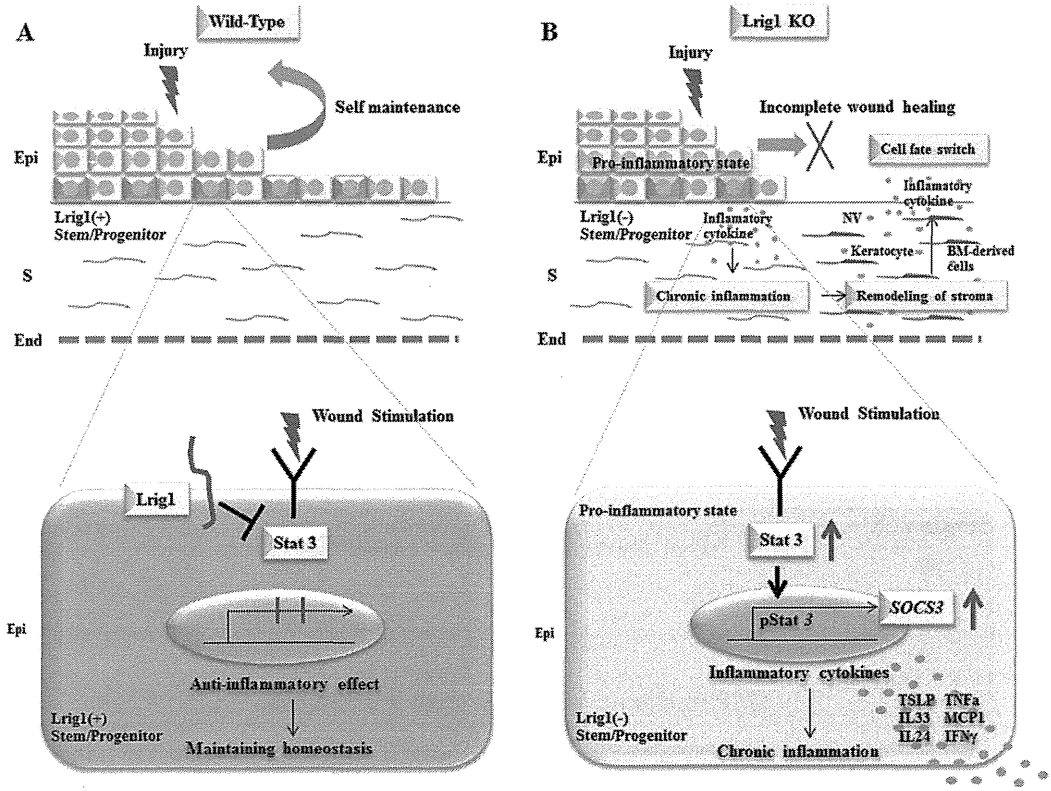

Fig. 13. Model of the function of Lrig1 in corneal homeostasis. (A) Normally, the cornea is self-maintained by Lrig1 (+) corneal stem/progenitor cells post-injury. Corneal transparency is maintained via negative regulation of the Stat3-dependent inflammatory pathway by Lrig1. (B) Loss of Lrig1 causes the pro-inflammatory state in Lrig1 (−) corneal stem/progenitor cells and impairs delayed/incomplete wound healing. Loss of Lrig1 activates the Stat3-dependent inflammatory pathway and induces chronic inflammation, resulting in remodeling of the corneal stroma. Inductive bone marrow-derived cells secrete inflammatory cytokines and cause cell-fate changes to keratinized epithelium. Epi: corneal epithelium; S: corneal stroma; End: corneal endothelium; NV: neovascularization. Modified with permission from Nakamura et al. (2014).

focus on pathogenic organisms in recent years, suitable sterilization of both native and dried AM is vital, and these developments will contribute to the next generation of AM, using tissue engineering techniques. Following strict regulation in Japan, we are now trying to set up an AM banking center to organize the preparation and spread of AM.

4.2. Fibrin and the temperature-sensitive dish

Another promising substrate for clinical use in OSR is fibrin and the temperature-sensitive culture dish. The usefulness of a serum-derived fibrin substrate for cultured corneal epithelial cells has previously been reported (Hirayama et al., 2012; Rama et al., 2001, 2010). As fibrin substrate is absorbed after CLET and COMET, this method has some clinical benefits, in that the cultivated epithelial sheet can be transplanted directly onto the corneal surface. Although fibrin has achieved successful clinical outcomes, safety and logistical problems remain, such as the risk of disease transmission after operations (e.g. human parvovirus B19, prions) (Hino et al., 2000).

Another unique tissue-engineered technique has been developed using a temperature-sensitive culture dish for corneal epithelial cells (Nishida et al., 2004a, 2004b). This is an original system that allows the cultivated epithelium to be detached from the culture dish by changing the temperature. Both of these substrates (fibrin and the temperature-sensitive dish) may be more suitable for transplantation than AM because AM will remain permanently on the transplanted area if severe postoperative inflammation does not occur. However, since the ocular surface (including epithelium and subjacent stroma) is severely damaged in patients with severe OSD, we need to reconstruct the subjacent corneal stroma as well as the epithelial layer. In considering the biological clinical aspects, AM can serve as both an epithelial layer and as a healthy substrate covering a damaged corneal stroma. Further comparative clinical studies are needed to establish which substrate is most effective.

4.3. Development of novel cultured substrates

Although AM, fibrin and the temperature-sensitive culture dish have been widely used for OSR, a variety of alternative substrates suitable for generating tissue-engineered cultured sheet have been examined in preclinical or clinical applications. Among biosynthetic scaffolds, the cross-linked collagen scaffold (Dravida et al., 2008), fibroin membrane (Chirila et al., 2008), myogel extracted from skeletal muscle (Francis et al., 2009), collagen vitrigel (McIntosh Ambrose et al., 2009), compressed collagen (Levis et al., 2010; Mi et al., 2010), keratin films (Reichl et al., 2011) and chitosan hydrogels (Grolik et al., 2012) have all been studied. In the area of synthetic scaffolds, medical-use contact lenses (Di Girolamo et al., 2009), nanofibers (Sharma et al., 2011; Zajicova et al., 2010) and electrospun 3D scaffolds (Ortega et al., 2013) have also been examined. In addition, biological scaffolds such as lens capsule (Galal et al., 2007) and decellular corneal stroma (Shafiq et al., 2012) have been investigated. All these proposed substrates have potential advantages and disadvantages, and careful investigation is again needed before treating patients with severe OSD using these substrates.

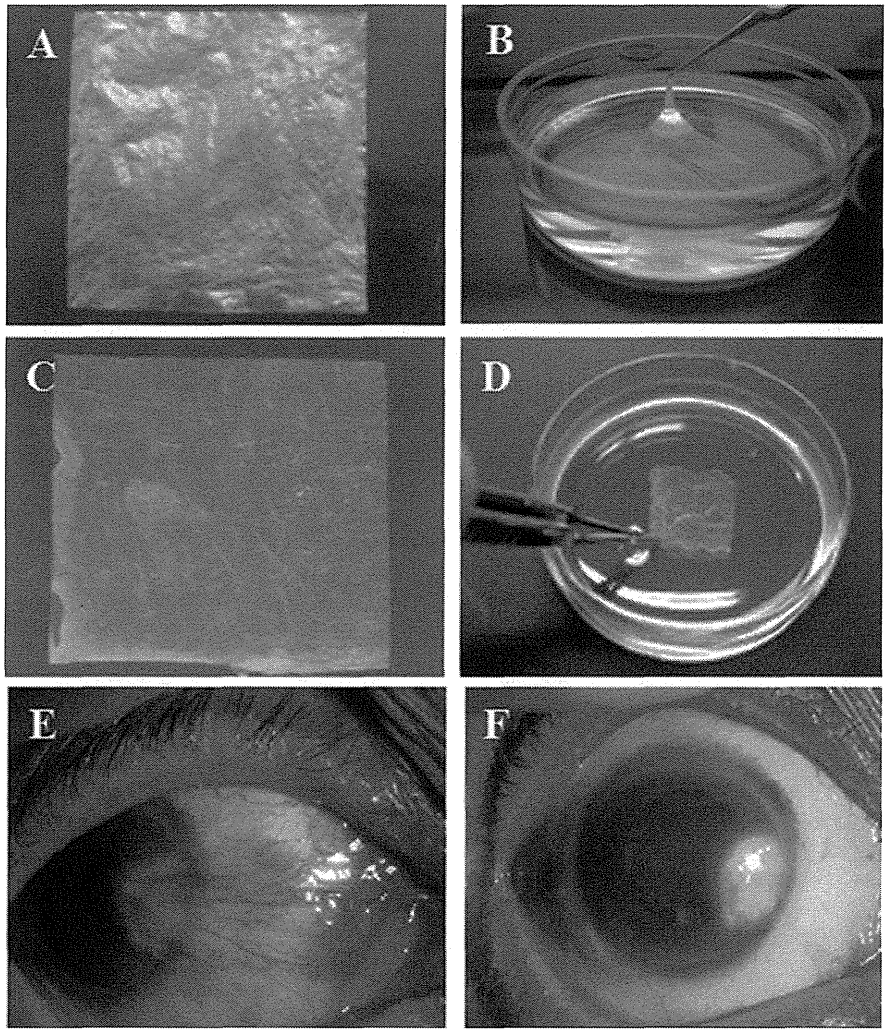

Fig. 14. Development of dried AM. (A) The sterilized, freeze-dried AM was wafer-like and very light and thin. (B) It became smooth and flexible on hydration. (C) Visually, trehalose-treated freeze-dried AM was similar to freeze-dried AM in the dry condition. (D) Trehalose-treated freeze-dried AM was smoother and more flexible than freeze-dried AM in the wet condition. Representative slit-lamp photographs taken before the sterilized, freeze-dried AM transplantation (E) and at 24 months after transplantation (F). Before transplantation, eyes manifested fibrovascular overgrowth of degenerative conjunctiva onto the cornea (E). At 24 months after transplantation, fibrosis was markedly suppressed, and the conjunctival surface was stable without inflammation (F). Modified with permission from Nakamura et al. (2004b), Nakamura et al. (2006b), Nakamura et al. (2008b).

4.4. Bio-adhesive

Among established OSR protocols, the most standard method of AM transplantation, CLET and COMET using AM carrier involve suturing, which is time-consuming and is associated with disadvantages that include suture abscesses, granuloma formation and tissue necrosis. To overcome these problems, sutureless transplantation, using proper tissue-engineered bio-adhesive, is ideal for developing next-generation OSR. There have previously been some studies of sutureless techniques for OSR using fibrin glue (Szurman et al., 2006); we initially developed a novel sutureless technique for AM transplantation by generating a fibrin glue-coated dried AM (Sekiyama et al., 2007) (Fig. 15). Fibrin is derived from serum, so if a non-biologic and defined bio-adhesive can be successfully developed, it would prove ideal for safe and simple OSR. On that basis, we developed AM transplantation for OSR using a chemically defined bio-adhesive that is safe, biocompatible and biodegradable (Takaoka et al., 2008) (Fig. 15). Based on those results, we foresee the use of chemically defined bio-adhesives in a range of sutureless transplantations, including CLET, COMET and lamellar keratoplasty (Takaoka et al., 2009). We are currently applying this technique for lamellar keratoplasty, and the clinical results are quite promising so far.

5. Growth factors

5.1. Feeder layer factor

Based on the pioneering culture method by Rheinwald and Green, long-term survival and serial expansion of epidermal stem cells is possible if the epithelial cells are co-cultured with mouse-derived 3T3 feeder layer (Rheinwald and Green, 1975). Since that time, 3T3 feeder cells have become the most widely-used for culturing corneal epithelial cells. The exclusion of animal material from the culture system offers significant clinical advantages for OSR, reducing the risk of transmission of animal-derived infections or unknown pathogens. Various candidate human-derived feeder cells such as MRC-5, amniotic epithelium,

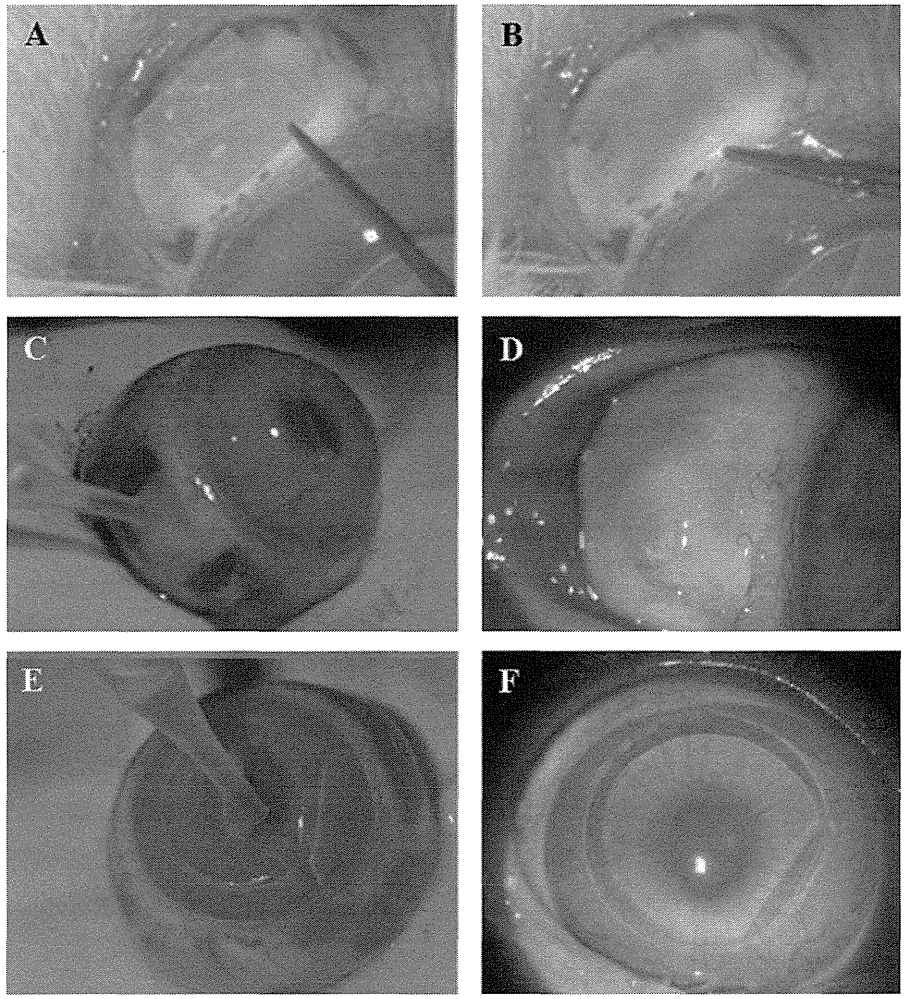

Fig. 15. Development of bio-adhesives. (A, B) Representative photos of fibrin glue-coated freeze-dried AM transplantation. FCFD-AM adhered immediately after transplantation onto the bare sclera, before fibrin glue-coated freeze-dried AM transplantation (A) and after fibrin glue-coated freeze-dried AM transplantation (B). Representative photographs of sutureless AM transplantation (C, D) and lamellar therapeutic keratoplasty (E, F) using chemically-defined bioadhesive. Modified with permission from Sekiyama et al. (2007), Takaoka et al. (2008), Takaoka et al. (2009).

adipose tissue, mesenchymal stem cells and dermal fibroblast have been examined for their usefulness in generating cultivated epithelial sheet (Chen et al., 2007; Notara et al., 2007; Oie et al., 2010; Omoto et al., 2009; Sugiyama et al., 2008). However, widespread use of these culture systems has been hindered by the standardization of culture protocols because of the variable culture conditions of feeder cells.

5.2. Serum factor

The previously preferred method of cultivating epithelial sheets also requires the use of xenobiotic materials such as fetal bovine serum (FBS) in the culture system. However, the use of FBS in the culturing system is a major clinical concern, as bovine spongiform encephalopathy cannot be detected by any known assay. The use of autologous serum (AS) as an alternative to FBS is therefore significantly safer, excluding the need for bovine material in the culture process. Initially, we tried to determine whether AS from patients with severe OSD was as effective in supporting cell proliferation and differentiation in cultivated corneal and oral mucosal epithelial cells as culture methods using FBS (Nakamura et al., 2006a) (Fig. 16). We found that an AS-supplemented culture protocol was effective in supporting the proliferation of human corneal and oral mucosal epithelial cells, as well as the development of transplantable cultivated corneal and oral mucosal epithelial sheets. Based on these findings, we adapted this method for clinical application and reported the successful clinical use of cultivated corneal and oral mucosal epithelial sheets (Ang et al., 2006; Nakamura et al., 2006c). These clinical reports make important suggestions and represent progress in the pursuit of completely xenobiotic-free tissue-engineered transplants. In addition, we know from our clinical work that AS sometimes has donor-dependent variations (e.g. disease or age) that must be kept in view.

5.3. Development of feeder-free and serum-free systems

In light of the above mentioned factors, the development of feeder-free and serum-free culturing systems might be ideal for the next generation of OSR using tissue-engineering techniques. Yokoo et al. reported the generation of a cultivated corneal epithelial cell sheet for OSR in a completely serum-free and feeder-free culture system containing epidermal growth factor and B-27 (Yokoo et al. 2008). More recently, Miyashita et al. reported the long-term

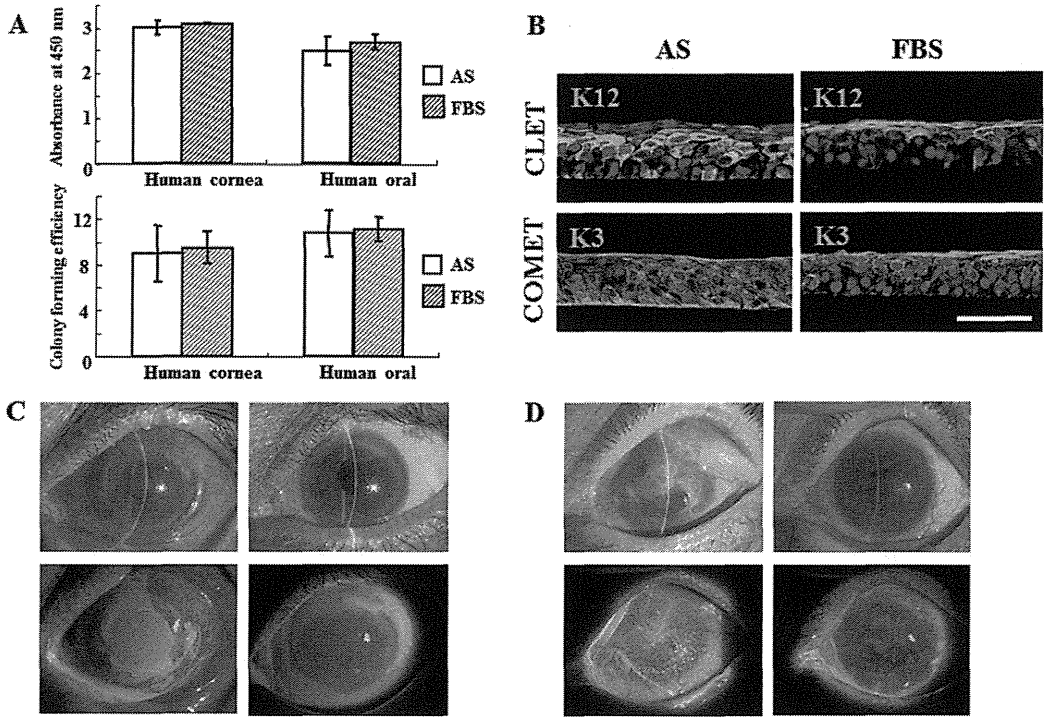

Fig. 16. Development of autologous serum-derived CLET and COMET. (A) BrdU proliferation assay and colony-forming efficiencies (CFE) showed that the proliferation indices and CFE of human corneal and oral mucosal epithelium cultivated using autologous serum (AS) and fetal bovine serum (FBS) were almost similar. (B) The expression patterns of K12 and K3 were similar in cultivated corneal and oral mucosal epithelial sheets derived from AS- and FBS-supplemented culture systems. (C) Representative clinical outcomes of autologous serum-derived CLET in patients with OCP. (D) Representative clinical outcomes of autologous serum-derived COMET in patients with OCP. Modified with permission from Nakamura et al. (2006a), Ang et al. (2006), Nakamura et al. (2006c).

Table 1
Upregulated genes in holoclone-type corneal keratinocytes (selected).

Gene symbol	Gene title	Proposed function
NLRP2	NLR family, pyrin domain containing 2	Activation of proinflammatory caspases
IL24	Interleukin 24	Anti-proliferative property
PTX3	Pentraxin-related gene, rapidly induced by IL-1 beta	Regulation of innate resistance to pathogens
CALD1	Caldesmon 1	Actin- and myosin-binding protein
KLK6	Kallikrein 6 (neurosin, zyme)	Serine protease
CRISP2	Cysteine-rich secretory protein 2	Regulation of ion channels' activity
JPH3	Junctophilin 3	Stabilization of the junctional membrane
MUM1	Melanoma associated antigen (mutated) 1	DNA damage response pathway
LRIG1	Leucine-rich repeats and Ig-like domains 1	Epidermal and intestinal stem cell marker
MTSS1	Metastasis suppressor 1	Cancer progression
KRT19	Keratin 19	Organization of myofibers
LRP11	Low density lipoprotein receptor-related protein 11	Receptor activity
DEFB4	Defensin, beta 4	Antibacterial activity
LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5	Intestinal and hair follicule stem cell marker
KRT24	Keratin 24	Structural constituent of cytoskeleton

maintenance of corneal epithelial stem/progenitor cells using Rho Kinase inhibitor and keratinocyte growth factor (Miyashita et al., 2013). We believe that these studies represent an important step in the development of real feeder-free and serum-free transplantable cultivated epithelial sheets for safe and ideal OSR.

6. Future directions

Corneal regeneration has long been one of the great challenges for ophthalmologists and vision scientists worldwide. With advances in basic research in regenerative medicine and tissue engineering, great progress has been made in the fundamental understanding and development of novel therapeutic modalities such as CLET and COMET. However, although CLET and COMET currently represent the safest and most reliable form of newly developed transplantation, several issues remain to be overcome. First, autologous CLET is to date the most promising treatment for reconstructing the ocular surface in cases of unilateral severe OSD. However, in our long-term follow-up, we certainly observed some incidence of mild conjunctivalization in peripheral corneas. The current cultivated corneal epithelial sheet could not absolutely reproduce the corneal limbal niche, and recreation of the functional corneal limbal niche using innovative tissue-engineering technology may be needed to properly develop this surgical tool. In contrast, treatment of patients with bilateral severe OSD requires either allogeneic CLET or autologous COMET, depending on patient variables (e.g. type of disease, age). In the case of CLET, the risk of postoperative rejection must be addressed, requiring a basic knowledge of the immunological background of allogenic OSR and an appropriate protocol for postoperative management, especially with regard to immunosuppressive therapy. In the case of COMET, we must exercise caution because the cultivated oral mucosal epithelial sheet is not identical to *in vivo* corneal epithelium. This requires a basic understanding of epithelial cell biology in trying to characterize the cell source to be used. One candidate approach is to identify a novel, non-ocular surface cell source for use in OSR. The other candidate approach is to develop innovative genetically-modified biotechnology using induced-pluripotent stem cells or

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direct reprogramming. We strongly believe that greater knowledge of proposed and established surgical modalities, stem cell behavior, the surrounding extracellular matrix and beneficial growth factors will provide a foundation for the further development of treatments for severe OSD.

References

Ang, L.P., Nakamura, T., Inatomi, T., Sotozono, C., Koizumi, N., Yokoi, N., Kinoshita, S., 2006. Autologous serum-derived cultivated oral epithelial transplants for severe ocular surface disease. Arch. Ophthalmol. 124, 1543–1551.

Ballen, P.H., 1963. Mucous membrane grafts in chemical (lye) burns. Am. J. Ophthalmol. 55, 302–312.

Barrandon, Y., Green, H., 1987. Three clonal types of keratinocyte with different capacities for multiplication. Proc. Natl. Acad. Sci. U. S. A. 84, 2302–2306.

Bath, C., Muttuvelu, D., Emmersen, J., Vorum, H., Hjortdal, J., Zachar, V., 2013. Transcriptional dissection of human limbal niche compartments by massive parallel sequencing. PLoS One 8, e64244.

Baylis, O., Figueiredo, F., Henein, C., Lako, M., Ahmad, S., 2011. 13 years of cultured limbal epithelial cell therapy: a review of the outcomes. J. Cell Biochem. 112, 993–1002.

Bi, Y., Bock, F., Zhou, Q., Cursiefen, C., 2013. Central corneal epithelium self-healing after ring-shaped glycerin-cryopreserved lamellar keratoplasty in Terrien marginal degeneration. Int. J. Ophthalmol. 6, 251–252.

Chang, C.Y., McGhee, J.J., Green, C.R., Sherwin, T., 2011. Comparison of stem cell properties in cell populations isolated from human central and limbal corneal epithelium. Cornea 30, 1155–1162.

Chen, Y.T., Li, W., Hayashida, Y., He, H., Chen, S.Y., Tseng, D.Y., Kheirkhah, A., Tseng, S.C., 2007. Human amniotic epithelial cells as novel feeder layers for promoting ex vivo expansion of limbal epithelial progenitor cells. Stem Cells 25, 1995–2005.

Chiou, A.G., Florakis, G.J., Kazim, M., 1998. Management of conjunctival cicatrizing diseases and severe ocular surface dysfunction. Surv. Ophthalmol. 43, 19–46.

Chirila, T., Barnard, Z., Zainuddin, Harkin, D.G., Schwab, I.R., Hirst, L., 2008. Bombyx mori silk fibroin membranes as potential substrata for epithelial constructs used in the management of ocular surface disorders. Tissue Eng. Part A 14, 1203–1211.

Cotsarelis, G., Cheng, S.Z., Dong, G., Sun, T.T., Lavker, R.M., 1989. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell. 57, 201–209.

De RÖTTH, A., 1940. Plastic repair of conjunctival defects with fetal membrane. Arch. Ophthalmol. 23, 522–525.

Di Girolamo, N., Bosch, M., Zamora, K., Coroneo, M.T., Wakefield, D., Watson, S.L., 2009. A contact lens-based technique for expansion and transplantation of autologous epithelial progenitors for ocular surface reconstruction. Transplantation 87, 1571–1578.

Di Girolamo, N., Sarris, M., Chui, J., Cheema, H., Coroneo, M.T., Wakefield, D., 2008. Localization of the low-affinity nerve growth factor receptor p75 in human limbal epithelial cells. J. Cell Mol. Med. 12, 2799–2811.

Dravida, S., Gaddipati, S., Griffith, M., Merrett, K., Lakshmi Madhira, S., Sangwan, V.S., Vemuganti, G.K., 2008. A biomimetic scaffold for culturing limbal stem cells: a promising alternative for clinical transplantation. J. Tissue Eng. Regen. Med. 2, 263–271.

Endo, K., Nakamura, T., Kawasaki, S., Kinoshita, S., 2004. Human amniotic membrane, like corneal epithelial basement membrane, manifests the alpha5 chain of type IV collagen. Invest. Ophthalmol. Vis. Sci. 45, 1771–1774.

Figueira, E.C., Di Girolamo, N., Coroneo, M.T., Wakefield, D., 2007. The phenotype of limbal epithelial stem cells. Invest. Ophthalmol. Vis. Sci. 48, 144–156.

Francis, D., Abberton, K., Thompson, E., Daniell, M., 2009. Myogel supports the ex-vivo amplification of corneal epithelial cells. Exp. Eye Res. 88, 339–346.

Friend, J., Kinoshita, S., Thoft, R.A., Eliason, J.A., 1982. Corneal epithelial cell cultures on stromal carriers. Invest. Ophthalmol. Vis. Sci. 23, 41–49.

Galal, A., Perez-Santonja, J.J., Rodriguez-Prats, J.L., Abad, M., Alio, J., 2007. Human anterior lens capsule as a biologic substrate for the ex vivo expansion of limbal stem cells in ocular surface reconstruction. Cornea 26, 473–478.

Gipson, I.K., Geggel, H.S., Spurr-Michaud, S.J., 1986. Transplant of oral mucosal epithelium to rabbit ocular surface wounds in vivo. Arch. Ophthalmol. 104, 1529–1533.

Gipson, I.K., Grill, S.M., 1982. A technique for obtaining sheets of intact rabbit corneal epithelium. Invest. Ophthalmol. Vis. Sci. 23, 269–273.

Griffith, M., Osborne, R., Munger, R., Xiong, X., Doillon, C.J., Laycock, N.L., Hakim, M., Song, Y., Watsky, M.A., 1999. Functional human corneal equivalents constructed from cell lines. Science 286, 2169–2172.

Grolik, M., Szczubialka, K., Wowra, B., Dobrowolski, D., Orzechowska-Wylegala, B., Wylegala, E., Nowakowska, M., 2012. Hydrogel membranes based on genipin-cross-linked chitosan blends for corneal epithelium tissue engineering. J. Mater Sci. Mater Med. 23, 1991–2000.

Grueterich, M., Espana, E.M., Touhami, A., Ti, S.E., Tseng, S.C., 2002. Phenotypic study of a case with successful transplantation of ex vivo expanded human limbal epithelium for unilateral total limbal stem cell deficiency. Ophthalmology 109, 1547–1552.

Hall, P.A., Watt, F.M., 1989. Stem cells: the generation and maintenance of cellular diversity. Development 106, 619–633.

Hayashi, R., Yamato, M., Saito, T., Oshima, T., Okano, T., Tano, Y., Nishida, K., 2008. Enrichment of corneal epithelial stem/progenitor cells using cell surface markers, integrin alpha6 and CD71. Biochem. Biophys. Res. Commun. 367, 256–263.

Hayashi, R., Yamato, M., Sugiyama, H., Sumide, T., Yang, J., Okano, T., Tano, Y., Nishida, K., 2007. N-Cadherin is expressed by putative stem/progenitor cells and melanocytes in the human limbal epithelial stem cell niche. Stem Cells 25, 289–296.

He, L., Hannon, G.J., 2004. MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. 5, 522–531.

Hino, M., Ishiko, O., Honda, K.I., Yamane, T., Ohta, K., Takubo, T., Tatsumi, N., 2000. Transmission of symptomatic parvovirus B19 infection by fibrin sealant used during surgery. Br. J. Haematol. 108, 194–195.

Hirayama, M., Satake, Y., Higa, K., Yamaguchi, T., Shimazaki, J., 2012. Transplantation of cultivated oral mucosal epithelium prepared in fibrin-coated culture dishes. Invest. Ophthalmol. Vis. Sci. 53, 1602–1609.

Homma, R., Yoshikawa, H., Takeno, M., Kurokawa, M.S., Masuda, C., Takada, E., Tsubota, K., Ueno, S., Suzuki, N., 2004. Induction of epithelial progenitors in vitro from mouse embryonic stem cells and application for reconstruction of damaged cornea in mice. Invest. Ophthalmol. Vis. Sci. 45, 4320–4326.

Horenstein, A.L., Sizzano, F., Lusso, R., Besso, F.G., Ferrero, E., Deaglio, S., Corno, F., Malavasi, F., 2009. CD38 and CD157 ectoenzymes mark cell subsets in the human corneal limbus. Mol. Med. 15, 76–84.

Inatomi, T., Nakamura, T., Kojyo, M., Koizumi, N., Sotozono, C., Kinoshita, S., 2006. Ocular surface reconstruction with combination of cultivated autologous oral mucosal epithelial transplantation and penetrating keratoplasty. Am. J. Ophthalmol. 142, 757–764.

Jensen, K.B., Watt, F.M., 2006. Single-cell expression profiling of human epidermal stem and transit-amplifying cells: Lrig1 is a regulator of stem cell quiescence. Proc. Natl. Acad. Sci. U. S. A. 103, 11958–11963.

Kaufman, H.E., 1984. Keratoepithelioplasty for the replacement of damaged corneal epithelium. Am. J. Ophthalmol. 97, 100–101.

Kawakita, T., Higa, K., Shimmura, S., Tomita, M., Tsubota, K., Shimazaki, J., 2011. Fate of corneal epithelial cells separated from limbus in vivo. Invest. Ophthalmol. Vis. Sci. 52, 8132–8137.

Kawashima, M., Kawakita, T., Yoshida, S., Shimmura, S., Tsubota, K., 2009. Nucleostemin as a possible progenitor marker of corneal epithelial cells. Mol. Vis. 15, 1162–1168.

Kenyon, K.R., Tseng, S.C., 1989. Limbal autograft transplantation for ocular surface disorders. Ophthalmology 96, 709–722 discussion 722–723.

Kim, J.C., Tseng, S.C., 1995. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea 14, 473–484.

Kinoshita, S., Adachi, W., Sotozono, C., Nishida, K., Yokoi, N., Quantock, A.J., Okubo, K., 2001. Characteristics of the human ocular surface epithelium. Prog. Retin Eye Res. 20, 639–673.

Kinoshita, S., Koizumi, N., Nakamura, T., 2004. Transplantable cultivated mucosal epithelial sheet for ocular surface reconstruction. Exp. Eye Res. 78, 483–491.

Kinoshita, S., Ohashi, Y., Ohji, M., Manabe, R., 1991. Long-term results of keratoepithelioplasty in Mooren's ulcer. Ophthalmology 98, 438–445.

Kobayashi, H., Ikada, Y., 1991. Corneal cell adhesion and proliferation on hydrogel sheets bound with cell-adhesive proteins. Curr. Eye Res. 10, 899–908.

Kobayashi, M., Nakamura, T., Yasuda, M., Hata, Y., Okura, S., Iwamoto, M., Nagata, M., Fullwood, N.J., Koizumi, N., Hisa, Y., Kinoshita, S., 2015. Ocular surface reconstruction with a tissue-engineered nasal mucosal epithelial cell sheet for the treatment of severe ocular surface diseases. Stem Cells Transl. Med. 4, 99–109.

Koizumi, N., Inatomi, T., Quantock, A.J., Fullwood, N.J., Dota, A., Kinoshita, S., 2000a. Amniotic membrane as a substrate for cultivating limbal corneal epithelial cells for autologous transplantation in rabbits. Cornea 19, 65–71.

Koizumi, N., Inatomi, T., Sotozono, C., Fullwood, N.J., Quantock, A.J., Kinoshita, S., 2000b. Growth factor mRNA and protein in preserved human amniotic membrane. Curr. Eye Res. 20, 173–177.

Koizumi, N., Inatomi, T., Suzuki, T., Sotozono, C., Kinoshita, S., 2001a. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. Ophthalmology 108, 1569–1574.

Koizumi, N., Inatomi, T., Suzuki, T., Sotozono, C., Kinoshita, S., 2001b. Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens-Johnson syndrome. Arch. Ophthalmol. 119, 298–300.

Ksander, B.R., Kolovou, P.E., Wilson, B.J., Saab, K.R., Guo, Q., Ma, J., McGuire, S.P., Gregory, M.S., Vincent, W.J., Perez, V.L., Cruz-Guilloty, F., Kao, W.W., Call, M.K., Tucker, B.A., Zhan, Q., Murphy, G.F., Lathrop, K.L., Alt, C., Mortensen, L.J., Lin, C.P., Zieske, J.D., Frank, M.H., Frank, N.Y., 2014. ABCB5 is a limbal stem cell gene required for corneal development and repair. Nature 511, 353–357.

Kulkarni, B.B., Tighe, P.J., Mohammed, I., Yeung, A.M., Powe, D.G., Hopkinson, A., Shanmuganathan, V.A., Dua, H.S., 2010. Comparative transcriptional profiling of the limbal epithelial crypt demonstrates its putative stem cell niche characteristics. BMC Genomics 11, 526.

Kusanagi, R., Umemoto, T., Yamato, M., Matsuzaki, Y., Nishida, K., Kobayashi, Y., Fukai, F., Okano, T., 2009. Nectin-3 expression is elevated in limbal epithelial side population cells with strongly expressed stem cell markers. Biochem. Biophys. Res. Commun. 389, 274–278.

Lajtha, L.G., 1979. Stem cell concepts. Differentiation 14, 23–34.

Langer, R., Vacanti, J.P., 1993. Tissue engineering. Science 260, 920–926.

Lavker, R.M., Sun, T.T., 1982. Heterogeneity in epidermal basal keratinocytes:

morphological and functional correlations. Science 215, 1239–1241.
Leblond, C.P., 1981. The life history of cells in renewing systems. Am. J. Anat. 160, 114–158.
Lee, S.K., Teng, Y., Wong, H.K., Ng, T.K., Huang, L., Lei, P., Choy, K.W., Liu, Y., Zhang, M., Lam, D.S., Yam, G.H., Pang, C.P., 2011. MicroRNA-145 regulates human corneal epithelial differentiation. PLoS One 6, e21249.
Levis, H.J., Brown, R.A., Daniels, J.T., 2010. Plastic compressed collagen as a biomimetic substrate for human limbal epithelial cell culture. Biomaterials 31, 7726–7737.
Lin, D., Halilovic, A., Yue, P., Bellner, L., Wang, K., Wang, L., Zhang, C., 2013. Inhibition of miR-205 impairs the wound-healing process in human corneal epithelial cells by targeting KIR4.1 (KCNJ10). Invest. Ophthalmol. Vis. Sci. 54, 6167–6178.
Ma, Y., Xu, Y., Xiao, Z., Yang, W., Zhang, C., Song, E., Du, Y., Li, L., 2006. Reconstruction of chemically burned rat corneal surface by bone marrow-derived human mesenchymal stem cells. Stem Cells 24, 315–321.
Majo, F., Rochat, A., Nicolas, M., Jaoude, G.A., Barrandon, Y., 2008. Oligopotent stem cells are distributed throughout the mammalian ocular surface. Nature 456, 250–254.
McIntosh Ambrose, W., Salahuddin, A., So, S., Ng, S., Ponce Marquez, S., Takezawa, T., Schein, O., Elisseeff, J., 2009. Collagen vitrigel membranes for the in vitro reconstruction of separate corneal epithelial, stromal, and endothelial cell layers. J. Biomed. Mater Res. B Appl. Biomater. 90, 818–831.
Meyer-Blazejewska, E.A., Call, M.K., Yamanaka, O., Liu, H., Schlotzer-Schrehardt, U., Kruse, F.E., Kao, W.W., 2011. From hair to cornea: toward the therapeutic use of hair follicle-derived stem cells in the treatment of limbal stem cell deficiency. Stem Cells 29, 57–66.
Mi, S., Chen, B., Wright, B., Connon, C.J., 2010. Ex vivo construction of an artificial ocular surface by combination of corneal limbal epithelial cells and a compressed collagen scaffold containing keratocytes. Tissue Eng. Part A 16, 2091–2100.
Minami, Y., Sugihara, H., Oono, S., 1993. Reconstruction of cornea in three-dimensional collagen gel matrix culture. Invest. Ophthalmol. Vis. Sci. 34, 2316–2324.
Miyashita, H., Yokoo, S., Yoshida, S., Kawakita, T., Yamagami, S., Tsubota, K., Shimmura, S., 2013. Long-term maintenance of limbal epithelial progenitor cells using rho kinase inhibitor and keratinocyte growth factor. Stem Cells Transl. Med. 2, 758–765.
Monteiro, B.G., Serafim, R.C., Melo, G.B., Silva, M.C., Lizier, N.F., Maranduba, C.M., Smith, R.L., Kerkis, A., Cerruti, H., Gomes, J.A., Kerkis, I., 2009. Human immature dental pulp stem cells share key characteristic features with limbal stem cells. Cell Prolif. 42, 587–594.
Nakamura, T., Ang, L.P., Rigby, H., Sekiyama, E., Inatomi, T., Sotozono, C., Fullwood, N.J., Kinoshita, S., 2006a. The use of autologous serum in the development of corneal and oral epithelial equivalents in patients with Stevens-Johnson syndrome. Invest. Ophthalmol. Vis. Sci. 47, 909–916.
Nakamura, T., Endo, K., Cooper, L.J., Fullwood, N.J., Tanifuji, N., Tsuzuki, M., Koizumi, N., Inatomi, T., Sano, Y., Kinoshita, S., 2003a. The successful culture and autologous transplantation of rabbit oral mucosal epithelial cells on amniotic membrane. Invest. Ophthalmol. Vis. Sci. 44, 106–116.
Nakamura, T., Endo, K., Kinoshita, S., 2007a. Identification of human oral keratinocyte stem/progenitor cells by neurotrophin receptor p75 and the role of neurotrophin/p75 signaling. Stem Cells 25, 628–638.
Nakamura, T., Hamuro, J., Takaishi, M., Simmons, S., Maruyama, K., Zaffalon, A., Bentley, A.J., Kawasaki, S., Nagata-Takaoka, M., Fullwood, N.J., Itami, S., Sano, S., Ishii, M., Barrandon, Y., Kinoshita, S., 2014. LRIG1 inhibits STAT3-dependent inflammation to maintain corneal homeostasis. J. Clin. Invest. 124, 385–397.
Nakamura, T., Inatomi, T., Cooper, L.J., Rigby, H., Fullwood, N.J., Kinoshita, S., 2007b. Phenotypic investigation of human eyes with transplanted autologous cultivated oral mucosal epithelial sheets for severe ocular surface diseases. Ophthalmology 114, 1080–1088.
Nakamura, T., Inatomi, T., Sekiyama, E., Ang, L.P., Yokoi, N., Kinoshita, S., 2006b. Novel clinical application of sterilized, freeze-dried amniotic membrane to treat patients with pterygium. Acta Ophthalmol. Scand. 84, 401–405.
Nakamura, T., Inatomi, T., Sotozono, C., Amemiya, T., Kanamura, N., Kinoshita, S., 2004a. Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. Br. J. Ophthalmol. 88, 1280–1284.
Nakamura, T., Inatomi, T., Sotozono, C., Ang, L.P., Koizumi, N., Yokoi, N., Kinoshita, S., 2006c. Transplantation of autologous serum-derived cultivated corneal epithelial equivalents for the treatment of severe ocular surface disease. Ophthalmology 113, 1765–1772.
Nakamura, T., Inatomi, T., Sotozono, C., Koizumi, N., Kinoshita, S., 2004b. Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease. Acta Ophthalmol. Scand. 82, 468–471.
Nakamura, T., Kinoshita, S., 2003. Ocular surface reconstruction using cultivated mucosal epithelial stem cells. Cornea 22, S75–S80.
Nakamura, T., Koizumi, N., Tsuzuki, M., Inoki, K., Sano, Y., Sotozono, C., Kinoshita, S., 2003c. Successful regrafting of cultivated corneal epithelium using amniotic membrane as a carrier in severe ocular surface disease. Cornea 22, 70–71.
Nakamura, T., Ohtsuka, T., Sekiyama, E., Cooper, L.J., Kokubu, H., Fullwood, N.J., Barrandon, Y., Kageyama, R., Kinoshita, S., 2008a. Hes1 regulates corneal development and the function of corneal epithelial stem/progenitor cells. Stem Cells 26, 1265–1274.
Nakamura, T., Sekiyama, E., Takaoka, M., Bentley, A.J., Yokoi, N., Fullwood, N.J., Kinoshita, S., 2008b. The use of trehalose-treated freeze-dried amniotic membrane for ocular surface reconstruction. Biomaterials 29, 3729–3737.
Nakamura, T., Sotozono, C., Bentley, A.J., Mano, S., Inatomi, T., Koizumi, N., Fullwood, N.J., Kinoshita, S., 2010. Long-term phenotypic study after allogeneic cultivated corneal limbal epithelial transplantation for severe ocular surface diseases. Ophthalmology 117, 2247–2254.
Nakamura, T., Takeda, K., Inatomi, T., Sotozono, C., Kinoshita, S., 2011. Long-term results of autologous cultivated oral mucosal epithelial transplantation in the scar phase of severe ocular surface disorders. Br. J. Ophthalmol. 95, 942–946.
Nakamura, T., Yoshitani, M., Rigby, H., Fullwood, N.J., Ito, W., Inatomi, T., Sotozono, C., Shimizu, Y., Kinoshita, S., 2004c. Sterilized, freeze-dried amniotic membrane: a useful substrate for ocular surface reconstruction. Invest. Ophthalmol. Vis. Sci. 45, 93–99.
Nakatsu, M.N., Vartanyan, L., Vu, D.M., Ng, M.Y., Li, X., Deng, S.X., 2013. Preferential biological processes in the human limbus by differential gene profiling. PLoS One 8, e61833.
Nishida, K., Adachi, W., Shimizu-Matsumoto, A., Kinoshita, S., Mizuno, K., Matsubara, K., Okubo, K., 1996. A gene expression profile of human corneal epithelium and the isolation of human keratin 12 cDNA. Invest. Ophthalmol. Vis. Sci. 37, 1800–1809.
Nishida, K., Yamato, M., Hayashida, Y., Watanabe, K., Maeda, N., Watanabe, H., Yamamoto, K., Nagai, S., Kikuchi, A., Tano, Y., Okano, T., 2004a. Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface. Transplantation 77, 379–385.
Nishida, K., Yamato, M., Hayashida, Y., Watanabe, K., Yamamoto, K., Adachi, E., Nagai, S., Kikuchi, A., Maeda, N., Watanabe, H., Okano, T., Tano, Y., 2004b. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N. Engl. J. Med. 351, 1187–1196.
Notara, M., Haddow, D.B., MacNeil, S., Daniels, J.T., 2007. A xenobiotic-free culture system for human limbal epithelial stem cells. Regen. Med. 2, 919–927.
Oie, Y., Hayashi, R., Takagi, R., Yamato, M., Takayanagi, H., Tano, Y., Nishida, K., 2010. A novel method of culturing human oral mucosal epithelial cell sheet using post-mitotic human dermal fibroblast feeder cells and modified keratinocyte culture medium for ocular surface reconstruction. Br. J. Ophthalmol. 94, 1244–1250.
Okabe, M., Kitagawa, K., Yoshida, T., Suzuki, T., Waki, H., Koike, C., Furuichi, E., Katou, K., Nomura, Y., Uji, Y., Hayashi, A., Saito, S., Nikaido, T., 2014. Hyperdry human amniotic membrane is useful material for tissue engineering: physical, morphological properties, and safety as the new biological material. J. Biomed. Mater Res. A 102, 862–870.
Omoto, M., Miyashita, H., Shimmura, S., Higa, K., Kawakita, T., Yoshida, S., McGrogan, M., Shimazaki, J., Tsubota, K., 2009. The use of human mesenchymal stem cell-derived feeder cells for the cultivation of transplantable epithelial sheets. Invest. Ophthalmol. Vis. Sci. 50, 2109–2115.
Ortega, I., Ryan, A.J., Deshpande, P., MacNeil, S., Claeyssens, F., 2013. Combined microfabrication and electrospinning to produce 3-D architectures for corneal repair. Acta Biomater. 9, 5511–5520.
Ouyang, H., Xue, Y., Lin, Y., Zhang, X., Xi, L., Patel, S., Cai, H., Luo, J., Zhang, M., Yang, Y., Li, G., Li, H., Jiang, W., Yeh, E., Lin, J., Pei, M., Zhu, J., Cao, G., Zhang, L., Yu, B., Chen, S., Fu, X.D., Liu, Y., Zhang, K., 2014. WNT7A and PAX6 define corneal epithelium homeostasis and pathogenesis. Nature 511, 358–361.
Pajoohesh-Ganji, A., Pal-Ghosh, S., Simmens, S.J., Stepp, M.A., 2006. Integrins in slow-cycling corneal epithelial cells at the limbus in the mouse. Stem Cells 24, 1075–1086.
Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., Ponzin, D., McKeon, F., De Luca, M., 2001. p63 identifies keratinocyte stem cells. Proc. Natl. Acad. Sci. U. S. A. 98, 3156–3161.
Pellegrini, G., Golisano, O., Paterna, P., Lambiase, A., Bonini, S., Rama, P., De Luca, M., 1999. Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. J. Cell Biol. 145, 769–782.
Pellegrini, G., Traverso, C.E., Franzi, A.T., Zingirian, M., Cancedda, R., De Luca, M., 1997. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet 349, 990–993.
Potten, C.S., Loeffler, M., 1990. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. Development 110, 1001–1020.
Powell, A.E., Wang, Y., Li, Y., Poulin, E.J., Means, A.L., Washington, M.K., Higginbotham, J.N., Juchheim, A., Prasad, N., Levy, S.E., Guo, Y., Shyr, Y., Aronow, B.J., Haigis, K.M., Franklin, J.L., Coffey, R.J., 2012. The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. Cell. 149, 146–158.
Qi, H., Li, D.Q., Shine, H.D., Chen, Z., Yoon, K.C., Jones, D.B., Pflugfelder, S.C., 2008. Nerve growth factor and its receptor TrkA serve as potential markers for human corneal epithelial progenitor cells. Exp. Eye Res. 86, 34–40.
Rama, P., Bonini, S., Lambiase, A., Golisano, O., Paterna, P., De Luca, M., Pellegrini, G., 2001. Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. Transplantation 72, 1478–1485.
Rama, P., Matuska, S., Paganoni, G., Spinelli, A., De Luca, M., Pellegrini, G., 2010. Limbal stem-cell therapy and long-term corneal regeneration. N. Engl. J. Med. 363, 147–155.
Reichl, S., Borrelli, M., Geerling, G., 2011. Keratin films for ocular surface reconstruction. Biomaterials 32, 3375–3386.
Reza, H.M., Ng, B.Y., Gimeno, F.L., Phan, T.T., Ang, L.P., 2011. Umbilical cord lining stem cells as a novel and promising source for ocular surface regeneration. Stem Cell Rev. 7, 935–947.

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Rheinwald, J.G., Green, H., 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell. 6, 331–343.
Sangwan, V.S., Basu, S., MacNeil, S., Balasubramanian, D., 2012. Simple limbal epithelial transplantation (SLET): a novel surgical technique for the treatment of unilateral limbal stem cell deficiency. Br. J. Ophthalmol. 96, 931–934.
Sangwan, V.S., Vemuganti, G.K., Iftekhar, G., Bansal, A.K., Rao, G.N., 2003. Use of autologous cultured limbal and conjunctival epithelium in a patient with severe bilateral ocular surface disease induced by acid injury: a case report of unique application. Cornea 22, 478–481.
Satake, Y., Higa, K., Tsubota, K., Shimazaki, J., 2011. Long-term outcome of cultivated oral mucosal epithelial sheet transplantation in treatment of total limbal stem cell deficiency. Ophthalmology 118, 1524–1530.
Schermer, A., Galvin, S., Sun, T.T., 1986. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J. Cell Biol. 103, 49–62.
Schwab, I.R., Reyes, M., Isseroff, R.R., 2000. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. Cornea 19, 421–426.
Sekiyama, E., Nakamura, T., Kurihara, E., Cooper, L.J., Fullwood, N.J., Takaoka, M., Hamuro, J., Kinoshita, S., 2007. Novel sutureless transplantation of bioadhesive-coated, freeze-dried amniotic membrane for ocular surface reconstruction. Invest. Ophthalmol. Vis. Sci. 48, 1528–1534.
Shafiq, M.A., Gemeinhart, R.A., Yue, B.Y., Djalilian, A.R., 2012. Decellularized human cornea for reconstructing the corneal epithelium and anterior stroma. Tissue Eng. Part C Methods 18, 340–348.
Shalom-Feuerstein, R., Serror, L., De La Forest Divonne, S., Petit, I., Aberdam, E., Camargo, L., Damour, O., Vigouroux, C., Solomon, A., Gaggioli, C., Itskovitz-Eldor, J., Ahmad, S., Aberdam, D., 2012. Pluripotent stem cell model reveals essential roles for miR-450b-5p and miR-184 in embryonic corneal lineage specification. Stem Cells 30, 898–909.
Sharma, S., Mohanty, S., Gupta, D., Jassal, M., Agrawal, A.K., Tandon, R., 2011. Cellular response of limbal epithelial cells on electrospun poly-epsilon-caprolactone nanofibrous scaffolds for ocular surface bioengineering: a preliminary in vitro study. Mol. Vis. 17, 2898–2910.
Solomon, A., Rosenblatt, M., Monroy, D., Ji, Z., Pflugfelder, S.C., Tseng, S.C., 2001. Suppression of interleukin 1alpha and interleukin 1beta in human limbal epithelial cells cultured on the amniotic membrane stromal matrix. Br. J. Ophthalmol. 85, 444–449.
Sotozono, C., Ang, L.P., Koizumi, N., Higashihara, H., Ueta, M., Inatomi, T., Yokoi, N., Kaido, M., Dogru, M., Shimazaki, J., Tsubota, K., Yamada, M., Kinoshita, S., 2007. New grading system for the evaluation of chronic ocular manifestations in patients with Stevens-Johnson syndrome. Ophthalmology 114, 1294–1302.
Sotozono, C., Inatomi, T., Nakamura, T., Koizumi, N., Yokoi, N., Ueta, M., Matsuyama, K., Miyakoda, K., Kaneda, H., Fukushima, M., Kinoshita, S., 2013. Visual improvement after cultivated oral mucosal epithelial transplantation. Ophthalmology 120, 193–200.
Sugiyama, H., Maeda, K., Yamato, M., Hayashi, R., Soma, T., Hayashida, Y., Yang, J., Shirakabe, M., Matsuyama, A., Kikuchi, A., Sawa, Y., Okano, T., Tano, Y., Nishida, K., 2008. Human adipose tissue-derived mesenchymal stem cells as a novel feeder layer for epithelial cells. J. Tissue Eng. Regen. Med. 2, 445–449.
Suzuki, Y., Miura, H., Tanemura, A., Kobayashi, K., Kondoh, G., Sano, S., Ozawa, K., Inui, S., Nakata, A., Takagi, T., Tohyama, M., Yoshikawa, K., Itami, S., 2002. Targeted disruption of LIG-1 gene results in psoriasiform epidermal hyperplasia. FEBS Lett. 521, 67–71.
Szurman, P., Warga, M., Grisanti, S., Roters, S., Rohrbach, J.M., Aisenbrey, S., Kaczmarek, R.T., Bartz-Schmidt, K.U., 2006. Sutureless amniotic membrane fixation using fibrin glue for ocular surface reconstruction in a rabbit model. Cornea 25, 460–466.
Takacs, L., Toth, E., Losonczy, G., Szanto, A., Bahr-Ivacevic, T., Benes, V., Berta, A., Vereb, G., 2011. Differentially expressed genes associated with human limbal epithelial phenotypes: new molecules that potentially facilitate selection of stem cell-enriched populations. Invest. Ophthalmol. Vis. Sci. 52, 1252–1260.
Takeda, K., Nakamura, T., Inatomi, T., Sotozono, C., Watanabe, A., Kinoshita, S., 2011. Ocular surface reconstruction using the combination of autologous cultivated oral mucosal epithelial transplantation and eyelid surgery for severe ocular surface disease. Am. J. Ophthalmol. 152, 195–201.
Takaoka, M., Nakamura, T., Sugai, H., Bentley, A.J., Nakajima, N., Fullwood, N.J., Yokoi, N., Hyon, S.H., Kinoshita, S., 2008. Sutureless amniotic membrane transplantation for ocular surface reconstruction with a chemically defined bioadhesive. Biomaterials 29, 2923–2931.
Takaoka, M., Nakamura, T., Sugai, H., Bentley, A.J., Nakajima, N., Yokoi, N., Fullwood, N.J., Hyon, S.H., Kinoshita, S., 2009. Novel sutureless keratoplasty with a chemically defined bioadhesive. Invest. Ophthalmol. Vis. Sci. 50, 2679–2685.
Thoft, R.A., 1977. Conjunctival transplantation. Arch. Ophthalmol. 95, 1425–1427.
Thoft, R.A., 1984. Keratoepithelioplasty. Am. J. Ophthalmol. 97, 1–6.
Thoft, R.A., Friend, J., 1983. The X, Y, Z hypothesis of corneal epithelial maintenance. Invest. Ophthalmol. Vis. Sci. 24, 1442–1443.
Thoft, R.A., Friend, J., 1979. The Ocular Surface. International Ophthalmology Clinics.
Trelford, J.D., Trelford-Sauder, M., 1979. The amnion in surgery, past and present. Am. J. Obstet. Gynecol. 134, 833–845.
Tsai, R.J., Li, L.M., Chen, J.K., 2000. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. N. Engl. J. Med. 343, 86–93.
Tsai, R.J., Tseng, S.C., 1994. Human allograft limbal transplantation for corneal surface reconstruction. Cornea 13, 389–400.
Tseng, S.C., 1989. Concept and application of limbal stem cells. Eye 3 (Pt 2), 141–157.
Tseng, S.C., Li, D.Q., Ma, X., 1999. Suppression of transforming growth factor-beta isoforms, TGF-beta receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix. J. Cell Physiol. 179, 325–335.
Tsubota, K., Satake, Y., Ohyama, M., Toda, I., Takano, Y., Ono, M., Shinozaki, N., Shimazaki, J., 1996. Surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens-Johnson syndrome. Am. J. Ophthalmol. 122, 38–52.
Utheim, T.P., Raeder, S., Olstad, O.K., Utheim, O.A., de La Paz, M., Cheng, R., Huynh, T.T., Messelt, E., Roald, B., Lyberg, T., 2009. Comparison of the histology, gene expression profile, and phenotype of cultured human limbal epithelial cells from different limbal regions. Invest. Ophthalmol. Vis. Sci. 50, 5165–5172.
Wang, H., Tao, T., Tang, J., Mao, Y.H., Li, W., Peng, J., Tan, G., Zhou, Y.P., Zhong, J.X., Tseng, S.C., Kawakita, T., Zhao, Y.X., Liu, Z.G., 2009. Importin 13 serves as a potential marker for corneal epithelial progenitor cells. Stem Cells 27, 2516–2526.
Watanabe, K., Nishida, K., Yamato, M., Umemoto, T., Sumide, T., Yamamoto, K., Maeda, N., Watanabe, H., Okano, T., Tano, Y., 2004. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. FEBS Lett. 565, 6–10.
West, J.D., Dorà, N.J., Collinson, J.M., 2015. Evaluating alternative stem cell hypotheses for adult corneal epithelial maintenance. World J. Stem Cells 7, 281–299.
Wong, V.W., Stange, D.E., Page, M.E., Buczacki, S., Wabik, A., Itami, S., van de Wetering, M., Poulsom, R., Wright, N.A., Trotter, M.W., Watt, F.M., Winton, D.J., Clevers, H., Jensen, K.B., 2012. Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. Nat. Cell Biol. 14, 401–408.
Yang, X., Moldovan, N.I., Zhao, Q., Mi, S., Zhou, Z., Chen, D., Gao, Z., Tong, D., Dou, Z., 2008. Reconstruction of damaged cornea by autologous transplantation of epidermal adult stem cells. Mol. Vis. 14, 1064–1070.
Yoshida, S., Shimmura, S., Kawakita, T., Miyashita, H., Den, S., Shimazaki, J., Tsubota, K., 2006. Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. Invest. Ophthalmol. Vis. Sci. 47, 4780–4786.
Yokoo, S., Yamagami, S., Usui, T., Amano, S., Araie, M., 2008. Human corneal epithelial equivalents for ocular surface reconstruction in a complete serum-free culture system without unknown factors. Invest. Ophthalmol. Vis. Sci. 49, 2438–2443.
Yu, J., Ryan, D.G., Getsios, S., Oliveira-Fernandes, M., Fatima, A., Lavker, R.M., 2008. MicroRNA-184 antagonizes microRNA-205 to maintain SHIP2 levels in epithelia. Proc. Natl. Acad. Sci. U. S. A. 105, 19300–19305.
Zajicova, A., Pokorna, K., Lencova, A., Krulova, M., Svobodova, E., Kubinova, S., Sykova, E., Pradny, M., Michalek, J., Svobodova, J., Munzarova, M., Holan, V., 2010. Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. Cell Transpl. 19, 1281–1290.
Zhao, Y., Ma, L., 2015. Systematic review and meta-analysis on transplantation of ex vivo cultivated limbal epithelial stem cell on amniotic membrane in limbal stem cell deficiency. Cornea 34, 592–600.

Influence of Eyelid Pressure on Fluorescein Staining of Ocular Surface in Dry Eyes

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• PURPOSE: To determine the relationship between eyelid pressure during blinking and the fluorescein staining of the cornea and conjunctiva in dry eye patients.
• DESIGN: Cross-sectional prospective study.
• METHODS: The pressure of the upper and lower eyelids was measured with a specially designed blepahro-tensiometer in 130 eyes of 65 dry eye patients (D group) and in 58 eyes of 31 normal controls (N group). The correlations between the location and degree of ocular surface staining scores and the eyelid pressure were calculated for the D group.
• RESULTS: The pressures of the upper and lower eyelids were significantly higher in the D group than in the N group (upper $P < .0001$, lower $P = .0040$). The lower eyelid pressure was significantly correlated with the ocular surface staining scores for the inferior cornea ($r = 0.19$, $P = .0307$) and conjunctiva ($r = 0.19$, $P = .0252$).
• CONCLUSIONS: The significant correlation between the eyelid pressure and the ocular surface staining suggests that mechanical friction on the ocular surface by the eyelids may be one of the factors that affects the fluorescein staining of the inferior ocular surface. (Am J Ophthalmol 2015;160(4):685–692.

DRY EYE DISEASE HAS BEEN DEFINED AS A DISORDER of the tear film caused by a deficiency in tear formation or excessive evaporation of the tears. The presence of dry eyes can damage the ocular surface and is manifested by a variety of signs and symptoms. In 2007, the International Dry Eye WorkShop defined dry eye as a "multifactorial disease of the tear and ocular surface that results in symptoms of discomfort, visual disturbances, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface."[1]

It is well recognized that blinking and eyelid dynamics play important roles in the distribution of tears and in the maintenance of the integrity of the ocular surface.[2,3] It is also known that functional or anatomic abnormalities of the eyelids can cause ocular surface disorders, and oculoplastic surgery for abnormal eyelids can restore ocular surface integrity.[4,5]

Blepharospasm is a blinking disorder characterized by repeated forceful spasmodic contractions of the orbicularis oculi muscle, and many patients with blepharospasm suffer from dry eye symptoms. However, it has not been determined whether there is a causal relationship between dry eye and blepharospasm.[6–8] On the other hand, Mathers and Lemp used specular microscopy to demonstrate that the shearing force of blinking removed cells from corneal epithelium, and they suggested that the shearing forces of the eyelid movements alter the migration and turnover of epithelial cells by increasing exfoliation.[9]

Recently, Cher proposed a new term, "blink-related microtrauma," for ocular surface disorders arising from the mechanical friction or lubrication disorders of the eyes. Superior limbic keratoconjunctivitis (SLK) was an example of this condition.[10] Korb and associates proposed another term, "lid-wiper epitheliopathy," for the epitheliopathy that is characterized by staining of the conjunctival epithelium at the eyelid margin by fluorescein and/or rose bengal. They suggested that higher shear stress is generated at this region.[11] Thus, the behavior of the eyelid on the ocular surface during blinking should have a large influence on the flux of the tears produced beneath the eyelids.

Eyelid tension or pressure is defined as the tension or pressure exerted by the eyelids on the cornea and conjunctiva. In 1869, Snellen first proposed the concept of eyelid tension and reported that it could alter the shape of the cornea.[12] Since then, eyelid tension or pressure has been measured by various instruments that measure the tension of the eyelid on the ocular surface.[13–15] However, a standardized method of measuring the eyelid tension or pressure has not been established because the methods and measuring devices were not easy to use and were not accurate.

To overcome these problems, we have developed a simple and easy-to-use eyelid pressure measurement system called a blepahro-tensiometer, which uses tactile pressure sensors. Measurements of the eyelid pressure with the blepahro-tensiometer have shown that reliable and valid measurements of the pressure of the eyelids on the ocular surface can be obtained.[16,17]

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The purpose of this study was to determine the effect of eyelid pressure elicited by blinking on the ocular surface, and to determine which area of the ocular surface will be affected by blinking.

METHODS

• STUDY DESIGN: The study was approved by the Institutional Review Board of Ehime University (No. 0701006) and University Hospital Medical Information Network Clinical Trials Registry (ID number UMIN000004256). An informed consent for the examinations was obtained from all subjects, and the procedures used conformed to the tenets of the Declaration of Helsinki. This was a prospective, cross-sectional study designed to determine the relationship between eyelid pressure during blinking and the fluorescein staining of the cornea and conjunctiva in dry eye patients.

• SUBJECTS: The eyelid pressure was measured in 130 eyes of 65 dry eye patients and 58 eyes of 31 normal controls between March 2008 and November 2008 at the Ehime University School of Medicine. The dry eye group (D group) consisted of 13 men and 52 women whose average ± standard deviation age was 58.7 ± 15.0 years with a range from 19 to 91 years. The normal control group (N group) consisted of 14 men and 17 women whose average age was 51.1 ± 17.3 years with a range from 20 to 85 years.

Dry eye was diagnosed according to the 2006 revised Japanese Dry Eye Diagnostic Criteria.[18] The revised criteria for dry eyes were: (1) presence of dry eye symptoms; (2) presence of either qualitative or quantitative disturbances of the tear film (Schirmer I test ≤5 mm or fluorescein tear break-up time [BUT] ≤ 5 s); and (3) presence of conjunctivocorneal epithelial damage (fluorescein staining score ≥3 points or rose bengal staining score ≥3 points or lissamine green staining score ≥3 points). The presence of all 3 criteria is required to establish a diagnosis of definite dry eye. Individuals with 2 positive criteria among the 3 are diagnosed with probable dry eye. Individuals with the presence of either 1 or no positive criteria are diagnosed as normal.

Cases of allergic conjunctivitis, eyelid closure failure, deformed eyelids, conjunctival concretion, or abnormal blinking disorders and cases with a history of any type of eye surgery were excluded. The differences in the age and sex distribution in the N and D groups were not significant.

• EYELID PRESSURE MEASUREMENTS BY BLEPHAROTENSIOMETER: We developed a simple and easy-to-use eyelid pressure measurement system using tactile pressure sensors (DigiTacts Single Point Sensors; Pressure Profile Systems, Inc, Los Angeles, California, USA).[16,17] The pressures of the upper and lower eyelids were measured using the same sensor for each subject, as described in detail.[16,17] Briefly, a sterile disposable soft contact lens (Focus Dailies -3.0 diopter; Chiba Vision, Duluth, Georgia, USA) was placed on the cornea after the eye was anesthetized with topical 0.4% oxybuprocaine (Santen, Osaka, Japan) to protect the cornea. The pressure sensor with the protective polyurethane cap (Okamoto, Tokyo, Japan) was inserted between the soft contact lens and the inner surface of the eyelid. The sensor was placed at the center of the upper eyelid and at the nasal region of the lower eyelids. The subjects were asked to close their eyes and keep their eyes closed for at least 5 seconds for the measurements. The measured pressure was divided into 2 phases: an increasing phase and a plateau phase. Then 2 best-fitting lines were drawn to fit the 2 phases, and the intersecting point was determined. The eyelid pressure was defined as the average of 150 tension values obtained during the 5 seconds after the intersection point. An ophthalmic technician was trained on the protocol for measuring the eyelid pressure, and after she became proficient with the device, she was instructed to perform the measurements.

• EVALUATION OF OCULAR SURFACE STAINING: After instillation of 2 μL of 1% fluorescein Na solution into the cul-de-sac, the location and degree of the ocular surface staining were determined by slit-lamp microscopy with a yellow stop filter (Carl Zeiss Meditec, San Francisco, California, USA).[19] The cornea and conjunctiva were divided into 7 sections (Figure), and the ocular surface staining was scored in each area on a scale of 0–3. Examples of the ocular surface staining scores are shown in the Figure. The corneal fluorescein staining was denoted as KFS while that of the conjunctiva was denoted as CFS. The ocular surface staining was evaluated before eyelid pressure measurements.

• EVALUATION OF OTHER FACTORS USED TO ASSESS OCULAR SURFACE: We calculated the correlations between the location and scores of the ocular surface staining and other factors used to assess the condition of the ocular surface. These factors included the Schirmer I test scores (mm), fluorescein tear film BUT, meibomian gland dysfunction (MGD; grade 0–3 after Shimazaki and associates[20]), tear meniscus height (TMH; low/medium/high), superior conjunctivochalasis (grade 0–3 after Yokoi and associates[21]), inferior conjunctivochalasis (grade 0–3 after Hirotani and associates[22]), superior or inferior lid wiper epitheliopathy (LWE; grade 0–3 after Yamamoto and associates[17]), eyelid shape (1 fold/2 folds in the eyelid), and eyelid ptosis (present/absent) (Table 1).

• STATISTICAL ANALYSES: All data are presented as the means ± standard deviations. Statistical analyses were performed with the Student *t* tests, Pearson coefficient of

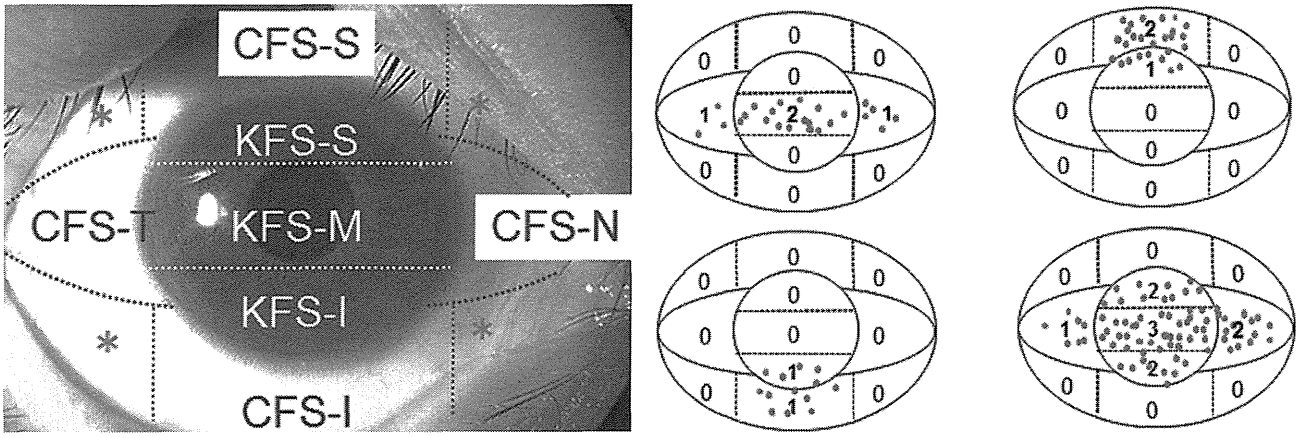

FIGURE. Location and degree of ocular surface staining by fluorescein. (Left) Cornea and conjunctiva are divided into 7 sections. (Right) Examples of ocular surface staining scores. KFS = corneal (kerato) fluorescein staining; CFC = conjunctival fluorescein staining. *: Excluded from the analysis because fluorescein staining scores in these areas were negligible.

TABLE 1. Factors Used to Assess the Correlations Between the Location and Scores of Ocular Surface Staining

Other Tests for Evaluating	Score
Schirmer I test	(mm)
BUT	(second)
MGD	(0–3)
TMH	(low / medium / high)
Superior conjunctivochalasis	(0–3)
Inferior conjunctivochalasis	(0–3)
Superior LWE	(0–3)
Inferior LWE	(0–3)
Eyelid shape	(1 fold / 2 folds in the eyelid)
Eyelid ptosis	(+/–)

BUT = fluorescein tear film break-up time; LWE = lid wiper epitheliopathy; MGD = meibomian gland dysfunction; TMH = tear meniscus height.

correlation, and multiple linear regression analyses. A P value <.05 was considered statistically significant. All analyses were done with JMP for Windows, Version 7 (SAS Institute, Cary, North Carolina, USA).

RESULTS

• **EYELID PRESSURE IN NORMAL EYES AND DRY EYES:** The mean eyelid pressure for the normal eyes (N) group was 16.25 ± 6.18 mm Hg for the upper lid and 16.39 ± 6.82 mm Hg for the lower lid. For the dry eyes (D) group, the mean eyelid pressure was 20.23 ± 5.73 mm Hg for the upper lid and 19.55 ± 6.58 mm Hg for the lower lids. The values for both eyelids were significantly higher in the D group than in the N group (Table 2; upper $P < .0001$, lower $P = .0040$). When the eyelid pressures were examined by age, no significant difference was observed between ages <39 years and and 40–49 years in the N and D groups. However, the eyelid pressures were significantly higher in the D group than the N group for the older ages, especially ages of 50–59 years (Table 2; upper $P = .0068$, lower $P = .0127$) and 60–69 years (Table 2; upper $P = .0007$, lower $P = .0230$). In the N group, all of the eyelid pressure values decreased with increasing age, while this trend was not observed in the D group, where the values for each age group were not significantly different, although no significant difference was observed between N and D groups in lower eyelid pressure for ages over 70 years (Table 2).

• **EFFECT OF EYELID PRESSURE AND OTHER OCULAR FACTORS ON OCULAR SURFACE STAINING SCORES IN DRY EYE PATIENTS:** The ocular surface staining scores in each region are shown in Table 3. The correlations between the ocular surface staining scores and the eyelid pressure values of each region are shown in Table 4. The ocular surface staining scores of the inferior cornea (KFS-I) and inferior conjunctiva (CFS-I) were significantly correlated with the higher pressures in the lower eyelid ($r = 0.19$ and $r = 0.20$, and $P = .0307$ and $P = .0252$, respectively). The correlations between the upper and lower eyelid pressure values and the staining scores for the superior and middle cornea and the intrapalpebral conjunctiva were not significant (Table 4).

The Schirmer I test scores were significantly correlated with the CFS-T and CFS-N scores ($r = -0.21$ and $r = -0.25$, and $P = .0224$ and $P = .0068$, respectively), and the BUT values were significantly correlated with the KFS-S, CFS-S, and CFS-I scores ($r = -0.22$, $r = -0.27$, $r = -0.25$; and $P = .0131$, $P = .0018$, $P = .0043$, respectively). The MGD values were significantly correlated with the KFS-S, KFS-I, CFS-T, CFS-N,

TABLE 2. Eyelid Pressure in Normal Eyes and Dry Eyes by Age

Age		≤39	40–49	50–59	60–69	70+	Total	
Upper eyelid pressure (mm Hg)	N	21.36 ± 5.03	18.86 ± 5.77	14.14 ± 3.95	11.22 ± 5.95	11.54 ± 3.08	16.25 ± 6.18	$P < .0001^{b,c}$
	D	21.22 ± 4.20	19.38 ± 5.93	19.25 ± 5.24	21.03 ± 5.95	20.28 ± 6.73	20.23 ± 5.73	$P = .9515^{b}$
		$P = .9351^{a}$	$P = .8232^{a}$	$P = .0068^{a,c}$	$P = .0007^{a,c}$	$P = .0002^{a,c}$	$P < .0001^{a,c}$	
Lower eyelid pressure (mm Hg)	N	21.72 ± 7.23	15.86 ± 6.41	13.00 ± 4.75	13.52 ± 7.09	15.43 ± 5.47	16.39 ± 6.82	$P = .0009^{b,c}$
	D	21.91 ± 6.11	17.85 ± 6.99	18.34 ± 5.94	20.83 ± 6.90	18.90 ± 6.83	19.55 ± 6.58	$P^{b} = .5527$
		$P = .9364^{a,c}$	$P = .4573^{a}$	$P = .0127^{a,c}$	$P = .0230^{a,c}$	$P^{a} = .1383$	$P = .0040^{a,c}$	
	N	15	14	12	6	11	58	
	D	19	12	35	34	30	130	(eyes)

D = dry eyes; N = normal eyes.
[a]*P* values between dry eyes and normal eyes.
[b]Regression analysis by age.
[c]Significant correlations.

TABLE 3. Ocular Surface Staining Scores in 7 Sections of Cornea and Conjunctiva

Section	Mean ± SD Score
KFS-S	0.19 ± 0.48
KFS-M	0.59 ± 0.73
KFS-I	0.85 ± 0.79
CFS-S	0.42 ± 0.76
CFS-T	0.99 ± 0.74
CFS-N	1.00 ± 0.77
CFS-I	0.51 ± 0.66

CFS = conjunctival fluorescein staining; I = inferior; KFS = corneal (kerato) fluorescein staining; M = middle; N = nasal; S = superior; T = temporal.

and CFS-I scores (r = 0.30, r = 0.19, r = 0.21, r = 0.20, and r = 0.19; and *P* = .0012, *P* =.0435, *P* =.0251, *P* =.0263, and *P* =.0371, respectively), and superior conjunctivochalasis values were significantly correlated with the CFS-S (r = 0.36, *P* < .0001) (Table 4).

To determine how the eyelid pressure was correlated with other factors that measure the ocular surface status, we performed multivariate analyses. The results are summarized in Table 5, with the ocular surface staining score divided into 3 parts: that of the superior conjunctiva and cornea (KFS-S + CFS-S), that of the temporal and nasal intrapalpebral conjunctiva (CFS-T + CFS-N), and that of the inferior conjunctiva and cornea (KFS-I + CFS-I). The analyses showed that the ocular surface staining scores of KFS-S + CFS-S were strongly correlated with the presence of superior conjunctivochalasis and BUT. The ocular surface staining scores of the CFS-T + CFS-N were also strongly correlated with the Schirmer I test and MGD. The ocular surface staining scores of KFS-I + CFS-I were strongly correlated with the lower eyelid pressure and MGD (Table 5).

DISCUSSION

WE HAVE DEVELOPED A SIMPLE AND RELIABLE EYELID PRESsure measuring system using tactile pressure sensors.[16,17] We used this system to measure the eyelid pressure in dry eye patients and normal volunteers and found that the eyelid pressure values were significantly higher in dry eye patients than in normal subjects, especially those older than 50 years. When we examined the correlations between the eyelid pressure and the location of the fluorescein ocular surface staining scores, the ocular surface staining scores for the inferior cornea and conjunctiva were significantly correlated with the higher pressure in the lower eyelid.

Eyelid tension or pressure has been measured by different instruments,[13–16] and although the instruments were different for each laboratory, there was general agreement that the eyelid tension or pressure decreased with increasing age in normal eyes, as was found in our normal subjects. Interestingly, this significant decrease in the eyelid pressure was not found in the patients with dry eye. Although our study was not designed to examine the effect of age, we found that the eyelid pressure did not decrease with increasing age in the dry eye patients. However, these findings do not provide any evidence on whether the higher eyelid pressure was due to the dry eye conditions, or dry eye conditions were due to the higher eyelid pressure. We cannot explain this difference at this time, and future studies will be designed to resolve this issue.

Our focus in this study was to determine whether the shear stress generated by blinking can affect the ocular surface and, if it can, which part of the ocular surface will be affected by blinking. Therefore, we determined whether significant correlations existed between the location of the fluorescein ocular surface staining and the eyelid pressure as a marker of the force generated by blinking.

TABLE 4. Correlation Between Location of Ocular Surface Staining and All Evaluating Items in Dry Eye Patients

	KFS-S	KFS-M	KFS-I	CFS-S	CFS-T	CFS-N	CFS-I
Upper eyelid pressure							
r	−0.14	−0.04	0.10	−0.07	0.11	0.17	0.06
P	.1260	.6288	.2805	.4537	.2338	.0647	.5380
Lower eyelid pressure							
r	0.04	0.06	0.19	−0.05	0.10	0.13	0.20
P	.6774	.5333	.0307[a]	.6151	.2858	.1459	.0252[a]
Schirmer I test scores							
r	−0.11	−0.14	−0.03	−0.10	−0.21	−0.25	−0.04
P	.2234	.1265	.7489	.2738	.0224[a]	.0068[a]	.6655
BUT							
r	−0.22	−0.04	0.02	−0.27	−0.09	−0.02	−0.25
P	.0131[a]	.6803	.8564	.0018[a]	.3154	.8170	.0043[a]
MGD							
r	0.30	0.18	0.19	−0.11	0.21	0.20	0.19
P	.0012[a]	.0507	.0435[a]	.2387	.0251[a]	.0263[a]	.0371[a]
TMH							
r	0.13	−0.02	0.00	−0.06	−0.11	−0.05	−0.02
P	.1499	.8195	.9596	.5038	.2331	.6065	.7949
Superior conjuctivochalasis							
r	0.12	0.00	−0.03	0.36	0.02	−0.01	0.03
P	.1954	.9764	.7448	<.0001[a]	.8361	.9016	.7741
Inferior conjunctivochalasis							
r	−0.06	−0.05	0.06	−0.01	0.04	0.03	0.03
P	.5237	.5843	.5152	.9316	.6218	.7712	.7371
Superior LWE							
r	−0.01	−0.06	0.11	−0.17	0.06	0.18	0.02
P	.8880	.5277	.2054	.0507	.5098	.0475[a]	.7935
Inferior LWE							
r	−0.01	0.05	0.07	−0.18	0.09	0.09	0.02
P	.9045	.5925	.4335	.0435[a]	.3076	.2954	.8360
Eyelid shape							
r	−0.03	0.12	0.03	−0.08	0.04	0.10	−0.06
P	.7406	.1802	.7792	.3715	.6787	.2735	.5005
Eyelid ptosis							
r	0.17	−0.07	−0.19	0.23	−0.14	−0.14	−0.10
P	.0632	.4406	.0429[a]	.0122[a]	.1420	.1441	.3040

BUT = fluorescein tear film break-up time; CFS = conjunctival fluorescein staining; I = inferior; KFS = corneal (kerato)fluorescein staining; LWE = lid wiper epitheliopathy; M = middle; MGD = meibomian gland dysfunction; N = nasal; S = superior; T = temporal; TMH = tear meniscus height.

[a]Significant correlations.

Contrary to our expectations, the surface staining scores of the superior cornea and conjunctiva were not significantly correlated with the eyelid pressure values of the upper eyelid. Cher proposed a new term, "blink-related microtrauma," for ocular surface disorders arising from mechanical friction or lubrication such as that in SLK.[10] Thus, we expected that the ocular surface staining score of the superior cornea and conjunctiva might be significantly correlated with the eyelid pressure of the upper eyelid. But this was not found, although the number of patients who had SLK was small. Most of our dry eye patients had staining scores of 0 for the KFS-S and CFS-S, and thus the statistical analyses may not detect significant differences in the subjects with scores of 0–3 in the KFS-S and CFS-S area. However, when we examined the staining score for the KFS-S and CFS-S area excluding those eyes with a score of 0, the ocular staining scores were significantly correlated with the upper eyelid pressure ($P < .05$). Thus, eyes with SLK may have higher eyelid pressure for the upper eyelid, and this matter can be resolved by a study with a larger number of patients with SLK.

We found that the ocular surface staining scores of the inferior cornea and conjunctiva (KFS-I + CFS-I) were

TABLE 5. Multivariate Analysis for Effect of Eyelid Pressure and Other Factors on Ocular Surface Staining Scores

	Estimated Value	SE	t Value	*P* Value (Prob > |t|)
KFS-S + CFS-S[a]				
Intercept	0.742	0.361	2.05	.0421[d]
Upper eyelid pressure	−0.011	0.014	−0.80	.4279
BUT	−0.183	0.055	−3.30	.0013[d]
Superior conjunctivochalasis	0.435	0.123	3.54	.0006[d]
KFS-I + CFS-I[b]				
Intercept	0.698	0.384	1.82	.0715[d]
Lower eyelid pressure	0.036	0.017	2.17	.0325[d]
BUT	−0.122	0.075	−1.63	.1064
MGD	0.379	0.180	2.11	.0371[d]
CFS-T + CFS-N[c]				
Intercept	2.089	0.270	7.74	<.0001[d]
Schirmer I test scores	−0.034	0.017	−2.10	.0383[d]
BUT	−0.087	0.098	−0.89	.3771
MGD	0.424	0.210	2.01	.0467[d]

BUT = fluorescein tear film break-up time; CFS = conjunctival fluorescein staining; I = inferior; KFS = corneal (kerato)fluorescein staining; M = middle; MGD = meibomian gland dysfunction; N = nasal; S = superior; SE = standard error; T = temporal.

[a]KFS-S + CFS-S = 0.742 − 0.011 × upper eyelid pressure − 0.183 × BUT + 0.435 × superior conjunctivochalasis.

[b]KFS-I + CFS-I = 0.698 + 0.036 × lower eyelid pressure − 0.122 × BUT + 0.379 × MGD.

[c]CFS-T + CFS-N = 2.089 − 0.034 × Schirmer I test scores − 0.087 × BUT + 0.424 × MGD.

[d]Significant values.

significantly correlated with the lower eyelid pressure. Doane measured the movement of eyelids and globe and reported that there was a significant correlation between the posterior movement of the globe and the pressure on the ocular surface during blinking.[23] To further validate the evidence of the eyelid pressure, we recently measured the movement of eyelids or globes during blinking and found a significant correlation between the lower eyelid pressure and the horizontal movement of the lower eyelid, and the posterior movement of the globe during blinking.[17] However, the correlation between the upper eyelid pressure and horizontal eyelid movements was not significant.[17] These findings indicate that the lower eyelid probably has a greater shearing force or pressure exerted on the ocular surface during blinking.

During a blink, the upper eyelid has large vertical excursion while the lower lid has a nasalward movement.[17,23] Because of the large excursion of the upper eyelid, most investigators have paid more attention to the upper eyelid movements and tension than to the lower eyelid. Among the limited number of studies that examined the lower eyelid movement, Shore reported that the decrease in lower eyelid movement with aging was closely correlated with the increase in eyelid laxity.[24]

It should also be remembered that the lower eyelid moves horizontally, meaning that the eyelid margin rubs on the same area of the ocular surface. Thus, the friction of the eyelid movements on the restricted area of the ocular surface might be greater by the lower eyelid than by the upper eyelid. More attention should be paid on the direction of movement of the lower eyelids, and further investigations should be focused on the stress induced by eyelid movements. Our results indicate that higher eyelid pressure is significantly correlated with higher staining scores of the inferior cornea and inferior conjunctiva but not for other areas of the ocular surface.

We were concerned about the reliability of the results. Therefore, we conducted a multiple regression analysis to determine how the eyelid pressure and other factors associated with ocular surface staining of dry eye patients were correlated. The results of the multiple regression analysis showed that the staining score of the intrapalpebral conjunctiva was significantly correlated with Schirmer I test scores. Our findings also showed that the Schirmer I test scores were not significantly correlated with the staining score of the upper and lower areas of the ocular surface that are usually covered by the eyelids. The staining score of the intrapalpebral area is known to be high in dry eye patients. Our dry eye patients were diagnosed based on the 2006 revised Japanese criteria for dry eye, which include BUT scores ≤5 s and Schirmer I test score ≥5 mm. Our findings showed that the mean value of Schirmer I test score was 6.93 ± 0.48 mm and BUT was 2.34 ± 1.36 s. Therefore, to consider the effect of aqueous tear deficiency in the analysis, we also analyzed the relationship between eyelid pressures and the ocular surface staining scores in eyes with Schirmer I test score ≤5 mm and those with >5 mm Schirmer I test scores. However, there was no significant difference in the results in the 2 groups. These findings indicate the reliability of our results. The staining scores of the superior conjunctiva and cornea were strongly correlated with the superior conjunctivochalasis. These results are in good agreement with earlier reports that the reduction of laxity of conjunctivochalasis was highly effective in eyes with SLK.[25–28]

It has been well documented that meibomian gland dysfunctions are associated with ocular irritation and ocular surface staining.[20,29–31] Our multiple regression analysis showed that the staining score of the inferior conjunctiva and cornea were correlated with the meibomian gland dysfunction scores. These results were not too surprising because these regions are frequently in contact with the lid margins during blinking. These regions may be easily affected by inflammatory or infectious meibomian glands, resulting in the higher staining scores.

Thus, the significant correlations found between the lower eyelid pressure and the staining scores of the inferior cornea and conjunctiva by multiple regression analysis also support the idea that higher eyelid pressure may be related

to the ocular surface staining score of the inferior area of the ocular surface.

There are limitations to our study because we examined the eyelid pressure as an alternative to shear stress generated by blinking. Thus, further examinations to measure the shear stress generated by blinking will be needed to determine the effect of blinking on the ocular surface.

In conclusion, we have found that the eyelid pressure on the ocular surface was higher in dry eyes than in normal eyes. The highly significant correlation between eyelid pressure and the fluorescein ocular surface staining score of the inferior area of the ocular surface suggests that mechanical friction on the ocular surface by the eyelids may be a risk factor for ocular surface disorders.

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REFERENCES

1. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007). *Ocul Surf* 2007;5(2):75–92.
2. Bron AJ, Tiffany JM, Gouveia SM, Yokoi N, Voon LW. Functional aspects of the tear film lipid layer. *Exp Eye Res* 2004;78(3):347–360.
3. Goto E, Tseng SC. Kinetic analysis of tear interference images in aqueous tear deficiency dry eye before and after punctal occlusion. *Invest Ophthalmol Vis Sci* 2003;44(5):1897–1905.
4. Kakizaki H, Zako M, Mito H, Iwaki M. Filamentary keratitis improved by blepharoptosis surgery: two cases. *Acta Ophthalmol Scand* 2003;81(6):669–671.
5. Qian JG, Wang XJ, Wu Y. Severe cicatrical ectropion: repair with a large advancement flap and autologous fascia sling. *J Plast Reconstr Aesthet Surg* 2006;59(8):878–881.
6. Horwath-Winter J, Bergloeff J, Floegel I, Haller-Schober EM, Schmut O. Botulinum toxin A treatment in patients suffering from blepharospasm and dry eye. *Br J Ophthalmol* 2003;87(1):54–56.
7. Price J, O'Day J. A comparative study of tear secretion in blepharospasm and hemifacial spasm patients treated with botulinum toxin. *J Clin Neuroophthalmol* 1993;13(1):67–71.
8. Spiera H, Asbell PA, Simpson DM. Botulinum toxin increases tearing in patients with Sjogren's syndrome: a preliminary report. *J Rheumatol* 1997;24(9):1842–1843.
9. Mathers WD, Lemp MA. Morphology and movement of corneal surface cells in humans. *Curr Eye Res* 1992;11(6):517–523.
10. Cher I. Blink-related microtrauma: when the ocular surface harms itself. *Clin Experiment Ophthalmol* 2003;31(3):183–190.
11. Korb DR, Greiner JV, Herman JP, et al. Lid-wiper epitheliopathy and dry-eye symptoms in contact lens wearers. *Clao J* 2002;28(4):211–216.
12. Snellen H. Die Richtung der Hauptmeridiane des astigmatischen Auges. *Albrecht von Grafes Arch Ophthamol* 1869;2:199–207.
13. Ehrmann K, Francis I, Stapleton F. A novel instrument to quantify the tension of upper and lower eyelids. *Cont Lens Anterior Eye* 2001;24(2):65–72.
14. Francis IC, Stapleton F, Ehrmann K, Coroneo MT. Lower eyelid tensometry in younger and older normal subjects. *Eye (Lond)* 2006;20(2):166–172.
15. Vihlen FS, Wilson G. The relation between eyelid tension, corneal toricity, and age. *Invest Ophthalmol Vis Sci* 1983;24(10):1367–1373.
16. Sakai E, Shiraishi A, Yamaguchi M, Ohta K, Ohashi Y. Blepharo-tensiometer: new eyelid pressure measurement system using tactile pressure sensor. *Eye Contact Lens* 2012;38(5):326–330.
17. Yamamoto Y, Shiraishi A, Sakane Y, Ohta K, Yamaguchi M, Ohashi Y. Involvement of eyelid pressure in lid-wiper epitheliopathy. *Curr Eye Res* 2015. forthcoming.
18. Uchino Y, Uchino M, Dogru M, Ward S, Yokoi N, Tsubota K. Changes in dry eye diagnostic status following implementation of revised Japanese dry eye diagnostic criteria. *Jpn J Ophthalmol* 2012;56(1):8–13.
19. Koh S, Watanabe H, Hosohata J, et al. Diagnosing dry eye using a blue-free barrier filter. *Am J Ophthalmol* 2003;136(3):513–519.
20. Shimazaki J, Sakata M, Tsubota K. Ocular surface changes and discomfort in patients with meibomian gland dysfunction. *Arch Ophthalmol* 1995;113(10):1266–1270.
21. Yokoi N, Komuro A, Maruyama K, Tsuzuki M, Miyajima S, Kinoshita S. New surgical treatment for superior limbic keratoconjunctivitis and its association with conjunctivochalasis. *Am J Ophthalmol* 2003;135(3):303–308.
22. Hirotani Y, Yokoi N, Komuro A, Kinoshita S. [Age-related changes in the mucocutaneous junction and the conjunctivochalasis in the lower lid margins]. *Nippon Ganka Gakkai Zasshi* 2003;107(7):363–368.
23. Doane MG. Interactions of eyelids and tears in corneal wetting and the dynamics of the normal human eyeblink. *Am J Ophthalmol* 1980;89(4):507–516.
24. Shore JW. Changes in lower eyelid resting position, movement, and tone with age. *Am J Ophthalmol* 1985;99(4):415–423.
25. Dal Pizzol MM, Roggia MF, Kwitko S, Marinho DR, Rymer S. [Use of fibrin glue in ocular surgery]. *Arq Bras Oftalmol* 2009;72(3):308–312.
26. Chun YS, Kim JC. Treatment of superior limbic keratoconjunctivitis with a large-diameter contact lens and Botulium Toxin A. *Cornea* 2009;28(7):752–758.

27. Kheirkhah A, Casas V, Esquenazi S, et al. New surgical approach for superior conjunctivochalasis. *Cornea* 2007; 26(6):685–691.
28. Yokoi N, Inatomi T, Kinoshita S. Surgery of the conjunctiva. *Dev Ophthalmol* 2008;41:138–158.
29. Macri A, Pflugfelder S. Correlation of the Schirmer 1 and fluorescein clearance tests with the severity of corneal epithelial and eyelid disease. *Arch Ophthalmol* 2000;118(12):1632–1638.
30. Souchier M, Joffre C, Gregoire S, et al. Changes in meibomian fatty acids and clinical signs in patients with meibomian gland dysfunction after minocycline treatment. *Br J Ophthalmol* 2008;92(6):819–822.
31. Yamaguchi M, Kutsuna M, Uno T, Zheng X, Kodama T, Ohashi Y. Marx line: fluorescein staining line on the inner lid as indicator of meibomian gland function. *Am J Ophthalmol* 2006;141(4):669–675.

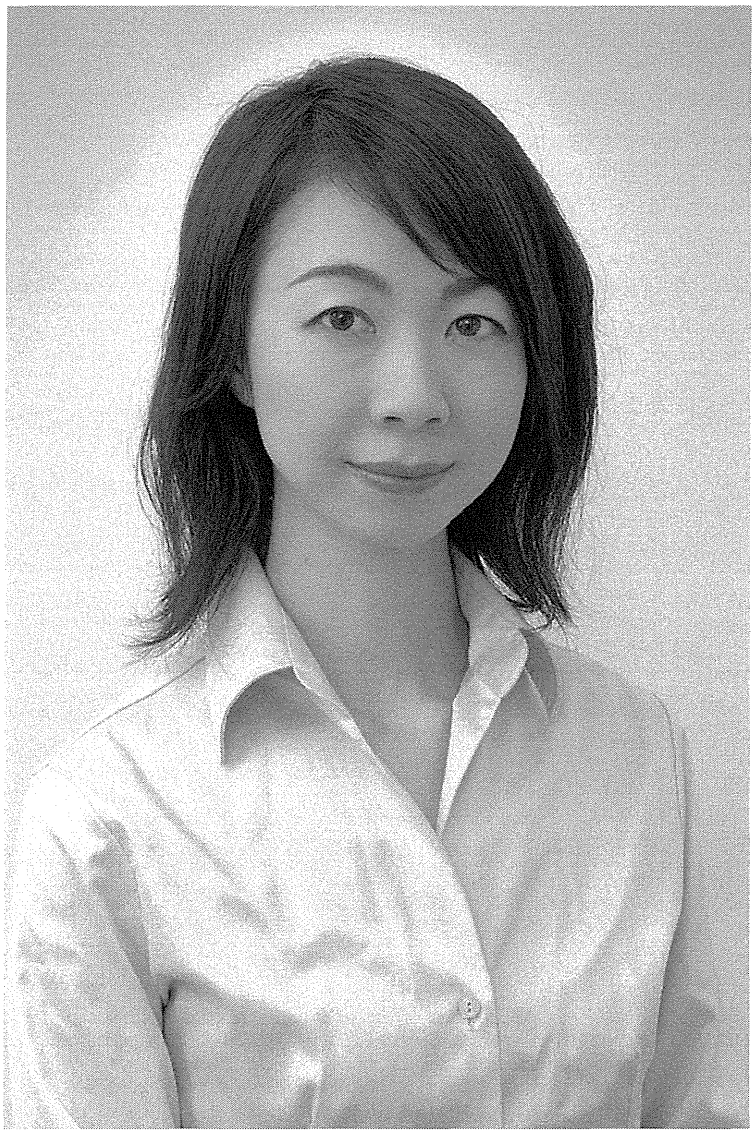
REPORTING VISUAL ACUITIES

The AJO encourages authors to report the visual acuity in the manuscript using the same nomenclature that was used in gathering the data provided they were recorded in one of the methods listed here. This table of equivalent visual acuities is provided to the readers as an aid to interpret visual acuity findings in familiar units.

Table of Equivalent Visual Acuity Measurements

Snellen Visual Acuities				
4 Meters	6 Meters	20 Feet	Decimal Fraction	LogMAR
4/40	6/60	20/200	0.10	+1.0
4/32	6/48	20/160	0.125	+0.9
4/25	6/38	20/125	0.16	+0.8
4/20	6/30	20/100	0.20	+0.7
4/16	6/24	20/80	0.25	+0.6
4/12.6	6/20	20/63	0.32	+0.5
4/10	6/15	20/50	0.40	+0.4
4/8	6/12	20/40	0.50	+0.3
4/6.3	6/10	20/32	0.63	+0.2
4/5	6/7.5	20/25	0.80	+0.1
4/4	6/6	20/20	1.00	0.0
4/3.2	6/5	20/16	1.25	−0.1
4/2.5	6/3.75	20/12.5	1.60	−0.2
4/2	6/3	20/10	2.00	−0.3

From Ferris FL III, Kassoff A, Bresnick GH, Bailey I. New visual acuity charts for clinical research. Am J Ophthalmol 1982;94:91–96.



Biosketch

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難渋症例にチャレンジ！

眼科診断トレーニング

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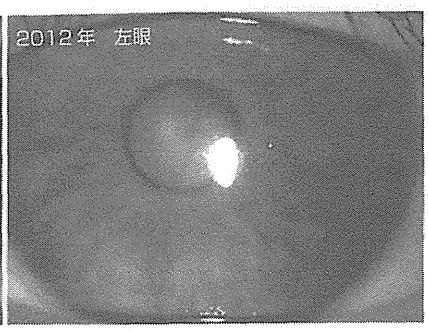

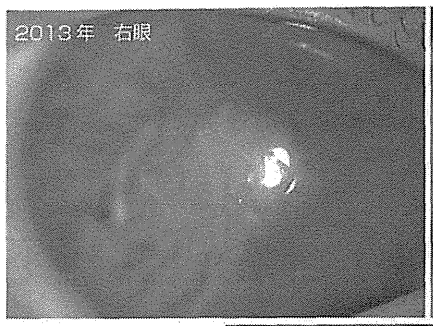

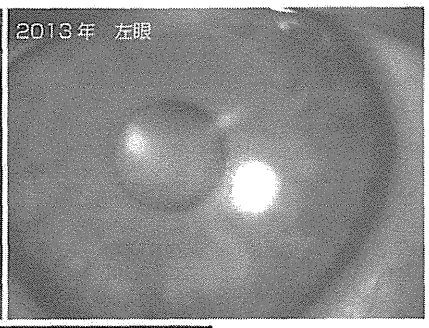

症　例

56歳　女性

主　訴

目がかすむ．ゴロゴロする．

経　過

2012年3月に，「目がかすむ」という訴えで眼科受診した．午前中，とくに起床時に自覚症状が強かった．そのときは白内障とドライアイと診断され，経過観察となった．

その後，次第に右眼の霧視，視力低下が進み，眼痛の頻度も多くなってきたため，2013年8月に眼科を再受診した．

既往歴

特になし．また，外傷歴もなし．

検査結果

視　力（2012年3月）
R：0.3（0.7×+3.5D cyl −0.5D Ax 90°）
L：0.1（0.9×+4.0D cyl −0.5D Ax 60°）
眼　圧（2012年3月） RT：15mmHg，LT：16mmHg

視　力（2013年8月）
R：0.1（0.4×+2.5D cyl −0.5D Ax 90°）
L：0.1（0.9×+4.5D cyl −0.5D AX50°）
眼　圧（2013年8月） RT：15mmHg，LT：16mmHg

観察のポイント

①程度の差はあるが，両眼性に角膜中央部の浮腫性混濁を認める

②2013年の右眼には，上皮下の水疱形成もみられる

③2013年の左眼のスリット写真のように，拡大して鏡面反射法で観察すると……？

問題点の整理

両眼性に角膜中央部の浮腫性混濁があり，右眼はこの1年で進行してきた女性の症例である．霧視と視力低下だけでなく，眼痛も進行してきている．外傷歴はない．

memo

診断名 フックス角膜内皮ジストロフィ

解　説

本症例は，初期の段階から1年かけて徐々に進行してきたフックス角膜内皮ジストロフィである．

フックス角膜内皮ジストロフィは常染色体優性遺伝形式をもち，滴状角膜（guttata cornea）という特徴的所見を伴い，原発性に角膜内皮が障害され，進行性に内皮細胞数の減少をきたす角膜内皮ジストロフィの一つである．

フックス角膜内皮ジストロフィには民族差があり，白人に多く日本人ではまれとされる．家系調査をすると常染色体優性遺伝形式をとることがわかっているが，罹患率の調査をすると明らかに女性のほうが高い．

滴状角膜は，細隙灯顕微鏡検査では角膜中央部に集中した，角膜内皮層の多数の微細，透明な光輝点として観察できる．拡大して観察すると，角膜後面沈着物ではなく，Descemet膜の異常な肥厚により，いぼ状の病変が前房側に突出した状態であることがわかる．2013年の左眼のスリット写真は，滴状角膜の鏡面反射法による撮影像である．

さらに，滴状角膜の観察には，スペキュラーマイクロスコピー検査が非常に有用である．スペキュラーマイクロスコピーでは，孤発性ないし融合して拡大したdark areaとして観察される（**図1**）．滴状角膜の位置と一致して，角膜内皮細胞密度減少に伴う角膜浮腫も中央部から始まる．

初期の症状としては，一日のうちで朝の起床時にもっとも強く霧視を訴えるが，起床後しばらくすると改善してくる．これは夜間睡眠時の閉瞼により角膜の浮腫が強くなり，起床直後はこの影響が残っているためだが，その後，開瞼状態の持続により，角膜の乾燥と酸素不足が解消されることで浮腫が改善され，角膜の透明性が増して視力が回復するためと考えられる．

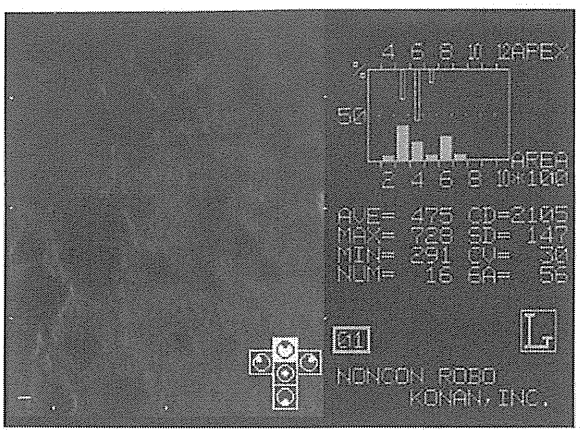

図1…スペキュラーマイクロスコピー検査の結果

進行すると水疱性角膜症に至り，視力低下だけでなく，上皮下水疱の形成とその破裂による眼痛を生じる．

治療は，全層角膜移植あるいは角膜内皮パーツ移植であるDSEAK（descemet's stripping automated endothelial keratoplasty）が良い適応である．進行した症例で血管侵入や実質の瘢痕性混濁が生じている症例では全層角膜移植の適応となる．本症例は右眼にDSEAKを行い，術後経過良好である．

Roles of Epithelial Cell–Derived Type 2–Initiating Cytokines in Experimental Allergic Conjunctivitis

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Purpose. To clarify the possible involvement of the type 2-initiating cytokines interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP) in the pathophysiology of allergic conjunctivitis, we evaluated ragweed (RW)-induced experimental allergic conjunctivitis (EAC) models by using IL-25 knockout (KO), IL-33 KO, and TSLP receptor (TSLPR) KO mice.

Methods. Interleukin-25 KO, IL-33 KO, TSLPR KO, and BALB/c wild-type mice were sensitized twice with RW in alum and then challenged with RW in eye drops. Clinical scores and eosinophil infiltration were evaluated. Expression levels of serum immunoglobulin E (IgE) and cytokines in the conjunctival tissues were quantified and immunohistochemical analysis was carried out.

Results. Significant reductions in clinical scores and numbers of infiltrating eosinophils were observed in the RW-EAC model using IL-33 KO mice. There were no significant differences in clinical scores and numbers of infiltrating eosinophils among IL-25KO, TSLPR KO, and wild-type mice. Serum IgE concentration was upregulated after RW challenges, and there were no differences among the mouse genotypes. Expression levels of of *il4*, *il5*, *il13*, and *ccl5* mRNA were diminished in the conjunctivae of the RW-EAC model using IL-33 KO mice compared to those in wild-type mice. Interleukin-33 expression was upregulated as early as 1 hour after RW eye-drop challenge. The number of infiltrating basophils in the conjunctivae of the RW-EAC model using IL-33 KO mice was diminished compared to that in wild-type mice.

Conclusions. Among the type 2-initiating cytokines, IL-33 may play a major role in conjunctival inflammation in an RW-EAC model.

Keywords: experimental allergic conjunctivitis, IL-25, IL-33, thymic stromal lymphopoietin, type 2-initiating cytokines

Type 2 immune responses are inflammatory conditions associated with parasitic infections[1] and atopic diseases like asthma, atopic dermatitis, and atopic keratoconjunctivitis (AKC).[2] These type 2 immune responses are characterized by activation of $CD4^+$ T-helper type 2 (Th2) cells and production of typical type 2 immunity-associated cytokines (e.g., interleukin-4 [IL-4], IL-5, and IL-13). Although various external stimuli (including pollen, house dust mites [HDM], food allergens, and parasites) can induce type 2 responses, these antigens cannot directly activate Th2 cells because they are too large to be phagocytosed by antigen-presenting cells.[3] Epithelial cell-derived type 2-initiating cytokines (IL-25, IL-33, and thymic stromal lymphopoietin [TSLP]) were characterized recently as indispensable for initiating type 2 immune responses stimulated by these type 2 immunity-related antigens.[3]

We previously reported the expression of IL-33[4] and TSLP[5] mRNA and protein in giant papillae obtained from patients with vernal keratoconjunctivitis (VKC) and AKC. Our study group also established an IL-33 knockout (KO) mouse and reported that IL-33 has an essential role in papain-induced lung inflammation, which is considered to be an innate immune system-dependent type 2 inflammation.[6] The role of IL-33 was also reported in an ovalbumin (OVA)-induced asthma model,[7–9] an established model for acquired immune system-dependent type 2 inflammation. Ragweed (RW)-induced experimental allergic conjunctivitis (EAC) has been used as a common model for T-cell (acquired immunity)-dependent allergic conjunctivitis.[10] Matsuba-Kitamura et al.[11] reported that addition of recombinant IL-33 at the time of RW eye-drop challenge augmented eosinophil infiltration in the conjunctival tissue in their RW-EAC model.

Thymic stromal lymphopoietin is produced by epithelial cells in response to various protein allergens (e.g., OVA) and protease allergens (e.g., pollen and papain).[12] It activates dendritic cells through the TSLP receptor (TSLPR)–IL-7Rα receptor heterodimer complex.[13] Thymic stromal lymphopoie-