博士論文（要約）

Establishment of a limbus-derived canine corneal epithelial cell line and application of its cell sheet for corneal regenerative therapy

（犬輪部由来角膜上皮細胞株の樹立と
細胞シートによる角膜再生療法への応用）

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Chapter 2.

Establishment of a canine corneal epithelial cell line and investigation of the maintenance mechanism of the proliferative properties in canine corneal epithelial cells

General Introduction

Structure and biology of the cornea

The cornea is a transparent and avascular tissue, acting as a barrier between the eye and the environment, and refracts light to form an image on the retina. The cornea consists of the epithelium, stroma, Descemet's membrane, and endothelium. The surface of cornea is covered by non-keratinized stratified squamous epithelium, consisting of the layers of columnar basal cells lying on the basement membrane, intermediate wing cells, and squamous superficial cells. The epithelial basement membrane, which anchors epithelial cells to the stroma, is mainly composed of type IV collagen, laminin, and proteoglycans. The stroma occupies 90% of corneal thickness, primarily composed of stromal cells which are called as keratocytes, collagen fibrils, and proteoglycans. The anterior stroma is a thin and acellular zone known as the Bowman's layer, which is absent in carnivores [1], while the posterior stroma, called as the Descemet's membrane, is the basement membrane for the endothelial cells, which are single layer of flattened cells located in the innermost of cornea.

Corneal epithelial stem cells have an important role in the maintenance of corneal epithelial homeostasis via the continuous renewal. It is widely accepted that corneal epithelial stem cells reside in the basal epithelium of the limbus, a narrow transitional zone between the cornea and the bulbar conjunctiva. This theory is supported by the evidence that limbal epithelial cells contain slow cycling cells [2], lack differentiation marker [3], and have higher proliferative potential than peripheral corneal epithelial cells [4]. Corneal epithelial stem cells

comprise less than 10% of the total limbal basal epithelial cell population, producing proliferative daughter cells known as transient amplifying cells [2, 5]. Transient amplifying cells migrate from limbus toward the central cornea to replace the terminally differentiated cells desquamating from the corneal surface [6] (Figure). This process keeps corneal transparency by preventing both conjunctival cell ingrowth and superficial neovascularization, which cause corneal opacity.

Corneal epithelial cells are isolated from the limbal tissue because cells with proliferative properties are absent in the central cornea [7]. There are many studies which investigated the biological phenotypes of the corneal epithelial cells and corneal epithelial stem cells using *in vitro* culture system. Although the definitive markers of corneal epithelial stem cells have not been identified, several markers such as p63, ABCG2, N-cadherin, and cytokeratin 15 (K15) have been suggested as putative stem/progenitor cell markers based on the preferential expression in the cells located in the limbal basal epithelium [8-11]. The expression of these markers is absent in differentiated corneal epithelial cells, which express the markers such as cytokeratin 3 (K3), cytokeratin 12 (K12), and connexin 43 [3, 9].

Corneal diseases and their treatments in dogs

Corneal diseases are very common in dogs due to their conformational features. For example, prominent eyes and nasal folds, often found in brachycephalic dogs, have been considered as risk factors for corneal ulcers [12]. A variety of etiologies including trauma, infection, tear film deficiencies and neurological, immunological, genetic, or metabolic disorders may result in corneal diseases. One of the major problems associated with corneal diseases is corneal opacity that can lead to low or loss of vision.

Corneal opacity is caused by various abnormalities. Pigmentation is a nonspecific biological response to corneal stimulation and may develop associating with corneal wound healing [13]. Melanocytic pigment deposits in the basal epithelial cells and the anterior stroma [14]. Neovascularization is a normal reparative response after injury but it causes disruption of corneal architecture and result in corneal opacity [14]. Stromal scar is formed as a result of epithelial-stromal wound healing process after trauma, inflammation, and infection. These stimuli induce the differentiation of the quiescent keratocytes into α-smooth muscle actin (αSMA)-positive myofibroblasts, which are opaque and secrete disorganized extracellular matrix, resulting in fibrotic scar and loss of corneal transparency [15, 16]. Corneal edema also leads to corneal opacity, caused by damage of epithelium and endothelium, which serve as a barrier against fluid influx [14].

Depending on the underlying causes and their severity, corneal diseases are treated

by topical medication or surgical treatment. In case of mild corneal injury, topical antibiotics with an anti-collagenase agent or hyaluronic acid usually provide a healing in a short term and restoration of corneal transparency. Surgical intervention is needed when the corneal damage is severe and the topical medication is not effective. Conjunctival graft has been commonly performed to provide tectonic support and fibrovascular tissue as well as blood supply, but could leave corneal scar that may interfere with vison [14, 17]. Other grafting materials such as equine, porcine, and canine amniotic membrane have been developed and investigated, but it is often difficult to acquire these materials [18-20]. In addition, there is a concern regarding the safety of the use of xenogeneic materials. Although corneal transplantation as a curative treatment for severe corneal injury has been reported in dogs, it is not practical for veterinary medicine because the eye bank for dogs has been developed poorly and donor cornea is difficult to obtain [21, 22]. Although the incidence of corneal graft rejection in dogs is controversial due to the limited information, corneal transplantation in dogs often result in graft opacity such as pigmentation and edema [21, 22]. Therefore, a novel therapy that can restore corneal transparency is required for the dogs with severe corneal injury.

Corneal transplantation and corneal regenerative therapy using a cultivated corneal epithelial cell sheet

In humans, corneal blindness is the third leading cause of blindness, following cataract and glaucoma [23]. Ten million people are suffered from bilateral corneal blindness in the world [23]. For patients with corneal blindness, corneal transplantation has been performed widely in order to restore the corneal transparency and visual function. Although allograft rejection is the major cause of graft failure, success rate of corneal transplantation is high in human because cornea is considered to be rather immunotolerant as compared with other organs due to immune privilege including weak expression of major histocompatibility complex (MHC) antigens, the relative lack of mature antigen-presenting cells, and the presence of immunomodulating molecules in the anterior chamber fluid [24]. However, the shortage of donor tissue limits the number of patients who can receive the corneal transplantation [23, 25].

In 1997, Pellegrini et al. initially reported a successful transplantation of corneal epithelial cell sheets harvested and expanded from autologous limbal tissue of patients with severe corneal disease [26]. In the study, cells were cultured from a 1-mm^2 biopsy sample taken from the limbus of the opposite healthy eye in two patients with corneal alkali burns. The cell sheets were transplanted onto the damaged eye resulting in improvement of corneal transparency and visual acuity [26]. To date, many researchers have reported that

transplantation of corneal epithelial cell sheets fabricated from autologous or allogeneic limbal epithelial tissue may be a curative treatment choice for patients with limbal stem cell deficiency [27-32]. Thus, the transplantation of corneal epithelial cell sheets is expected to be a novel regenerative therapy for severe corneal injury which is refractory to conventional therapies. Various improvements have been tried in culture method of corneal epithelial cell sheets by use of scaffolds such as amniotic membrane, fibrin glue, and collagen gel in order to facilitate the cell growth and graft procedure [27-32]. From the results obtained in these researches, it was suggested that the inclusion of p63-positive cells in the grafted sheet is associated with successful transplantation [32].

In veterinary medicine, cultivation of canine corneal epithelial cell sheets using canine amniotic membrane or type I atelocollagen gel as scaffolds has been reported [33, 34]. The atelocollagen gel was considered to be an optimal scaffold for cultivating canine cell sheets because it supported the adhesion and proliferation of the cells and maintenance of the higher expression of p63 in the cell sheets compared to amniotic membrane [34]. Although transplantation of canine corneal epithelial cell sheets has not been reported, it would be a promising treatment choice for intractable corneal injury in dogs as well as in human.

Limitations for clinical application of canine corneal epithelial cell sheets

Although the transplantation of corneal epithelial cell sheets is expected to be a novel regenerative therapy for severe corneal injury in dogs, there are some limitations for clinical application. Generally, co-culture with feeder cells and the use of growth promoting additives have been commonly employed for *in vitro* expansion of various stratified squamous epithelial tissues including skin, oral mucosa, and cornea [26, 35, 36]. Feeder cells such as mitotically inactivated 3T3 murine fibroblasts help to maintain the stem/progenitor phenotypes of epithelial cells probably by secreting soluble factors [37-42]. The absence of feeder cells has been shown to limit the growth potential of epithelial cells and prevent continuous culture [43-45]. Feeder cells have been used when corneal epithelial cells are cultured to obtain cell sheets in dogs as well as human and rabbits [26, 33, 34, 46]. However, the use of xenogeneic feeder cells or xenoantigen derived from feeder cells give rise to ethical, immunological, and safety issues. Therefore, the use of alternative autologous cell sources or development of the novel cultivation method without feeder cells is desired.

In addition, growth promoting additives, e.g. epidermal growth factor (EGF), insulin, transferrin, cholera toxin, isoproterenol, and hydrocortisone, were also used in most of the previous studies to facilitate corneal epithelial cell growth [47-49]. Although the combination use of these factors enabled successful culture of corneal epithelial cells without feeder cells in human [48, 50], it takes a high cost for the clinical use.

Canine severe corneal injury often needs early surgical intervention. However, it is difficult to transplant a corneal epithelial cell sheet to the patients immediately after injury because cultivation of cell sheets from autologous tissue usually needs about two to three weeks. In human, allogeneic transplantation of corneal epithelial cell sheets has been used when autologous tissue is not available due to bilateral corneal diseases [30, 31]. Allogeneic limbal tissue is usually obtained from eye bank or living-related donor, but application of these resources is not practical in dogs. Therefore, other resource such as canine corneal epithelial cell line is expected to be established.

Species-specific maintenance mechanism of proliferative properties in canine corneal epithelial cells

It has been reported that canine oral mucosal epithelial cells exhibited a prominent proliferative potential under a feeder cell-free condition [51]. A mechanism of high proliferative potential without feeder cells has been suggested that canine oral mucosal epithelial cells secrete growth promoting factors in an autocrine manner which spontaneously maintain superior cell proliferative properties [52]. Recently, oral mucosal epithelial cells have been explored as alternative cell sources to corneal epithelial cells for cultivating corneal epithelial cell sheets in humans due to their biological similarity [36]. These findings indicate that canine corneal epithelial cells may also have the spontaneous maintenance of proliferative properties and some underlying mechanism as in canine oral mucosal epithelial cells. If canine corneal epithelial cells proliferate spontaneously without feeder cells or growth promoting additives, it may become possible to fabricate canine corneal epithelial cell sheets more safely and at lower cost. Further, if there is some maintenance mechanism of proliferative properties in canine corneal epithelial cells, a canine corneal epithelial cell line could be established by serial culture. Successful establishment of a canine corneal epithelial cell line would enable a constant supply of the cells for the corneal epithelial cell sheets, and the feasibility of its transplantation to canine corneal injury which require the emergency treatment will be expanded.

Purposes of this study

Based on the background described above, the purposes of this study were to investigate whether canine corneal epithelial cells possess the proliferative properties independent of feeder cells and growth promoting additives, to establish a canine corneal epithelial cell line, and to evaluate the feasibility of corneal epithelial cell sheets fabricated from the established cell line. First, I compared the proliferation of canine and rabbit corneal epithelial cells, and the influence of feeder cells and growth promoting additives on their proliferative properties was evaluated (Chapter 1). Then, I tried to establish a spontaneously derived canine corneal epithelial cell line by long-term serial culture, and the maintenance mechanism of the proliferative properties in canine corneal epithelial cells was investigated using the obtained cell line (Chapter 2). Next, I investigated whether transplantable cell sheets can be fabricated from the cell line (Chapter 3). Finally, transplantation of the cell line-derived sheet to a canine corneal injury model was performed to evaluate the safety and efficacy as a corneal regenerative therapy (Chapter 4).

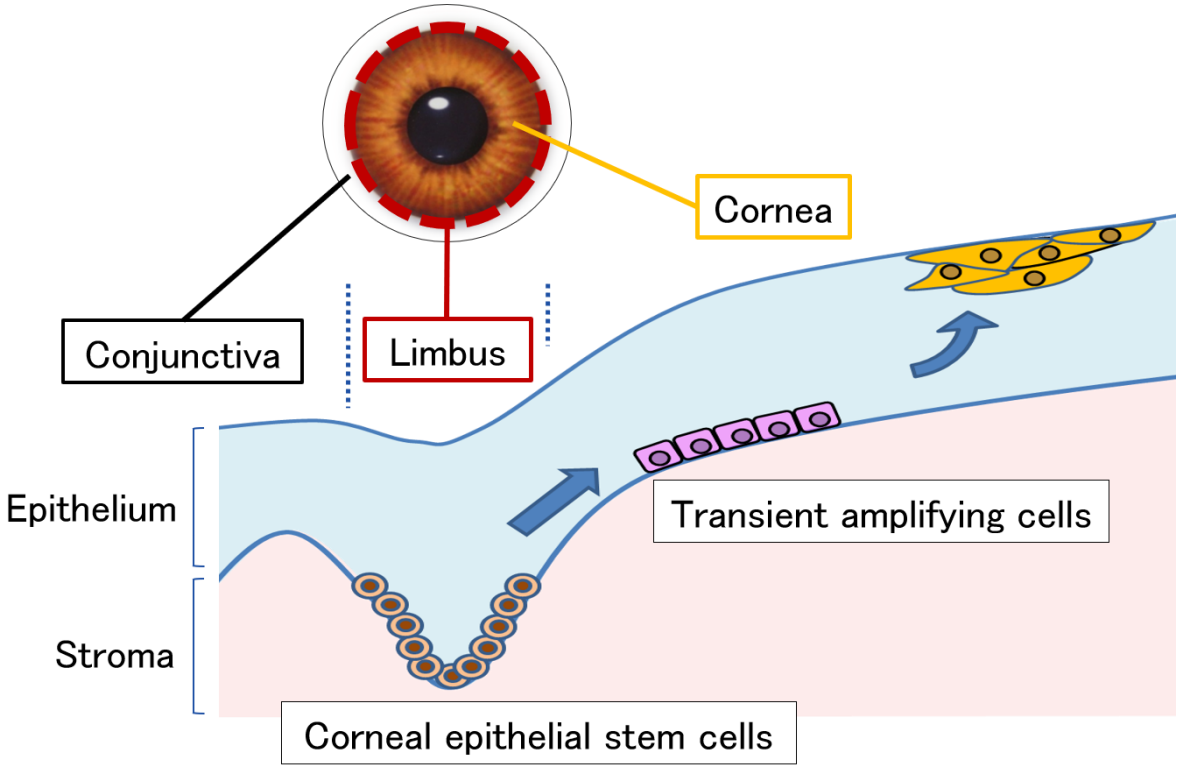

FIGURE. Maintenance of corneal epithelial homeostasis. Corneal epithelial stem cells located in the limbus produce transient amplifying cells. Transient amplifying cells migrate centripetally to replace the terminally differentiated cells desquamating from the corneal surface.

Chapter 1.

The influence of feeder cells and growth promoting additives on the proliferation of canine and rabbit corneal epithelial cells

Introduction

Corneal diseases are very common in dogs. In case of severely injured cornea, irreversible abnormalities such as pigmentation and stromal scar could remain on the ocular surface even after the surgical treatments and lead to permanent corneal opacity resulting in loss of vision. Corneal transplantation is widely accepted for ocular surface reconstruction in humans, but it is not practical in dogs due to the poor development of eye bank system. Recently, cultivation of canine corneal epithelial cell sheets has been reported and clinical application of the cell sheet transplantation is expected [33, 34]. However, since successful culture of corneal epithelial cells usually requires the use of feeder cells and growth promoting additives, there are concerns regarding the safety issues and the cost. The stable proliferation of corneal epithelial cells without feeder cells or growth promoting additives may make the corneal epithelial cell sheet transplantation more feasible in dogs.

It has been reported that canine oral mucosal epithelial cell growth is more prominent under feeder-free condition than those cells derived from other animals including humans, pigs, rabbits, rats, and mice [51, 52]. Using some growth promoting additives without feeder cells, primary canine oral mucosal epithelial cells exhibited a high proliferation, whereas primary rabbit cells proliferate poorly and failed to reach confluency [51, 52]. Oral mucosal epithelial cells share the similar characteristics with corneal epithelial cells such as cytokeratin 3 (K3) expression and absence of keratinization [36, 53]. Therefore,

canine corneal epithelial cells are expected to have a maintenance mechanism of proliferative properties as in canine oral mucosal epithelial cells and could be cultured without using feeder cells and growth promoting additives. Likewise, the proliferation of rabbit corneal epithelial cells is considered to be limited without these factors. Actually, the expression of proliferation marker in rabbit corneal epithelial cells is decreased under feeder-free condition [54].

The purpose of this chapter was to investigate whether canine corneal epithelial cells possess the proliferative properties independent of feeder cells and growth promoting additives. For this purpose, the influence of feeder cells and growth promoting additives on the proliferation of canine corneal epithelial cells was compared with rabbit corneal epithelial cells. The cell number and the percentage of cell proliferation marker positive cells were compared between canine and rabbit corneal epithelial cells when cultured with or without 3T3 feeder cells and growth promoting additives. Serial passages of canine and rabbit corneal epithelial cells were also conducted with or without growth promoting additives to evaluate whether canine cells maintain the proliferative properties unlike rabbit cells.

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Chapter 2.

Establishment of a canine corneal epithelial cell line and investigation of the maintenance mechanism of the proliferative properties in canine corneal epithelial cells

Introduction

Transplantation of corneal epithelial cell sheets is expected to be a novel regenerative therapy for severe corneal injury in dogs. However, the cell sheets cultivated from autologous limbal tissue could not be immediately applied to patients due to its preparation period for two to three weeks. Canine corneal epithelial cell line is one of the promising cell sources to solve this problem.

Several studies have reported the establishment of an immortalized rabbit and human corneal epithelial cell lines using oncogene transfection with the simian virus 40 large T antigen and human papilloma virus 16 E6/E7 [66-69], but these manipulations often cause genetic instability and changes in the cell growth and differentiation properties [70]. Human telomerase reverse transcriptase (hTERT) overexpression has also been reported to immortalize several types of mammalian cells, including corneal epithelial cells [71]. Although hTERT-immortalized cells are thought to be genetically stable and possess normal growth and differentiation characteristics, it has been reported that potentially malignant genetic changes appear during long term culture [72]. Therefore, it is difficult to use genetically immortalized cells in clinical settings. There have been a few studies on the establishment of a spontaneous corneal epithelial cell line from rabbit and human sources by serial culture [73, 74]. Although these spontaneously derived cell lines still require the use of xenogeneic murine 3T3 feeder cells or growth promoting additives to maintain their growth,

close similarities were shown between the cell lines and population in primary cell culture. Thus, the cell lines obtained by serial culture are advantageous compared to those established by genetical engineering.

In the previous chapter, it was suggested that canine corneal epithelial cells would have a maintenance mechanism of proliferative properties. Corneal epithelial cells cultured from about a half of dogs (47.8%) showed stable growth and reached P10 without using feeder cells and growth promoting additives. Therefore, it is expected that a canine corneal epithelial cell line would be established by further serial passages. Successful establishment of a cell line enables a constant supply of resource not only for cultivating canine corneal epithelial cell sheets but also for studies in biology of canine corneal epithelial cells.

The purposes of this chapter were to establish a spontaneously derived canine corneal epithelial cell line and to investigate the maintenance mechanism of the proliferative properties in canine corneal epithelial cells. First, serial passages of the canine corneal epithelial cells were conducted to establish a cell line and the biological and morphological characteristics were investigated (Section 1). Then, based on the hypothesis that canine corneal epithelial cells maintain the proliferative properties by species-specific autocrine secretion of growth promoting factor, the effect of conditioned medium of canine corneal epithelial cells (primary cells and the established cell line) on the growth of rabbit corneal epithelial cells was evaluated and vice versa. Comprehensive gene expression profile of

canine and rabbit corneal epithelial cells was also compared to explore the candidates of soluble factors providing sustained growth of canine corneal epithelial cells (Section 2).

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Chapter 3.

Cultivation of canine corneal epithelial cell sheets with using the corneal epithelial cell line

Introduction

In humans, transplantation of corneal epithelial cell sheets has been performed for reconstruction of ocular surface in some patients with severe corneal injury as an alternative treatment of corneal transplantation [26-32]. These reports indicate that successful ocular surface reconstruction requires inflammation control, good tear function, and retention of stem/progenitor cells in the cell sheets as well as robust structure with stratified layers.

Corneal epithelial cell sheet is usually cultivated from primary or P1 cells which are seeded on a scaffold such as amniotic membrane, fibrin glue, and collagen gel in order to facilitate cell growth and to maintain stem/progenitor phenotypes [26-32]. Air-lifting technique is commonly applied to fabricate corneal epithelial cell sheets after the cells reach confluency by lowering the level of culture medium so that the cells are exposed to air. Stimulation of air exposure promotes cell differentiation and stratification, and confers robustness to cell sheets [95]. Successful cultivation of canine corneal epithelial cell sheets with robust structure and retention of progenitor cells expressing p63 using the techniques described above has been reported [34]. In the previous study, corneal epithelial cell sheets were cultivated from P1 canine corneal epithelial cells seeded on porcine type I atelocollagen gel as a scaffold, using 3T3 feeder cells and growth promoting additives (human recombinant EGF and insulin) with 5 days of air-lifting [34]. However, using these materials gives rise to safety issues and takes a high cost. Moreover, to prepare P1 cells from limbal tissue needs

one to two weeks and delays cell sheet transplantation for patients requiring immediate treatment.

In the previous chapter, a spontaneously derived canine corneal epithelial cell line (cCEpi) was established without using feeder cells and growth promoting additives. Since the cell line maintained similar characteristics to normal canine corneal epithelial cells, cell sheets which are comparable to P1 cell-derived sheets would be cultivated with using the cell line. Canine corneal epithelial cell sheets cultivated from the cell line would shorten the waiting time for transplantation and increase its feasibility. Further, it is expected that cell sheets can be cultivated without feeder cells or growth promoting additives due to their prominent proliferative properties. Cultivation without these materials would improve the safety of transplantation and reduce the cost.

The purpose of this chapter was to investigate whether canine corneal epithelial cell sheets can be cultivated from the established cell line. Cell sheets were fabricated with or without growth promoting additives under feeder-free condition. Then, the structural phenotype of the cell line-derived sheets was compared with that of P1 cell-derived sheets, and the quality as a graft material was evaluated.

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Chapter 4.

Evaluation of safety and efficacy of allogeneic transplantation with cell line-derived canine corneal epithelial cell sheets

Introduction

Transplantation of corneal epithelial cell sheets is a promising treatment for severe corneal injury in dogs. Since patients usually require immediate treatment, it is not practical to transplant the cell sheets cultivated from autologous limbal tissue. In the previous chapter, canine corneal epithelial cell sheets with robust structure and retaining of stem/progenitor cells were successfully fabricated from cCEpi cell line and were expected to shorten the waiting time for transplantation. Moreover, the cell line-derived sheets had basement membrane component as well as P1 cell-derived sheets. Therefore, suture-less transplantation of the cell line-derived sheets may be available for reconstruction of injured cornea. However, there is a concern about immunological rejection because cell line-derived sheet is an allogeneic graft. Several clinical signs including linear epithelial defect at the host-graft junction, conjunctival congestion, corneal neovascularization, and detachment of grafted cell sheet were demonstrated as immunological rejection after allogeneic cell sheet transplantation [30, 99, 100]. Although the mechanism of rejection has not been completely explained, T cell-mediated rejection, in which grafts are infiltrated with T cells, macrophages, natural killer (NK) cells, and neutrophils, is considered to cause acute rejection after transplantation of cell sheets as well as corneal tissue [24, 100].

Despite the potential risk of immunological rejection, allogeneic transplantation of cell sheets would be clinically more valuable than autologous transplantation because of

several advantages. First, it is possible to prepare cell sheets in shorter period as described above. Shortening the waiting time for transplantation is essential for canine severe corneal injury because it often needs early surgical intervention. Second, the use of allogeneic tissue would relieve the burden of harvesting autologous normal tissue from patients. Finally, allogeneic transplantation can reduce the cost. Cell line is considered as a useful cell source for allogeneic cell sheet transplantation. Cell line-derived sheet transplantation would offer a novel treatment choice for canine corneal injury.

The purpose of this chapter was to evaluate the safety and efficacy of allogeneic transplantation of cell line-derived canine corneal epithelial cell sheets. Canine corneal injury models were received transplantation of fluorescent labeled cell sheets without suture, and the therapeutic effects and immunologic rejection were assessed by ophthalmic evaluation and histopathology.

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Conclusion

Transplantation of corneal epithelial cell sheets has been expected to be a novel regenerative therapy for intractable corneal injury in dogs. Cultivation of the cell sheets usually requires the use of feeder cells and growth promoting additives. However, there are concerns regarding the safety issues and the high cost of using these materials. In addition, the cell sheets cultivated from autologous limbal tissue could not be applied to patients who need immediate treatment. Other resources such as canine corneal epithelial cell line with spontaneous proliferative properties are desired to solve these problems. Recently, it has been reported that canine oral mucosal epithelial cells exhibit a prominent proliferative potential under feeder-free condition probably due to autocrine secretion of growth promoting factors [51, 52]. Since corneal epithelial cells share the similar characteristics with oral mucosal epithelial cells, canine corneal epithelial cells may maintain the spontaneous proliferative properties in a similar mechanism. Moreover, it is expected that a canine corneal epithelial cell line could be established by serial culture. Therefore, in this study, I investigated whether canine corneal epithelial cells possess the proliferative properties independent of feeder cells and growth promoting additives. Then, a spontaneously derived canine corneal epithelial cell line was established and the maintenance mechanism of the proliferative properties in canine corneal epithelial cells was investigated using the cell line. After that, corneal epithelial cell sheets were cultivated from the established cell line. Finally, the cell line-derived sheet was transplanted to a canine corneal injury model to evaluate the safety and efficacy as a corneal

regenerative therapy.

In chapter 1, the proliferation of canine corneal epithelial cells was compared with rabbit corneal epithelial cells when cultured with or without feeder cells and growth promoting additives. Canine corneal epithelial cells could proliferate independent of these materials unlike rabbit cells, and could be passaged multiple times in feeder-free condition, whereas rabbit cells failed to maintain the proliferation. It was suggested that canine corneal epithelial cells would have a unique maintenance mechanism of proliferative properties such as autocrine secretion of growth promoting factors as well as canine oral mucosal epithelial cells.

In chapter 2, a canine corneal epithelial cell line (cCEpi) was established and characterized through over 100 times of passage without feeder cells and growth promoting additives. Although some property changes were observed, cCEpi mostly maintained the similar characteristics to normal canine corneal epithelial cells initially cultured and could be a promising resource for studies of cell biology and for cultivating corneal epithelial cell sheets. Then, the maintenance mechanism of the proliferative properties in canine corneal epithelial cells was investigated focusing on autocrine secretion of soluble factors. Conditioned media of cCEpi cells significantly increased rabbit cell growth, but that of rabbit corneal epithelial cells significantly inhibited the growth of canine corneal epithelial cells and cCEpi cells. EGFR ligands including NRG1 and HB-EGF were highly expressed in canine

corneal epithelial cells and cCEpi. On the other hand, highly expression of soluble factors relating to TGF-β signaling including CTGF and TGF-β2 was observed in rabbit corneal epithelial cells. These results suggested that canine corneal epithelial cells secrete growth promoting factors such as NRG1 and HB-EGF in an autocrine manner, and have low potential to secrete growth inhibitory factors such as CTGF and TGF-β2. These mechanisms would play important roles in maintenance of the proliferative properties in canine corneal epithelial cells.

In chapter 3, canine corneal epithelial cell sheets were cultivated from cCEpi cells and the quality as a graft material was evaluated. Although cultivation of the cell sheets required the support of growth promoting additives, cell line-derived sheets which had robust structure and retained stem/progenitor cells were successfully cultivated without feeder cells. The structural phenotype of the cell line-derived sheets was mostly similar to P1 cell-derived sheets. Therefore, cell line-derived sheets were considered to be available as a graft material for canine corneal injury.

In chapter 4, the cell line-derived canine corneal epithelial cell sheets were transplanted to a canine corneal injury model. Although the histological structure was irregular compared to the normal tissue, transplantation of the cell sheets achieved early re-epithelialization of corneal epithelium. Moreover, reduction of corneal opacity via inhibition of differentiation of keratocytes into myofibroblasts was observed. Clinical signs

and histopathological findings indicating acute rejection were not identified. Although more long-term observation is necessary to evaluate the safety, these results suggested that transplantation of the cell line-derived canine corneal epithelial cell sheets is effective for corneal injury in dogs.

In conclusion, this study demonstrated that canine corneal epithelial cells can maintain their proliferative properties independent of feeder cells and growth promoting additives. Autocrine secretion of growth promoting factors and low secretion potential of growth inhibitory factors are suggested to be the underlying mechanism. Moreover, it becomes possible to constantly supply transplantable canine corneal epithelial cell sheets using the cell line, which was established by long-term serial culture. Cultivation method without feeder cells would improve the safety of transplantation. Transplantation of the cell line-derived sheets could achieve early reconstruction of corneal epithelial tissue and reduction of corneal opacity, and would be useful as a regenerative therapy for canine corneal injury. It is necessary to perform further study to establish a preservation method of cell sheets with high viability to apply transplantation of cell line-derived sheets for patients requiring immediate treatment. Combination of regenerative therapy for corneal stroma and endothelium is also desired in order to develop the cell sheet transplantation therapy with more convenience and higher therapeutic effect.

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