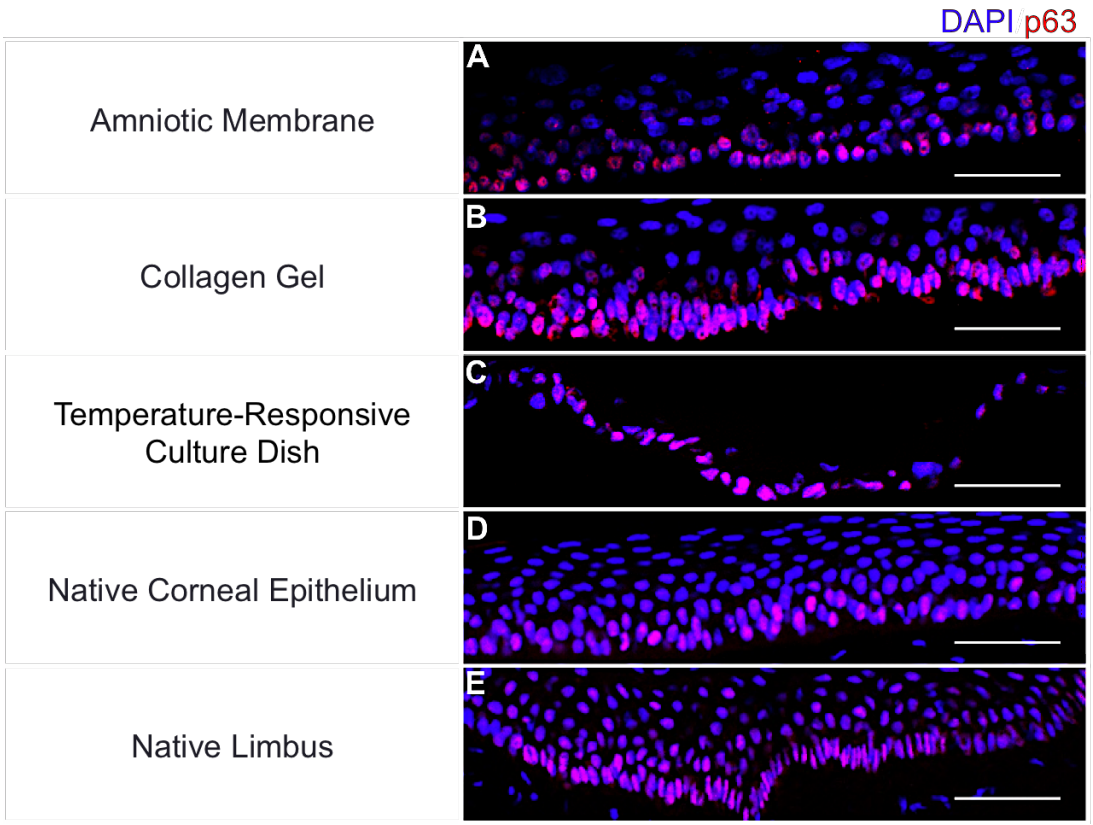

Fig. 2-6 Immunohistochemistry for p63 in the cultivated corneal epithelial cell sheets and normal canine cornea.

The expression of p63, was observed in the basal layer of the cell sheet cultivated on the amniotic membrane (A) and the temperature-responsive culture dish (C). However, the cultivated sheet on the collagen gel contained more cells positive stains of p63 in two to three layers including the basal layer (B). In normal canine cornea, p63 was expressed in the basal layer of both limbus (D) and central corneal epithelium (E) and strongly positive cells were crowded around limbal basal area. Bar scale: 50 μm.

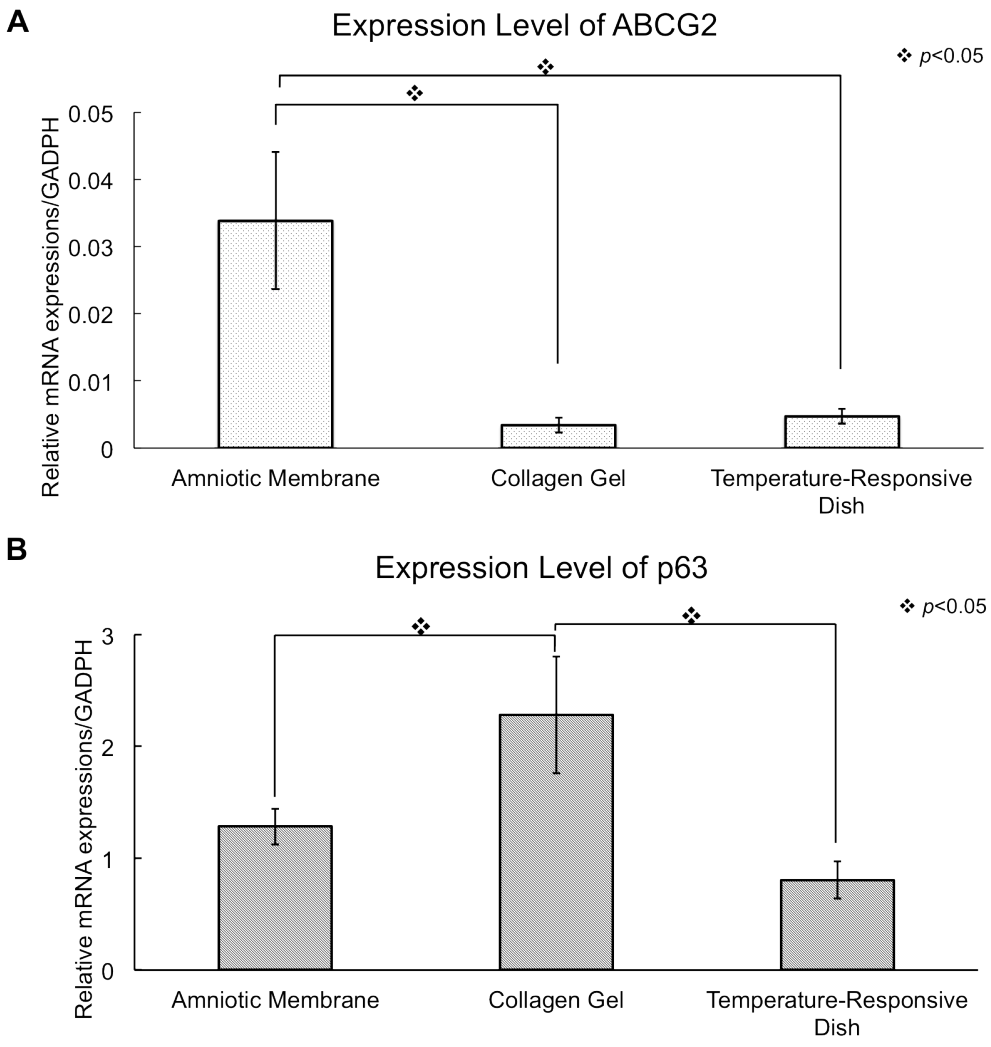

Fig. 2-7. The real-time RT-PCR analysis for the expression of ABCG2 and p63 mRNA in the corneal epithelial sheets cultivated on amniotic membrane, collagen gel and temperature-responsive culture dish.

The real-time RT-PCR revealed that the mRNA expression of ABCG2 in the sheet cultivated on the amniotic membrane was 9.9 and 7.2 times greater and significantly higher than that in the sheet cultivated on the collagen gel and the temperature-responsive culture dish, respectively (A). The expression level of p63 in the sheet on the collagen gel was 2.8 and 3.2 times greater and significantly higher than that in the sheets cultivated on the amniotic membrane and the temperature-responsive culture dish, respectively (B).

Chapter 3

Optimization of corneal epithelial cell sheets cultivated on collagen gel designed for transplantation in clinical settings

Introduction

In the previous chapter, it was demonstrated that the collagen gel supported development of the cell sheet that had similar structure with the normal canine corneal epithelium and preserved stem/progenitor cells. Air exposure is a cultivation method of exposing the cultivated tissue to air by lowering the medium level as described in chapter 2. An air-liquid interface stimulates corneal epithelial cell differentiation and stratification by setting the environment close to natural tissue development (77). This method allows the cultivated corneal epithelial stem/progenitor cells to mature into the terminally differentiated corneal epithelial cells expressing K3 and to increase proliferative capacity, and results in stratification of corneal epithelial cell sheet (78-80). However, irreversible clonal conversion by terminal differentiation of the stem/progenitor cells in the cultivated sheet cannot avoid reduction of p63 expression that indicates regenerative property of corneal epithelium (75). Successful transplantation of the cell sheet requires retention of stem/progenitor cells in the cultivated sheet and stable structure enough to transplant. Therefore, optimal period of air exposure methods which provide corneal epithelial sheet with maximal expression of p63 and structural stiffness should be investigated.

On the other hand, corneal opacity caused by the collagen gel after transplantation were concerned due to incomplete degradation of the gel (81). In addition, direct adhesion between the basal layer in the cell sheet and extracellular matrix of exposed host stroma lead to rapid and stable adhesion after removing defected corneal epithelium and transplantation of the corneal epithelial cell sheet (63). Therefore, transplantation of a carrier free sheet is safe way to avoid side effects caused by substrates and reinforce the stability of the sheet on the cornea.

In this chapter, the optimal period of air exposure and condition to degrade collagen gel substrate were investigated by evaluating the p63 expression and

structural phenotype of the cultivated sheet to develop the most suitable cultivated method for transplantation in clinical settings.

Materials and methods

Preparation of collagen gel

Methods of preparation of collagen gel were the same as described in chapter 2.

Preparation and evaluation of stratified corneal epithelial cell sheet by air exposure method

Limbal epithelial tissues were collected from the intact eyes of four healthy beagle dogs, which had been euthanized after they had been used for other research purposes. Methods of tissue collection and cultivation were the same as described in chapter 2. The inserts were submerged in culture medium until confluence, and the medium was replaced every other day. Then, confluent epithelial layers on the collagen gel were harvested without air exposure or with 3, 5, and 7 days of air exposure by lowering the level of the medium in the six-well plate to promote differentiation and stratification of the epithelial cells. Methods of immunofluorescence and H&E stain were the same as described in chapter 2. For immunofluorescence, anti-rabbit Ki-67 (1: 100), anti-mouse K3 (1:50), and anti-mouse p63 (1:50) were applied to the prepared sections. The expression of p63 mRNA in the sheet with 0, 3, 5, and 7 days of air-lift cultivation was evaluated by real time quantitative RT-PCR in the same methods as described in chapter 2.

Conditioning for degradation of collagen gel substrate

Collagen gel substrate (120µl) was prepared in six-well cell culture insert according to the same methods as described in chapter 2. Collagenase type I (50 Unit/ml) in PBS was added to the six-well plate up to the bottom of insert and incubated at 37°C. After that, the weight of collagen gel on the insert was measured

every 10 minutes until complete degradation of collagen gel. Three independent trials were performed in the same condition.

Preparation and evaluation of carrier-free corneal epithelial cell sheet

The inserts with confluent cell sheet (n=3) after 5 days of air exposure were soaked with 2 ml collagenase type I in PBS in the six-well plate to degrade collagen gel substrate for 80 minutes at 37°C. After that, the collagenase type I was eliminated and the culture medium were supplied to the plate to stop the collagenase activity. Then, the sheet was subjected to H&E staining and immunofluorescence staining against Ki-67 (1:100), K3 (1:50), ABCG2 (1:100) and p63 (1:50) in the same methods as described in chapter 2. The simultaneously cultivated sheet without collagenase degradation was also subjected as a control.

Statistical analysis

The statistical analysis was performed using Excel (Microsoft) and all the data shown by real time RT-PCR analysis was demonstrated by the mean values of samples and the standard deviations. A one-way ANOVA followed by the Tukey-Kramer test was used to determine the statistical differences in the p63 expression of the sheet exposed to air for each period. Significance was accepted at $p < 0.05$.

Results

The corneal epithelial cells adhered and proliferated on the collagen gel in the same manner described in chapter 2. After confluency of cells, the cultivated tissue was exposed to air by lowering the medium.

H&E staining revealed that the cell sheet without air exposure had single layer (Fig. 3-1-A). Two to three layers were shown in the cell sheet with 3 days air exposure (Fig. 3-1-B). The cell sheets with 5 and 7 days of air exposure similarly consisted of 4 to 5 layers (Fig. 3-1-C, D). And positive expressions of Ki-67 were observed in the sheets with 3 and 5 days of air exposure by immunofluorescence (Fig. 3-2-B, C). However, the cell sheet without air exposure and with 7 days of air exposure rarely expressed Ki-67 (Fig. 3-2-A, D). Immunofluorescence analysis revealed that K3 was expressed in all the cell sheets with or without air exposure (Fig. 3-3). The cell sheet without air exposure (Fig. 3-4-A) and with 7 days of air exposure (Fig. 3-4-D) showed slightly positive expression of p63. The basal layer of the cell sheet with 3 days of air exposure showed weekly positive expression of p63 (Fig. 3-4-B). On the contrary, cells in the basal and the suprabasal layer in the sheet with 5 days of air exposure were strongly positive for p63 (Fig. 3-4-C). Real time RT-PCR analysis also revealed that the highest mRNA expression of p63 in the sheet with 5 days of air exposure. The expression of p63 in the sheet with 5 days of air exposure was 6.72, 3.37, and 27.99 times greater than that in the sheet with 0, 3, 7 days of air exposure, respectively, and significantly higher than that in the sheet with 0 and 7 days of air exposure (Fig. 3-5).

The collagen gel was easily degraded by collagenase type I and the 120-μl of collagen gel which was used as a substrate to cultivate corneal epithelial cell sheet in this study was fully decomposed in 80 minutes (Fig. 3-6).

The cell sheets before and after degradation of the collagen gel that were exposed to air for 5 days were compared to investigate the effect of degradation of the collagen gel to the cell sheet. After degradation of the collagen gel, no morphological and immunohistological difference was observed in the cultivated sheet compared to before degradation (Fig. 3-7, 3-8). The cell sheet degraded collagen gel also showed 4 to 5 layers including a basal layer and similar expression of Ki-67, K3, ABCG2 and p63 with the cell sheet before degradation.

Discussion

The number of cell layers in the sheet increased with air exposure and the sheets with 5 and 7 days of air exposure showed similar morphology to normal cornea with 4 to 5 cell layers. K3, the differentiation marker of the corneal epithelium, was observed in all sheets with or without air exposure. However, the expressions of p63 and Ki-63 increased in the sheet with 5 days of air exposure and steeply decreased in the sheet with 7 days of air exposure. In normal corneal surface, young TACs, which differentiate from limbal corneal stem cells and strongly express p63 gene, proliferate vigorously and are involved in renewal of corneal epithelial cells (82). The increase of p63 expression in the sheet suggested that air exposure activated the conversion of stem/progenitor cells in the sheet into the cell like as TACs, and contributed to the proliferation and stratification. However, too much air exposure could not promote stratification and even resulted in downregulation of p63 expression in the sheet. Terminally differentiated cells that have no ability of proliferation do not express p63 (10, 11). Moreover, Ki-67, the proliferation marker, was rarely observed in the sheet with 7 days of air exposure. Therefore, 7 days of air exposure deprived the corneal epithelial cells of stem/progenitor cell-like phenotypes including proliferation ability. The result in the present study revealed that 5 days of air exposure could produce optimal canine corneal epithelial sheet on collagen gel substrate with morphological stiffness and retention of cells in immature state for promising transplantation in clinical settings.

Additionally, the condition of collagen gel degradation was investigated in expectation of clinical application. The degradation of the collagen gel exposes the basal layer of the cultivated sheet and allows the sheet to attach to the extracellular matrix of host stroma directly and stably (63). Any effects on structural and immunohistological phenotypes of the sheet were not observed after collagen gel

degradation for 80 minutes. Ke et al. (67) reported that the rabbit corneal epithelial cell sheets cultivated on collagen gel could also retained their structure after degradation of the collagen gel and basement membrane components, such as type IV collagen and laminin were also expressed, indicating that the cell sheets retained these components throughout the process of collagen degradation. Although the detail of the basement membrane components was not investigated in the present study, the basal layer of the sheet consisted of a population of close-packed basal cells similar to native cornea even after collagen degradation. Therefore, it was suggested that cultivated canine corneal epithelial cells also formed basement membrane component similar to normal cornea and prevented enzymatic activity of collagenase type I to the cultivated sheet. Even though the basal membrane of the cultivated sheet exhibited resistant to collagenase type I, excessive digestion might affect the cell sheet on the collagen gel. Therefore, 80 minutes of digestion was thought to be the optimal period in the present study. In this chapter, the cell sheet treated with 5 days of air exposure had stratified cell layers and retained more stem/progenitor cells than the cell sheet without air exposure or with other periods of air exposure. Moreover, the degradation of the collagen gel could not affect the phenotypes of the cell sheet cultivated on the collagen gel. Therefore, the culture methods, 5 days of air exposure and the degradation of the collagen gel, may be suitable for the cell sheet for transplantation in clinical settings. Further study should be investigated whether the cultivate cell sheet actually showed enough stiffness to endure the surgical procedure or stability after transplantation.

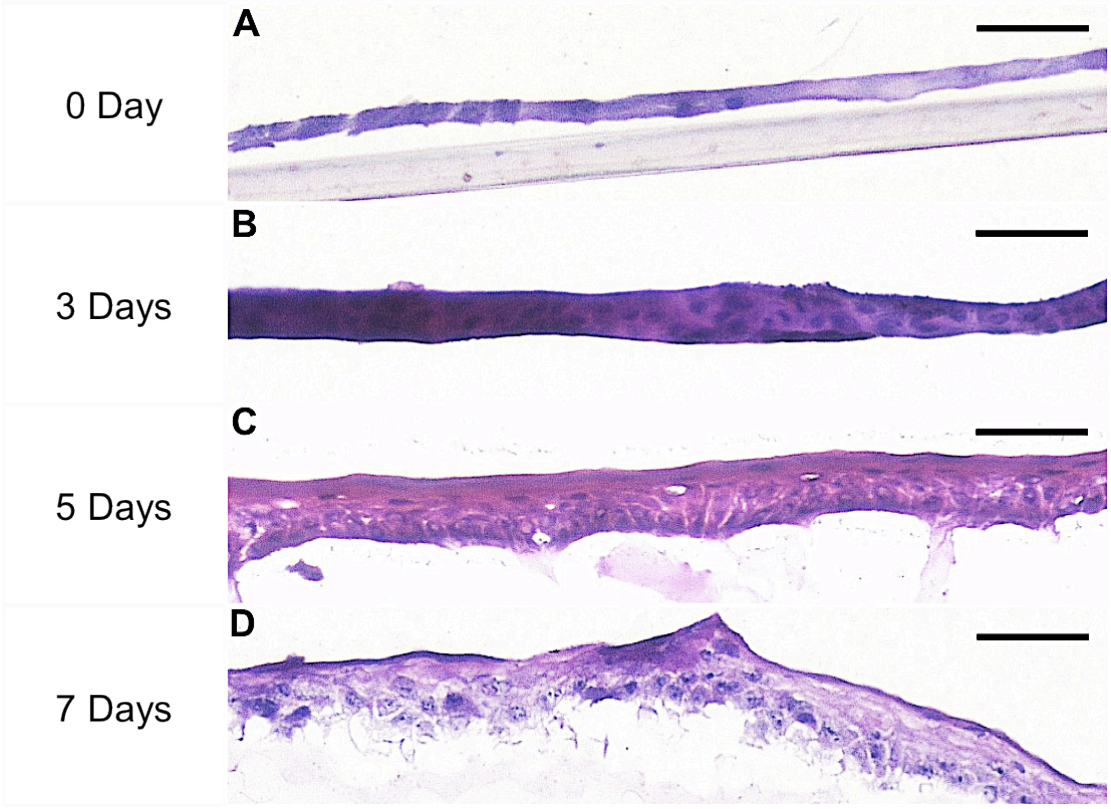

Fig. 3-1 Histological analysis by H&E stain of the corneal epithelial cell sheet cultivated on the collagen gel with air exposure for different periods.

The cell sheet without air exposure had single layer (A). Two to three layers were shown in the cell sheet with 3 days air exposure (B). The cell sheets with 5 (C) and 7 days (D) of air exposure similarly consisted of 4 to 5 layers. Bar scale: 50 μm.

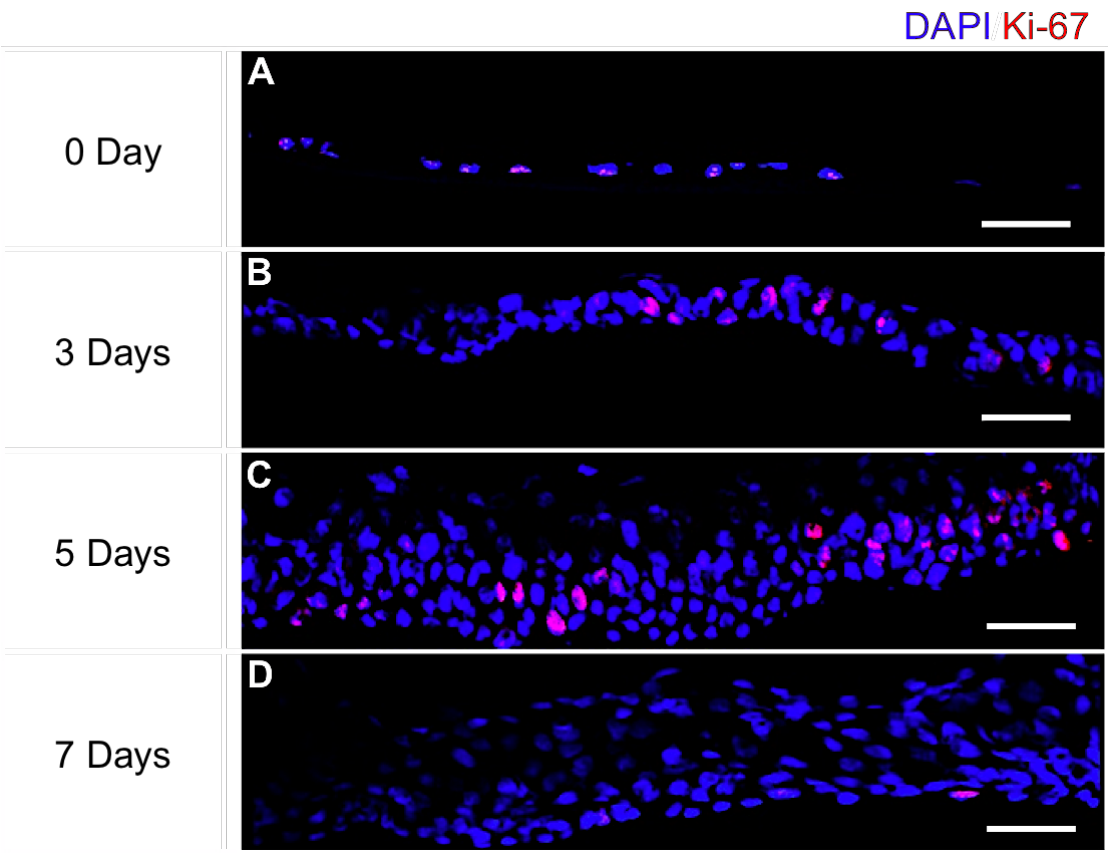

Fig. 3-2 Immunohistochemistry for Ki-67 in the corneal epithelial cell sheet cultivated on the collagen gel with air exposure for different periods.

Positive expressions of Ki-67 were observed in the sheets with 3 and 5 days of air exposure by immunofluorescence (B, C). However, the cell sheet without air exposure and with 7 days of air exposure rarely expressed Ki-67 (A, D). Bar scale: 50 μm.

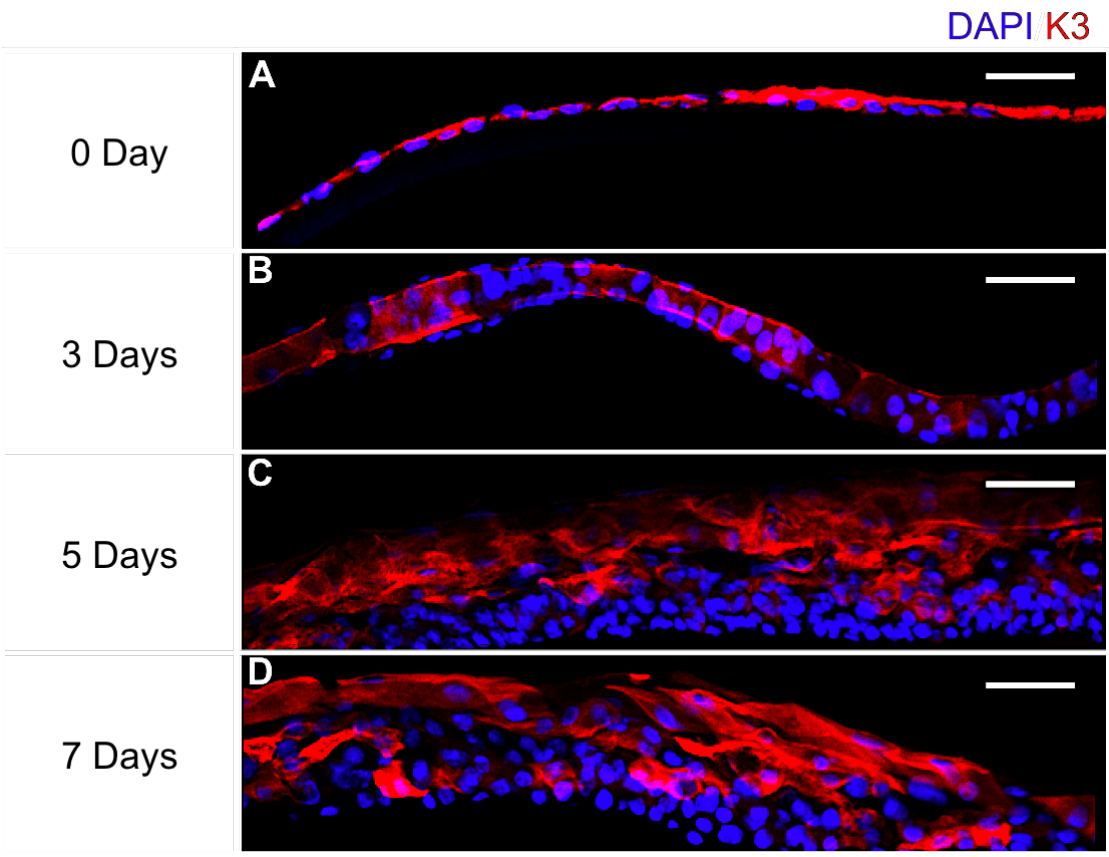

Fig. 3-3 Immunohistochemistry for K3 in the corneal epithelial cell sheet cultivated on the collagen gel with air exposure for different periods.

The immunofluorescence revealed that K3 was expressed in all layers of cell sheets with or without air exposure. Bar scale: 50 μm.

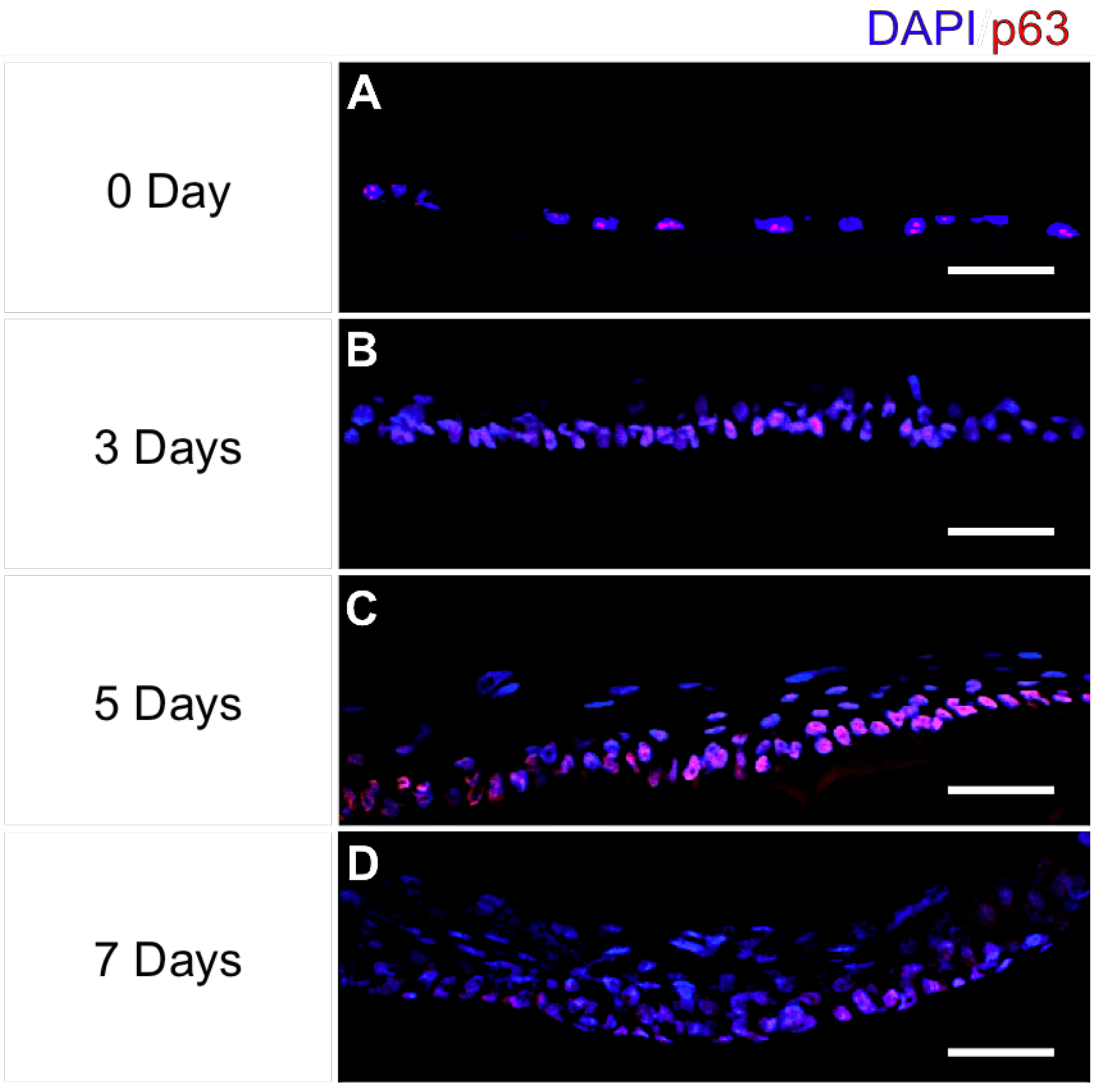

Fig. 3-4 Immunohistochemistry for p63 in the corneal epithelial cell sheet cultivated on the collagen gel with air exposure for different periods.

The cell sheet without air exposure (A) and with 7 days air exposure (D) showed slightly positive expression of p63. The basal layer of the cell sheet with 3 days of air exposure showed weekly positive expression of p63 (B). On the contrary, more cells strongly positive for p63 were observed in the basal and suprabasal layer of the sheet with 5 days of air exposure (C). Bar scale: 50 μm.

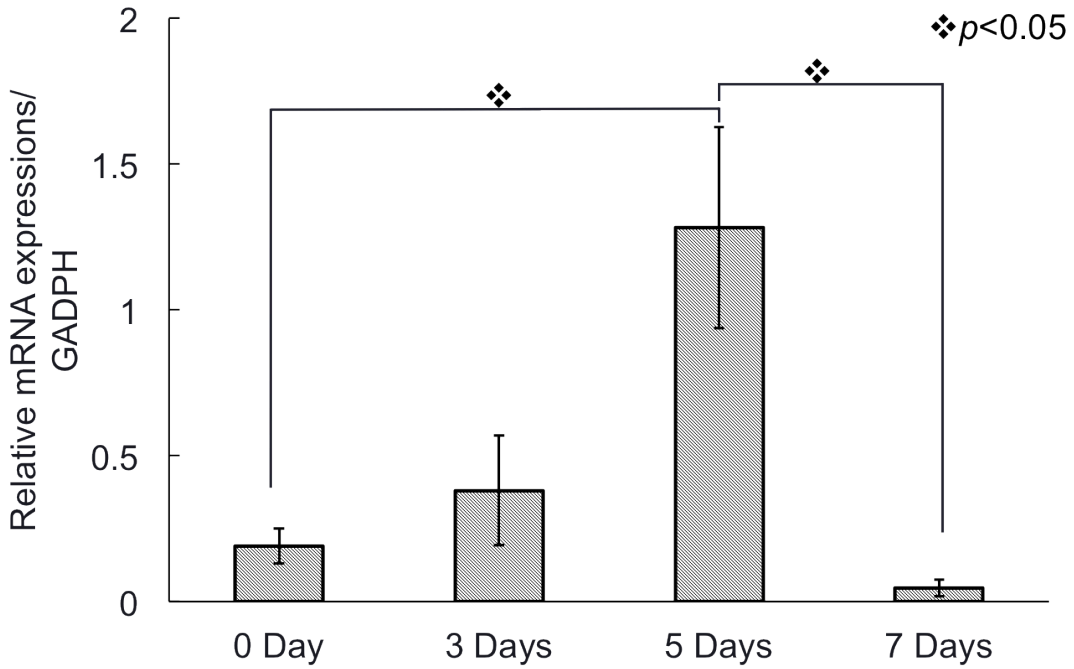

Fig. 3-5 Real time RT-PCR analysis for p63 expression in corneal epithelial sheets cultivated on collagen gel with air exposure for different periods.

The highest mRNA expression of p63 was observed in the sheet with 5 days of air exposure and 6.72, 3.37, and 27.99 times greater than that in the sheet with 0, 3, 7 days of air exposure, respectively. The expression of p63 in the sheet with 5 days of air exposure was significantly higher than that in the sheet with 0 and 7 days of air exposure.

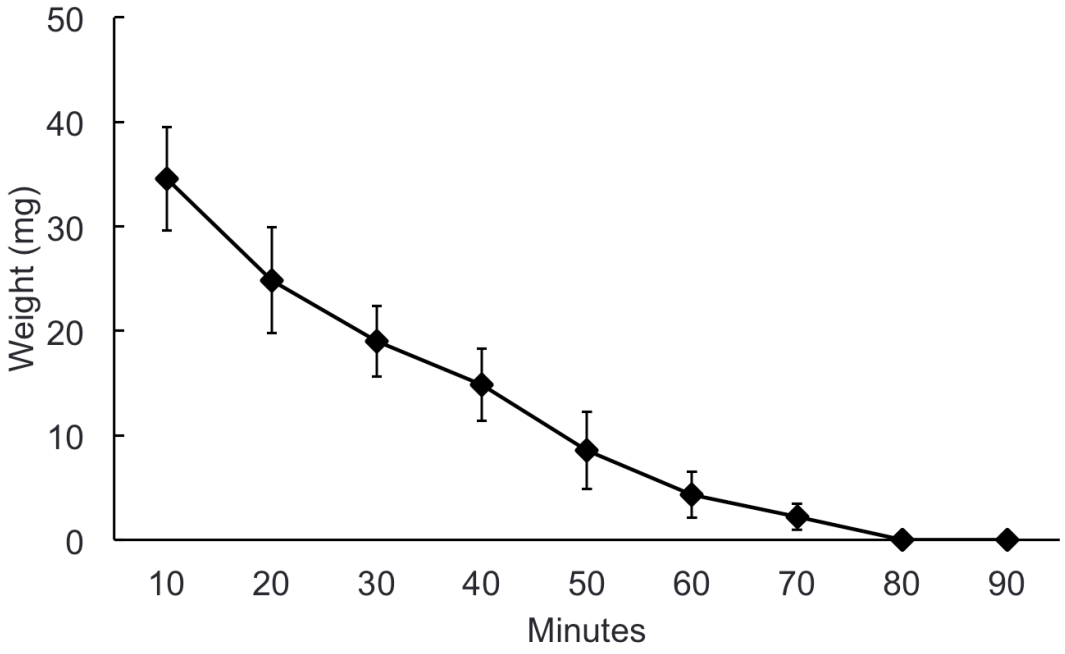

Fig. 3-6 Degradation of collagen gel substrate.

The collagen gel was easily degraded by collagenase type I and the 120-μl of collagen gel which was used as a substrate to cultivate corneal epithelial cell sheet in this study was fully decomposed in 80 minutes.

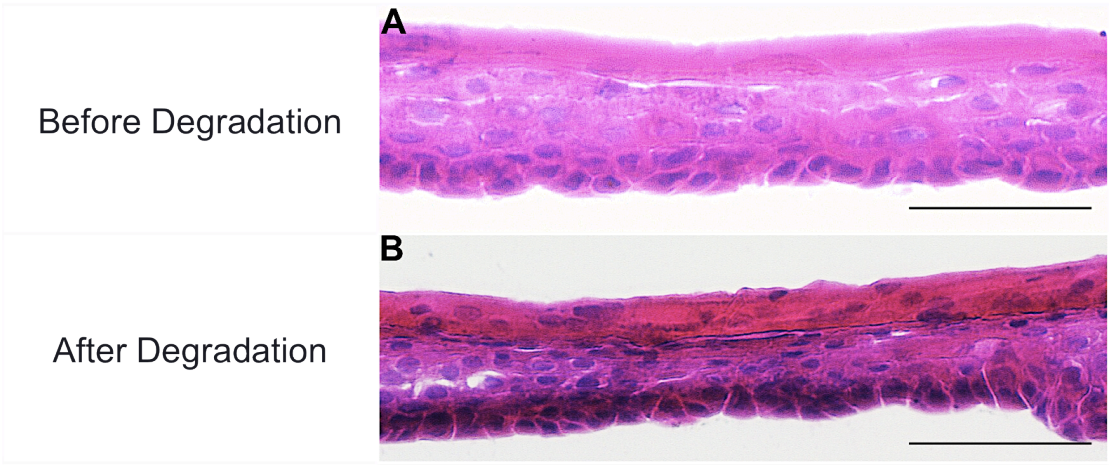

Fig. 3-7 H&E stain of the corneal epithelial cell sheet cultivated on the collagen gel with 5 days of air exposure before and after degradation of the collagen gel.

The cell sheet cultivated on the collagen gel with 5 days of air exposure showed 4 to 5 layers including a basal layer (A). Even after degradation of collagen gel, the sheet also showed the same morphology as before degradation. Bar scale: 50 μm.

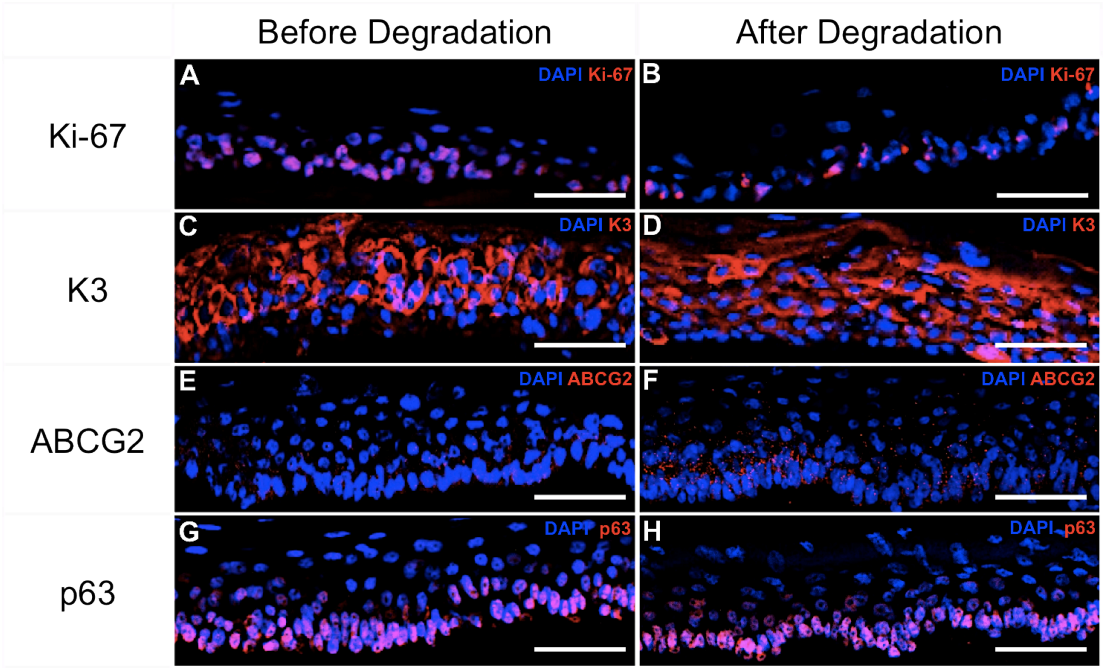

Fig. 3-8 Immunohistochemistry of the corneal epithelial cell sheet cultivated on the collagen gel before and afterdegradation of the collagen gel.

Immunofluorescence of the cell sheet after degradation of the collagen gel showed similar expression of Ki-67, K3, ABCG2 and p63 (B, D, F, H) to the cell sheet before degradation (A, C, E, G). Bar scale: 50 μm.

Chapter 4

Transplantation of the cultivated autologous corneal epithelial cell sheet in canine corneal injury models

Introduction

Corneal injury caused by trauma, infection, or diseases are very common and can lead to pain and corneal opacity, ulcers, perforations, or even vision loss. Ulcerative keratitis caused by foreign bodies, or malformation of eyelids or eyelashes, and spontaneous chronic corneal epithelial defects are common presenting disease in clinical cases and give rise to erosions in the corneal epithelium. However, in case the injury is severe, only medical therapy using eye-drops often fails to achieve complete recovery and aggressive surgical techniques such as nictitating membrane flap or conjunctival autograft are applied (83, 84). Nictitating membrane flap covers and protects ocular surface using the third eyelid which is a thin sheet of tissue in the medial canthus in dogs and enable to treat injury penetrated to the midstroma as well as the corneal epithelium. However, the nictitating membrane flap just protects cornea from further damages to the defected area, and does not support healing of corneal surface by blood supply (20). In addition, this technique can remain vascular and conjunctival tissue that obstruct vision after restoration of cornea injury depending on the ulcer size, depth, and position (21).

Corneal transplantation is an essential rescue technique for the treatment of severe corneal damage in human to restore the corneal transparency. Although the corneal transplantation has been reported, the number of canine cases is very limited due to insufficient resources of cornea because eye bank as in human is poorly equipped. Additionally, it was reported to be very difficult to control graft rejections or failures resulting in corneal severe vascularization or edema in dogs (22).

Cultivated corneal epithelial cell sheets have been used to regenerate ocular surface and to restore corneal transparency in patients with limbal cell deficiency in human ophthalmology (62, 85). Because the limbal cell deficiency causes intrusion of

conjunctival tissue and vascular into cornea, the corneal epithelial cell sheet is transplanted to stromal bed exposed by elimination of the conjuntival tissue on cornea.

The conjunctival tissue usually remains on the ocular surface after wound healing of severe corneal injury, and results in visual impairment in dogs. Therefore, the corneal epithelial cell sheet can be clinically valuable in dogs with irreversible corneal impairment and overcome the limitations of the traditional treatments.

In previous chapters, the corneal epithelial cell sheet cultivated from canine limbal epithelial cells and the collagen gel was an optimal substrate to support adhesion and proliferation of corneal epithelial cells and to preserve the stem/progenitor phenotypes of the cells including in the corneal epithelial cell sheet. In addition, the corneal epithelial cell sheet with 5 days of air exposure showed similar morphology to normal cornea and degradation of collagen gel did not affect the structure or preservation of stem/progenitor cells in the cultivated corneal epithelial cell sheet. Therefore, it was considered that the corneal epithelial cell sheet cultivated on collagen gel with 5 days of air exposure and additional degradation of the collagen gel could bring most promising effect on transplantation to canine corneal injury.

The purpose of study in this chapter is to evaluate the safety and efficacy of transplantation of corneal epithelial cell sheets cultivated from autologous limbal stem cells on collagen gel using corneal injury model in dogs.

Materials and methods

The Animal Care Committee of the Graduate School of Agricultural and Life Science at the University of Tokyo approved the animal experiments in this chapter.

Collection of limbal tissue and preparation of the corneal injury model

Four Beagle dogs, three years old, about 10kg, one male dog and three female dogs, were used in this study. Three of those dogs were used for transplantation and one as a control. All the animals underwent ophthalmic examinations, physical examination, radiography, and blood chemistry analysis and showed no evidence of abnormalities. As a preanesthetic medication, 25 μg/kg of atropine sulfate was injected subcutaneously. Additionaly, 20 mg/kg of cefazolin sodium was intravenously injected. All animals were anesthetized with propofol and anesthesia was maintained with isoflurane (2.0%) in oxygen after intubation. Fentanyl citrate (5–20 μg/kg/hr) was used to alleviate pain in the perioperative period.

Under the general anesthesia, center of the cornea of left eye in each dog was trephinated over a 7.5-mm diameter and 200-μm depths by Castroviejo corneal trephine (Inami, Tokyo, Japan), and corneal tissue was removed with a crescent knife (MANI, Tochigi, Japan). A piece of limbal epithelial segments (2 × 2 mm) were obtained from the right eye globe in three dogs in transplantation group to cultivate a corneal epithelial cell sheet. After the operation, a soft contact lens (Meni-one, Nagoya, Japan) were used to the trephinated site for one week and 0.3% ofloxacin was topically administered to manipulated eyes four times daily and 2.2 mg/kg of carprofen were applied orally two times daily for 14 days.

Prepation of the collagen gel and 3T3 cells

Methods of preparation of collagen gel and 3T3 feeder cells were the same as those described in chapters 2 and 3.

Cultivation of the epithelial cell sheet on the collagen gel

Collected limbal segment was washed three times in PBS containing gentamycin and then was placed with the epithelial side up on collagen-coated plastic dish. Culture medium and the methods for expansion of corneal epithelial cells were the same as described in chapter 2.

After confluency, limbal epithelial cells were passaged at a density of approximately 3.5×10^4 cells/cm^2 on the collagen gel in the six-well culture insert. The inserts were submerged in culture medium until confluency again, and the medium was replaced every other day. Then, confluent epithelial monolayer on the collagen gel was exposed to air for 5 days by lowering the level of the medium in the six-well plate to promote differentiation and stratification of the epithelial cells. After air exposure cultivation method, the collagen gel was degraded by collagenase I (50 Unit/ml) in PBS at 37°C. After one hour, collagenase solution was removed, and the culture medium including FBS was added to the plate to inhibit the collagenase activity. The corneal epithelial cell sheet was separated from the collagen gel substrate and stored at 4°C until the transplantation.

Transplantation

An autologous corneal epithelial cell sheet was transplanted in three dogs 21 days after corneal injury was induced. General anesthesia was performed as described above and fentanyl citrate (5–20 μg/kg/hr) was also used to alleviate pain in the perioperative period of transplantation. After debridement of scar tissue on injured cornea by trephine and crescent knife, a corneal epithelial cell sheet was transplanted and sutured to remaining cornea covering over injured area with 9-0

nylon sutures. A soft contact lens was covered for one week and 0.3% ofloxacin was topically administrated four times daily. Carprofen were also applied orally for 14 days. The sutures were removed after one week in one dog, and two weeks in two dogs. One dog without undergoing transplantation was subjected as a control.

Ophthalmic evaluation

An ophthalmic examination by a slit lamp was performed once a week after transplantation or scar debridement for 60 days in all dogs. A handheld retinal camera (Kowa, Nagoya, Japan) was used to take a picture of ocular surface of all dogs. Neovascularization and corneal opacity were respectively scored (0-4 point) based on a scale according to the previous studies (86, 87) (Table. 4-1). Opaque area was measured with image software (Image J version 1.37; National Institutes of Health, Bethesda, MD), and the percentage of the opaque area to the entire cornea was measured. In a control dog, all the evaluation was similarly performed after corneal injury for 60 days. After observation period, all dogs were humanly euthanized and entire cornea induced injury were collected and subjected to histopathological examination.

Histopathological and immunohistopathological examinations of cornea

The collected cornea was fixed and embedded in OCT compound. Tissues were frozen in liquid hexane and stored at −80°C until sectioning. Methods of H&E staining and immunohistochemical staining using antibodies against Ki-67, K3, ABCG2 and p63 were the same as described in chapter 2 and 3.

Results

Neovascularization in the cornea disappeared up to 35 days after the injury in a control dog. In the transplantation group, neovascularization was not observed on 21 days after the injury, just before transplantation in all three dogs. After the corneal epithelial sheets transplantation, neovascularization was induced within 7 days in response to the sutures. However, no obvious neovascularization into the transplanted sheets was observed. The neovascularization gradually disappeared by removing the sutures and the score of the neovascularization decreased below one in the transplantation group 21 days after transplantation (Fig. 4-1-A).

No invasion of the conjunctival tissue was observed in all dogs. On day 21 after the injury, just before transplantation of the corneal epithelial cell sheet, moderate haze that partially hindered observation of iris detail was comparably observed in all dogs. In a control dog, similar corneal opacity remained for 28 days and the mild haze was maintained up to 60 days. In the transplantation group, the score of corneal opacity gradually decreased after fourth week of transplantation. Up to 60 days, the cornea of all the dogs in the transplantation group showed improvement of transparency as compared with that of a control dog (Fig. 4-1-B).

The opaque area in the transplantation group was greatly decreased as compared with a control dog (Fig. 4-2-A). The diminution of opaque area was 4.12% in 60 days of observation period in a control dog, while the diminution of opaque area in the transplantation group was 17.93% in average (26.71%, 14.27%, and 12.81% in each dog) (Fig. 4-2-B).

H&E staining revealed that injured corneal area was filled with the epithelial-like cells in a control dog. However, lots of cells that had cytoplasmic vacuolization were observed in all layers (Fig. 4-3-A). On the other hand, in the transplantation

group, defected cornea was reconstructed by epithelial cells without vacuolization. However, reconstructed epithelial layer consisted of 6 to 13 layers of epithelium, which were likely to be caused by hyperplasia of epithelium. And hypercellularity in stroma under the transplanted area was observed in the transplantation group but not in a control dog (Fig. 4-3-B).

Immunofluorescence staining revealed that no positive expression of Ki-67 was observed in the injured area in a control dog (Fig. 4-4-A). In contrast, a few cells slightly positive for Ki-67 were occasionally observed in the basal layer of the transplanted area in the transplantation group (Fig. 4-4-B). The expression of K3 was also not observed in all layers in the injured area of a control dog (Fig. 4-5-A), while the transplanted area strongly expressed K3 (Fig. 4-5-B). ABCG2 expression was not detected throughout the cornea both in a control dog and transplanted dogs (Fig. 4-6-A, B). However, significantly different was observed in the expression of p63, another stem/progenitor marker, between a control dog and transplanted dogs. There was no positive expression of p63 in a control dog (Fig. 4-7-A). On the other hand, the basal layer of transplanted area clearly expressed p63 similar to normal cornea in the transplantation group (Fig. 4-7-B)

Discussion

In this chapter, canine corneal injury model was produced by widespread resection of the central corneal epithelium. Although the intrusion of the conjunctival tissue into the cornea did not appear, angiogenesis into the cornea and corneal opacity was observed after the injury. The moderate haze of the defected area and the neovascularization suggested that all the layers of corneal epithelium were removed completely and the superficial stroma was also eliminated. In addition, the mild haze persistently remained up to 60 days after the injury in a control dog even though all the dogs were received ophthalmic treatment with contact lens or eye-drop. Hence, it was supposed that the canine corneal injury model produced in the present study successfully represented the refractory corneal opacity caused by corneal injury.

Excision of the limbal epithelium induces partial limbal deficiency and can lead invasion of conjunctival tissue in cornea (88). Small piece of limbal tissue excised from opposite limbal epithelium was sufficient to cultivate the autologous corneal epithelial cell sheet to cover the injured area. However, local invasion of conjunctival tissue into the defected limbal area was observed. Although the intrusion was limited to the excised area and did not affect the cornea, downscale in size of the limbal segment may be needed to reduce the encroachment of the conjunctival tissue (89).

Corneal epithelial cell sheet was sutured to the remaining cornea around the injured area in the present study, and inflammatory response to the suture material was observed. It was reported that application of topical immunosuppressants including betamethasone was recommended to reduce the irritation when the transplantation of corneal epithelial cell sheet was sutured (90). Therefore, if possible, suture material may be better to be removed earlier to avoid inflammatory reaction and promote healing of corneal surface.

The cell sheet obtained in this study had enough strength to endure the surgical manipulation and the sutures to the cornea surface, and any rupture or deterioration of the sheet was not observed during transplantation. Although the sheet has adequate stiffness for transplantation, a soft contact lens was used to prevent the friction between the motions of the third eye lid and the sheet. However, contact lens originally contribute to protect the corneal defects and promote healing of injured cornea (91). Therefore, it is considered that healing of injured cornea in a control dog was also promoted by contact lens to some extent.

Inflammation and graft rejection are typical failure in corneal transplantation (92). Other than inflammation in response to the sutures, any inflammation response caused by the cell sheet was not observed, even though no immunosuppressant was administered after transplantation. Therefore, it is considered the autologous corneal epithelial cell sheet is safe and adequate to transplant for treatment of the corneal injury.

Epithelial-like cells with cytoplasmic vacuolization covered the defected area of cornea in a control dog and K3 expression was not observed in these cells. These phenotypes are similar to Meesmann's corneal dystrophy, which is a type of corneal dystrophy due to the mutations of the either *KRT3* or *KRT12* gene (93). K3 coding by *KRT3* gene consists intermediate filament and structural framework of corneal epithelial cells. Therefore, a lack of K3 expression in a control dog suggested that the corneal injury model established in the present study inhibited normal regeneration of corneal epithelial cells and lead to abnormal cell sequence resulted in corneal opacity.

Although any cytoplasmic vacuolization was not observed in the transplantation group, substantially thick epithelium including 6 to 13 layers expressing K3 was observed in transplanted area. Hyperplasia of the corneal epithelium is usually observed in normal process of corneal healing (94). Although the

expression of proliferation marker, Ki-67 was occasionally observed in the transplantation group, the expression level was similar to or less than normal cornea. Therefore, the hyperplasia observed in transplantation group was thought to be within normal reaction and expected to recover to normal structure by following turnover of corneal epithelial cells.

In the transplantation group, basal layer of the corneal epithelium in the reconstructed area was intact and tightly adhered to the stromal bed of the host eye. The intact basement membrane of cornea not only contributes to regeneration of epithelial cells but also works as the epithelial barrier that prevents cytokines and growth factors from tear film (95). For example, the transforming growth factors from tear film stimulate keratocytes to differentiate into myofibroblasts and lead corneal opacity during stromal recovery (96). Further, hypercellularity in stroma under the transplanted area was observed in the transplantation group but not in a control dog. The hypercellularity in stroma is also usually observed when the injury involved corneal stromal layer (97). Some reports suggest that IGF-II transforms hypercellular stromal cells to fibroblasts that regenerate extracellular matrix and restore corneal transparency in wound healing (98). It was also reported that canine corneal epithelial cells could release IGF-II in vitro (99). Therefore, it was suggested that the transplantation of the cultivated cell sheet promoted wound healing in both epithelial and stromal restoration resulted in the corneal transparency via the secretion of some growth factors including IGF-II.

The progenitor or immature cells with abundant proliferative property such as young TACs maintained in the sheet are essential to encourage long-term graft survival, reepithelization, and restoration of transparency by preventing the invasion of conjunctival epithelial cells (28-31). In the previous chapter, corneal epithelial cell sheet cultivated on collagen gel and exposed to air for five days slightly expressed

ABCG2 in the basal layer. However, after 60 days from transplantation, the expression of ABCG2 was not observed in the reconstructed corneal epithelium. ABCG2 expression is generally localized in the limbal basal cells and believed to give rise to TACs. Therefore, it was suggested that the cells with ABCG2 expression in the cultivated sheet contributed to reepithelization by producing highly proliferative cells like as TACs and resulted in hyperplasia of the epithelium in the transplantation group. Thus, the depletion of the cells positive for ABCG2 in the transplanted area indicated that aggressive proliferation of corneal epithelial cells had marked the end until the observation point.

Rama et al. (32) suggested that amount of cells with strong expression of p63, which indicated high potential of self-renewal and proliferation, was significantly associated with successful transplantation. In the previous chapter, the corneal cell sheet on collagen gel with five days of air exposure was selected as an optimal graft because of the highest level of p63 expression. After 60 days of observation period, no expression of p63 was observed in a control dog, while definitive expression was observed in transplanted area similar to normal cornea in the transplantation group. However, unapparent expression of proliferative marker Ki-67 at the observation point indicated that the cells with positive expression of p63 in the transplanted area were not in proliferation phase at the observation point. Although the expression of p63 is observed whole of the canine normal cornea, higher expression is observed in the limbus than central cornea. The reconstructed epithelium in the transplantation group supposedly consisted of proliferated tissue from the cells, which were highly expressed p63, in the corneal epithelial cell sheet. Therefore it was suggested that the cells strongly positive for p63 including in the sheet proliferated and then, the expression of p63 gradually decreased to the level of normal central cornea with the suspension of the proliferative ability.

In this study, the corneal epithelial cell sheet cultivated on the collagen gel with 5 days of air exposure was transplanted to the canine corneal injury model. Restoration of corneal transparency was achieved without any immune response caused by the transplanted cell sheet. In contrast, opaque area with moderate haze persistently remained in a control dog. Therefore, the transplantation of the autologous corneal epithelial cell sheet can be used for recovery of corneal transparency in dogs with corneal opacity caused by severe corneal injury. Further, reconstruction of superficial stroma as well as the corneal epithelium can be available by transplantation of the corneal epithelial cell sheet. The observation period longer than 60 days should be needed to identify influence of hyperplasia of reconstructed epithelium on corneal transparency for the future application of the canine corneal epithelial cell sheet in clinical cases.

Table. 4-1 Evaluation standards for neovascularization (A) and corneal opacity (B)

A

	Neovascularization
0	No neovascularization
1	Up to 3 clock hours
2	3 to 6 clock hours
3	6 to 9 clock hours

B

	Corneal Opacity
0	Completely clear cornea
1	Faint corneal haze
2	Mild haze easily visible on slit-beam illumination
3	Moderate haze partially obscuring iris detail
4	Marked haze that obscure iris detail

Evaluation standards for neovascularization (A) from “Cultivated human conjunctival epithelial transplantation for total limbal stem cell deficiency”. Ang, L. P., Tanioka, H., Kawasaki, S. et al. 2010.*Invest Ophthalmol Vis Sci* **51**: 758-764.

Evaluation standards for corneal opacity (B) from “Amniotic membrane patching promotes healing and inhibits proteinase activity on wound healing following acute corneal alkali burn.” Kim, J. S., Kim, J. C., Na, B. K. et al. 2000. *Exp Eye Res* **70**: 329-337.

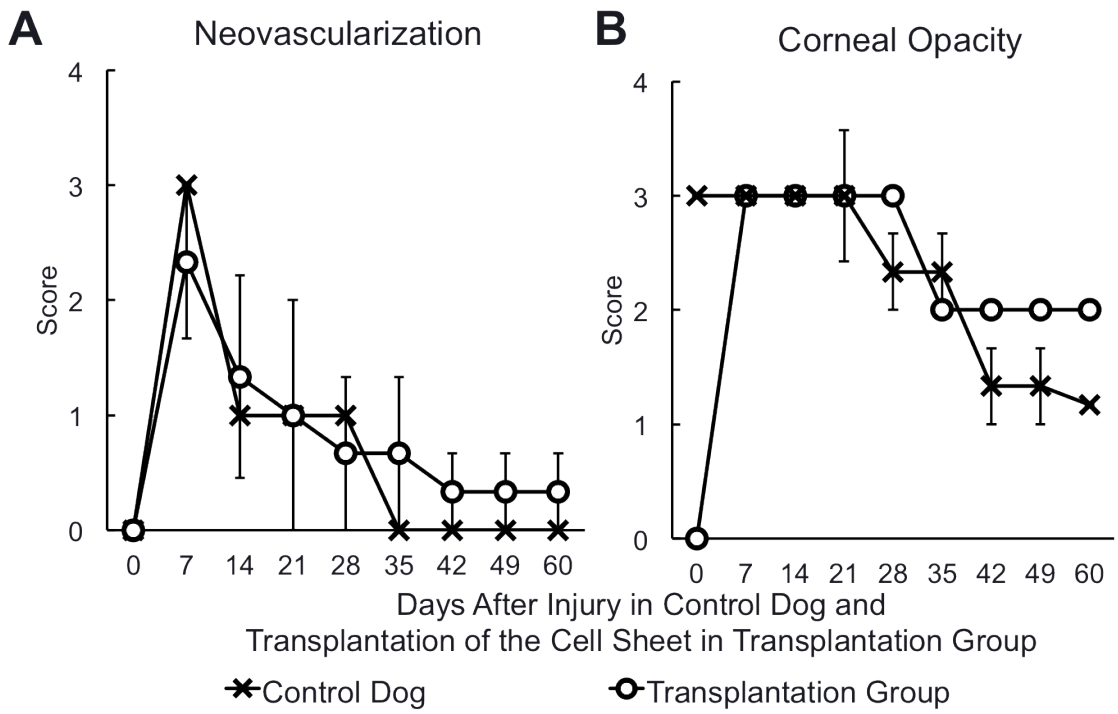

Fig. 4-1 Scores of neovascularization and corneal opacity

Neovascularization in the cornea disappeared up to 35 days after the injury in a control dog. After the corneal epithelial sheets transplantation, neovascularization was induced up to 7 days after transplantation in response to the sutures. By removing the sutures, the score of the neovascularization decreased below one in the transplantation group 21 days after transplantation (A). In evaluation of corneal opacity, moderate haze that partially hindered observation of iris detail was comparably observed in all dogs 21 days after the injury, just before transplantation of the corneal epithelial cell sheet. In a control dog, similar corneal opacity remained for 28 days and the mild haze was maintained up to 60 days. In the transplantation group, the score of corneal opacity gradually decreased after fourth week of transplantation and showed improvement of transparency as compared with that of a control dog up to 60 days after transplantation (B).

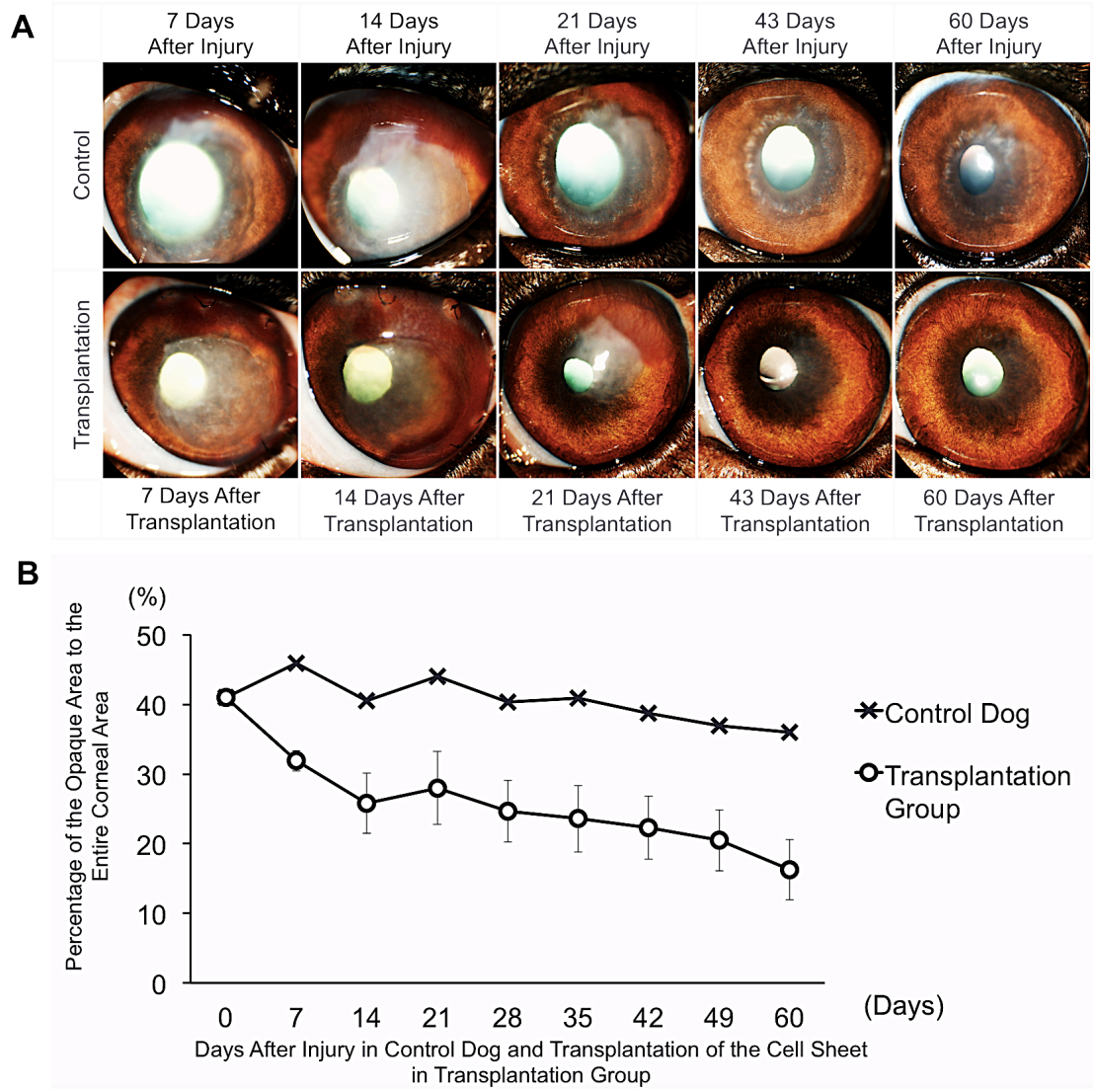

Fig. 4-2 Gross appearance of ocular surface and the changes in a percentage of opaque cornea/whole corneal area

A percentage of opaque area in the transplantation group was greatly decreased as compared with a control dog (A). The diminution of opaque area was 4.12% in 60 days of observation period in a control dog, while the diminution of opaque area in the transplantation group was 17.93% in average (26.71%, 14.27%, and 12.81%) (B).

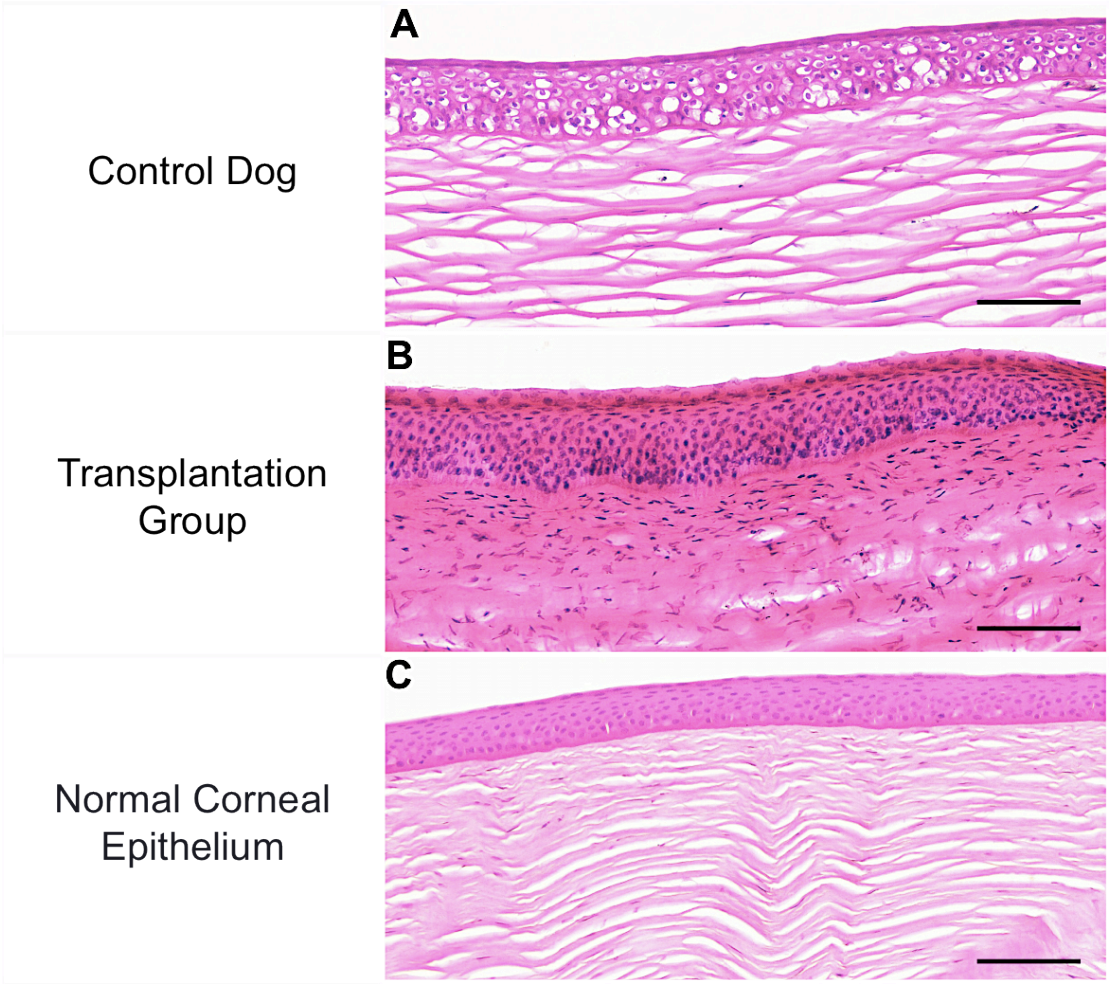

Fig. 4-3 H&E staining of the injured area of a control dog, the transplanted area of the transplantation group and the normal canine corneal epithelium.

H&E staining revealed that injured corneal area was filled with the epithelial-like cells with cytoplasmic vacuolization in a control dog (A). In the transplantation group, defected cornea was reconstructed by epithelial cells without vacuolization and 6 to 13 epithelial layers. And hypercellularity in stroma under the transplanted area was observed in the transplantation group but not in a control dog (B). Canine Normal corneal epithelium (C). Bar scale: 100 μm.

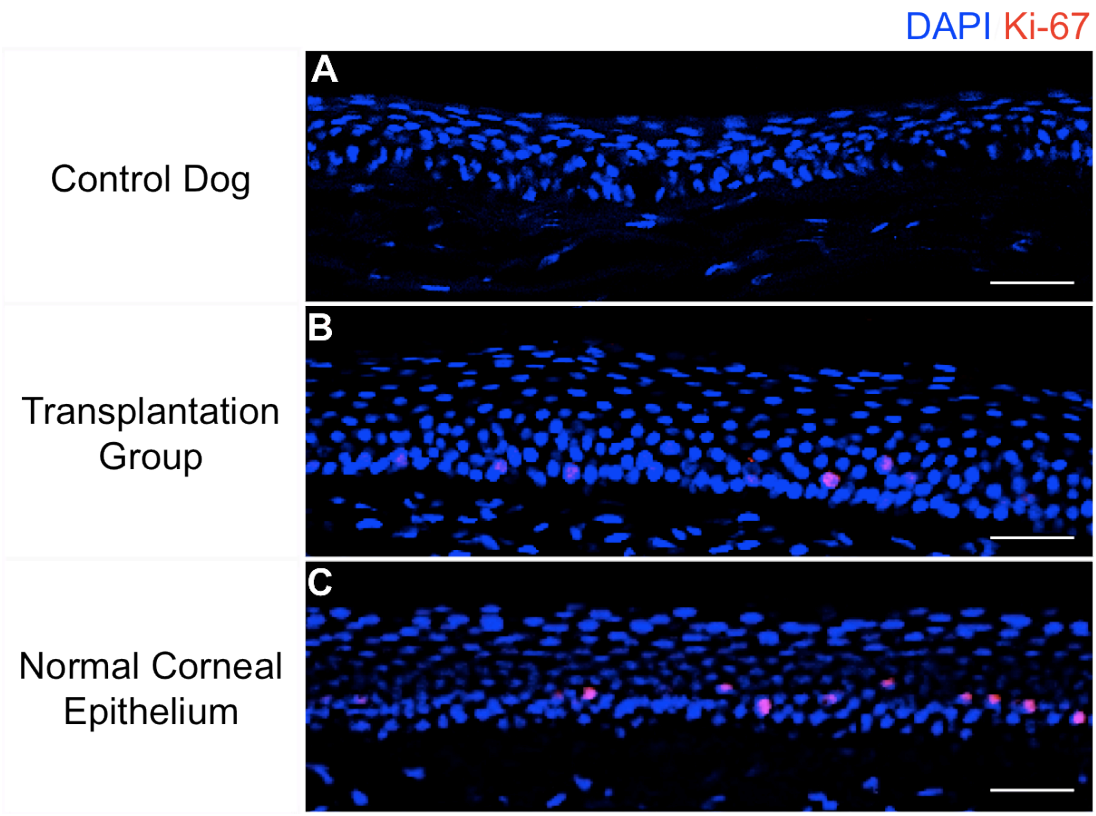

Fig. 4-4 Immunohistochemistry for Ki-67 of the injured area in a control dog, the transplanted area of the transplantation group and normal canine corneal epithelium.

No positive expression of Ki-67 was observed in the injured area in a control dog (A). In contrast, a few cells slightly positive for Ki-67 were occasionally observed in the basal layer of the transplanted area in the transplantation group (B). The expression of Ki-67 in normal canine corneal epithelium (C). Bar scale: 50 μm.

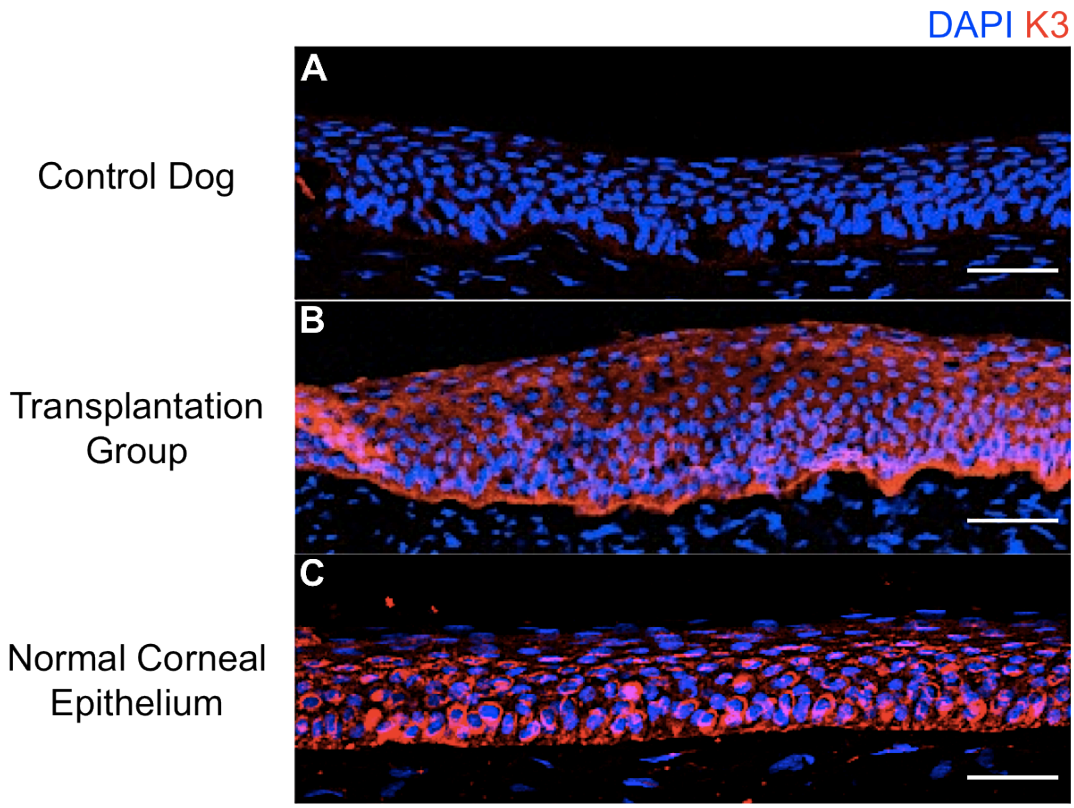

Fig. 4-5 Immunohistochemistry for K3 of the injured area in a control dog, the transplanted area of the transplantation group and normal canine corneal epithelium.

The expression of K3 was also not observed in all layers in the injured area of a control dog (A), while the transplanted area strongly expressed K3 (B). The expression of K3 in normal canine corneal epithelium (C). Bar scale: 50 μm.

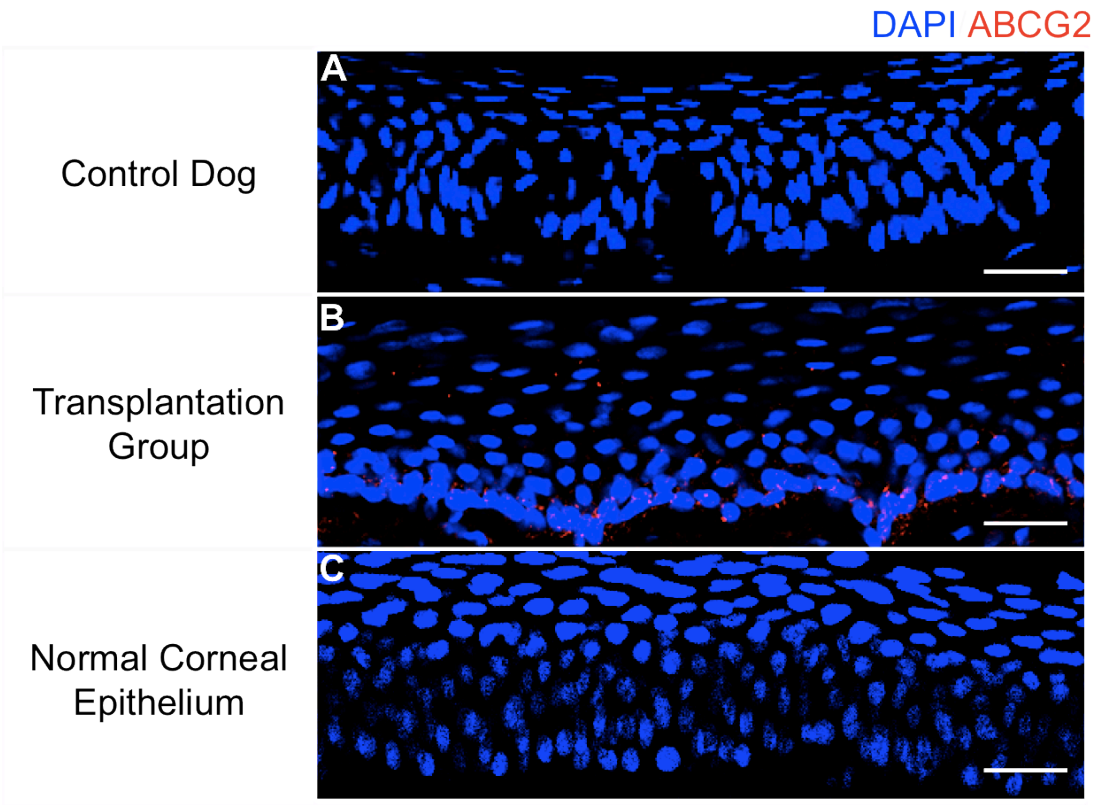

Fig. 4-6 Immunohistochemistry for ABCG2 of the injured area in a control dog, the transplanted area of the transplantation group and normal canine corneal epithelium.

ABCG2 expression was not detected throughout the cornea both in a control dog and transplanted dogs (A, B) The expression of ABCG2 was not observed in canine normal cornea. Bar scale: 25 μm.

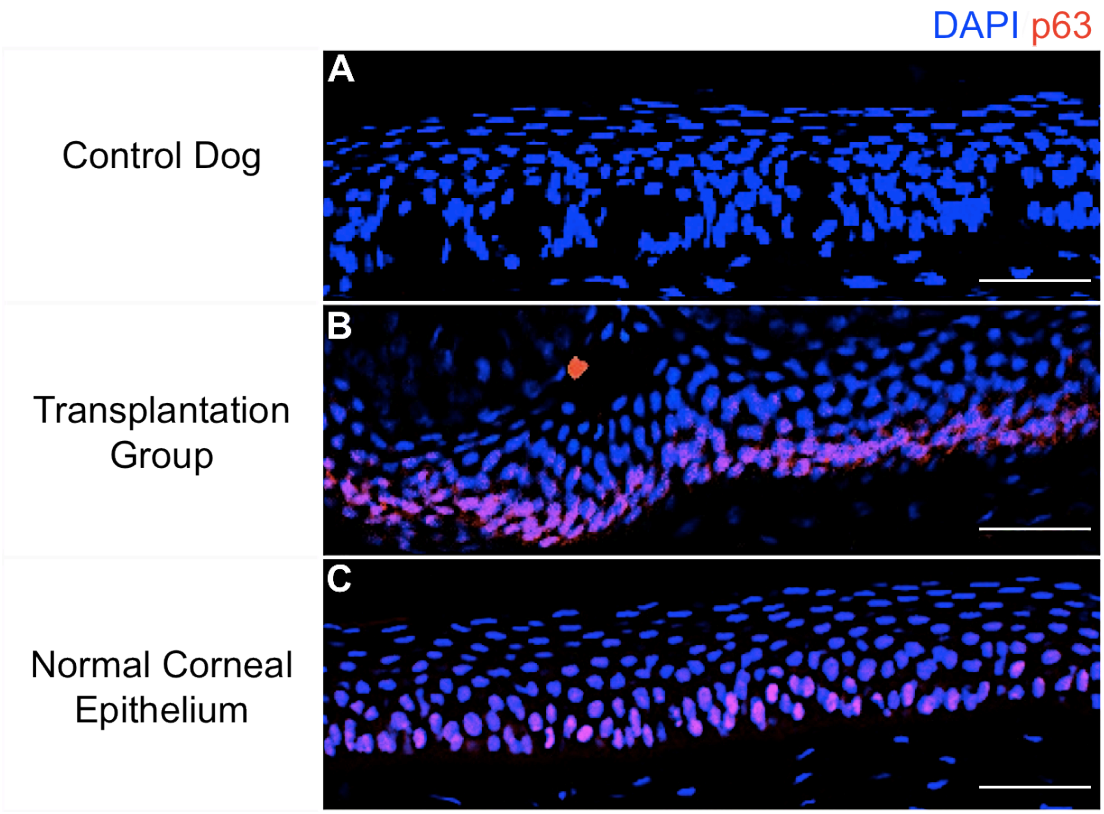

Fig. 4-7 Immunohistochemistry for p63 of the injured area in a control dog, the transplanted area of the transplantation group and normal canine corneal epithelium.

There was no positive expression of p63 in a control dog (A). In the transplantation group, the basal layer of transplanted area clearly expressed p63 similar to normal corneal epithelium (B, C). Bar scale: 50 μm.

Chapter 5

Conclusion

Corneal injury caused by trauma, infection, or diseases are very common in dogs. However, healing of epithelial injuries may be insufficient in severe cases resulting in corneal opacity, ulcers, perforations, or even vision loss. In humans, successful transplantations using with the corneal epithelial sheets cultivated from limbal corneal stem cells have been reported for treatment of severe corneal injury. Transplantation of autologous corneal epithelial cell sheet can solve problems such as insufficiency of a proper donor for the corneal transplantation and immunological rejection. In the present study, canine corneal epithelial cell sheet using corneal epithelial stem cells derived from limbus was cultivated using various materials and evaluated to investigate the method to produce most promising canine corneal epithelial cell sheet. After that, the safety and efficacy of the transplantation of the cell sheet was demonstrated using canine corneal injury model as a preliminary trial for clinical application.

In chapter 2, corneal epithelial cell sheets were successfully cultivated using by the three different materials, those were, the amniotic membrane, collagen gel and the temperature-responsive culture dish. The cells cultured on the collagen gel showed the highest ability of colony formation and proliferation. Stratified layers that were morphologically most similar to a normal corneal epithelium on the collagen gel and amniotic membrane and one or two layers on the temperature-responsive culture dish appeared. The expressions of corneal epithelial stem cell markers, p63 and ABCG2, were limited in the basal layer of the sheet cultivated on amniotic membrane and the temperature-responsive culture dish. On the contrary, the expression of p63 was appeared in two or three layers including basal layer in the sheet cultivated on collagen gel. Real-time RT-PCR analysis revealed that the cultivated sheet on collagen gel showed the highest value of p63 gene expression and the lowest value was detected in the sheet on the amniotic membrane. It is considered that the extracellular matrix of the collagen gel could promote differentiation from stem cells to

progenitor cells with preservation of stem/progenitor cells. Additionally, the corneal epithelial cell sheet cultivated on the collagen gel can be transplanted to cornea without the substrate because the collagen gel is easily degraded by collagenase. Therefore, collagen gel may be the most suitable material among the three to promise the effective and safe transplantation to canine corneal injury in clinical cases.

In chapter 3, the optimal period of air exposure was evaluated by comparing the cell sheets treated with air exposure for 0, 3, 5, and 7 days. The sheet with 5 days of air exposure showed similar morphology to normal cornea and exhibited the highest expression of Ki-67 and p63. However, longer than 5 days air exposure not only failed to promote stratification of the sheet and but also resulted in downregulation of p63 expression in the sheet. Any adverse effects on structural and immunohistological phenotypes of the sheet were not observed after degradation of collagen gel. Therefore, air exposure for 5 days and degradation of the collagen gel could be a most suitable culture method to develop the corneal epithelial cell sheet with similar morphology to normal cornea and retention of cells in immature state for promising transplantation in clinical settings.

In chapter 4, the corneal epithelial cell sheet was transplanted to the corneal injury model in dogs. Canine corneal epithelial cell sheet was cultivated according to the results in chapter 2 and 3, and was autologously transplanted to the corneal injury model. The persistent corneal opacity remained in a control dog, while restoration of corneal transparency was observed in the transplantation group. However the sutures used to transplant the sheet caused the moderate reactive inflammatory, the immune response caused by the cell sheet and the graft failure were not observed without administration of any immunosuppressant for 60 days after transplantation. The inflammation caused by the sutures diminished after elimination of the sutures. Histopathological analysis revealed that the defected area of cornea in the control

dog was coved by cells with cytoplasmic vacuolization in all layers and showed a lack of expression of K3, Ki-67 and p63. In contrast, the corneal epithelium in the transplantation group was reconstructed by cells with normal morphology and consisted 6 to 13 layers supposedly caused by hyperplasia of epithelium. In addition, hypercellularity in stroma under the transplanted sheet was observed and suggested that reconstruction of superficial stroma as well as the corneal epithelium can be available by transplantation of the corneal epithelial cell sheet. The transplanted area showed positive expressions of K3 in all the layers, and Ki-67 and p63 in the basal layer, while the lack of expression was detected in the control dogs. Although longer observation should be needed to identify the influence of hyperplasia of reconstructed epithelium, the transplantation of the autologous corneal epithelial cell sheet achieved the reconstruction of corneal epithelia morphologically and immunohistologically similar to the normal cornea and restored the corneal transparency.

The purpose of this study was to develop the corneal epithelial cell sheet using limbal stem cells for corneal regenerative therapy in dogs. This study suggested that the collagen gel was the most suitable material to cultivate corneal epithelial cells and preserve stem/progenitor cells. In addition, air exposure for five days and degradation of the collagen gel revealed that these culture methods could be used to produce most suitable cell sheet for transplantation. Finally, the corneal epithelial cell sheet demonstrated its safety and efficacy by transplantation to the corneal defect model in dogs. The result of this study encourage that the corneal epithelial cell sheet cultivated on the collagen gel can be a new treatment for corneal injury in the clinical practice.

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