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Citation	Japanese Journal of Veterinary Research, 66(2), 83-92
Issue Date	2018-05
DOI	10.14943/jjvr.66.2.83
Doc URL	http://hdl.handle.net/2115/70494
Type	bulletin (article)
File Information	p083-092 Naoki_Fujita.pdf



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A pilot study of transplantation of an autologous corneal epithelial cell sheet in a canine model of corneal injury

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Received for publication, November 16, 2017; accepted, December 25, 2017

Abstract

Corneal transplantation is the most effective method of restoring corneal transparency. However, graft rejection due to immunoreaction may occur and prevent proper healing of the wounded corneal surface. To address this issue, we transplanted an autologous corneal epithelial sheet into the wounded corneal surface of a dog and evaluated the sheet's efficiency and safety for clinical use. Four dogs with a wounded corneal surface— three experimental dogs receiving transplanted corneal epithelial cell sheets and a control dog who did not receive the transplant. We removed the entire layers of corneal epithelium and superficial stroma from each dog. Corneal epithelial sheets were cultivated from autologous limbal segments and were transplanted 21 days after removing the three dogs' corneal surfaces. The dog that did not undergo transplantation was euthanized 60 days after receiving corneal injury. The corneas of dogs in the transplantation group were collected 60 days after the transplant. Results: Corneal transparency was restored in the transplantation group. One dog in the transplantation group showed immunoreaction to the sutures. However, corneal opacity and neovascularization were negligible 60 days after the transplant in the transplantation group. In addition, the control dog showed opacity of the corneal surface. The transplanted areas each showed hyperproliferation in the epithelium and hypercellularity in the stroma. The stem/progenitor markers for a healing corneal surface also showed patterns similar to a normal cornea. We believe that the transplantation of an autologous sheet may restore corneal transparency and prevent irreversible opacity caused by severe injury.

Key Words: Corneal regeneration, corneal epithelial cell sheet, canine corneal injury, collagen gel, autologous transplantation

Introduction

Corneal injuries caused by trauma, infections, or diseases are very common in dogs, and patients with a severe corneal injury usually

cannot sufficiently recover corneal transparency. Corneal transplantation is the most effective and aggressive method of restoring corneal transparency, but success rates are hindered by immunological graft rejections and other

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doi: 10.14943/jjvr.66.2.83

complications that can result in severe corneal vascularization and edema. Consequently, such transplantation may not be recommended for the treatment of wounded corneas in dogs^{7,8)}. Recently, xenografts such as porcine intestines have been used as a material for corneal transplants in dogs³⁾. There is concern, however, that porcine intestines can cause graft rejections¹⁶⁾. As a result, it remains difficult to efficiently and safely restore corneal transparency after severe injuries in dogs.

Cultivated corneal epithelial cell sheets have been used to regenerate ocular surfaces and restore corneal transparency in human patients with limbal cell deficiency¹⁰⁾. Corneal epithelial cell sheets can be cultivated from a small piece of the limbal epithelium, which contains corneal stem/progenitor cells. Therefore, using them for transplantation may resolve the primary concerns with canine corneal transplantation: graft rejection and the lack of resources for corneal repair and development.

In a previous study of ours, a corneal epithelial cell sheet was successfully cultivated from canine limbal epithelial cells on collagen gel. The gel was an optimal substrate for the corneal sheet, as it supported the adhesion and proliferation of corneal epithelial cells and it sustained corneal epithelial stem/progenitor cells¹¹⁾. The phenotypes of the stem/progenitor corneal epithelial cells suggested that the sheet would demonstrate strong healing effects¹²⁾. Thus, it was proposed that the transplantation of a corneal epithelial cell sheet cultivated from the autologous limbal epithelium on collagen gel could bring about a better outcome in cases of canine corneal injury.

In this study, we cultivated canine corneal epithelial cell sheets on collagen gel and transplanted them onto experimentally injured canine corneal surfaces. The effectiveness and safety of this transplantation were evaluated by ophthalmic examination and histological evaluation.

Materials and methods

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

Collection of limbal tissue and preparation of the corneal injury model: One male and three female beagle dogs, at three years of age and weighing about 10 kg, were used in this study. Three dogs were used for the transplantation group and one was used as a control. All of the animals underwent, a physical examination, radiography analysis, and blood biochemistry analysis before surgery to examine whether dogs were healthy enough to tolerate general anesthesia. An ophthalmic examination was also performed and no abnormalities were found. For pre-anesthetic medication, 25 µg/kg of atropine sulfate was injected subcutaneously along with 20 mg/kg of cefazolin sodium. All of the animals were anesthetized with propofol and isoflurane (2.0%) in oxygen after intubation. Fentanyl citrate (5–20 µg/kg/hr) was used to alleviate pain in the perioperative period up to 12 hours after the surgery.

All procedures were performed by veterinarians using aseptic technique at a dedicated surgical room. Under general anesthesia, the center of the left cornea of each dog was trephined over a diameter of 7.5 mm and a depth of 200 µm using a Castroviejo corneal trephine (Inami, Tokyo, Japan). Next, the corneal tissue was removed with a crescent knife (MANI, Tochigi, Japan). To reduce dogs' unnecessary stress caused by continuous anesthesia and surgeries, a small segment of the limbus (2 × 2 mm) was simultaneously obtained from the right eye of each dog in the transplantation group for cultivation of corneal epithelial cell sheets. After the operation, the removed corneal surface of the both group was covered with a soft contact lens (Meni-one, Nagoya, Japan) for one week. To reduce pain and prevent infection, the

affected eyes were administered 0.3% ofloxacin topically four times a day and the dogs were given 2.2 mg/kg of carprofen orally twice a day before the surgery and for 14 days after the procedure. An Elizabethan collar was applied to all dogs of the both groups for two weeks after the surgery.

Preparation of the collagen gel and 3T3 cells: In accordance with our previous study¹¹⁾, collagen gel and 3T3 feeder cells were prepared to cultivate the corneal epithelial cell sheets. 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 ratio of 1:1:8 by volume⁴⁾. The solution was supplemented with 0.1% glutaraldehyde (Wako, Tokyo, Japan) for crosslinking and gently mixed at 4°C. One hundred twenty µl of the blended reagent was then spread onto the surface of the culture, which was inserted in the six-well plate and stored at 37°C for two hours. The collagen gel was washed twice with PBS and immersed in sterilized PBS overnight at 37°C to wash out any remaining glutaraldehyde before use.

In order to cultivate the corneal epithelial cell sheets, 3T3 fibroblast cells (Japanese Collection of Research Bioresources, Tokyo, Japan) were used as feeder cells to promote cell proliferation and stem/progenitor cell retention. Confluent 3T3 fibroblasts were incubated with 4 µg/ml of mitomycin C (Wako, Tokyo, Japan) for two hours at 37°C. Then, single cells of 3T3 fibroblasts were placed onto six-well cell culture dishes at a density of 3.3×10^4 cells/cm².

Cultivation of the epithelial cell sheet on collagen gel: Primary canine corneal epithelial cells were cultured with an explant culture method according to a previous report, with only a minor modification¹⁵⁾. The obtained limbal epithelial graft was attached to a type I collagen coated dish, cultured in DMEM/Ham's F12 mixture

(3:1;Invetrogen), and then supplemented with 10% FBS, 10 ng/ml human recombinant epidermal growth factor, 10 µg/ml human recombinant insulin, 100 U penicillin, 100 µg/ml streptomycin sulfate, and 2.5 µg/ml amphotericin B (Invitrogen). The culture medium was changed every other day. The cells were passaged at a density of approximately 3.5×10^4 cells/cm² on the collagen gel in the six-well culture insert. The inserts were submerged in culture medium until confluence was achieved. Then, the level of the medium in the six-well plate was lowered for five days so as to expose the confluent epithelial monolayer to air. This promoted the differentiation and stratification of the epithelial cells. After that, the collagen gel was degraded by collagenase I (50 U/ml) in PBS at 37°C⁴⁾. One hour later, the collagenase solution was removed. The culture medium was added to the plate to inhibit collagenase activity. The corneal epithelial cell sheet was kept at 4°C and harvested from the insert membrane just before transplantation.

Transplantation of the cultivated limbal epithelial cell sheet: An autologous corneal epithelial cell sheet was transplanted into three dogs 21 days after removing their corneal surfaces. All procedures were performed by veterinarians using aseptic technique at a dedicated surgical room. General anesthesia was performed as described above. Fentanyl was also used to alleviate pain in the perioperative period up to 12 hours after the transplantation. After debriding the surface of injured cornea with a trephine and a crescent knife to expose stromal bed, an autologous corneal epithelial cell sheet was sutured onto the corneal surface using 9-0 nylon sutures. A soft contact lens covered the operated area for one week (Fig. 1). Ofloxacin and carprofen were also applied before the surgery and for 14 days after the procedure. The collar was also applied to all dogs of the both groups for two weeks after the second surgery.

Ophthalmic evaluation: For the control dog, day 0

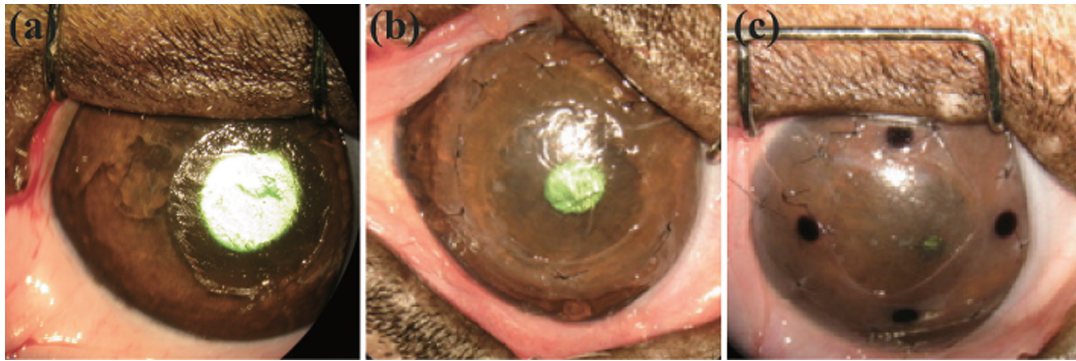


Fig. 1. Transplantation of the autologous corneal epithelial cell sheet to the injured canine cornea. The corneal epithelium and superficial stroma were removed by a trephine with a diameter of 7.5 mm (a). The corneal epithelial sheet was sutured onto the corneal surface (b). The soft contact lens was placed and sutured onto the cornea (c).

describes the day of the corneal epithelium injury. For the transplantation group, day 0 indicates the day the corneal sheet was transplanted. An ophthalmic examination was performed using a slit lamp once a week until day 60. A fundus camera (Kowa, Nagoya, Japan) was used to take pictures of the ocular surface. Neovascularization and corneal opacity were scored (0–4 points) based on the evaluation standards for each according to previous studies (Tables 1 and 2)^{1,5)}. The opaque area was measured with imaging software (Image J version 1.37; National Institutes of Health, Bethesda, MD). Then, the ratio of the opaque area to the injured area was calculated. After 60 days of observation period, all the dogs were humanely euthanized. The corneal tissues were collected and subjected to histopathological examination.

Histopathological and immunohistopathological examinations of the cornea: The collected corneal tissue was analyzed via hematoxylin and eosin (H&E) staining and immunohistochemical staining. The tissue was fixed in paraformaldehyde and then embedded in OCT compound (Sakura Finetek, Tokyo, Japan). It was then frozen in liquid hexane and stored at -80°C until sectioning. Frozen sections, 4- and 7- μm in thickness, were prepared using a cryomicrotome for H&E staining and immunofluorescence staining, respectively. For immunofluorescence

staining, sections were washed three times for five minutes each time in PBS and blocked with 10% normal goat serum for one hour at room temperature. Subsequently, anti-rabbit Ki-67 (1:100; Abcam, Cambridge, UK), anti-mouse keratin 3 (K3, 1:50; Progen, San Francisco, CA, USA), and anti-mouse p63 (1:50; Abcam) were applied and incubated overnight at 4°C . After washing the slides, the sections were treated with either Cy3-conjugated goat anti-mouse IgG antibody (Zymed, San Francisco, CA, USA) or Cy3-conjugated goat anti-rabbit IgG antibody (Zymed) for 45 minutes. Finally, 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), and examined with a laser confocal microscopy (Olympus, Tokyo, Japan). Corneal segments were collected from uninjured eye globes and stained to be used as references for normal canine corneas.

Results

To create the severe corneal injury model, we removed approximately 40% of the total area of the cornea. The eliminated corneal tissue included all layers of the corneal epithelium and the superficial corneal stroma. Because a moderate haze persisted on the corneal surface

Table 1. Evaluation standards for neovascularization

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

Evaluation standards for neovascularization from “Cultivated human conjunctival epithelial transplantation for total limbal stem cell deficiency”. Ang, L.P.K., Tanioka, H., Kawasaki, S., Ang, L.P.S., Yamasaki, K., Do, T.P., Thein, Z.M., Koizumi, N., Nakamura, T., Yokoi, N., Komuro, A., Inatomi, T., Nakatsukasa, M., and Kinoshita, S. 2010. *Investigative Ophthalmology & Visual Science* 51: 758–764.

Table 2. Evaluation standards for corneal opacity

	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 corneal opacity 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., Jeong, J.M., and Song, C.Y. 2000. *Exp Eye Res* 70: 329–337.

in the transplantation group until just before the transplantation, it was determined that a sufficient corneal injury had been produced.

To evaluate the safety and efficiency of the autologous sheet transplantation, we performed ophthalmic examinations for 60 days (Fig. 2a–j). Although excessive lacrimation was observed on day 1, tear production normalized by day 2. Sheet sutures in the transplantation group were removed on day 14, except for one dog, whose were removed on day 7 because they appeared to be causing inflammation. The inflammation disappeared shortly after suture removal. However, throughout the entire observation process, there was neither inflammation nor graft failure due to immunologic rejection around the sutures.

To confirm whether the corneal epithelial cell sheet transplantation was effective, we estimated neovascularization and corneal transparency. Neovascularization was observed around the injured or grafted area within seven

days in both groups. Neovascularization gradually decreased after suture removal in the transplantation group, with the score falling below one after day 21. The average score of neovascularization in the transplantation group was slightly higher than that of the control, because mild neovascularization around the suture persisted in one of the three dogs (Fig. 3a). Before the procedure, all dogs in the transplantation group showed a moderate haze (score 3) that was similar in appearance to the control dog at day 21. Although corneal opacity in the control dog was slightly decreased by day 35, no improvement in transparency was observed between then and day 60. On the other hand, the transplantation group showed a gradual recovery of corneal transparency between day 28 and day 60 (Fig. 3b). The opaque area in the transplantation group was significantly reduced as compared to the control dog, with an average diminution rate by day 60 of 59.18% for

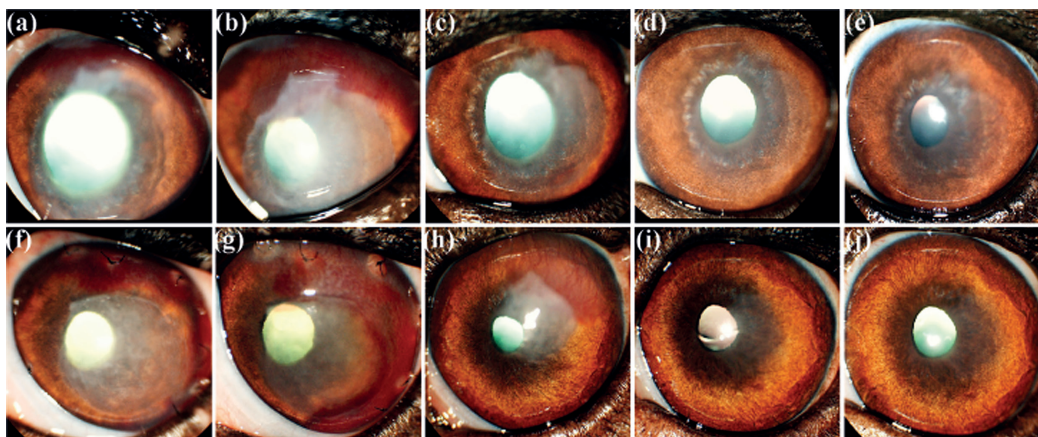


Fig. 2. Comparison of ophthalmic evaluations between the control dog and the transplantation group. Photographs of ocular surface at 7 days (a), 14 days (b), 21 days (c), 42 days (d), and 60 days (e) after injury in the control dog and 7 days (f), 14 days (g), 21 days (h), 42 days (i), and 60 days (j) after the transplantation of the corneal epithelial sheet in the transplantation group.

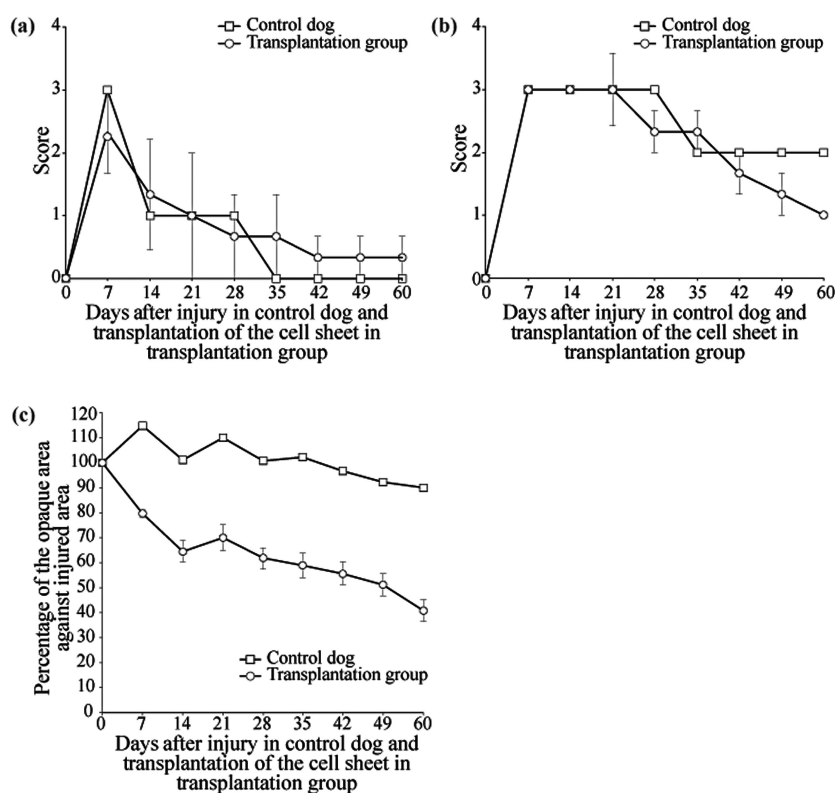


Fig. 3. Score of neovascularization and corneal opacity, and change of opaque area. Compared with the control dog, the transparency of cornea was improved in the transplantation group. Neovascularization scores in the transplantation group were slightly higher than that of the control dog due to the neovascularization around the suture (a). Corneal opacity decreased in the transplantation group for 60 days after the transplantation of the sheet (b). The percentage decline in the total opaque area in the transplantation group was higher than the control dog (c).

the transplantation group and 4.12% for the control (Fig. 3c). These results indicate that sheet transplantation helped to recover corneal

transparency.

Differences in the healing processes of the grafted cornea and the control were examined

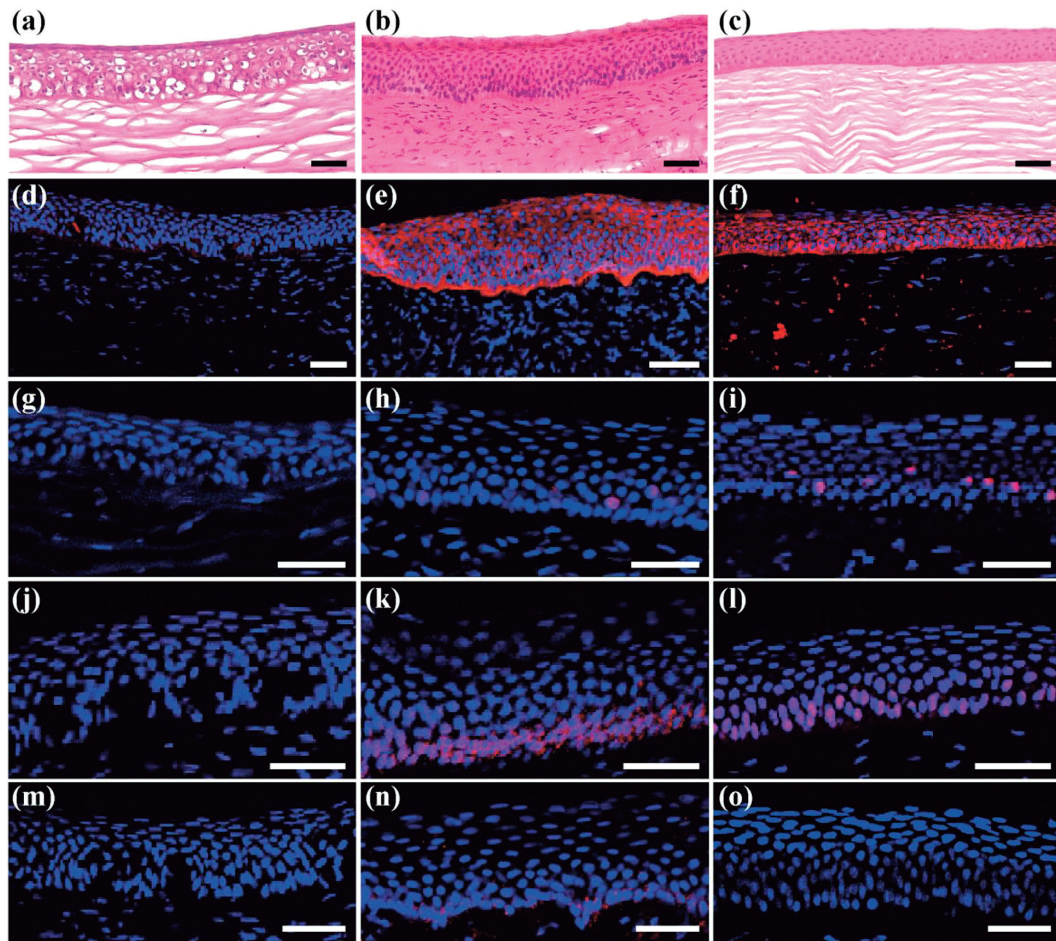


Fig. 4. Histopathological evaluation of the cornea. H&E staining of the injured area of the control dog (a), the transplanted area of the transplantation group (b), and the normal canine corneal epithelium (c). Cytoplasmic vacuolization in the corneal epithelium was observed in the control dog (a). Hyperplasia in the epithelium and hypercellularity in the stroma were observed in the transplantation group (b). Although the expression of K3 (red) was rare in the control dog (d), the transplanted area (e) and the normal corneal epithelium (f) expressed K3 in all layers. No positive expression of Ki-67 (red) appeared in the control dog (g). The transplanted area (h) showed expression of Ki-67 in the basal layer in a manner similar to the normal corneal epithelium (i). The expression of p63 was observed in the basal layer of the transplanted area (k) and normal epithelium (l), whereas no positive expression of p63 in the control dog was detected (j). Scale bars: 50 μ m

using cross sections. The control dog had epithelial-like cells with cytoplasmic vacuolization in all layers (Fig. 4a). On the other hand, the transplanted areas were covered by a range of 6 to 13 epithelial cell layers, all without vacuoles. In addition, corneal stroma hypercellularity was observed just under the transplantation site (Fig. 4b). The expression of K3, a specific marker of the corneal epithelium, was not observed in all layers of the injured area of the control dog (Fig. 3d), but was strongly expressed in the transplanted area (Fig. 4e). A few cells slightly

positive for Ki-67, a marker of proliferation, were discovered in the basal layer of the transplanted area (Fig. 4h) but not in the epithelium of the control dog (Fig. 4g). This phenotype is similar to a normal cornea (Fig. 4i). The basal layer of the transplanted area clearly showed p63, a marker of stem/progenitor corneal epithelial cells (Fig. 4k), similar to the center of a normal cornea in the transplantation group (Fig. 4l). However, the expression of p63 was not observed in the control dog (Fig. 4j).

Discussion

In this study, we cultured canine limbal cells on collagen gel and created corneal epithelial cell sheets for transplantation onto injured corneas. In a previous study, we observed that corneal epithelial sheets cultivated on collagen gel were suitable for transplantation because they have a structure similar to a normal corneal epithelium and contain abundant stem/progenitor cells¹¹. Though we degraded the collagen gel before the transplantation, the sheet had sufficient strength to endure the surgical procedures without the substrate. In addition, no ruptures on the sheet were observed in the transplantation group. To avoid detachment of the sheet from the corneal surface during washing process of fluorescein stain, we did not perform the fluorescence staining right after the transplantation. After removal of the lens on the corneal surface with the transplanted sheet, we preformed the fluorescence staining and could not find any stain on the cornea (data not shown). This result might indicate that corneal epithelial sheet successfully attached to the corneal surface.

No immunoreaction was observed after the transplantation. We eliminated the porcine collagen gel from the cultivated autologous material before the transplantation. Hence, the wound was able to heal and graft rejection was not a concern. It has been reported that sutures on the corneal surface may cause inflammation and neovascularization². Although this was observed in the present study, the complications were immediately relieved after suture removal on day 7. Despite this suture removal, the transplanted sheet did not move from the corneal surface. This suggests that the autologous epithelial corneal sheet successfully attached to the injured corneal surface soon after transplantation. Therefore, it may be not only permissible, but also advisable to remove sutures earlier in order to prevent inflammatory reactions. In addition, all dogs in the transplantation group showed less intense opacity and a reduced area

of opacity after the operation when compared to the control dog. Therefore, we submit that autologous corneal epithelial sheet transplantation is clinically safe and effective in restoring corneal transparency.

The corneal surface in the transplantation group seemed reconstructed by the corneal epithelium after transplantation. In a previous study, the corneal cell sheet on collagen gel was selected as an optimal graft because the sheet expressed high levels of p63 and Ki-67, which suggested a greater potential for self-renewal and proliferation¹¹. Several reports have suggested that cells expressing p63 are strongly associated with successful transplantation because they encourage long-term graft survival, reepithelialization, and transparency restoration by preventing the invasion of conjunctival epithelial cells^{12,13,14}. Consequently, we considered that the regeneration of the corneal epithelium in the transplantation group was mediated by cells expressing p63 and Ki-67.

Epithelial hyperproliferation was observed in the reconstructed corneas of the transplantation group. This might be a positive outcome because such hyperproliferation is typically observed in the normal corneal healing process and disappears after full wound repair¹⁷. Furthermore, Ki-67, a marker of proliferation, showed a negligibly positive expression in the epithelium. The expression levels of Ki-67 and p63 were similar to those of a normal cornea. Therefore, corneal stem/progenitor cells in the transplanted sheet may be responsible for inducing hyperproliferation and helping the transplanted corneal epithelium achieve a normal structure. Although hypercellularity were also observed in the stroma, we could not discern a type of the cells. However, it is supposed that these reaction might be one of regeneration processes in the stroma because cell migration in the stroma, which usually occurs when an injury involves the corneal stromal layer¹⁸. Furthermore, the hypercellularity also appeared under the transplanted area but not in the control. It has been reported that canine

corneal epithelial cells could release IGF-II *in vitro*⁶⁾. Since IGF-II transforms hypercellular stromal cells into fibroblasts that regenerate the extracellular matrix and restore corneal transparency during wound healing⁹⁾. Therefore, we suggest that the transplantation of the cultivated cell sheet with some growth factors such as IGF-II might promote wound healing in both the epithelium and the stroma. An observation period longer than 60 days is necessary to evaluate the importance of epithelial hyperplasia and stromal hypercellularity for future clinical use.

Contrary to the transplantation group, the control dog showed epithelial-like cells with cytoplasmic vacuolization and a lack of K3 expression in the affected area. These results suggest that abnormal cell phenotypes induced corneal opacity in the corneal injury model. However, the present study used only one dog to reduce number of dogs in animal experiments. Unfortunately, we could not explain the reason why the control dog showed abnormal pathological appearance on corneal surface. We supposed that wound including entire epithelial layers and superficial stroma might cause unusual degeneration of corneal epithelial tissue. Therefore, further study is needed to characterize the cellular mechanisms of control dogs in more numbers of corneal injury models.

In this study, autologous corneal epithelial cell sheets were derived from a small piece of the limbal epithelium, cultivated in collagen gel, and transplanted into canine corneal injury models. The wounded corneas were healed and showed restored transparency 60 days after the transplantation. In addition, we supposed that the superficial stroma was reconstructed by the transplanted corneal epithelial cell sheet. Because the sheet was cultivated from autologous cells, no adverse effects were observed. For safer transplantation, subjects must be observed for a longer period of time and a method must be established to prevent inflammation induced by sutures. In addition, more numbers of control

dogs would be needed to compare the effect of the transplantation of sheet. In conclusion, autologous canine corneal epithelial cell sheet transplantation might be used as a safe and effective treatment for recovering corneal transparency. The procedure's benefit is that it provides proliferative resources for the graft and prevents immunological issues that often occur when xenogenous materials are transplanted. This technique could be offer a new perspective on veterinary ophthalmology and improves the likelihood of recovery for dogs suffering from irreversible corneal opacity caused by injury.

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