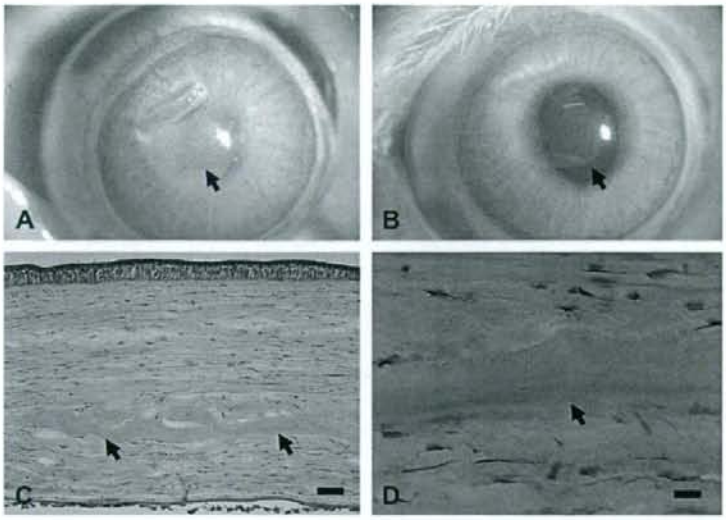

Fig. 6. Representative slit-lamp photographs of one rabbit taken just after intracorneal transplantation (A) and 1 month after transplantation (B) of TT-FDAM. A cross-section of the cornea 1 month after TT-FDAM (C, D). One month after intracorneal TT-FDAM transplantation, there was no evidence of neovascularization or stromal edema on the corneal surface and the clarity of the TT-FDAM was markedly improved (B). The transplanted TT-FDAM adapted well to the host corneal stroma, with no evidence of subepithelial cell infiltration or stromal edema (C, D). Scale bar: 50 μm (C) and 10 μm (D).

the transplanted TT-FDAMs adapted well to the host corneal stroma, with no evidence of subepithelial cell infiltration or stromal edema (Fig. 6C,D). Immunohistochemical analysis regarding the inflammatory reaction using several markers (CD4/CD8 (lymphocyte), CD68 (macrophage), neutrophil elastase (neutrophil)) indicated that TT-FDAM did not induce the significant inflammatory reaction *in vivo* (Supplementary data, Fig. 2).

Both TT-FDAM and FDAM were successfully transplanted onto the bare rabbit sclera with sutures as shown in Fig. 7. The secured AMs had been fixed without loss or dislocation for 4 weeks. The grade of epithelialization and hyperemia in the surgical area was evaluated by slit-lamp microscopy, with and without fluorescein (Fig. 7). Though FDAM transplantation did not epithelialize within 1 week (Fig. 7B), the surface of the TT-FDAM was covered with conjunctival epithelium at 1 week after transplantation (Fig. 7E). In regards to the hyperemia, there was no significant difference between FDAM and TT-FDAM (Fig. 7C,F).

3.8. Characterization of epithelial cells on TT-FDA

One month after transplantation of FDAM and TT-FDAM onto the bare sclera, we compared the characterization of the migrating

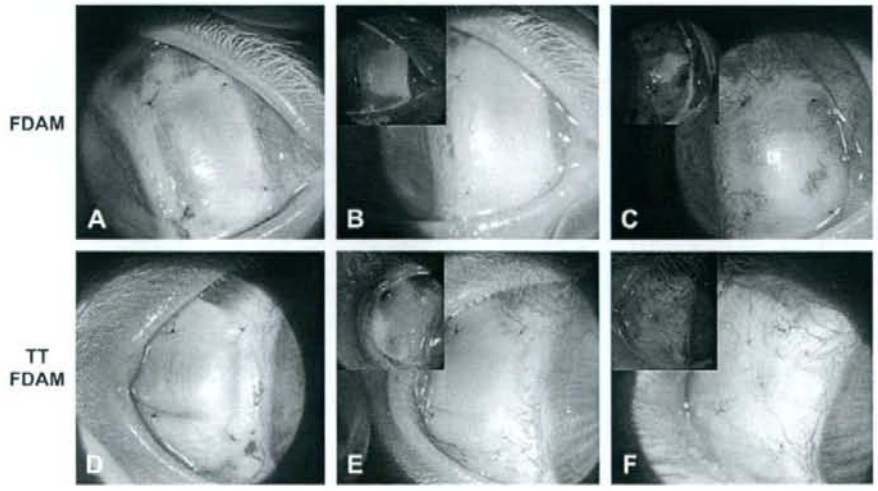

Fig. 7. Representative slit-lamp photographs of rabbit eyes taken just after transplantation (A, D), 7 days after transplantation (B, E), and 1 month after transplantation (C, F) using FDAM (A–C) and TT-FDAM (D–F). Inserts: representative fluorescein images. FDAM transplantation did not epithelialize within 1 week (B), and the surface of the TT-FDAM was covered with conjunctival epithelium at 1 week after transplantation (E). In regards to the hyperemia, there was no significant difference between FDAM and TT-FDAM (C, F).

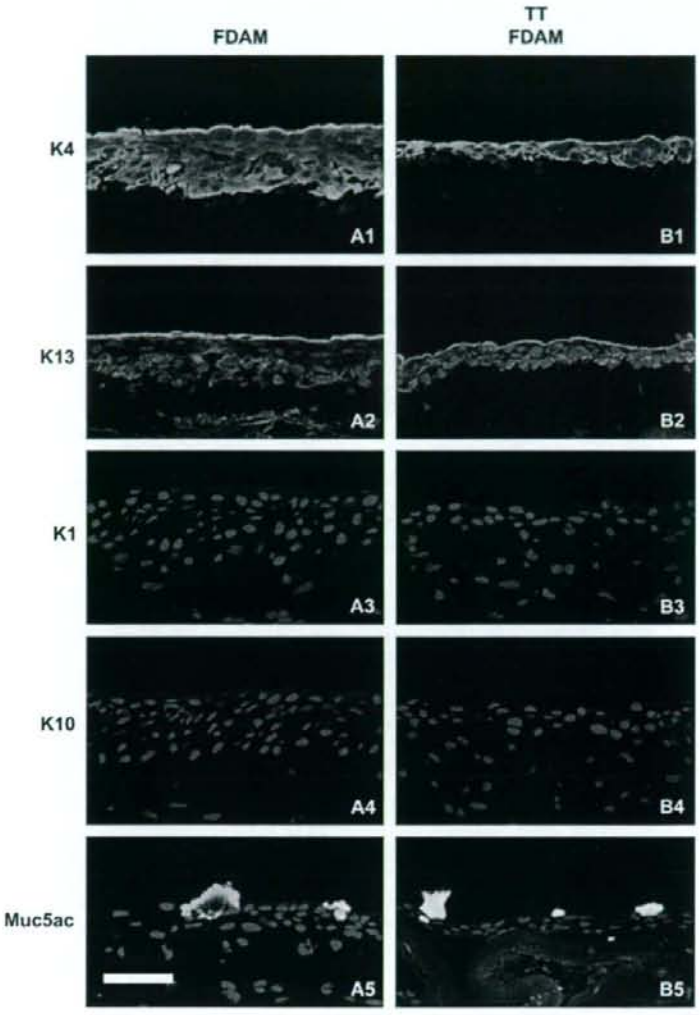

Fig. 8. Representative immunohistochemical staining of K4, K13, K1, K10, and Muc5ac in FDAM (A1–A5) and TT-FDAM (B1–B5). K4, K13, and Muc5ac were expressed at the epithelial cell layer of FDAM (A1, A2, and A5) and TT-FDAM (B1, B2, and B5). In contrast, K1 and K10 were not expressed in any of the FDAM (A3, A4) or TT-FDAM (B3, B4) samples. Scale bar: 50 μm.

conjunctival epithelial cells on them by immunohistochemistry for keratins 1, 4, 10, 13, and Muc5ac. The staining for keratins 4, 13, and Muc5ac was detected at the epithelial cell layer of both TT-FDAM and FDAM (Fig. 8A1,A2,A5,B1,B2,B5). We were unable to detect any staining for keratins 1 and 10 (Fig. 8A3–A4,B3–B4). These staining patterns were similar to that of *in vivo* normal rabbit conjunctival epithelium [14].

4. Discussion

Currently, most ophthalmologists throughout the world use cryopreserved AM under conditions that are as sterile as possible, however, safe and complete sterilization cannot be guaranteed with present procedures. To overcome this problem, we previously developed the prototype of FDAM by preserving AM in a vacuum-dried state and used gamma-irradiation for sterilization [9]. To further improve the biological and practical quality of FDAM, we devised a novel manufacturing procedure using a simple trehalose treatment and found that trehalose significantly improved the quality of FDAM. To the best of our knowledge, this is the first study to present the novel concept of using trehalose-treated biomaterials for ocular surface reconstruction.

Our examinations of the physical properties of AM were of particular interest. From our results, the freeze-drying process definitely reduced the thickness of AM, however, SS tests disclosed no significant differences in the mechanical properties of AM, FDAM, or TT-FDAM. Most interestingly, the adaptability of TT-FDAM is markedly improved as compared to FDAM, and it is almost identical to native AM. These findings clearly suggest that the physical properties of TT-FDAM are superior to those of FDAM, and that it has an excellent capacity for wider clinical applications in a variety of research fields.

We thus used several methods to determine the physical properties of TT-FDAM, however, we think that these methods are not accurate enough and more appropriate methods should be selected to determine its exact physical property. For example, haze was measured by turbidimeter, but if a spectroradiometer had been used to determine the coefficients of scattering, absorption, extinction, and translucence using Kubelka–Munk or another type of algorithm, we could have examined its exact transparency. By using a rheometer, it is possible to determine the elastic or viscoelastic behavior of TT-FDAM, along with the dynamic modulus and other relevant parameters of the material. In addition, the method used in this study for the determination of TT-FDAM's adaptability was a subjective method. Therefore, although we were extremely careful with regards to the interpretation of our results, we feel that further investigations using these types of assay are needed to clarify these points.

We previously reported that the organization of the extracellular matrix molecules plays a crucial role in the physical and biological properties of AM. In this study, we examined various kinds of molecular organization and found that collagens 1, 3–5, and fibronectin are expressed throughout the entire TT-FDAM, whereas collagen 7 and laminin 5 are observed in its basement membrane side. These results are almost identical to those of AM and FDAM [9]. Moreover, our electron microscopy results for AM, FDAM, and TT-FDAM showed that the detailed morphological appearance of the TT-FDAM is more similar to that of AM than to FDAM. Even though the expression pattern of basement membrane components and morphological appearance in the three AM types were somehow contradictory, we presume that all AMs examined in this study have the original basement membrane components yet their detailed morphology may change during the freeze-drying process without trehalose treatment. Thus, these physical, immunohistochemical, and morphological examinations confirmed that the simple trehalose treatment worked well for protecting the AM matrix from the process of freeze-drying and irradiation, and well maintained the physical or biological properties of the AM.

To use TT-FDAM as a biomaterial, it is important to examine its biocompatibility. Since trehalose is a kind of sugar, it may induce an inflammatory reaction *in vivo* and may compromise the anti-inflammatory property of AM. Therefore, we examined its biocompatibility by intracorneal transplantation. All transplanted TT-FDAMs examined in this study adapted well to the host corneal stroma, with no evidence of subepithelial cell infiltration, stromal edema, or neovascularization. Nor was there any evidence of infection or rejection on the corneal surface. Our immunohistochemical analysis regarding the inflammatory reaction using several markers indicated that TT-FDAM did not induce the significant inflammatory reaction *in vivo*. We think that trehalose is a chemically inactive and non-permeating agent, which explains why the residual trehalose in TT-FDAM was minimal, affected the surrounding tissues less, and immediately disappeared after transplantation. To further confirm these results, we also examined the biocompatibility by scleral-surface transplantation, and found that TT-FDAM-transplanted eyes showed an almost identical course to FDAM-sutured eyes. Even though our biocompatibility assay was only a 1-month examination, further long-term follow-up studies are needed to confirm their exact biocompatibility.

When TT-FDAM is used for clinical applications, what is of key importance is that it has little influence on the surrounding tissues, especially on migrating conjunctival epithelial cells. We examined the expression of CK1, CK4, CK10, and CK13 in the transplanted TT-FDAM covered with conjunctival epithelium. The epithelium covering expressed the mucosal-specific K4 and K13, and did not express keratinized-marker K1 or K10, indicating that TT-FDAM does not affect the differentiation of the migrating conjunctival epithelium. These results are consistent with the previous report regarding AM and FDAM [9]. From these results, we are confident that the TT-FDAM we produced shows reasonable biocompatibility with ocular surface tissues.

Recently, AM has been widely used as a substrate for cultivating corneal, conjunctival, and oral mucosal epithelium [17,18]. Therefore, we first examined if TT-FDAM is a suitable substrate for culturing epithelial cells. Adherent cells assay indicated that the cell adhesion property of TT-FDAM was better than that of FDAM, and almost identical to that of AM. Moreover, the cultivated corneal epithelial cells on TT-FDAM showed 4–5 layers of stratification, were well differentiated, and demonstrated immunoreactivity for cornea-specific keratin 3, but not keratins 10 and 13. These findings indicated that TT-FDAM supported normal differentiation of the cells and consequently is an ideal substrate for culturing epithelial cells.

Thus, trehalose has a variety of beneficial effects, and therefore it has been clinically applied in other biological fields for human application. For example, Chen et al. reported that trehalose-containing organ preservation solution is useful for clinical lung transplantation [19]. Moreover, Matsuo et al. reported that trehalose solution was an effective and safe eye drop for the treatment of moderate to severe dry-eye syndrome in their group of patients [20]. Even though trehalose is a natural sugar and is a very safe material, it should be used carefully because sugar may cause the growth of bacteria and mold, etc. In addition, because of the current technical difficulties, we could not fully examine the longevity of trehalose in TT-FDAM. Further investigation into this subject using special procedures and instruments (e.g. special gas chromatography, Fourier-transform infrared analysis, etc.) is needed to clarify these points.

5. Conclusions

In conclusion, our study is the first to demonstrate the usefulness of TT-FDAM for ocular surface reconstruction on the basis of several experiments evaluating physical, morphological, and biological properties. We have shown that compared to FDAM, the TT-FDAM we produced more greatly retains the characteristics of native AM. On the basis of these results, we are in the process of using this biomaterial for ocular surface reconstruction in patients with severe ocular surface diseases and are carefully evaluating the long-term clinical usefulness of TT-FDAM.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2008.05.023.

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Sutureless amniotic membrane transplantation for ocular surface reconstruction with a chemically defined bioadhesive

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ABSTRACT

The purpose of this study was to evaluate the efficiency and safety of a sutureless amniotic membrane transplantation (AMT) for ocular surface reconstruction with a chemically defined bioadhesive (CDB). The CDB was synthesized from aldehyded polysaccharides and ε-poly(L-lysine), two kinds of medical and food additives, as starting materials. Biocompatibility assay indicated that the CDB showed excellent biocompatibility with *in vitro* and *in vivo* ocular surface tissues and most of the CDB was histologically degraded within 4 weeks. Sutureless AMT using the CDB was safely and successfully performed onto a rabbit scleral surface. Transplanted amniotic membrane (AM) evaluated by histological, electron microscopic- and immunohistochemical examination indicated that the CDB did not affect normal differentiation of the cells or the integrity of the surrounding tissue. Thus, this newly developed CDB was found to be very useful for sutureless AMT for ocular surface reconstruction, without considering the risk of infection. It has the ability to fix AM to the ocular surface for a long time-period without additional inflammation, scarring, or damage to the surrounding tissues.

1. Introduction

Human amniotic membrane (AM) has been shown to possess various kinds of biological effects such as anti-inflammatory [1,2], antifibroblastic [3], anti-microbial [4], and anti-angiogenic properties [5], and promote epithelialization by facilitating the migration of epithelial cells [6], reinforcing adhesion of basal epithelial cells, and promoting epithelial differentiation [7]. It also produces growth factors that promote epithelial cell growth [8]. Due to these desirable properties, AM has been used as surgical material in a wide variety of operations. Since Kim and Tseng achieved successful corneal re-epithelialization in chemical burns by amniotic membrane transplantation (AMT) in 1995 [9], AM has been widely used in eyes with ocular surface diseases such as persistent corneal epithelial defects [10,11], pterygium [12], symblepharon [13,14], and stem cell deficiency [9,13,15–18].

AM is usually sutured onto the ocular surface using 10-0 nylon sutures to fix [19]. Although the suturing method makes for a secure attachment, it inflicts trauma to the ocular surface. Moreover, a prolonged operative time and technical skill are needed for effective suture placement. Sutures can not only cause postoperative pain and discomfort which is a significant problem for patients [20], but can also be associated with suture-related complications such as suture abscesses [21,22], granuloma formation [23], and tissue necrosis [24]. To solve these problems, sutureless techniques have been applied for various kinds of operations on the ocular surface including conjunctival closure after surgery [25,26], pterygium surgery [20,27,28], corneal stem cell transplantation [29,30], lamellar keratoplasty [31], and conjunctivochalasis [32].

Recently, we developed a sutureless AMT using bioadhesive-coated freeze-dried AM for ocular surface reconstruction [33]. This AM is based on our previous report regarding sterilized, freeze-dried AM [34]. Being coated by a minimum dose of fibrin glue, bioadhesive-coated freeze-dried AM can be readily used after opening the package. It can be kept sterilized at room temperature (RT) for long periods without deterioration so that it can easily be stored, transported, and used. This 'ready-to-use' AM has enabled AMT to be easily performed in every hospital in the world.

Although these newly developed sutureless methods using fibrin glue achieved successful outcomes, safety and logistical problems still remain. Some viruses, such as parvovirus B19 (HPV

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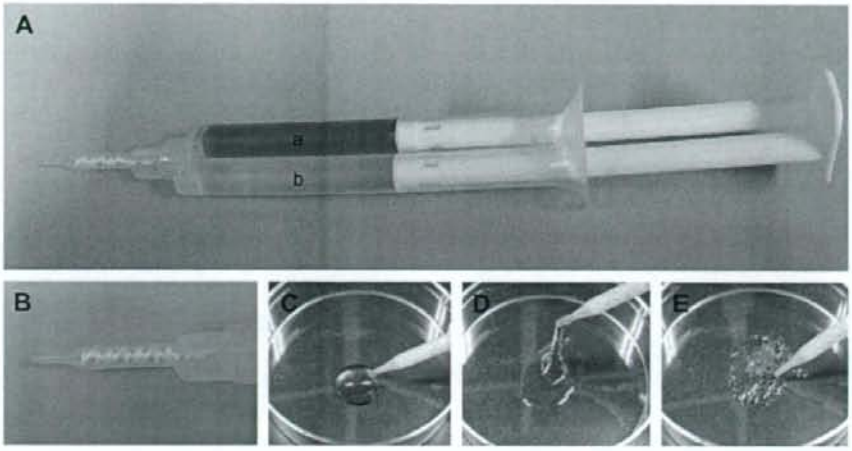

Fig. 1. (A) This glue prepared with syringe-like container with two cylinders (a, b); one cylinder (a) is filled with 2 ml of 14 w/w% aldehyded dextran solution with aldehyde introduced ratio of 0.43 per sugar unit, and the other (b) with 2 ml of 7 w/w% ε-poly(L-lysine) solution containing 2.1% w/w% acetic anhydride. The container has special mixing tip which can mix two solutions each other by equal volume as passing through it (B). The mixed adhesive is gradually gelatinized (C–E). The tip is disposable because the adhesive had been gelatinized in the tip before next application.

B19) are particularly difficult to totally remove or inactivate, and human infection has been reported after the use of fibrin glue that had been prepared in house from pooled plasma [35]. Moreover, in thoracic surgery, epidemiological evidence suggests that more than 20% of uninfected people were subsequently infected with HPV B19 by use of commercially available fibrin sealant during the procedure [36]. In addition, there is a potential for the transmission of prions originating even from commercially available human plasma [37]. Although the risks of both diseases are extremely low, patients should be informed before surgery. Some other tissue adhesives using collagen or gelatin of animal sources have been previously developed and experimentally evaluated, but like with fibrin glue, the risks of infection have still remained. If non-biologic and chemically defined bioadhesives can be successfully applied, it would prove ideal for safe and simple ocular surface reconstruction.

In this report, we present our newly developed CDB for sutureless AM transplantation. It was made from antibiotic food additives and characterized by its self-degradability, low toxicity, and stronger bonding property. To the best of our knowledge, there have been no reports investigating ocular surface reconstruction using safe and effective CDB.

2. Materials and methods

2.1. Preparation of CDB

The mechanism of CDB gelation is based on Schiff base formation between oxidized and aldehyded polysaccharides and ε-poly(L-lysine), two kinds of antibacterial additives for medicine or food [38]. CDB is prepared with a syringe-like container with two cylinders (Fig. 1A). One cylinder is filled with 2 ml of 14% w/w aldehyded dextran solution (molecular weight: 75K Da) with aldehyde introduced ratio of 0.43 per sugar unit, and the other with 2 ml of 7% w/w ε-poly(L-lysine) solution (molecular weight: 4K Da) containing 2.1% w/w acetic anhydride. CDB was used after filtration sterilization of both solutions using a syringe filter with 0.2 μm pore size. When the end of the syringe is pushed, the two solutions are mixed together in equal volumes as they pass through the tip (Fig. 1B) and then gradually gelatinize (Fig. 1C–E). Gel formation time, which can be altered by aldehyde introduction in dextran, was approximately 31 seconds at the temperature of 37 °C, counted by the same method as in the previous report [38]. Degradation speed can also be altered by acetic anhydride concentration in ε-poly(L-lysine). The CDB in this report was self-degradable within 4 days at the temperature of 37 °C *in vitro*.

2.2. Preparation of AM

AMs were prepared according to our previously reported method [39]. Briefly, after obtaining proper informed consent in accordance with the tenets of the Declaration of Helsinki, and with approval by the Institutional Review Board of Kyoto Prefectural University of Medicine, human AM was obtained under a sterile condition from seronegative donors after an elective caesarean section. The AM was washed with sterile phosphate buffered saline (PBS) (Nissui, Tokyo, Japan) containing antibiotics and antimycotics. The chorion was peeled off manually and epithelial cells were removed by incubation with 0.02% ethylene diamine tetra-acetic acid (EDTA) (Nacalai, Kyoto, Japan) at 37 °C for 2 h. Denuded AM was cut into approximately 4 × 4 cm pieces, and cryopreserved at −80 °C in a sterile vial containing Dulbecco's modified Eagle medium and glycerol in a volume ratio of 1:1. Before use, the AM was thawed by warming the vial to RT.

2.3. Examination of biocompatibility of CDB

Before using the CDB in this assay, we have already investigated each cytotoxicity test of aldehyded dextran and poly(L-lysine) using mouse established cell line L929 [38]. IC50 (sample concentration in culture medium which suppresses the cell viability down to 50%) of them was 6000 and >10,000 μg/ml, respectively. These values are 1000 times as much as those of formaldehyde and glutaraldehyde (1.7 and 3.9 μg/ml, respectively), which suggests quite the low toxicity of both components. Because the difficulty in cytotoxic evaluation of hydrogel materials *in vitro*, *in vivo* biocompatibility test of gelation sample was carried out. All experiments in this study were performed in accordance with the Committee for Animal Research at Kyoto Prefectural University of Medicine and according to the ARVO statement on the Use of Animals in Ophthalmic and Vision Research. To investigate the biocompatibility of CDB with the ocular surface, it was injected into the subconjunctival

Table 1
Antibodies and their sources

Specificity	Immunized	Dilution	Sources	Cat. number
Cytokeratin 1	Mouse, mAb	20×	Novocastra	NCL-CK1
Cytokeratin 4	Mouse, mAb	200×	Novocastra	NCL-CK4
Cytokeratin 10	Mouse, mAb	50×	Novocastra	NCL-CK10
Cytokeratin 13	Mouse, mAb	200×	Novocastra	NCL-CK13
MUC5AC	Mouse, mAb	200×	Zymed	18-2261

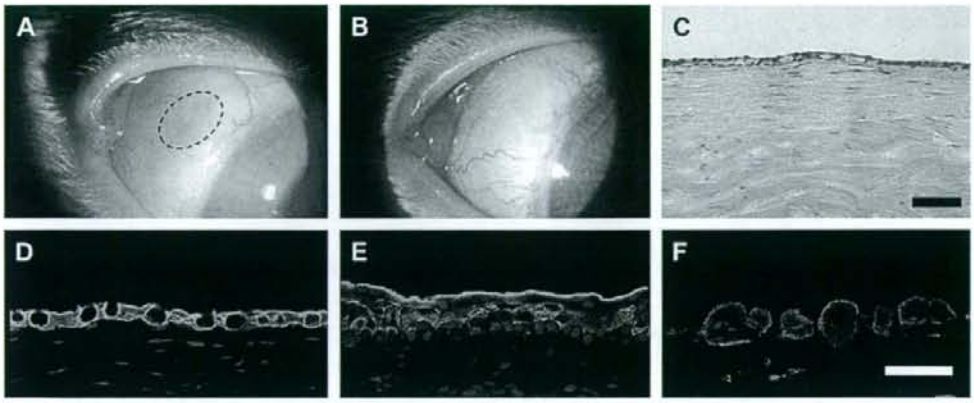

Fig. 2. Representative finding of slit lamp examination immediately (A) and 4 weeks (B) after the subconjunctival injection of the bioadhesive. Injected area could be identified by blue area translucent through conjunctiva (dashed circle). H&E staining (C) and immunohistochemical staining of CK4 (D) and 13 (E), and MUC5AC (F) in injected area revealed that they were similar to normal tissue. Nuclei were stained with propidium iodide (*red*). Scale bars: 50 μm.

space of a rabbit eye. A 27-gauge needle was attached at the tip of the syringe and a small amount of CDB (approximately 0.1 ml) was subconjunctively injected. Four weeks after injection, tissues were removed and cryostat sections were stained with hematoxylin and eosin.

2.4. Sutureless transplantation

To investigate the adherence and biocompatibility of the CDB to the ocular surface, AM was transplanted onto the rabbit sclera using CDB. AMT with sutures and simple recession of the sclera without AMT were also performed as a control.

First, the rabbit conjunctiva (15 × 10 mm) was removed with surgical scissors and the severed edge of the conjunctiva was secured onto the sclera with 9-0 silk so that the sufficient size of sclera was exposed. For the sutureless AMT, a drop of CDB was put onto the bare sclera from the tip of the syringe, and then the squarely trimmed AM was transferred into place with the epithelial basement membrane side up. Excess fluid that extruded from the interface was rubbed off with a sponge and approximately 3 min elapsed before the AM was fixed. For the conventional AMT, the AM was secured at the corner of the membrane onto the bare sclera using 10-0 nylon sutures. The operated eye was patched with a topical antibiotic ointment (0.3% ofloxacin), and a topical steroid/antibiotic ointment was applied once daily for a week. Operations were performed on three rabbits for each group.

At 2, 4, 8, and 12 weeks after transplantation, the grade of epithelialization and hyperemia in the surgical area was examined by slit lamp microscopy. The rabbit was euthanized by the phleboclysis of 1 ml pentobarbital sodium, and the tissue was embedded in OCT compound (Tissue-Tek®; Miles Inc., Elkhart, IN, USA) and frozen with liquid nitrogen. Cryostat sections were stained with hematoxylin and eosin. The interface between the sclera and AM was also checked by electron microscopy 1 day after transplantation. To investigate the biological effect of CDB on cells and surrounding tissues, collected tissues were

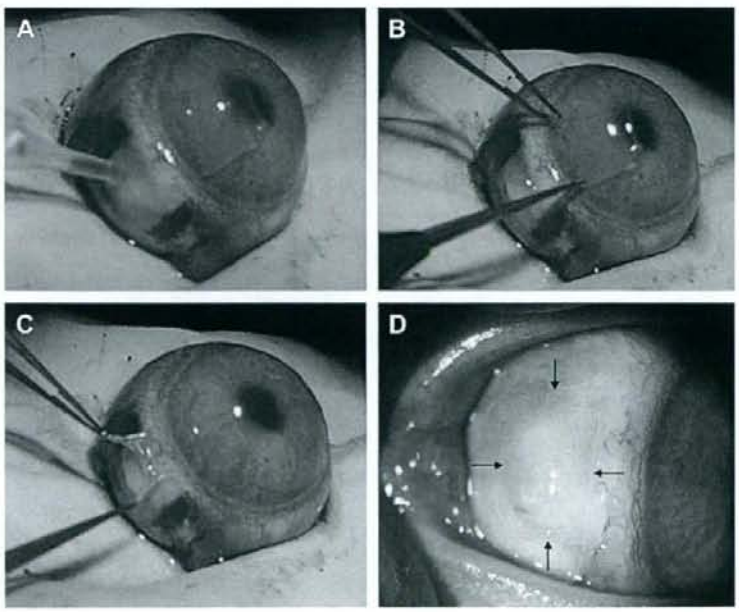

Fig. 3. Representative photos of sutureless AMT. A drop of the bioadhesive was put onto the bare sclera from the tip of the syringe (A), and then the squarely trimmed AM was transferred into the place with the epithelial basement membrane side up (B and C). Excess fluid that extruded from the interface was rubbed off with a sponge and approximately 3 min elapsed before the AM was fixed. Immediately after sutureless AMT, AM was firmly secured on the bare sclera (D).

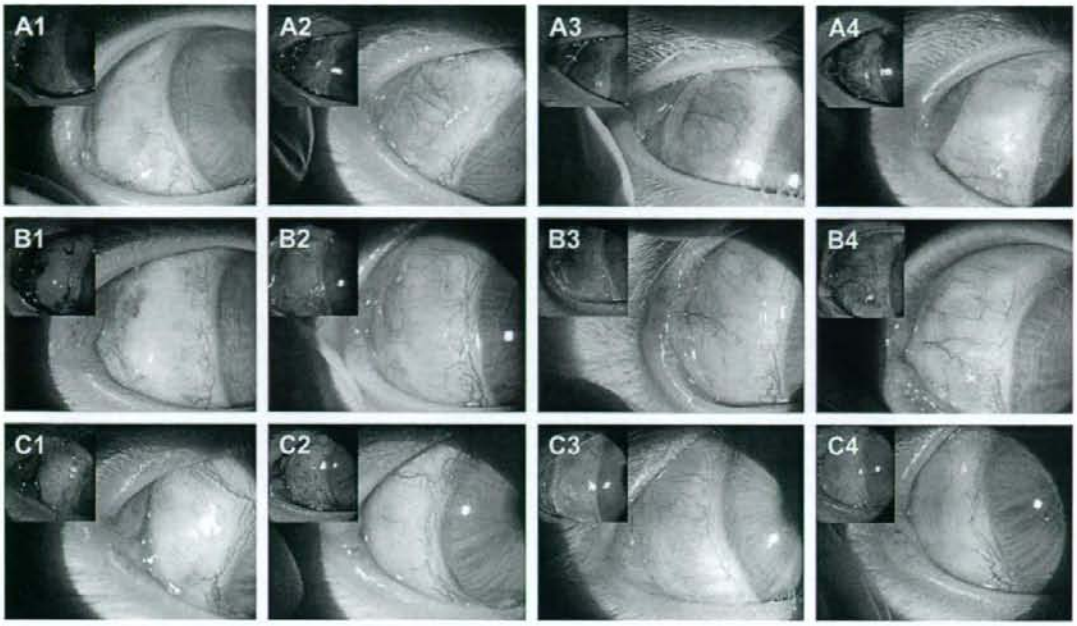

Fig. 4. Representative slit-lamp photographs of rabbits taken 2 (A1–C1), 4 (A2–C2), 8 (A3–C3), and 12 (A4–C4) weeks after sutureless transplantation with bioadhesive (A1–A4), conventional AMT (B1–B4), and no graft (C1–C4). Though the bare sclera without AM transplantation failed to epithelialize within 2 weeks (C1), the surface of AM (A1 and B1) was completely covered with epithelium at 2 weeks after transplantation. The covered epithelium on AM was stable and there was no significant difference between sutureless AMT and conventional AMT eyes in regards to hyperemia (A and B).

histologically, ultrastructurally, and immunohistochemically examined on the 12-week samples.

2.5. Electron microscopic examination

2.5.1. Transmission electron microscopy

Samples were fixed in 2.5% glutaraldehyde in PBS buffer prior to processing for transmission electron microscopy. They were dissected into small pieces and washed three times in PBS for 15 min. Samples were post-fixed in 2% osmium tetroxide for 2 h and washed again in PBS before being passed through a graded ethanol series. Specimens were then transferred to propylene oxide twice for 20 min each time. They were placed in a solution containing 50% propylene oxide and 50% Araldite resin (Agar Scientific, Stansted, Essex, UK) overnight after which they were transferred to 100% resin and infiltrated overnight under agitation. The samples were embedded in moulds containing fresh resin and polymerized at 60 °C for 24–36 h. Ultra-thin sections (50–70 nm thick) were cut on a microtome (Ultracut E; Reichert, Depew, NY, USA), collected on naked copper grids and counterstained prior to examination on a transmission electron microscope (JEM 1010; JEOL Ltd., Tokyo, Japan).

2.5.2. Scanning electron microscopy

Samples were fixed in 2.5% glutaraldehyde in PBS buffer prior to processing for transmission electron microscopy. They were dissected into small pieces and washed three times in PBS for 15 min. Samples were post-fixed in 2% osmium tetroxide for 2 h and washed again in PBS before being passed through a graded ethanol series. Samples were then transferred to hexamethyldisilazane (HMDS) for 20 min then air-dried. The samples were then mounted on aluminum stubs and sputter coated with gold using an S150A sputter coater (Edwards, Crawley, West Sussex, UK). Samples were examined on a scanning electron microscope (JSM 5600; JEOL Ltd., Tokyo, Japan).

2.6. Immunohistochemistry

Immunohistochemical studies were performed on three groups of the samples at 12 weeks after operation using our previously described method [40]. Briefly, semi-thin (8 μm) cryostat sections were obtained from unfixed tissue embedded in Tissue-Tek® OCT compound. After fixing with cold acetone for 10 min, the sections were incubated with 1% bovine serum albumin for 30 min. Subsequently, the sections were incubated at RT for 1 h with the primary antibody (Table 1) and washed

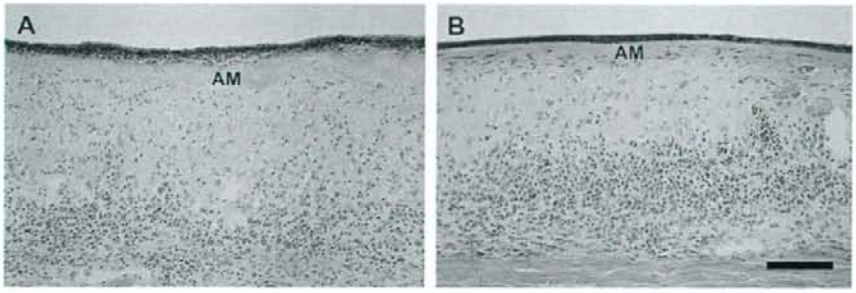

Fig. 5. Representative H&E staining for cryosections of the sutureless (A) and sutured (B) AMT models at 12 weeks after surgery. The transplanted membranes adapted well to the host scleral surface and migrating conjunctival epithelium on AM were well stratified and differentiated. Inflammatory cells were accumulated beneath both AMs, however, there were no significant differences between suture and non-suture models regarding the inflammatory reactions. Scale bar: 50 μm. AM: amniotic membrane.

three times in PBS containing 0.15% Triton X-100 (PBST) for 10 min. In the negative controls we replaced the primary antibody with the appropriate non-immune IgG. The sections were then incubated at RT for 1 h with appropriate secondary antibodies; Alexa Fluor 488 conjugated anti-mouse IgG antibody (Molecular Probes Inc., Eugene, OR, USA). After several washings with PBS, the sections were coverslipped using anti-fading mounting medium containing propidium iodide (Vectashield; Vector, Burlingame, CA, USA) and examined by confocal microscopy (TCS-SP2; Leica, Tokyo, Japan).

3. Results

3.1. Biocompatibility of CDB

In vitro cytotoxicity tests of aldehyded dextran and poly(L-lysine) indicated quite a low toxicity of both CDB components. However, due to the difficulty in cytotoxic evaluation of hydrogel materials *in vitro*, an *in vivo* toxicity test of the gelation sample was carried out using the rabbit subconjunctival injection model. Just after the injection, it was possible to identify the CDB beneath the conjunctiva due to its light blue color as seen through the translucent conjunctiva (Fig. 2A). The CDB is characterized by its self-degradability, and is known to gradually turn brown as it degrades. The ocular surface was expected to look brown had the CDB remained in that area, however, we were unable to find any brown discoloration under the conjunctiva at 4 weeks after the injection (Fig. 2B). Histological examination showed no apparent inflammation or scarring (Fig. 2C), and immunohistochemical findings in the conjunctival epithelial cells showed positive expressions of keratins 4 and 13 and mucin type 5AC (MUC5AC) (Fig. 2D–F), which are all expressed in normal conjunctival epithelial cells [41].

3.2. Sutureless transplantation

Cryopreserved AM was successfully transplanted onto the bare rabbit sclera without sutures as shown in Fig. 3A–D. The secured AMs had been fixed without loss or dislocation for 12 weeks. The grade of epithelialization and hyperemia in the surgical area was evaluated by slit lamp microscopy, with and without fluorescein (Fig. 4). Although the bare sclera without AMT was not epithelialized within 2 weeks, the color surface was covered within 2 weeks after sutured and sutureless AMT (Fig. 4A1, B1, C1). In regard to the hyperemia, there was no significant difference between suture models and non-suture models (Fig. 4A and B). The conjunctival fornix in the vicinity of the transplanted area was shortened only in the eyes that received simple recession of the conjunctiva (Fig. 4C), while it was sufficiently deep in the eyes with conventional and sutureless AMT (Fig. 4A and B).

3.3. Histological examination

Histological examinations of the 12-week samples of the suture and non-suture models showed that the transplanted membranes adapted well to the host scleral surface and that the migrating conjunctival epitheliums on the AM of both model types were well stratified and differentiated. It also showed that the inflammatory cells were accumulated beneath the AM in both models, however, there were no significant differences between the suture and non-suture models in regard to inflammatory reactions (Fig. 5).

3.4. Electron microscopic examination

The stromal side of the AM was coated with the CDB by dropping the solution onto the AM and then allowing a sufficient amount of elapsed time for the surface to dry. Scanning electron microscopic examination of the CDB-coated side of the AM showed a smooth surface that was relatively featureless (Fig. 6A). Transmission electron microscopic investigation of the AMT after 1 day showed a firm attachment of the AM to the sclera (Fig. 6B).

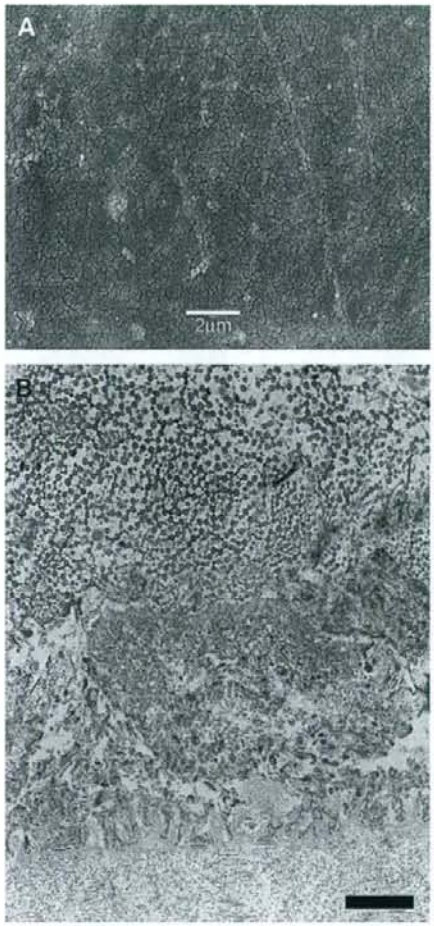

Fig. 6. Scanning electron microscopic investigation revealed that AM was fully covered by the bioadhesives (A). Transmission electron microscopic investigation of the AMT after 1 day showed a firm attachment of the AM to the sclera (B). Scale bars: 2 μm. S: sclera, asterisk: CDB.

3.5. Morphological findings of the conjunctival epithelial cells on AM

Scanning electron microscopic examination of the conjunctival epithelial cells on the AM after sutureless AMT showed a continuous layer of normal looking flat, irregular-shaped polygonal cells. The cells appeared completely normal since they had distinct borders and their surfaces were covered with microvilli (Fig. 7A and B). Examination of the conjunctival epithelial cells by transmission electron microscopy showed normal looking cells forming 6–7 layers, from basal cells to wing and superficial cells, with neighboring cells having numerous desmosomal cell junctions (Fig. 7C and D). These cells were also well attached to the AM through frequent hemidesmosomal contacts (Fig. 7E and F).

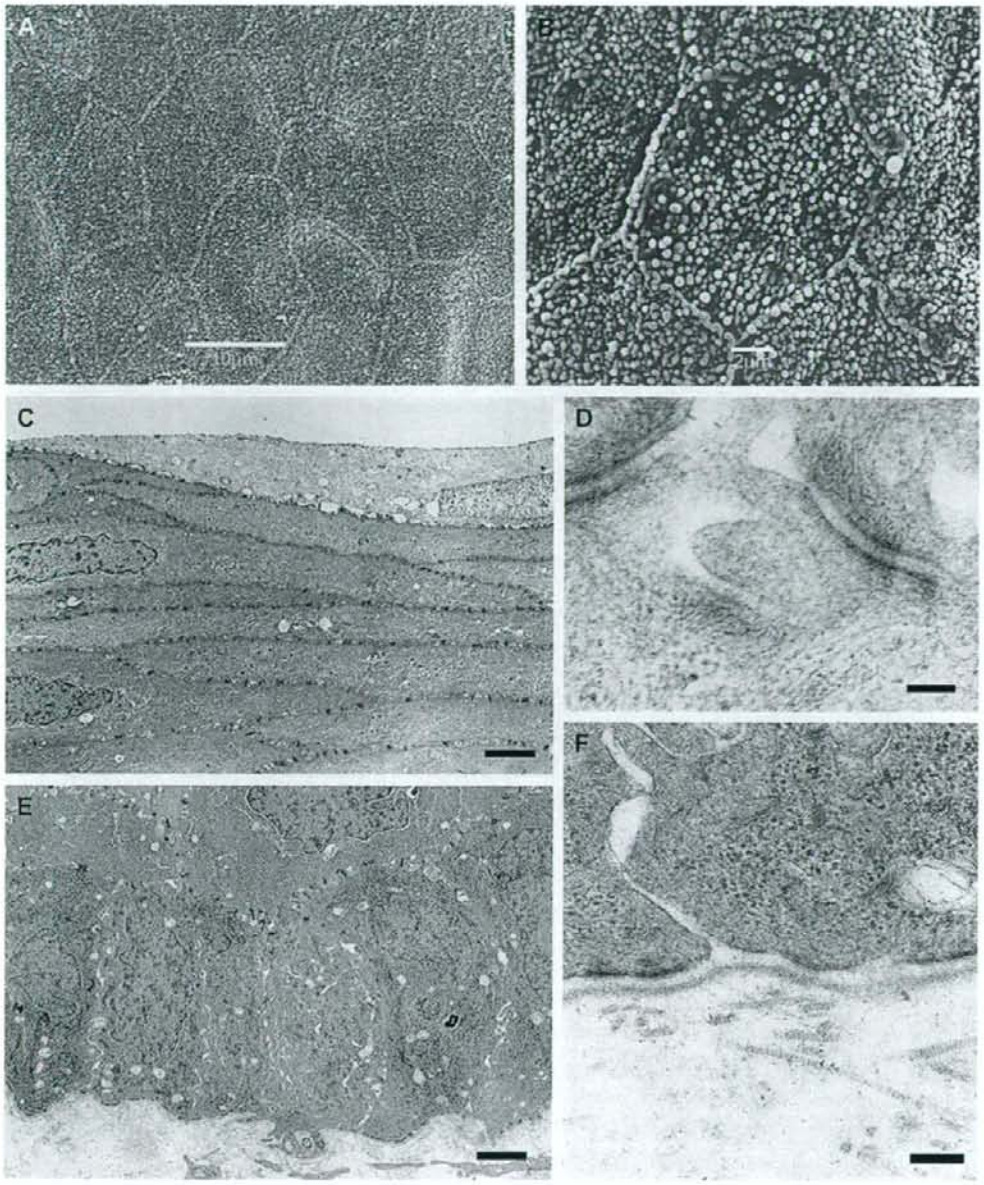

Fig. 7. Scanning electron microscopic examination of the conjunctival epithelial cells on the AM after sutureless AMT showed a continuous layer of normal looking flat irregular-shaped polygonal cells. The cells had distinct borders and their surfaces were covered with microvilli which they appeared completely normal (A and B). Examination of the conjunctival epithelial cells by transmission electron microscopy showed normal looking cells forming 6–7 layers, from basal cells to wing and superficial cells, with neighboring cells having numerous desmosomal cell junctions (C–E). These cells were also well attached to the AM through frequent hemidesmosomal contacts (F). Scale bars: (A) 10 μm, (B–D) 2 μm, (E and F) 100 nm.

3.6. *Characterization of the conjunctival epithelial cells on AM*

The characterization of the epithelial cells on three groups of samples was compared at 12 weeks by immunohistochemistry for cytokeratins (CK) (types 1, 4, 10, and 13), and MUC5AC (Fig. 8). The staining for CK4 and 13, and MUC5AC was detected at the epithelial cell layer on the slides from each group. There was no detectable staining for CK1 and 10. These staining patterns were similar to that of normal rabbit conjunctival epithelial cells [41].

4. Discussion

Many sutureless techniques have been previously developed using fibrin glue for ocular surface reconstruction [20,25–29,31,32],

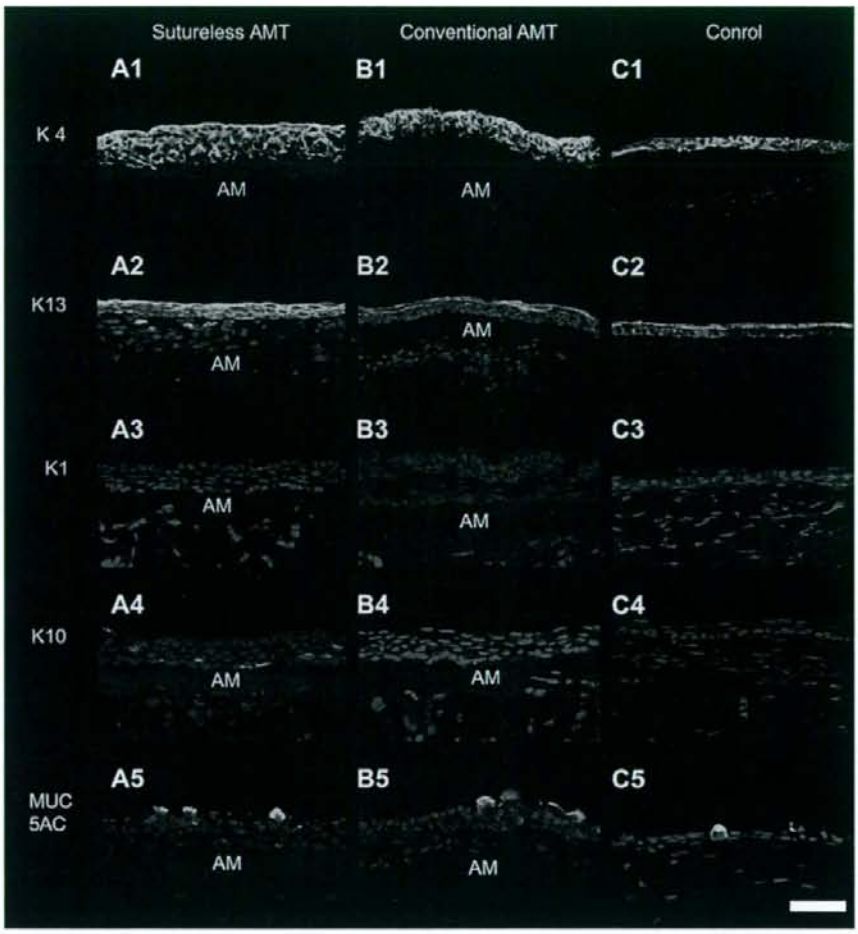

Fig. 8. Representative immunohistochemical staining of CK4, 13, 1, 10 and MUC5AC in sutureless AMT (A1–A5), conventional AMT (B1–B5) and no graft (control) (C1–C5). CK4, 13 and MUC5AC were expressed at the epithelial cell layer of sutureless AMT (A1, A2 and A5) and conventional AMT (B1, B2 and B5). In contrast, CK1 and 10 were not expressed in any of sutureless AMT (A3 and A4) and conventional AMT (B3 and B4). These staining patterns were similar to that of simple recession of conjunctiva (C1–C5). Scale bar: 50 μm.

however, the risk of disease transmission had come into question after the operations using fibrin glue [35–37]. To overcome that risk, we devised a sutureless transplantation using a newly developed bioadhesive that is chemically defined, self-degradable, and biocompatible with the ocular surface. To the best of our knowledge, this is the first attempt to apply a chemically defined adhesive that is strong, safe, biocompatible, and biodegradable for ocular surface reconstruction.

Many tissue adhesives have been improved to overcome the problems that are encountered with sutures. Cyanoacrylates are one of the synthetic adhesives that have traditionally been applied to ocular surfaces since the 1960s for the repair of corneal perforations [42]. They have also been used successfully for cataract wound closure [43], leaking blebs [44], and conjunctival wound closure [45]. However, chronic inflammation and delay of wound healing have occurred because of their toxicity [46], and thereby they are far from safe for the ocular surface. Other disadvantages have also been previously reported related to its inflexibility, inability to be reabsorbed, and lack of transparency [47]. Recently, synthetic and non-biologic adhesives have been developed such as biodendrimer [48], hydrogel adhesives [49], and chondroitin sulfate aldehydes [50], for ocular surface. They have been shown to be effective for sealing corneal incisions, however, in regard to toxicity, biocompatibility, and biodegradability, they have yet to be fully examined.

To perform sutureless AMT successfully, it is essential that the adhesive can be easily handled. The adhesive in this report was easy to handle for sutureless AMT without unexpected gelatinization or a complicated technique. Immediately after exiting the syringe, our newly devised, liquid-form CDB was properly spread on the ocular surface, gradually gelatinized, and then finally fixed the AM firmly onto the ocular surface for a long time. The CDB had a smooth rubbery texture when gelled and showed sufficient flexibility even after becoming totally gelled. Moreover, and adding to the effectiveness of this new adhesive, the gel formation time can be altered so that operators can match an appropriate gel formation time to the required handling time.

Previously synthesized adhesives have been associated with problems such as chronic inflammation and delayed wound healing due to their residual toxicity and degradation properties. Our

new CDB has already shown to be of low cytotoxicity *in vitro*, and moreover, it degraded into the surrounding tissue within 4 weeks, with no additional inflammation and no influence on the character of keratin and MUC5AC expression in nearby conjunctiva. These findings indicate that the CDB is sufficiently biocompatible and biodegradable for use on the ocular surface.

We successfully performed the sutureless AMT with this adhesive on rabbit sclera. Histological and electron microscopic examination showed that the AM was firmly attached to the sclera, and slit lamp examination also showed good fixation without loss or dislocation for at least 3 months. For a firm attachment, stable crosslinking between the molecules is needed. The formation of Schiff base strongly depends on the solution pH, with a higher pH enhancing the reaction. However, the solution pH of aldehyded dextran and poly(L-lysine) are around 5 and 9, respectively, and the pH of the mixture is approximately 7 (neutral). High reactivity of poly(L-lysine) might cause the effective gel formation even at the neutral pH (data not shown). Although we did not exactly show whether the crosslinkings with the Schiff base was modified *in vivo* or not, the fixation on the ocular surface lasted at least 3 months. The period of epithelialization and degree of hyperemia in eyes with sutureless AMT were similar to those associated with sutured AM, and both methods revealed prompt epithelialization when compared to non-transplanted eyes. These findings show that our sutureless AMT technique using CDB is equally effective and useful for ocular surface reconstructions.

When bioadhesives are applied for sutureless AMT, what is of key importance is that they are non-toxic and thus have little influence on the surrounding tissues, especially on conjunctival epithelial cells. In this study, the ultrastructure of conjunctival epithelial cells on the transplanted AM showed the normal features of conjunctiva. On the surface, hexagonal cells had regular microvillis and were closely attached to neighboring cells with numerous desmosomal cell junctions, and in the basal layers had frequent hemidesmosomal contacts to the AM. These findings are compatible to the previous report that AM has been shown to promote a predominantly conjunctival non-goblet epithelial phenotype with expression of microvilli, intercellular junction, and increased density of desmosomes and hemidesmosomes [51]. We did not exactly compare the ultrastructural feature to either the sutured model or normal conjunctiva, however, the conjunctiva on the AM was at least similar to normal even in the presence of CDB nearby. Furthermore, the epithelium expressed CK4 and 13, which is specific for conjunctival epithelium, and did not express keratinized marker K1 or 10, thus indicating that the CDB did not affect normal differentiation of the conjunctival epithelial cells. Taken together, these results suggest that the CDB has no, or if any, very little negative influence on surrounding tissues and is sufficiently safe for use on the ocular surface.

This study has shown that our newly devised CDB is safe and useful for sutureless AMT. Based on our results, we expect the CDB to be applied in a variety of sutureless operations on the ocular surface including conjunctival closure after several kinds of operations, cultivated corneal or oral mucosal epithelium transplantation, and lamellar keratoplasty. We are currently in the process of investigating a variety of applications.

5. Conclusions

Our study demonstrates a safe and simple technique for sutureless AMT using a chemically defined bioadhesive which promotes a secure and rapid adhesion onto the sclera *in vivo* without the need for suturing. If this technique is applied for human ocular surface reconstruction, we expect it to achieve safe and satisfactory results while simultaneously reducing postoperative pain and discomfort. Further investigation is still needed for its clinical use.

Acknowledgments

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Rheology of Tear Film Lipid Layer Spread in Normal and Aqueous Tear–Deficient Dry Eyes

Norihiko Yokoi,[1] Hideaki Yamada,[1] Yutaka Mizukusa,[2] Anthony J. Bron,[3] John M. Tiffany,[3] Takahisa Kato,[4] and Shigeru Kinoshita[1]

Purpose. To analyze the relationship between tear volume and tear film lipid layer (TFLL) spread.

Methods. Twenty-nine eyes from 22 subjects, including normal eyes and eyes with aqueous tear-deficient dry eye, were enrolled in this study. In all eyes, the radius of curvature (R: mm) of the central lower tear meniscus was measured with a videomeniscometer, and interference images from the TFLL were recorded with a video-interferometer. Interference images were captured as still images every 0.05 second, and the relationship between the acquisition time for each image after a blink and the averaged heights of the spreading TFLL in the upstroke of the blink were calculated.

Results. In all cases, the time-dependent changes in TFLL spread could be described by the expression $H(t) - H(0) = \rho[1 - \exp(-t/\lambda)]$, where $H(t)$ is the averaged height in millimeters at time t, $H(0)$ is the averaged height at $t = 0$, ρ is a constant, t is time in seconds, and λ is the characteristic time in seconds. A statistically significant correlation was found between those changes and the initial upward velocity of the spreading TFLL [$H'(0) = dH(0)/dt$] and R ($r = 0.573$; $P = 0.003$).

Conclusions. This study demonstrated that the time-dependent changes of TFLL spread are compatible with the Voigt model of viscoelasticity and that the initial velocity of TFLL spread after a blink decreased in proportion to the decrease of tear volume. There is potential interest in using this parameter to diagnose and evaluate the severity of aqueous tear deficiency. (*Invest Ophthalmol Vis Sci.* 2008;49:5319–5324) DOI:10.1167/iovs.07-1407

The aqueous layer of the tear film is covered by a thin lipid layer[1] that can be imaged noninvasively by interferometry.[2–4] We have reported elsewhere that graded interference patterns can be used as a parameter to screen dry eye and evaluate its severity.[5] Other studies have shown that after a blink, the tear film lipid layer (TFLL) spreads over the aqueous layer in a reproducible manner[2,6–9] and that its dynamics can be observed using an interferometer. Such studies[10,11] have noted that the time taken for the interference pattern to stabilize after a blink (defined as spreading time) is longer in aqueous-deficient dry eyes than in aqueous-sufficient normal eyes. Goto and Tseng[11] noted that the spreading time was shortened in aqueous tear–deficient dry eyes after punctal occlusion and proposed that spreading is affected by aqueous tear volume. This semiquantitative method offers a novel direct approach to the study of lipid-layer kinetics. A related report has analyzed the kinetic behavior of particles embedded in the tear film, probably located at the level of the TFLL.[12,13]

With the use of our interferometer, we found that in the upstroke of the blink, the speed of upward spread of the TFLL slows dramatically to reach a stable position in the normal eye, within approximately 1 second. This has also been observed by other authors.[9,11,12] This effect can be seen more clearly in eyes with aqueous tear deficiency because the speed of spreading is slower.

We hypothesized that this time-dependent behavior might reflect a viscoelastic property of the tear film. With this in mind, we conducted a study of TFLL dynamics. In addition, we were interested in the relationship between the TFLL spread and tear volume.

Subjects and Methods

Subjects

Twenty-nine eyes from 22 subjects (1 eye from 1 man and 28 eyes from 21 women) were enrolled in this study. The age of the subjects ranged from 42 to 87 years (64.6 ± 11.0 years; mean ± SD). According to the diagnostic criteria given below, there were 5 normal eyes of 4 healthy subjects (54.8 ± 4.7 years) and 24 aqueous tear-deficient dry eyes of 18 patients (66.8 ± 10.9 years). Of the 24 aqueous tear-deficient dry eyes, 15 eyes were from 11 patients with Sjögren's-syndrome dry eye (SSDE), and 9 eyes were from 7 patients with non-Sjögren's-syndrome dry eye (NSDE).[14]

Tear tests were performed on all subjects before enrollment. These included the Schirmer I test[15] (abnormal value, ≤5 mm/5 min), the measurement of fluorescein break-up time (BUT)[16] (abnormal value, ≤5 seconds), and the grading of corneal staining with fluorescein[17] and of ocular surface staining with rose Bengal.[18] Abnormal scores for fluorescein were A1D1 and greater; A and D representing area and density, respectively, were graded from 0 to 1 (mild), 2 (moderate), and 3 (severe). An abnormal score for rose Bengal, based on the van Bijsterveld criteria, was ≥3.

The aqueous tear-deficient dry eyes enrolled in the study met the inclusion criteria, among those being at least one abnormal Schirmer I test value and abnormal scores for either fluorescein or rose Bengal staining. All eyes also showed abnormal BUT values. Patients with aqueous tear-deficient dry eye were categorized as SSDE or NSDE, with the diagnosis of SSDE based on the criteria of Fox et al.[19] Healthy eyes in this study met the following criteria: normal Schirmer I test value, normal scores for fluorescein and rose Bengal staining, and normal BUT values. Exclusion criteria were meibomian gland dysfunction, punctal-plug occlusion or surgical punctal occlusion, previous corneal surgery, previous or current corneal disease (excluding aqueous tear-deficient dry eye), and hard or soft contact lens wear by the subject.

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FIGURE 1. Representative sequential images of the spreading tear-film lipid layer, captured every 0.05 second after a blink (case 2).

In all subjects, reflected images from the central lower tear meniscus were first digitally recorded in the interblink period, during natural blinking with a video-meniscometer.[20–25] Immediately after that, interference images from the TFLL were digitally recorded just after a blink with a video-interferometer (DR-1; Kowa, Tokyo, Japan).[5,25]

This research was approved by the Committee for Ethical Issues on Human Research of Kyoto Prefectural University of Medicine (C-240) and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all dry eye patients and healthy subjects after explanation of the nature and possible consequences of participation in the study.

Evaluation of the Radius of the Lower Tear Meniscus Using the Video-Meniscometer

The radius of curvature (R: mm) of the tear meniscus at the central lower eyelid was measured by video-meniscometry.[20–25] With this, a real-time reflected image of a target consisting of a pair of horizontal, black-and-white stripes was captured digitally, and R was calculated using the concave mirror formula.[20–22] In our video-meniscometry, the R of the lower tear meniscus is measured as the average of three consecutive measurements (Sugita J, et al. *IOVS* 2002;43 ARVO E-Abstract 95). In the model, R is assumed to be constant over the duration of the interblink, as demonstrated by Palakuri et al.[26] for the upper and lower tear meniscus using optical coherence tomography. However, it should be noted that Johnson et al.,[27] using the video slit-lamp technique, reported an increase of R at the end of the interblink period. The tear meniscus is reportedly responsible for 75% to 90% of the total tear volume,[28] and the value of R reportedly reflects the total tear volume over the ocular surface.[25]

Rheologic Analysis of Interference Images from the Precorneal Tear Film Lipid Layer by Use of the Video-Interferometer

The video-interferometer (DR-1; Kowa) provides information about lipid layer thickness and lipid layer spread after a blink[5,10,11,24,25] and is equipped with low- and high-magnification viewing modes that allow observation of 7-mm and 2-mm circular areas in the central cornea, respectively.[24] In this study, the low-magnification mode was the primary mode selected to obtain information regarding the behavior of the precorneal TFLL.

Interference images obtained by the video-interferometer were recorded noninvasively, in real time, using a digital video recorder. Images were sequentially captured into a computer as still images every 0.05 second (Fig. 1) with the use of specially developed software. Regions of the spreading lipid layer after a blink were clipped along the delineated border using graphic software (Photoshop CS, version 8.0.1; Adobe Systems, San Jose, CA; Fig. 2). Given that only

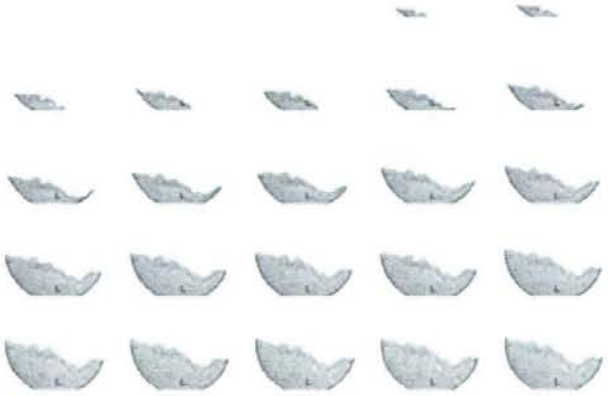

FIGURE 2. Regions of spreading lipid layer clipped along the border.

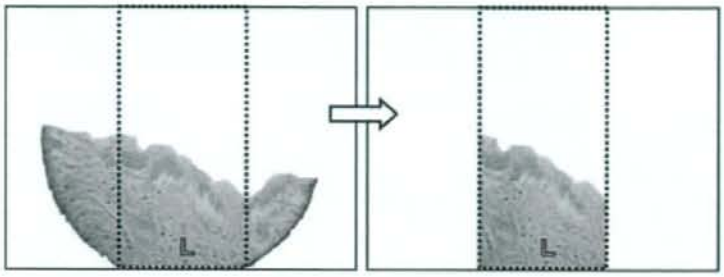

FIGURE 3. The central rectangular area (200 pixels horizontal × 480 pixels vertical) was cropped from the clipped spreading lipid image as the representative region for the whole tear-film lipid layer over the cornea and the averaged height of spreading lipid within the area were calculated.

upward spreading was considered and not the associated widening of the TFLL within the viewing window, only the area of lipid film within a rectangle 200 pixels (horizontal) × 480 pixels (vertical) was considered (Fig. 3). The averaged heights of each spreading lipid sheet within the rectangular areas were calculated using specially developed software that permitted averaging of the length of spreading lipid along the vertical y-direction at each pixel point along the horizontal x-direction within the rectangular area. Images of the spreading TFLLs with ill-delineated upper borders and images with no spreading pattern, possibly because of extremely severe aqueous tear deficiency, were excluded. After calculating the averaged heights of the spreading lipid, the relationship between the acquisition time for the images after a blink and the calculated averaged heights of the spreading lipid were plotted. Curve-fitting to the plots satisfied an exponential equation in keeping with the simple rheological model of Voigt[29] describing the behavior of a viscoelastic material (see Discussion).

In all 29 eyes studied, the relationship between the radius of the central lower tear meniscus (R: mm), which was proportional to the total tear volume over the ocular surface,[23] and the initial upward velocity of the lipid layer spread were analyzed. The Spearman correlation coefficient by rank test was performed, and $P \leq 0.05$ was considered statistically significant.

RESULTS

In all eyes, the time-dependent changes in H of lipid layer spread were found to conform to an exponential model of the form $H(t) - H(0) = \rho[1 - \exp(-t/\lambda)]$, where $H(t)$ is the averaged heights in millimeters at time t, $H(0)$ is the averaged heights at time $t = 0$, ρ is a constant, t is time in seconds, and λ is characteristic time in seconds.

One representative example (case 2, Table 1) of spreading behavior is shown in Figure 1. Curve-fitting to the representative three examples—including cases 1, 2, and 3 in Table 1—is shown in Figure 4. The constants of the exponential equations and corresponding R values calculated from the video-meniscometer-detected images (Fig. 5) from the central lower tear meniscus in three representative cases are given in Table 1.

The relationship between R and $H'(0)$ is shown in Figure 6. There was a statistically significant correlation between R and $H'(0)$ ($r = 0.573$; $P = 0.003$; Spearman correlation coefficient by rank test). The relationship between R and the other parameters, including $H(0)$, ρ, and λ, was also analyzed. As a result, a similar and statistically significant correlation was found between R and ρ ($r = 0.573$; $P = 0.002$), and a weak, statistically insignificant correlation was found between R and $H(0)$ ($r = 0.318$; $P = 0.095$). However, there was no statistically significant correlation between R and λ ($r = -0.241$; $P = 0.194$).

TABLE 1. Constants of the Exponential Equations and the Corresponding R

Case	$H(0)$ (mm)	ρ (mm)	λ (s)	R (mm)	$H'(0)$ (mm/s)
1	0.08	1.54	0.68	0.09	2.25
2	0.02	4.00	0.79	0.14	5.04
3	2.96	2.85	0.14	0.45	20.52

DISCUSSION

In this study, images of the spreading TFLL were obtained noninvasively[5,10,11,25] at intervals of 0.05 second, allowing the dynamics of the TFLL spread to be analyzed quantitatively. The time dependence of the averaged height of the spreading TFLL, as determined from the captured images, was successfully fitted to an exponential formula of the form $H(t) - H(0) = \rho[1 - \exp(-t/\lambda)]$, where $H(t)$ is the averaged height in millimeters at time t, $H(0)$ is the averaged height at time $t = 0$, ρ is a constant, t is time in seconds, and λ is characteristic time in seconds. For the averaged height, H is first measured when the TFLL first appears in the viewing window. True $H(0)$ is the point in time at which the TFLL front first crosses the lower boundary of the viewing window, so that $H(0)$ as recorded is not zero and has a positive value. The size of this depends on the rate of movement of the TFLL and is higher when the rate of TFLL movement is high rather than when it is low (Table 1).

One of the simplest models of linear viscoelasticity is the Kelvin-Voigt (commonly referred to as the Voigt) model.[29] The applied stress σ can be related to the shear γ through the sum of elastic and viscous forces such that

$$\sigma = \kappa\gamma + \eta d\gamma/dt \qquad (1)$$

where κ is the coefficient of elasticity and η is the coefficient of viscosity. The elastic response can be represented by a

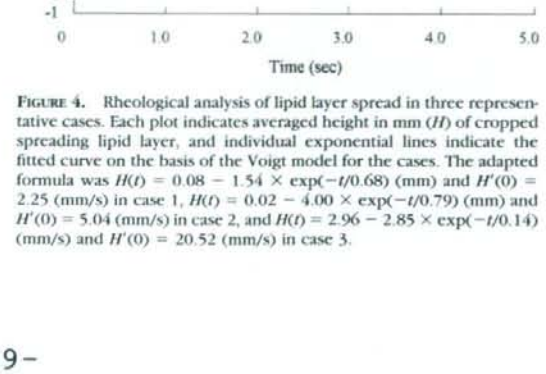

FIGURE 4. Rheological analysis of lipid layer spread in three representative cases. Each plot indicates averaged height in mm (H) of cropped spreading lipid layer, and individual exponential lines indicate the fitted curve on the basis of the Voigt model for the cases. The adapted formula was $H(t) = 0.08 - 1.54 \times \exp(-t/0.68)$ (mm) and $H'(0) = 2.25$ (mm/s) in case 1, $H(t) = 0.02 - 4.00 \times \exp(-t/0.79)$ (mm) and $H'(0) = 5.04$ (mm/s) in case 2, and $H(t) = 2.96 - 2.85 \times \exp(-t/0.14)$ (mm/s) and $H'(0) = 20.52$ (mm/s) in case 3.

FIGURE 5. Video-meniscometer–detected images from the central lower tear meniscus in three representative cases. R = 0.09 (mm) in case 1, R = 0.14 (mm) in case 2, and R = 0.45 (mm) in case 3.

spring and the viscous flow by a dashpot (i.e., a viscous damping system; Fig. 7). If a stress σ_1 is suddenly applied at time t = 0 and held constant thereafter, the linear differential equation can be solved to give

$$\gamma = (\sigma_1/\kappa)[1 - \exp(-t/\tau)] \quad (2)$$

where $\tau = \eta/\kappa$. That is, the shear does not rise instantaneously to a fixed value (as would occur with a purely elastic body) but rises asymptotically to this value. We can suppose that, in the case of the TFLL, the applied stress σ_1 is related to the surface tension forces pulling the lipid layer upward and that the resultant shear (or observed linear displacement) of the lipid front $H(t)$ corresponds to the shear parameter γ in the Voigt model. Hence, the resemblance between the exponential terms of the two expressions suggests a possible viscoelastic property of the tear film that will influence its spread. In relation to the above, Berger and Corrsin[12] analyzed the movement of particles in the tear film after a blink, taking them to be components of the TFLL. On this basis they concluded that immediately after the blink, the TFLL was displaced, like a spring being stretched, and that the spring (the TFLL) was then allowed to return to its equilibrium position. This approach gives rise to an exponential equation similar to the Voigt model but may better reflect what happens. In addition, it seems that there is no lipid layer above the boundary seen in Figure 1, but inspection of Figure 1 indicates that a thin lipid layer is in fact present. To explain this, it has been proposed that TFLL spread consists of two events: first, that in the upstroke of the blink, a polar (largely phospholipid) layer spreads over the aqueous subphase; and second, that the nonpolar lipids (chiefly cholesterol and sterol esters, the greater part of the meibomian secretion) spread over the phospholipid layer.[30,31] Therefore, it is reasonable to conclude that a finite surface tension gradient causes the upward velocity of the TFLL rather than a discontinuity of surface tension at the border, which would theoretically cause infinite velocity at that point.

Owens and Phillips[12] also reported that the spreading velocity of particles on the tear film is adequately described by a logarithmic function. Because the particles they observed appeared to be located in the lipid film, their report may be taken to reflect the velocity of the spreading TFLL.

Curve fitting to the data, shown in Figure 4, indicates that for each of the cases studied, the relationship is of the form $H(t) - H(0) = \rho[1 - \exp(-t/\lambda)]$. Differentiation of this with respect to time gives $H'(t) = \rho/\lambda \times \exp(-t/\lambda)$. The velocity of particles observed by Owens and Phillips[12] can also be described in exponential form as (velocity) = $k_1 \times \exp(-k_2 t)$, where k_1 and k_2 are constants. It can then readily be seen that this leads to a positional formula (position) = const.$[1 - \exp(-k_2 t)]$ similar to ours and that the particle motion follows the same form as our TFLL spread. This strengthens the suggestion of Owens and Phillips[12] that the particles are embedded in and move with the lipid layer, though the sizes of these particles suggest that they may also project into the aqueous layer. Although this might help to explain our observed connection between TFLL spreading rate and tear volume, we did not notice such particles in the image samples of the TFLL images we studied.

Statistical analysis of the relationship between R and the other parameters, including $H(0)$, ρ, and λ, showed a strong and statistically significant relationship between R and ρ. However, there was no statistically significant correlation between R and λ, yet there was a weak, though statistically insignificant, correlation in the relationship between R and $H(0)$. It can theoretically be expected that the time constant, λ, correlates with the meniscus radius, R, and that one might expect equilibrium to occur more quickly when the tear film is thicker (greater meniscus radius), yet no correlation was found in the cases we studied. However, the correlation with statistical significance similar to that between R and $H'(0)$ was found between R and ρ. This is reasonable, taking the Voigt model (equation 2) into consideration, because λ in our empiric equation corresponds to τ (i.e., η/κ of equation 2, where both η and κ are material, intrinsic properties of the TFLL and are unrelated to R). Moreover, considering $H'(0) = \rho/\lambda$ and the significant correlation between $H'(0)$ and R obtained from our result, a significant correlation between R and ρ is also reasonably expected, where ρ is concerned with the equilibrium value for lipid spread and implies how high the lipid layer can spread along the y-direction; a lower height for lipid layer spread can be expected when the tear film is thinner (lower meniscus radius) because a thicker aqueous layer would be expected to allow the lipid layer to be carried higher.

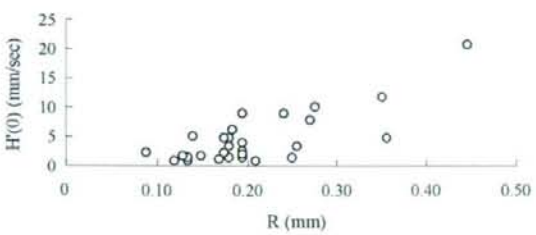

FIGURE 6. Relationship between the radius of the central lower tear meniscus (R) and the initial upward velocity of spreading TFLL, $H'(0)$, where $H'(0) = dH(0)/dt$. There was a statistically significant correlation between R and $H'(0)$ (r = 0.573; P = 0.003).

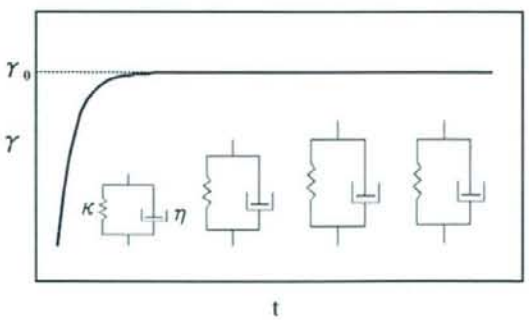

FIGURE 7. The Voigt model in rheology ($\gamma = \gamma_0[1 - \exp(-t/\lambda)]$, where γ = extent of stretch, $\gamma_0 = \gamma$ at $t = \infty$, t = time (s), λ = retardation time (s) = η/κ, η = viscosity, κ = elasticity), consisting of a spring (κ = elasticity) and a dashpot (η = viscosity) in juxtaposition.

The findings of this study show that the Voigt model, a simple mechanistic rheological model, could successfully be adopted for analysis of the time-dependent TFLL spread in all cases studied. This suggests that the tear film is a viscoelastic material, in accordance with data showing that meibomian oil has shear-dependent viscosity and demonstrates shear thinning, which is characteristic of some viscoelastic fluids (Tiffany JM, unpublished observations, 2000).

A study by Goto and Tseng[11] of the speed of lipid layer spreading indicated that the time required for the lipid layer to stabilize after a blink was longer than normal in aqueous tear–deficient dry eyes but was shortened after punctal occlusion. This implied a normalization of the speed of spreading by the restoration of tear volume. However, the diagnosis of dry eye in that study was made by a Schirmer-based fluorescein clearance test, and tear volume was not estimated.

In a previous study we were able to show that the meniscus radius is directly related to the total tear volume over the ocular surface.[23] Furthermore, the formula given by Creech et al.[32] suggests that the precorneal aqueous layer thickness is proportional to the meniscus radius. Our present study has shown a positive relationship between meniscus radius and initial velocity of the TFLL spread, which strongly supports the concept that this parameter is influenced by tear volume, particularly tear film thickness. Thus, it is likely that the slowed initial velocity of the TFLL spread in our patients with aqueous-deficient dry eye was related to reduced tear film thickness.

The relationship between the tear film thickness and the initial velocity of the TFLL can be explained based on the model by Berger and Corrsin.[12] Their analysis could be roughly described as a viscoelastic model in which the elastic component is provided by the TFLL, whereas the viscous component is attributed to the whole thickness of the tears, particularly the aqueous layer. They assume that surface tension is inversely related to the concentration of a surfactant (presumably related to lipid layer thickness); when the tear surface is stretched, the surfactant concentration is reduced and the surface tension increases. Thus, the lipid layer behaves like an elastic membrane whose tension increases when it is stretched. Moreover, according to their model, the force-per-unit area on the lipid layer is $dT/dx = \mu \cdot v/b$, where T is the surface tension, x is the vertical position on the cornea, μ is the viscosity of the (aqueous) tear film, v is the upward velocity of the lipid layer, and b is the thickness of the (aqueous) tears. Rearranged, $v = (b/\mu) \cdot dT/dx$, and upward velocity is thus proportional to tear film thickness.

If a low tear meniscus volume implies a low tear film thickness and a low tear film thickness is responsible for slow spreading of the TFLL, either the meniscus radius or the TFLL spreading rate could be used as an index of low aqueous volume and, therefore, of aqueous-deficient dry eye. In addition, because a low tear volume is considered to contribute to dry eye symptoms through a mechanism involving shear stress, which may increase friction during blinking,[33,34] it will be of interest to study the relationship between TFLL spread and dry eye symptoms.

Although our method of analysis provides a useful quantitative approach, some limitations must be overcome in the future. Thus, we can only measure the area of the spreading TFLL if the uppermost border of the layer is well defined; cases with poorly defined borders were excluded from the present study. It has been noted that the interference image profile changes from a series of horizontal wave fronts in healthy subjects to a vertical disposition in patients with meibomian gland disease (MGD).[10] Therefore, it could prove difficult to diagnose aqueous-deficient dry eye by such a method in the presence of MGD. In addition, analysis of the spreading using graphic software was time consuming. New image-processing computer algorithms and analytical techniques are in development and should permit analysis of all interference patterns automatically in the near future.

In conclusion, our study has demonstrated that the time-dependent changes of the TFLL spread are consistent with a simple Voigt rheological model, implying viscoelastic properties of the tear film. Moreover, the initial velocity of the TFLL spread after a blink increases steadily with increase of the radius of the tear meniscus, suggesting that the rheological behavior of the TFLL is influenced by aqueous tear film thickness over the cornea. Therefore, it may be expected that the initial velocity of movement of the spreading TFLL could provide a noninvasive and quantitative parameter for the screening of aqueous tear–deficient dry eye, and the rheological analysis of the TFLL spread could potentially open a new field of research of the TFLL.

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Tight Junction Transmembrane Protein Claudin Subtypes Expression and Distribution in Human Corneal and Conjunctival Epithelium

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