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Development of new therapeutic modalities for corneal endothelial disease focused on the proliferation of corneal endothelial cells using animal models

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ABSTRACT

This review describes our recent attempts to develop new therapeutic modalities for corneal endothelial disease using animal models including non-human primate model in which the proliferative ability of corneal endothelial cells is severely limited, as is the case in humans. First, we describe our attempt to develop new surgical treatments using cultivated corneal endothelial cells for advanced corneal endothelial dysfunction. It includes two different approaches; a "corneal endothelial cell sheet transplantation" with cells grown on a type-I collagen carrier, and a "cell-injection therapy" combined with the application of Rho-kinase (ROCK) inhibitor. Recently, it was reported that the selective ROCK inhibitor, Y-27632, promotes cell adhesion and proliferation and inhibits the apoptosis of primate corneal endothelial cells in culture. When cultivated corneal endothelial cells were injected into the anterior chamber of animal eyes in the presence of ROCK inhibitor, endothelial cell adhesion was promoted and the cells achieved a high cell density and a morphology similar to corneal endothelial cells *in vivo*. We are also trying to develop a novel medical treatment for the early phase of corneal endothelial disease by the use of ROCK inhibitor eye drops. In rabbit and monkey experiments using partial endothelial dysfunction models, corneal endothelial wound healing was accelerated by the topical application of ROCK inhibitor to the ocular surface, and resulted in the regeneration of a corneal endothelial monolayer with a high endothelial cell density. We are now trying to advance the clinical application of these new therapies for patients with corneal endothelial dysfunction.

The corneal endothelium is the innermost layer of the cornea, derived from the neural crest, and plays an essential role in the maintenance of corneal transparency via its barrier and pump functions. Since the human corneal endothelium is essentially non-regenerative *in vivo*, endothelial cell loss due to dystrophy, trauma, or surgical intervention is followed by a compensatory enlargement of the remaining endothelial cells. Thus, there is functional reserve. However, if cell loss is too great the outcome is often irreversible corneal endothelial dysfunction. For many years penetrating keratoplasty was the only realistic choice of surgery for visual loss due to corneal endothelial dysfunction, but it is not a risk-free treatment. To overcome the problems associated with penetrating keratoplasty, new surgical procedures (i.e. the posterior lamellar keratoplasties) which replace the endothelium without host corneal trephination have been developed (Gorovoy, 2006; Melles et al., 2000; Price and Price, 2005; Terry and Ousley, 2001). However, irrespective of the selected keratoplasty procedure, corneal endothelial cell loss can be a long-term problem following corneal transplantation using donor tissue (Price et al., 2011; Terry et al., 2008).

The ultimate goal of our research is to develop new surgical and medical treatments for corneal endothelial disease, which provide a healthy corneal endothelium with high cell density. To achieve this we are currently focusing on the proliferation of corneal endothelial cells. Currently, our efforts are aimed at developing feasible medical treatments for the early stage of corneal endothelial dysfunction, such as those that involve the use of ROCK inhibitor eye drops. We have also tried to develop surgical treatments for advanced corneal endothelial dysfunction, such as a cultivated corneal endothelial cell sheet transplantation using a type-I collagen carrier, or a cultivated Descemet-stripping automated endothelial keratoplasty (DSAEK) surgery using a human lamellar graft in animal bullous keratopathy models. At present, we are also investigating a form of cultivated corneal endothelial

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transplantation without the use of a carrier. In this review we report our recent progress toward the development of new therapeutic modalities for corneal endothelial disease focused on the proliferation of corneal endothelial cells using animal models.

1. Cultivated corneal endothelial cell sheet transplantation in a monkey model

Although human corneal endothelial cells are mitotically inactive and are arrested at the G1 phase of the cell cycle (Joyce, 2003), they retain the capacity to proliferate *in vitro* (Engelmann et al., 1988; Miyata et al., 2001; Senoo and Joyce, 2000; Zhu and Joyce, 2004). Some groups, ours included, have worked on developing cultivated human corneal endothelial cell sheet transplantation with (Ishino et al., 2004; Mimura et al., 2004) or without (Sumide et al., 2006) carrier materials, and have demonstrated *in vivo* functionality in a rabbit model. It is known that the proliferative ability of corneal endothelial cells varies among species, and that rabbit corneal endothelial cells proliferate very well even *in vivo*. In contrast, as in humans, the ability of monkey and feline corneal endothelial cells to proliferate is severely limited (Matsubara and Tanishima, 1982; 1983; Tsuru et al., 1984; Van Horn and Hyndiuk, 1975; Van Horn et al., 1977), rendering these species as representative models for corneal endothelial cell research. To this end, our laboratory developed a corneal endothelial dysfunction model in monkeys by mechanical scraping of the endothelium followed by trypan blue staining of the denuded Descemet's membrane. Thereafter we examined the feasibility of cultivated corneal endothelial transplantation. To the best of our knowledge, endothelial research programmes using monkey models for developing new corneal therapies are not established or in widespread use in other laboratories.

1.1. Cultivated monkey corneal endothelial sheets using collagen type-I as a carrier

Corneas were obtained from cynomolgous monkeys (3–5 years old: estimated comparable human age, 5–20 years) at euthanasia for other research purposes at NISSEI BILIS Co., Ltd. (Ohtsu, Japan), and KEARI Co., Ltd. (Wakayama, Japan). At all times the ARVO guidelines for the use of animals in ophthalmic research were adhered to, as were local and national ethical rules. We cultivated monkey corneal endothelial cells according to a modified protocol for human corneal endothelial cell culture (Ishino et al., 2004; Miyata et al., 2001). In brief, Descemet's membrane was stripped of intact monkey corneal endothelial cells and dissociated using Dispase II. The monkey corneal endothelial cells were cultivated on tissue culture plates coated with cell attachment reagent (FNC coating mix) in culture medium containing DMEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 ng/ml bFGF. Primary cultures formed confluent layers of hexagonal cells within 14 days, with an average cell density of more than 2500 cells/mm^2. After 3–5 passages on culture plates, confluent subculture cells were seeded onto rehydrated collagen type-I sheets (Fig. 1A, B) at a concentration of 5–10 $\times$ 10^2 cells/mm^2. After reaching confluence in one week, cells were kept in culture for an additional two weeks. Alizarin red staining revealed mainly hexagonal, homogeneous cells with an average density of 2240 $\pm$ 31 cells/mm^2 (mean $\pm$ S.E.) (Fig. 1C). Immunohistochemical staining of ZO-1 and Na^+/K^+-ATPase revealed that these functional proteins were located at the cell boundaries of the cultivated MCEC sheets (Fig. 1C). Examination by TEM showed a monolayer of endothelial cells similar to that seen in normal *in vivo* corneal endothelium of monkeys (Koizumi et al., 2007).

1.2. Transplantation of cultivated monkey corneal endothelial cells on type-I collagen sheets into monkey eyes

Six female cynomolgous monkeys (2.0–2.5 kg) were anesthetized intramuscularly with a mixture of ketamine hydrochloride (5 mg/kg; Sankyo, Tokyo, Japan) and xylazine (1 mg/kg, Bayer, Munich, Germany) followed by inhalation anesthesia with isoflurane. Surgery was carried out in an animal surgery room at the same levels of cleanliness as for human keratoplasty. During surgical procedures, animals were observed by veterinarians monitoring pulse, blood pressure, and partial pressure oxygen. To induce endothelial dysfunction 3-mm limbal-corneal incisions were made in the right eyes of the six monkeys, and then the corneal endothelia were removed by mechanical scraping using a 20G silicone needle, followed by 0.04% trypan blue staining to confirm that all endothelial cells were removed from Descemet's membrane. The scraped area measured at least 9 mm in diameter (the diameter of the cornea is approximately 10 mm). For the posterior graft, a 6 mm-diameter limbal-corneal incision was made and a 6-mm diameter disc of a cultivated monkey corneal endothelial cells on a sheet was brought into the anterior chamber in four eyes of four animals using a lens glide with the corneal endothelial side facing the anterior chamber. In one of the surgeries a DiI labeled cultivated monkey corneal endothelial cell sheet was used. In all cases the limbal-corneal incision was closed with 10-0 nylon interrupted sutures and the cultivated monkey corneal endothelial cell sheet attached to Descemet's membrane by air injection. As controls, a collagen sheet without monkey corneal endothelial cells was transplanted in one eye of one endothelial-dysfunctional animal, and a suspension of cultivated monkey corneal endothelial cells was injected into the anterior chamber in one eye of another. Following surgery we conducted a four-year follow up of corneal clarity (slit-lamp), corneal thickness (ultrasound pacymeter) and *in vivo* corneal endothelial assessment (non-contact specular microscopy).

After surgery, the monkey corneal endothelial cell sheet was attached to Descemet's membrane and remained attached in all experimental eyes (Fig. 2, 3rd day). In the two control eyes (i.e. sheet only, and cell-injection) severe corneal edema was observed after surgery. In the postoperative day 5–14 period in the operative group the monkey corneal endothelial cell sheets became detached from Descemet's membrane and dropped into the anterior chamber in all of three eyes. Nevertheless, these corneas achieved full clarity (Fig. 2, 14th day), which was maintained at least up to eight months after surgery (Fig. 2, 8 months). These experiments revealed that whereas irreversible corneal edema and neovascularization, similar to that seen in advanced bullous keratopathy in humans, occurred following endothelial scraping, eyes which received cultivated monkey corneal endothelial cell sheet transplantation recovered their clarity and became less edematous with time. Ours is the first study to investigate the feasibility of cultivated corneal endothelial sheet transplantation in a primate allograft model in which corneal endothelial cells have low *in situ* proliferative potential. Interestingly, in the successful post-surgery animals corneal endothelial cells more than 2000 cells/mm^2 were observed by specular microscopy six months postoperatively. In some additional experiments, we found DiI labeled donor corneal endothelial cells on the host Descemet's membrane outside of the sheet transplantation area in the early postoperative period (Koizumi et al., 2008). This was unexpected, and the mechanism of wound healing was not as we initially envisaged; i.e. we did not expect to see migration or proliferation of monkey corneal endothelial cells in the eye. This finding lead us to speculate that, once cultivated *in vitro*, monkey corneal endothelial cells might recover their proliferative ability and are able to migrate onto the host Descemet's membrane and proliferate *in vivo*. This provides us with

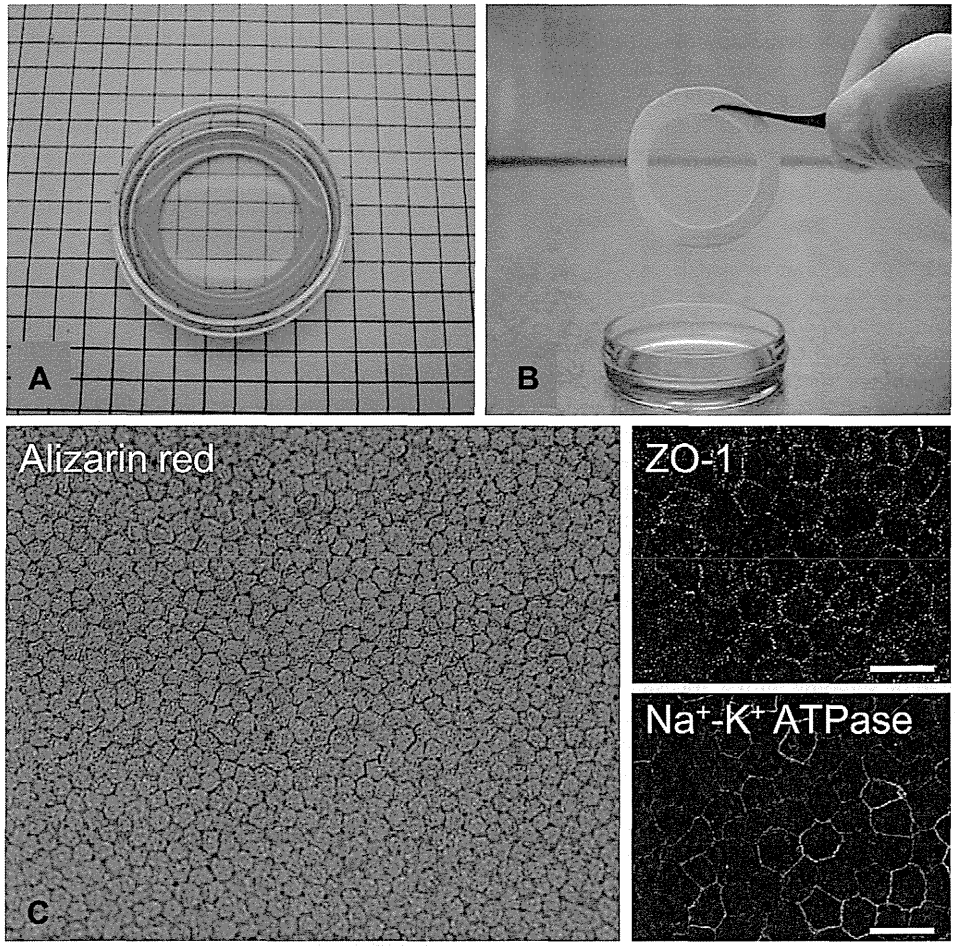

Fig. 1. Cultivated monkey corneal endothelial cell sheet on a collagen type-I carrier. (A, B) Primary culture of monkey corneal endothelial cells subcultured on a collagen type-I sheet. The cultivated corneal endothelial sheet is transparent and easy to handle. (C) Alizarin red staining of the cultures reveals mainly hexagonal, homogeneous cells with a density of 2800 cells/mm^2. The cultivated monkey corneal endothelial cells on a collagen type-I sheets expressed ZO-1 and Na^+/K^+-ATPase at their lateral cell membranes (green). Propidium iodide was used to visualize the cell nuclei (red). (Scale bars: 50 μm). (Reprinted with some modification from Koizumi et al. (2007) with permission from the Association for Research in Vision and Ophthalmology). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

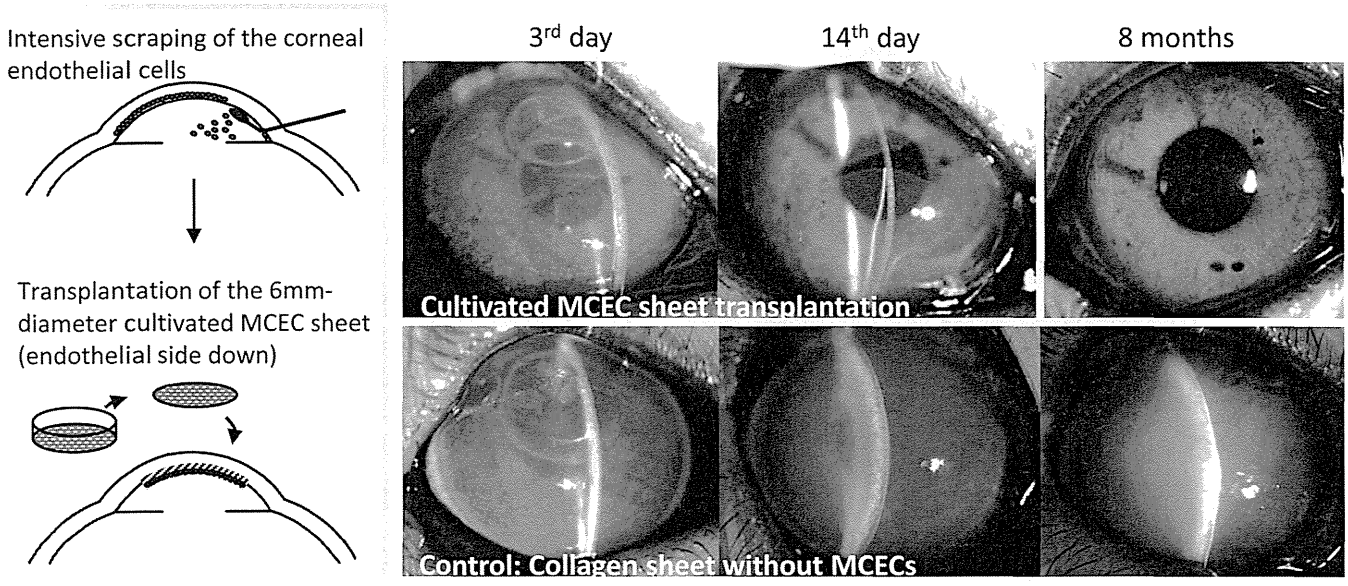

Fig. 2. Schematic image of the surgical procedure and slit-lamp photographs after cultivated monkey corneal endothelial cell sheet transplantation. In cultivated monkey corneal endothelial cell sheet transplanted eyes, the sheet was attached to Descemet's membrane on the 3rd day and a clear cornea was recovered by two weeks. The eyes remain clear up to the most recent observation, even though the sheet was detached from the posterior cornea.

a potential new concept for the treatment of corneal endothelial dysfunction, which involves not just transplantation of a cultivated corneal endothelial sheet, but the transplantation of endothelial cells which have the renewed ability to proliferate *in vivo*. Our long-term observation using non-contact specular microscopy suggest that corneal endothelial cell proliferation was stopped when the cells reached confluence probably due to contact-inhibition (Koizumi et al., 2008).

1.3. Cultivated-DSAEK surgery in an animal models

Another concept for corneal endothelial repair is the use of donor posterior stromal tissue as a carrier for cultivated corneal endothelial cells. We examined the feasibility of using human corneal lamellar graft tissue as a carrier for the cultivation of corneal endothelial cells. Descemet's membrane, with an intact corneal endothelium, was removed from human corneal tissue obtained from an American eye bank (SightLife, Seattle, WA) for research purposes. Corneal lamellar grafts (150–200 μm thick and 8 mm in diameter) were made from the posterior stroma using a Moria microkeratome. They were preserved in the freezer at −20 °C for four weeks before being seeded with monkey corneal endothelial cells (2×10^5 cells/graft) and cultivated for three weeks. Under general anesthesia, the corneal endothelium and Descemet's membrane were removed by scraping with a 20G silicone needle, and the lamellar grafts with monkey corneal endothelial cells were transplanted onto the posterior cornea of one monkey and one rabbit using a Busin glide in a similar procedure to DSAEK (Fig. 3A). The allograft in the monkey eye was performed for the long-term observation of the surgical outcome; the xenograft in the rabbit was performed for the short-term (up to 48 h) evaluation of donor endothelial cell damage during the graft insertion process. Histological examination showed that confluent monkey corneal endothelial cells were established on the human corneal lamellar graft at a density of 2240 cells/mm^2, and the protein expression of ZO-1 and Na^+/K^+-ATPase was confirmed by immunohistochemistry. The day after surgery, the graft was well attached to the host corneal stroma and mild corneal edema was observed (Fig. 3B). Histological examination of the rabbit eye with alizarin red staining showed no donor endothelial damage due to the graft insertion (Fig. 3C). A long-term observation in the monkey model indicated that the cornea recovered its clarity by postoperative week two. Pre-experimental corneal thickness of the monkey was 473 μm, and the corneal thickness one week postoperatively was 1042 μm, which decreased to 600 μm at the eight month time point. One month after surgery the cornea was clear and remained so seven months later (Fig. 4). No signs of rejection were detected with the use of minimal immunosuppressive treatment (steroid ointment applied once daily for one month). Control eyes from which corneal endothelial cells were scraped showed severe bullous keratopathy after surgery, which did not recover during the observation period. By non-contact specular microscopy, polygonal cells were observed at a density of 2178 cells/mm^2 at two months and 1841 cells/mm^2, 8 months after surgery (Fig. 4D). Though our results are still preliminary, they suggest the possibility of cultivated corneal endothelial cell transplantation using a corneal lamellar graft.

2. Cell-injection therapy using a selective Rho-kinase (ROCK) inhibitor

Direct transplantation of cultivated corneal endothelial cells onto the posterior cornea by "cell-injection into the anterior

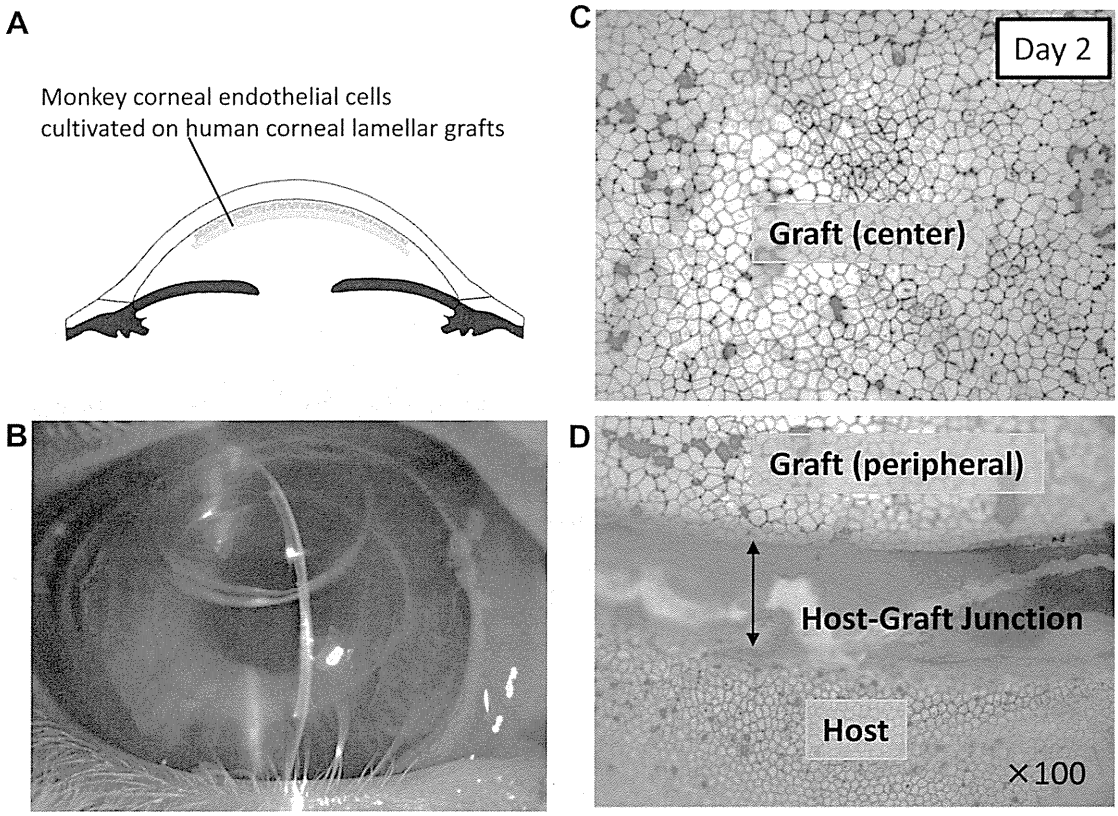

Fig. 3. Cultivated monkey corneal endothelial transplantation (cultivated-DSAEK) in a rabbit corneal endothelial dysfunction model. Using a microkeratome, a human corneal lamellar graft was created onto which monkey corneal endothelial cells were cultivated for 3 weeks. The graft was successfully transplanted into rabbit eyes. Donor monkey corneal endothelial cells were detected both at the center and peripheral part of the graft were not damaged by the graft insertion process. In addition, donor monkey corneal endothelial cells were clearly distinguished from host (rabbit) corneal endothelial cells with acellular area (host-graft junction).

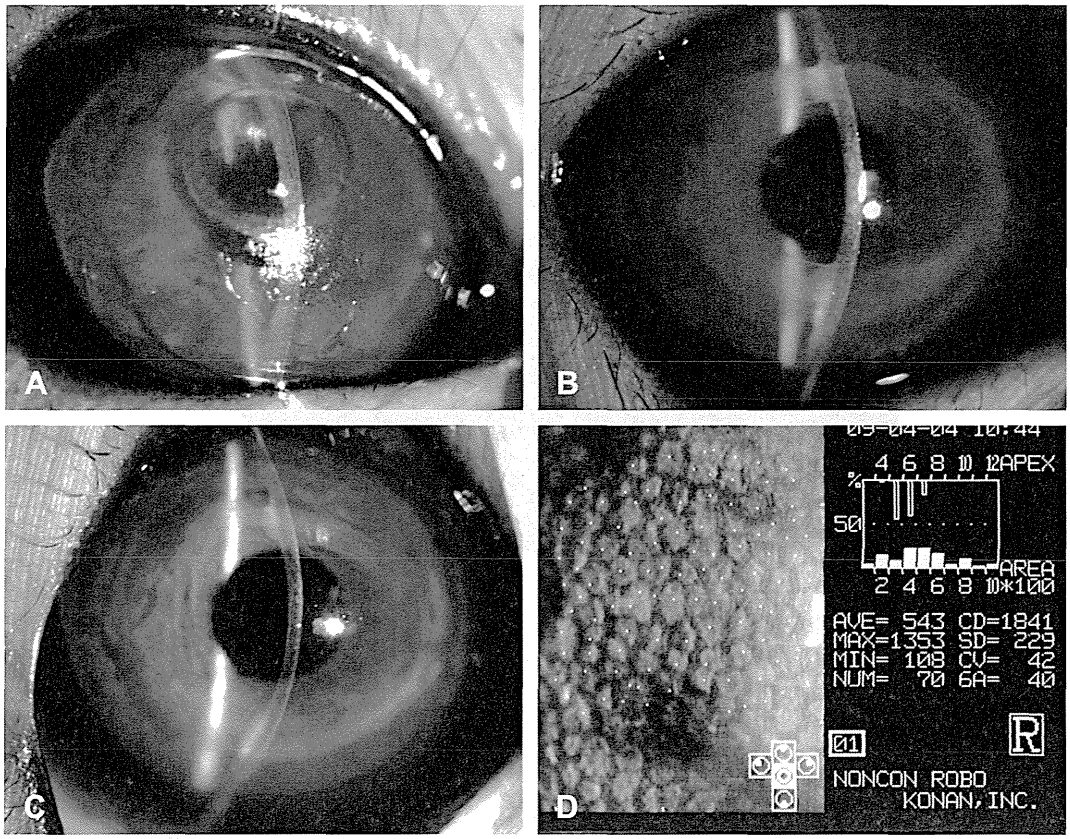

Fig. 4. Cultivated-DSAEK in a monkey corneal endothelial dysfunction model. DSAEK graft composed of monkey corneal endothelial cells cultivated on a human lamellar graft was transplanted into endothelially denuded monkey eyes. The graft was well-attached to the host corneal stroma 24 h after transplantation (A). One month after surgery, the cornea became clear (B) and remained so for up to 8 months (C). Corneal endothelial cells at a density of 1841 cells/mm^2 were observed (D).

chamber" has been considered an ideal method of reconstructing the corneal endothelial layer of patients with endothelial dysfunction. To develop an effective method to deliver cultivated corneal endothelial cells to the posterior cornea, magnetic attachment of iron-powder (Mimura et al., 2003; Mimura et al., 2005a) or superparamagnetic microspheres (Patel et al., 2009) incorporated in cultivated corneal endothelial cells has been attempted. These approaches work in a rabbit transplantation model or an organ culture model of the human eye, but have not yet been clinically applied. Now, we are trying to develop a cell-injection therapy combined with the use of a ROCK inhibitor which promotes corneal endothelial cell adhesion onto the posterior cornea.

2.1. ROCK inhibitor and corneal endothelial cells in vitro

Rho-kinase (ROCK) is a serine/threonine kinase, which serves as a target protein for Rho and has been initially characterized as a mediator of the formation of RhoA-induced stress fibers and focal adhesions. The Rho/ROCK pathway is involved in regulating the cytoskeleton, cell migration, cell proliferation, and apoptosis (Coleman et al., 2004; Hall, 1998; Olson et al., 1995; Riento and Ridley, 2003). In the cornea field, it is reported that ROCKs are involved in corneal epithelial differentiation, cell-cycle progression, and cell–cell adhesion (Anderson et al., 2002; Anderson and SundarRaj, 2001; SundarRaj et al., 1998). ROCKs also influence the phenotype of stromal cells, their cytoskeleton reorganization, and cell–matrix interactions (Anderson et al., 2004; Harvey et al., 2004; Kim et al., 2006; Kim and Petroll, 2007; Lakshman et al., 2007; Petroll et al., 2004). In terms of the corneal endothelium, the Rho/ROCK pathway has an influence on the wound healing and barrier function (D'Hondt et al., 2007; Satpathy et al., 2005; 2004).

In 2007, our collaborators reported that a selective ROCK inhibitor, Y-27632, diminished the dissociation-induced apoptosis of human embryonic stem cells (Watanabe et al., 2007). We subsequently examined the effect of Y-27632 on primate corneal endothelial cells *in vitro* and found that the inhibition of Rho/ROCK signaling by Y-27632 inhibited dissociation-induced apoptosis and promoted the adhesion and proliferation of monkey corneal endothelial cells (Okumura et al., 2009) (Fig. 5). We are now applying commercially available Y-27632 purchased from Wako Pure Chemical Industries (Osaka, Japan) to human corneal endothelial cells in culture, as well as developing cell-injection therapies. We have no commercial interest with the use of Y-27632 of this project.

2.2. Cell-injection therapy combined with ROCK inhibitor in animal models

Rabbit corneal endothelial cells were cultured as previously described and 2 x 10^5 cells were injected into the anterior chambers of rabbit eyes from which host corneal endothelial cells had been scraped off. Cells were injected with or without 100 μM of ROCK inhibitor, Y-27632. The eye of each animal was kept in the face-down position for 3 h following injection (Mimura et al., 2005b), and it was found that when Y-27632 was present the donor cells became nicely attached onto the host Descemet's membrane and the host cornea recovered its transparency. This attachment was not so advanced in the cell-injected eyes without the inclusion of Y-27632. Histological examination confirmed that cell adhesion

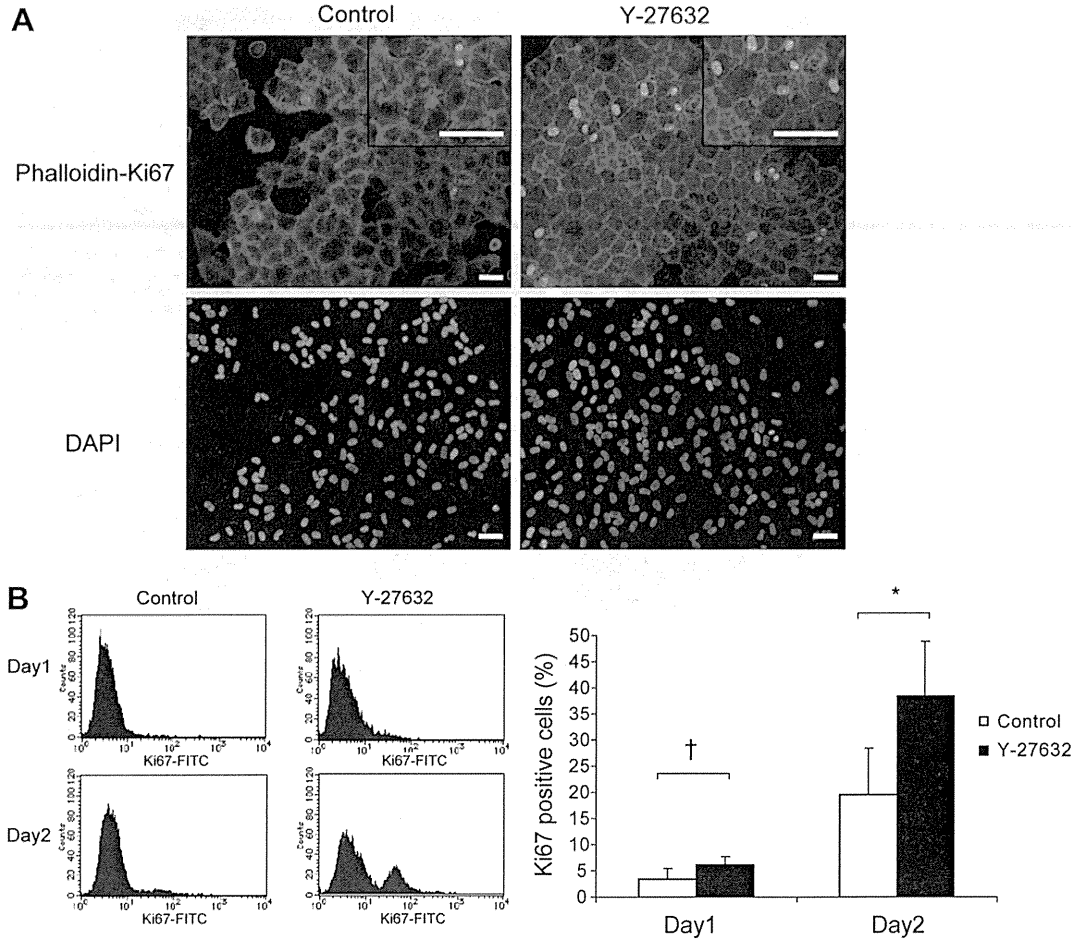

Fig. 5. ROCK inhibitor (Y-27632) promotes the proliferation of monkey corneal endothelial cells. (A) Double-immunostaining of Ki67 and actin fibers; passaged monkey corneal endothelial cells were cultured for 48 h and stained successively with Ki67 and phalloidin. Ki67 (green), actin (red), and DAPI (blue). Insets are higher magnification. Scale bars 250 μm. (B) Ki67 positive cells were analyzed by flow cytometry. Monkey corneal endothelial cells were subcultured for 1 or 2 days, and stained successively with Ki67. The numbers of Ki67 positive cells were significantly elevated in the presence of Y-27632 on both day 1 and 2 ($^{\dagger}P < 0.05$, $^{*}P < 0.01$). Data are expressed as the mean ± SE ($n = 6$). (Reprinted from Okumura et al. (2009) with permission from the Association for Research in Vision and Ophthalmology). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

was enhanced by Y-27632, and that the healthy polygonal monolayer was reconstructed in the cell-injected eyes with this selective ROCK inhibitor. Unlike in the Y-27632-treated eyes, corneal edema persisted in the cell-injected eyes without Y-27632 and most of the endothelial cells showed fibroblastic changes with elongated cell shapes (in submission). Stratification was also detected by phalloidin staining. Repeated experiments in monkeys with longer observation periods (in submission) have confirmed that the procedure results in a high density of corneal endothelial cells formed into healthy polygonal monolayers.

3. Eye drop treatment for corneal endothelial disease

A pure medical treatment for corneal endothelial disease has been sought for a long time by ophthalmologists and patients. It has been reported that human corneal endothelial cells in organ cultured corneas proliferate in response to wounding, as they are if released from contact-inhibition by EDTA (Senoo et al., 2000). It has also been reported by our group that connexin43 knockdown by siRNA promotes corneal endothelial proliferation and wound healing in a rat corneal endothelial injury model (Nakano et al., 2008). However, to the best of our knowledge no pharmacological agent is in use clinically for the treatment of corneal endothelial dysfunction.

3.1. ROCK inhibitor eye drop treatment in in vivo animal models

With the purpose of developing a pharmacological treatment for corneal endothelial dysfunction, we examined the effect of Y-27632 ROCK inhibitor eye drops on corneal endothelial cells using an animal corneal endothelial injury model. The target of the pharmacological treatment is the early phase of corneal endothelial disease in patients such as those with Fuchs' dystrophy, or those with corneal endothelial damage induced by intraocular surgeries who nevertheless retain some healthy corneal endothelial cells.

First, we made a partial endothelial injury by transcorneal freezing using a 7 mm diameter stainless-steel cryo-probe in rabbits. After injury, 10 mM of Y-27632 diluted in 50 μl of phosphate-buffered saline was applied topically in one eye of each animal six times daily for 2 days, while PBS was applied in the other eye as a control. In the Y-27632-treated eyes less corneal edema was observed by slit-lamp microscopy and ultrasound pachymetry. Histology showed that the mean wound area of Y-27632-treated eyes was significantly smaller than that of control eyes (Fig. 6). These results demonstrate that the topical administration of selective ROCK inhibitor, Y-27632, as an eye drop has the potential to enhance corneal endothelial wound healing (Okumura et al., 2011).

To establish the application of ROCK inhibitor eye drop in a clinical setting we have recently conducted a similar experiment

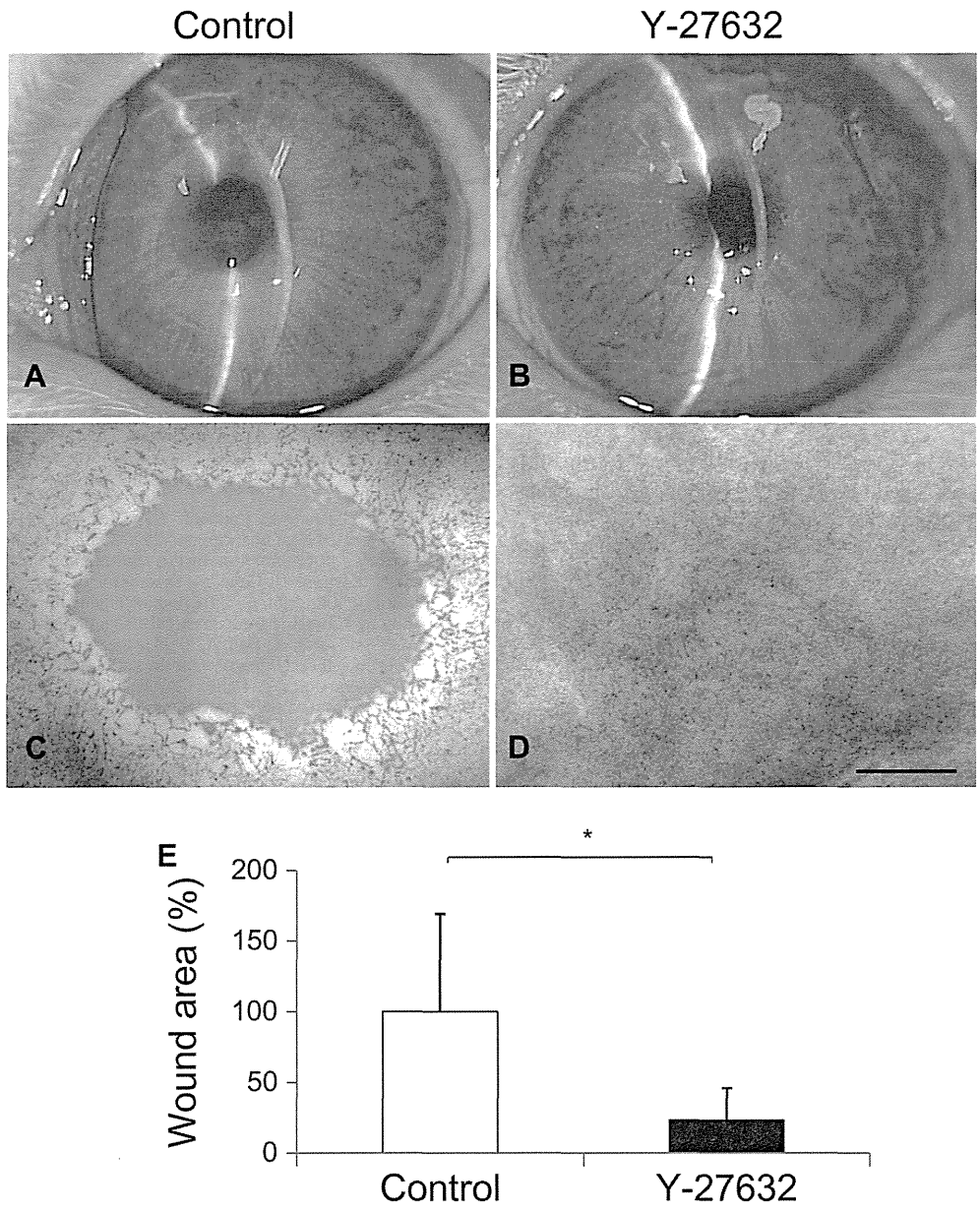

Fig. 6. *Effects of ROCK inhibitor Y-27632 eye drops in a rabbit model. The center of the corneal endothelium was damaged by transcorneal freezing, after which Y-27632 was applied topically for 2 days. Slit-lamp microscopy revealed that corneal transparency was higher in the Y-27632 group compared to the control group (A, B). Alizarin red staining shows that corneal endothelial wound healing was promoted in the Y-27632 group compared to the control group (D, E). The mean wound area of the Y-27632 group was significantly smaller than that of the control group after 48 h (23.1 ± 22.9% as a ratio of control; $^*P < 0.05$) (E). Scale bar: 500 μm. (Reprinted from Okumura et al. (2009) with permission from the British Journal of Ophthalmology). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

using a partial corneal endothelial dysfunction model in monkey. Early indications show that the topical application of Y-27632 following cryo-injury enables the corneal endothelium to retain a high cell density in cynomolgus monkeys during a 1-month observation period, with reproducibility confirmed in six eyes (in submission).

4. Discussion: toward the clinical application of new therapies

There are a numbers of research papers which report protocols for human corneal endothelial cell culture, however, it is still difficult for us to consistently expand usable amounts of human corneal endothelial cells which retain a healthy morphology and high cell density. Recently, we have used a selective ROCK inhibitor, Y-27632, in our human corneal endothelial cell culture and it has improved the culture results. Based on these findings, we are now planning to apply cell-injection therapy using human cultivated corneal endothelial cells combined with Y-27632 to advanced corneal endothelial dysfunction patients in clinical setting. In line with ethical considerations, endothelial cell expansion has great potential to be helpful in the reconstruction of the posterior cornea with possibilities for genetically-engineered endothelial cells or HLA matched corneal endothelial cells to help avoid the allograft rejection.

Regarding the Y-27632 eye drop treatment, we have obtained the approval of the Institutional Review Board of Kyoto Prefectural University of Medicine and have started a clinical pilot study of

ROCK inhibitor eye drop treatment for bullous keratopathy and have confirmed its safety and ability to recover corneal endothelial cell density in some patients with specific conditions. Currently, we are accumulating evidence regarding the mechanism of ROCK inhibitor eye drops; our current understanding is that it works by stimulating proliferation of the patients' corneal endothelium (unpublished data).

Given the burden on individuals and healthcare providers as a result of corneal endothelial dysfunction, the discovery and introduction into clinical practice of new pharmacological agents which are safe and effective is highly desirable. Such an achievement will potentially reduce the over-reliance on corneal transplantation and improve the quality of life and vision for many.

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眼合併症を伴う日本人Stevens-Johnson症候群のHLA class Ⅰ解析

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要　約

目　的：以前，我々は，眼合併症を伴う日本人Stevens-Johnson症候群(Stevens-Johnson syndrome：SJS)・中毒性表皮壊死融解症(toxic epidermal necrolysis：TEN)患者71人と非発症対照113人を対象に，HLA class Ⅰ-A，B，Cの解析を行い，SJS/TEN発症とHLA class Ⅰ，特にA*0206との間に強い相関があることを報告した．今回は，日本人SJS/TEN患者118人と前回と異なる日本人対照220人を対象に，HLA class Ⅰ-A，B，Cについての再検討を行ったので報告する．

対象と方法：京都府立医科大学附属病院眼科に通院するSJS/TEN患者118例を対象とし，新たに京都府立医科大学で血液を採取した日本人非発症者220人を対照として用いた．末梢血からDNAを採取し，polymerase chain reaction-sequence specific oligonucleotide probe(PCR-SSO)法を用いてHLA class Ⅰ-A，B，Cについて塩基配列レベルの解析を行い，遺伝子頻度(gene frequency：GF)と保持者頻度(carrier frequency：CF)を検討した．

結　果：A*0206が有意に増加していたが，p値は以前の報告よりさらに上昇した(p値はGF：$p=0.000000007$，CF：$p=0.0000000002$，オッズ比はGF：4.2，CF：5.2)．

結　論：眼合併症を伴うSJS/TENの発症には，前回の報告よりさらにHLA class Ⅰ A*0206が強く相関することが確認された．(日眼会誌116：581-587，2012)

キーワード：HLA-A*0206，Stevens-Johnson症候群(SJS)，中毒性表皮壊死融解症(TEN)，眼合併症，HLA

HLA-class Ⅰ Gene Polymorphisms in Japanese Stevens-Johnson Syndrome Patients with Ocular Surface Complications

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Abstract

Purpose：Our previous study of polymorphisms in the HLA-class Ⅰ genes of 71 Japanese SJS/TEN patients with ocular surface complications and 113 Japanese healthy controls showed that in the Japanese, HLA-A*0206 was strongly associated with SJS/TEN. In this study, we examined 118 Japanese SJS/TEN patients with ocular surface complications and a new control group consisting of 220 healthy Japanese volunteers, and investigated the association between HLA class Ⅰ antigens, HLA-A, B, C, and the SJS/TEN.

Methods：For HLA genotyping we enrolled 118 Japanese patients with SJS/TEN in the chronic or sub-acute phase at Kyoto Prefectural University of Medicine；all presented with ocular surface complications. We also enrolled 220 healthy Japanese volunteers. We performed polymerase chain reaction amplification followed by hybridization with sequence-specific oligonucleotide probes(PCR-SSO).

Results：HLA-A*0206 was most strongly associated with Japanese SJS/TEN patients with ocular surface complications (carrier frequency：$p=0.0000000002$，OR=5.2；gene frequency：$p=0.000000007$，OR=4.2).

Conclusion：HLA-A* 0206 is strongly associated with Japanese SJS/TEN patients with ocular surface complications.

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Key words：HLA-A*0206, Stevens-Johnson syndrome (SJS), Toxic epidermal necrolysis (TEN), Ocular surface complications, HLA

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I 緒 言

Stevens-Johnson症候群(Stevens-Johnson syndrome：SJS)，ならびにその重症型とされる中毒性表皮壊死融解症(toxic epidermal necrolysis：TEN)は，ともに突然の高熱と皮膚および粘膜の発疹，びらんで発症し，急速に全身に拡大する疾患である[1)2)]．日本では，皮疹の範囲が10%以下をSJS，それ以上の広範囲に及んだ場合はTENと診断される[3)4)]．両疾患は致死率が高く，医療体制の充実した本邦にあってもSJSで数%，TNEで約30%が敗血症，呼吸不全，多臓器障害などで亡くなっている．SJS/TENにおける眼障害合併率は約60%とされ[5)]，高熱や発疹の出現と同時，あるいはその数日前より両眼性に結膜充血を生じ，偽膜や角膜びらん，結膜びらんを伴う[6)7)](図a)．広範囲に眼表面のびらんを生じて角膜上皮幹細胞を喪失すると，角膜は周囲から伸展する結膜で被覆されて高度の角膜混濁，血管侵入を伴うようになる(図b)．救命できた場合の最大の問題は眼後遺症であり，高度のドライアイ，角膜混濁による視力障害が生涯に及ぶ．

SJS/TENのほとんどは発症前に何らかの薬剤を投与されており，両疾患は重症薬疹として分類される．発症機序は不明であるが，患者側の素因が関与すると考えられ，これまでに，SJS/TENの発症にはHLA型が関与するということが報告されている[8)9)]．HLA型は民族による違いが大きく，我々は日本人の眼合併症を伴うSJS/TEN 71例，非発症対照113例を比較し，HLA-A*0206が有意に相関していることを以前に報告した[10)11)]．発症に強く関与するHLA型が明らかになれば，発症予防や発症後の迅速な診断が可能となり，予後改善に寄与することができる．

そこで今回，眼合併症を伴う日本人SJS/TEN症例数を100例以上に増やし，かつ前回とは異なった日本人対照サンプルを用いてHLA class Ⅰ-A，B，Cの解析を行い興味ある結果を得たので報告する．

II 対象と方法

京都府立医科大学附属病院眼科に通院するSJS/TEN患者118例を対象とし，新たに京都府立医科大学で日本人非発症者220人より同意を得て採取した血液を対照として用いた．対照については，前回の解析に用いた東京都赤十字血液センターで採取したものとはまったく別グループのものである．末梢血からDNAを採取し，polymerase chain reaction-sequence specific oligonucleotide probe(PCR-SSO)法に基づきHLAタイピング試薬(湧永製薬)を用いて，WAKFlow systemにてHLA遺伝子のタイピングを行った[10)11)]．HLA class Ⅰ-A，B，Cについて塩基配列レベルの解析を行い，遺伝子頻度(gene frequency)と保持者頻度(carrier frequency)を検討した．

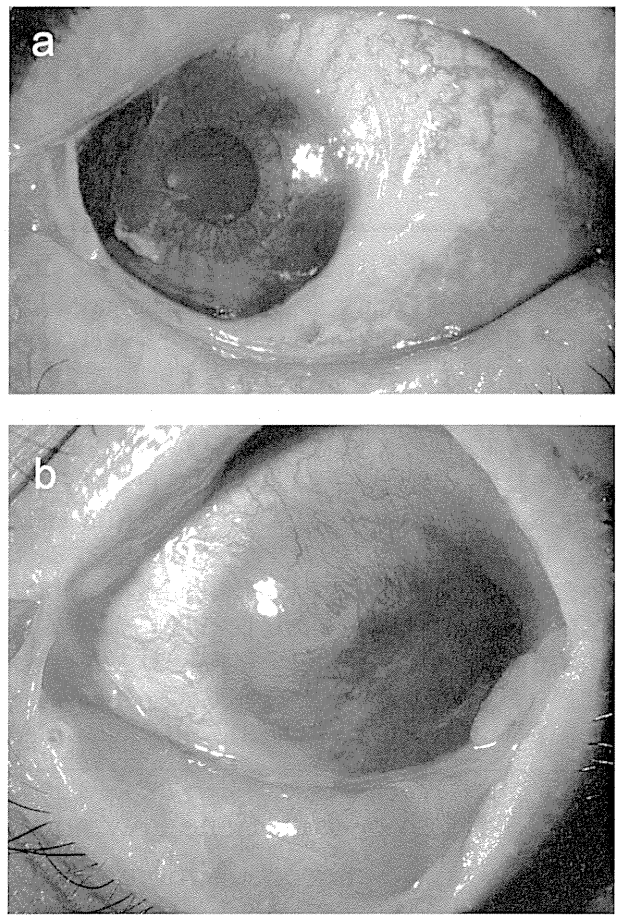

図 Stevens-Johnson症候群(SJS)/中毒性表皮壊死融解症(TEN)の眼所見．
a：急性期SJS/TENの眼所見．結膜充血，偽膜，角膜びらん，結膜びらんを認める．
b：慢性期SJS/TENの眼所見．角膜への結膜侵入，瞼球癒着を認める．この症例ではドライアイに対して，涙点プラグを挿入している．眼脂を認めることも多い．

III 結 果

表1にHLA class Ⅰ-A型の結果を示す．118人の患者を対象とした今回の解析において，前回の71人の患者を対象にした解析(保持者頻度：p=0.00004，オッズ比4.1．遺伝子頻度：p=0.0001，オッズ比3.2)以上に[10)11)]，HLA-A*0206と強い有意な相関が確認された(保持者頻度：p=0.0000000002，オッズ比5.16．遺伝子頻度：p=0.000000007，オッズ比4.15)．

また，HLA-A*1101(保持者頻度：p=0.02，オッズ比0.43．遺伝子頻度：p=0.03，オッズ比0.46)とHLA-A*2402(保持者頻度：p=0.008，オッズ比0.54，遺伝子頻度：p=0.001，オッズ比0.57)に相関を認めたが，HLA class Ⅰ-A型の数18で補正すると有意差はなくなり，弱い相関を示唆するのみとなった．HLA-A*1101については，前回の解析では，発症しにくさと相関することが推定されたが[10)]，症例数を71人から118人に増

表1　HLA class Ⅰ-Aの遺伝子頻度と保持者頻度

HLA-A alleles	保持者頻度				遺伝子頻度			
	SJS/TEN (n=118)	正常対照 (n=220)	p値 (χ^2)	オッズ比	SJS/TEN (n=236)	正常対照 (n=440)	p値 (χ^2)	オッズ比
*0101	0.0% (0/118)	1.4% (3/220)	0.20	—	0.0% (0/236)	0.7% (3/440)	0.20	—
*0201	26.3% (31/118)	20.5% (45/220)	0.22	—	14.4% (34/236)	11.1% (49/440)	0.22	—
*0206	44.9% (53/118)	13.6% (30/220)	1.91×10^{-10}	5.16	23.3% (55/236)	6.8% (30/440)	7.14×10^{-9}	4.15
*0207	8.5% (10/118)	7.7% (17/220)	0.99	—	4.2% (10/236)	3.9% (17/440)	0.81	—
*0210	0.0% (0/118)	0.9% (2/220)	0.30	—	0.0% (0/236)	0.5% (2/440)	0.30	—
*0301	2.5% (3/118)	0.9% (2/220)	0.24	—	1.3% (3/236)	0.5% (2/440)	0.24	—
*0302	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*1101	8.5% (10/118)	17.7% (39/220)	0.02	0.43	4.2% (10/236)	8.9% (39/440)	0.03	0.46
*2402	45.8% (54/118)	60.9% (134/220)	0.008	0.54	25.0% (59/236)	37.0% (163/440)	0.001	0.57
*2601	11.0% (13/118)	12.3% (27/220)	0.73	—	5.5% (13/236)	6.4% (28/440)	0.66	—
*2602	5.1% (6/118)	2.7% (6/220)	0.26	—	2.5% (6/236)	1.6% (7/440)	0.39	—
*2603	1.7% (2/118)	7.3% (16/220)	0.03	0.22	0.8% (2/236)	3.6% (16/440)	0.03	0.23
*2605	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*2901	0.0% (0/118)	1.8% (4/220)	0.14	—	0.0% (0/236)	0.9% (4/440)	0.14	—
*3001	0.8% (1/118)	0.5% (1/220)	0.65	—	0.4% (1/236)	0.2% (1/440)	0.65	—
*3101	14.4% (17/118)	18.1% (40/220)	0.38	—	7.2% (17/236)	9.5% (42/226)	0.30	—
*3201	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*3303	22.0% (26/118)	14.1% (31/220)	0.06	—	11.0% (26/236)	7.0% (31/440)	0.06	—

やすことにより，その相関は消失した．

表2にHLA class Ⅰ-B型の結果を示す．HLA-B*1501（保持者頻度：p=0.02，オッズ比0.43．遺伝子頻度：p=0.05，オッズ比0.50），HLA-B*4403（保持者頻度：p=0.004，オッズ比2.26．遺伝子頻度：p=0.004，オッズ比2.14），HLA-B*5201（保持者頻度：p=0.01，オッズ比0.44．遺伝子頻度：p=0.03，オッズ比0.51）とHLA-B*5401（保持者頻度：p=0.01，オッズ比0.36．遺伝子頻度：p=0.01，オッズ比0.37）に弱い相関を認めたが，HLA class Ⅰ-B型の数37で補正すると有意差は消失した．結果として，HLA class Ⅰ-B型には，重篤な眼合併症を伴うSJS/TENと有意な相関を認めるものはなかった．

表3にHLA class Ⅰ-C遺伝子の結果を示す．HLA-C*0304（保持者頻度：p=0.01，オッズ比1.96．遺伝子頻度：p=0.01，オッズ比1.81），HLA-C*1403（保持者頻度：p=0.005，オッズ比2.25．遺伝子頻度：p=0.007，オッズ比2.06）に弱い相関を認めたが，HLA class Ⅰ-C型の数18で補正すると有意差は消失した．結果として，HLA class Ⅰ-C型には，重篤な眼合併症を伴うSJS/TENと有意な相関を認めるものはなかった．

Ⅳ　考　　按

日本人の眼合併症を伴うSJS/TEN患者118人ならびに対照220人の解析により，眼合併症を伴うSJS/TENの発症には，HLA class Ⅰ A*0206が強く相関することが確認された．前回の患者71人，対照113人の解析（保持者頻度：p=0.00004，オッズ比4.1．遺伝子頻度：p

表 2 HLA class I-B の遺伝子頻度と保持者頻度

HLA-B alleles	保持者頻度				遺伝子頻度			
	SJS/TEN (n=118)	正常対照(n=220)	p 値(χ^2)	オッズ比	SJS/TEN (n=118)	正常対照(n=220)	p 値(χ^2)	オッズ比
*0702	8.5%(10/118)	11.0%(24/220)	0.48	—	4.2%(10/236)	6.6%(29/440)	0.21	—
*0705	0.0% (0/118)	1.8% (4/220)	0.14	--	0.0% (0/236)	0.9% (4/440)	0.14	—
*1301	6.8% (8/118)	2.7% (6/220)	0.07	—	3.4% (8/236)	1.4% (6/440)	0.08	—
*1302	0.0% (0/118)	1.4% (3/220)	0.20	—	0.0% (0/236)	0.7% (3/440)	0.20	—
*1501	8.5%(10/118)	17.7%(39/220)	0.02	0.43	4.7%(11/236)	8.9%(39/440)	0.05	0.50
*1502	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*1507	0.8% (1/118)	1.4% (3/220)	0.68	—	0.4% (1/236)	0.7% (3/440)	0.68	—
*1511	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*1518	3.4% (4/118)	1.0% (2/220)	0.10	—	1.7% (4/236)	0.5% (2/440)	0.10	—
*1527	0.8% (1/118)	0.0% (0/220)	0.17	—	0.4% (1/236)	0.0% (0/440)	0.17	—
*2704	0.8% (1/118)	0.5% (1/220)	0.65	—	0.4% (1/236)	0.2% (1/440)	0.65	—
*2705	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*3501	18.6%(22/118)	14.5%(32/220)	0.33	—	9.3%(22/236)	7.3%(32/440)	0.35	—
*3701	0.8% (1/118)	1.8% (4/220)	0.48	—	0.4% (1/236)	0.9% (4/440)	0.48	—
*3802	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*3901	7.6% (9/118)	5.9%(13/220)	0.54	—	3.8% (9/236)	3.0%(13/440)	0.55	—
*3902	0.8% (1/118)	1.0% (2/220)	0.95	—	0.4% (1/236)	0.5% (2/440)	0.95	—
*3904	0.0% (0/118)	1.0% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*4001	14.4%(17/118)	10.9%(24/220)	0.35	—	7.6%(18/236)	5.5%(24/440)	0.26	—
*4002	12.7%(15/118)	9.5%(21/220)	0.37	—	6.8%(16/236)	5.2%(23/440)	0.41	—
*4003	1.7% (2/118)	1.0% (2/220)	0.52	—	0.8% (2/236)	0.5% (2/440)	0.53	—
*4006	7.6% (9/118)	5.9%(13/220)	0.54	—	3.8% (9/236)	3.0%(13/440)	0.55	—
*4402	0.8% (1/118)	1.8% (4/220)	0.48	—	0.4% (1/236)	0.9% (4/440)	0.48	—
*4403	26.3%(31/118)	13.6%(30/220)	0.004	2.26	13.6%(32/236)	6.8%(30/440)	0.004	2.14
*4601	12.7%(15/118)	8.2%(18/220)	0.18	—	6.4%(15/236)	4.3%(19/440)	0.25	—
*4801	4.2% (5/118)	8.2%(18/220)	0.17	—	2.1% (5/236)	4.1%(18/440)	0.18	—
*5101	19.5%(23/118)	19.5%(43/220)	0.99	—	10.6%(25/236)	10.2%(45/440)	0.88	—
*5102	1.7% (2/118)	0.0% (0/220)	0.05	—	0.8% (2/236)	0.0% (0/440)	0.053	—
*5201	11.0%(13/118)	21.8%(48/220)	0.01	0.44	5.9%(14/236)	10.9%(48/440)	0.03	0.51
*5401	5.9% (7/118)	15.0%(33/220)	0.01	0.36	3.0% (7/236)	7.7%(34/440)	0.01	0.37
*5502	2.5% (3/118)	4.5%(10/220)	0.36	—	1.3% (3/236)	2.3%(10/440)	0.37	—
*5601	2.5% (3/118)	1.8% (4/220)	0.65	—	1.3% (3/236)	0.9% (4/440)	0.66	—
*5603	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*5801	2.5% (3/118)	1.8% (4/220)	0.66	—	1.3% (3/236)	0.9% (4/440)	0.66	—
*5901	7.6% (9/118)	4.5%(10/220)	0.24	—	3.8% (9/236)	2.3%(10/440)	0.25	—
*5904	0.8% (1/118)	0.0% (0/220)	0.17	—	0.4% (1/236)	0.0% (0/440)	0.17	—
*6701	1.7% (2/118)	3.6% (8/220)	0.31	—	0.8% (2/236)	1.8% (8/440)	0.32	—

=0.0001，オッズ比 3.2)からサンプルを増やすことにより，保持者頻度での p 値が 0.0000000002，オッズ比 5.16，遺伝子頻度での p 値が 0.000000007，オッズ比 4.15 と，さらに強い相関を確認できたことは大変に意義深い．

SJS/TEN の HLA 解析について以前の報告を振り返ってみると，1982 年にアメリカ人の眼科医 Mondino らが[8]，また，1986 年にフランス人の皮膚科医 Roujeau らが[9]，白人の SJS 患者では HLA-B12 血清型を有意に多く保有すると報告した．HLA-B12 血清型は，現在の遺伝子型

表3　HLA class Ⅰ-Cの遺伝子頻度と保持者頻度

HLA-C alleles	保持者頻度				遺伝子頻度			
	SJS/TEN (n=118)	正常対照 (n=220)	p値 (χ^2)	オッズ比	SJS/TEN (n=236)	正常対照 (n=440)	p値 (χ^2)	オッズ比
*0102	30.5% (36/118)	34.5% (76/220)	0.45	—	15.7% (37/236)	18.6% (82/440)	0.34	—
*0103	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*0302	2.5% (3/118)	1.8% (4/220)	0.66	—	1.3% (3/236)	0.9% (4/440)	0.66	—
*0303	2.3% (27/118)	21.3% (47/220)	0.75	—	11.4% (27/236)	10.9% (48/440)	0.83	—
*0304	32.3% (38/118)	19.5% (43/220)	0.01	1.96	17.8% (42/236)	10.7% (47/440)	0.01	1.81
*0401	8.5% (10/118)	9.5% (21/220)	0.75	—	4.2% (10/236)	4.8% (21/440)	0.75	—
*0501	2.5% (3/118)	1.8% (4/220)	0.66	—	1.3% (3/236)	0.9% (4/440)	0.66	—
*0602	0.8% (1/118)	3.2% (7/220)	0.18	—	0.4% (1/236)	1.6% (7/440)	0.18	—
*0701	0.8% (1/118)	0.0% (0/220)	0.17	—	0.4% (1/236)	0.0% (0/440)	0.17	—
*0702	18.6% (22/118)	25.0% (55/220)	0.18	—	10.6% (25/236)	14.1% (62/440)	0.20	—
*0704	2.5% (3/118)	0.0% (0/220)	0.02	0	1.3% (3/236)	0.0% (0/440)	0.02	0
*0801	8.5% (10/118)	13.6% (30/220)	0.16	—	4.2% (10/236)	6.8% (30/440)	0.18	—
*0803	2.5% (3/118)	3.6% (8/220)	0.59	—	1.3% (3/236)	1.8% (8/440)	0.59	—
*1202	11.9% (14/118)	21.4% (47/220)	0.03	0.50	6.4% (15/236)	10.7% (47/440)	0.06	—
*1402	12.7% (15/118)	14.1% (31/220)	0.72	—	6.8% (16/236)	7.5% (33/440)	0.73	—
*1403	25.4% (30/118)	13.2% (29/220)	0.005	2.25	12.7% (30/236)	6.6% (29/440)	0.007	2.06
*1502	8.5% (10/118)	5.9% (13/220)	0.37	—	4.2% (10/236)	3.0% (13/440)	0.38	—
*1505	0.0% (0/118)	1.8% (4/220)	0.14	—	0.0% (0/236)	0.9% (4/440)	0.14	—

ではHLA-B*4402とHLA-B*4403に当てはまる．しかしながら我々の解析ではHLA-B*4402は対照で1.8%，患者で0.8%と保持者頻度が少なく，また相関も認めなかった．HLA-B*4403については，対照で13.6%，患者で26.3%の頻度で認めたが，SJS/TEN発症との相関は確認されなかった．また，2004年にChungらが，抗てんかん薬カルバマゼピンにより発症した台湾のSJS/TEN患者では，100%がHLA-B*1502を保有していることを報告した[12]．しかし，Lonjouらは，白人ではHLA-B*1502保有率が低く，カルバマゼピン発症SJS/TEN患者とHLA-B*1502には相関を認めなかったと報告している[13]．日本人でもHLA-B*1502を保有していることはごくまれであり，SJS/TENの発症と相関を示さないことが報告されている[14]．我々の解析でも，HLA-B*1502は，対照でわずか1例認めたのみであった．また，重篤な眼合併症を伴うSJS/TENにおいては，抗てんかん薬による発症は大変少ない[15]．2005年にHungらは，高尿酸血症薬であるアロプリノールにより発症したSJS/TEN患者とHLA-B*5801との間に有意な相関があると報告した[16]．このアロプリノールとHLA-B*5801との相関は，白人でも[17]，日本人でも[18]報告されている．しかし，今回解析しているSJS/TEN患者118例のなかでアロプリノールによる発症はわずか3例であり，どの症例も眼後遺症は軽度であった．これらのことから，我々は，アロプリノールによる発症では，重篤な眼合併症は生じにくい可能性を考えている．

皮膚科医は，SJS/TEN症例の多くは，抗てんかん薬またはアロプリノールによる発症であると報告してい

る[19)20)]．しかし上述したように，眼障害を合併し後遺症を生じた患者では抗てんかん薬，アロプリノールによる発症は少なく，多くが総合感冒薬あるいは非ステロイド性抗炎症薬(non-steroidal anti-inflammatory drugs：NSAIDs)を契機に発症している[21)]．重篤なTENで眼障害のない症例もあれば，全身的に軽症で眼障害の高度なSJS症例もある．眼合併症を伴うSJS/TENは，SJS/TEN全体からみた単なる一部の症例群ではなく，独立した疾患カテゴリーになるのではないかと我々は考えている．

我々の調査では，重篤な眼合併症を伴うSJS/TEN患者では，急性結膜炎が皮疹に先行することが多く，口唇・口腔内の出血性びらん，爪囲炎が必発である[6)7)]．これらの患者の約8割は感冒様症状を最初に自覚し，その後に薬剤投与がなされて高熱，発疹を生じている[6)7)21)]．SJS/TENの発症機序は不明であるが，このような共通する病歴と初期症状から，最初に何らかのウイルス感染を生じ，その後に薬剤が契機となって眼表面，口唇・口腔内，爪囲を主座とする病変を生じていると考えられる．

HLAクラスⅡは，抗原提示細胞に発現しており，T細胞を介した免疫応答に大きく関与している．一方，HLAクラスⅠは，上皮細胞を含むほぼすべての細胞に発現しており，ウイルス抗原を提示することにより，ウイルス感染に対する生体反応に大きく関与する．SJS/TENの発症素因に，HLAクラスⅡではなく，HLAクラスⅠであるHLA-A*0206が大きく関与していることは，その発症にウイルス感染が何らかの形でかかわっている可能性があるという我々の考え[22)~24)]を支持する結果であると考えられる．

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利益相反：利益相反公表基準に該当なし

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ORIGINAL ARTICLE

A whole-genome association study of major determinants for allopurinol-related Stevens–Johnson syndrome and toxic epidermal necrolysis in Japanese patients

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Stevens–Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) are severe, cutaneous adverse drug reactions that are rare but life threatening. Genetic biomarkers for allopurinol-related SJS/TEN in Japanese were examined in a genome-wide association study in which Japanese patients ($n=14$) were compared with ethnically matched healthy controls ($n=991$). Associations between 890 321 single nucleotide polymorphisms and allopurinol-related SJS/TEN were analyzed by the Fisher's exact test (dominant genotype mode). A total of 21 polymorphisms on chromosome 6 were significantly associated with allopurinol-related SJS/TEN. The strongest association was found at rs2734583 in *BAT1*, rs3094011 in *HCP5* and GA005234 in *MICC* ($P=2.44\times10^{-8}$; odds ratio = 66.8; 95% confidence interval, 19.8–225.0). rs9263726 in *PSORS1C1*, also significantly associated with allopurinol-related SJS/TEN, is in absolute linkage disequilibrium with *human leukocyte antigen-B*5801*, which is in strong association with allopurinol-induced SJS/TEN. The ease of typing rs9263726 makes it a useful biomarker for allopurinol-related SJS/TEN in Japanese.
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Keywords: allopurinol; Stevens–Johnson syndrome; toxic epidermal necrolysis; human lymphocyte antigen; single nucleotide polymorphism; genome-wide association study

Introduction

Allopurinol is a xanthine oxidase inhibitor that prevents the production of uric acid to reduce plasma uric acid levels to a normal range. It is the most frequently used anti-hyperuricemic agent in the world due to its long-term pharmacological effect.[1] However, allopurinol is also one of the most frequent causes of a variety of delayed severe cutaneous adverse drug reactions (SCARs).[2] According to spontaneous reports of severe adverse drug reactions to the Ministry of Health, Labor, and Welfare of Japan, allopurinol-related SCARs accounted for about 11% of all reported SCAR cases in Japan in 2008.[3] Allopurinol-related SCARs include the drug-induced hypersensitivity syndrome, Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN).[4] SJS/TEN are characterized by high fever, malaise and rapid development of blistering exanthema, with macules and target-like lesions, accompanied by mucosal involvement.[5] Even though the incidence of SJS/TEN is extremely low, the mortality rate of TEN can be as high as 26%.[5] Therefore, SJS/TEN is a serious problem in allopurinol therapy, in spite of the ideal anti-hyperuricemic effect of allopurinol.

Although previous works have suggested that the development of SJS/TEN depends on an immune mechanism involving a drug-dependent cytotoxic cell response against epidermal cells,[5,6] the pathophysiology of SJS/TEN remains largely unknown. Susceptibility to such idiosyncratic reactions is thought to be genetically determined, and familial predisposition to allopurinol-induced SJS/TEN has been reported.[6] Therefore, the exploratory studies for genetic risk factors related to SJS/TEN are needed. A strong association has been observed between allopurinol-induced SCAR and the human lymphocyte antigen (*HLA*) allele B variant (*HLA-B*5801*) in the Han Chinese in Taiwan[7] and in the Thai population.[8] These studies showed that the *HLA-B*5801* allele is present in all patients with allopurinol-induced SCAR (51/51 of Han Chinese and 27/27 of Thai patients) and in only 12–15% of tolerant patients (20/135 and 7/54, respectively). The odds ratio (OR) was 580 (95% confidence interval , 34–9781; $P=4.7\times10^{24}$) for the Han-Chinese data[7] and 348.3 (95% confidence interval, 19.2–6336.9; $P=1.61\times10^{13}$) for the Thai study.[8] Although the association was confirmed in both Caucasian and Japanese subjects,[9,10] the OR in the Han-Chinese and Thai populations were much higher than those in the Caucasian (OR=80) and Japanese (OR=40) groups. These reports indicated that *HLA-B*5801* is the valid genetic biomarker for allopurinol-induced SJS/TEN in various ethnic groups, but the mechanisms by which *HLA-B*5801* is specifically involved in allopurinol-induced SJS/TEN progression and the strength of the association showed ethnic differences are unknown.

Currently, genotyping by high-density array scanning of the whole genome allows discovery of previously unsuspected genetic risk factors that influence the pathogenesis of serious adverse drug reactions.[11–13] Genome-wide association studies (GWASs) provide opportunities to uncover polymorphisms that influence susceptibility to allopurinol-induced SJS/TEN free of mechanistic hypotheses. Therefore, in addition to *HLA-B* typing as shown in our previous study,[10] we further conducted a retrospective pharmacogenetic case–control study using whole-genome single nucleotide polymorphism (SNP) data from high-density DNA microarrays in order to identify new and effective genetic biomarkers for allopurinol-related SJS/TEN in Japanese patients.

Materials and methods

Recruitment of study subjects

A total of 141 Japanese SJS/TEN patients from unrelated families were recruited from July 2006 to April 2010 from participating institutes of the Japan Severe Adverse Reactions (JSAR) research group and through a nationwide blood-sampling network system in Japan for SJS/TEN onset patients, operated by the National Institute of Health Sciences.[10] In all, 121 of these patients were diagnosed as defined SJS or TEN by JSAR research group's dermatological experts based on diagnostic criteria[4] that are currently used in Japan. Information was collected using a standardized case report form that includes medical records, co-administrated drug records, disease progress and involvement of systemic complications, as well as SJS/TEN treatment. Among the 141 SJS/TEN patients, 20 were diagnosed as probable SJS due to atypical or mild symptoms. TEN and SJS were defined as mucocutaneous disorders characterized by extensive erythema, blisters, epidermal detachment, erosions, enanthema and high fever. SJS was defined as skin detachment of 10% or less of the body surface area, and TEN as skin detachment of more than 10%, excluding staphylococcal scaled skin syndrome.[5] In all enrolled cases defined as SJS or TEN, allopurinol was regarded as the drug responsible for SJS or TEN if the onset of SJS/TEN symptoms occurred within the first 2 months of allopurinol exposure. For the retrospective pharmacogenetic case–control study, 991 healthy, ethnically matched subjects in the Tokyo metropolitan area were used as the control group. Healthy subjects were used as the control group instead of allopurinol-tolerant patients because the incidence of SJS/TEN is extremely low (0.4–6 per million per year).[3]

The ethics committees of the National Institute of Health Sciences, each participating institute of the JSAR research group and the Japan Pharmacogenomics Data Science Consortium (JPDSC) approved this study. Written informed consent was obtained from all cases and ethnically matched controls.

Whole-genome genotyping of SNPs

Genome-wide genotyping of the 14 allopurinol-related SJS/TEN patients and 991 ethnically matched controls was conducted using the Illumina Human 1M-Duo BeadChip (Illumina, San Diego, CA, USA), which contained 1 163 218 SNPs. SNPs were discarded from case–control association analysis if they exhibited a minor allele frequency <0.001 in the control group (237 890 SNPs), a call rate <0.95 for each SNP (32 640 SNPs) or a *P*-value <0.001 in the test of Hardy–Weinberg equilibrium among controls (2368 SNPs). These quality control steps removed a total of 272 897 SNPs. All samples had a call rate for each microarray above 0.99. Sample duplicates and hidden relatedness were investigated on the basis of pairwise identity-by-state analysis via PLINK;[14] however, there was no duplicate or hidden relatedness in the samples. This quality-control procedure ensured reliable genotyping data.

HLA genotyping and TaqMan genotyping of SNPs on chromosome 6

HLA A, *B* and *Cw* types were determined using sequencing-based methods, as described previously.[10] Representative SNPs of 6p21 (rs2734583, rs3099844, rs9263726 and rs3131643) were re-genotyped using TaqMan SNP Genotyping Assays (Life Technologies, Carlsbad, CA, USA) (ID; C__27465749_10, C__27455402_10, C__30352071_10, C__26778946_20) according to the manufacturer's instruction using 5 ng of genomic DNA. We did not genotype rs9267445 and rs1634776 because TaqMan SNP genotyping assays for these SNPs were not available. Measurement of the linkage disequilibrium (LD) coefficient was performed using

the *HLA* types and 6p21 SNPs of the 141 Japanese SJS/TEN cases and an additional 65 Japanese individuals (non-SJS/TEN patients). The LD coefficient was calculated as previously described.[15,16]

Association analysis

Genome-wide SNPs data from allopurinol-related SJS/TEN cases and ethnically matched controls were used for association analysis using the Fisher's exact test based on the dominant genotype mode and minor allele frequencies of each SNP. Because there are no homozygotes of minor alleles of SNPs, which have significantly related to allopurinol-related SJS/TEN except rs3099844 and rs3131643 in 'Case group', other association analysis models such as trend test (Cochran–Armitage analysis) or recessive model analysis were not applied in this study. All association analyses were carried out with PLINK.[14] *P*-values were corrected for multiple testing according to the Bonferroni's correction. *P*-values $<5.62\times10^{-8}$ were regarded as statistically significant.

Results

Characteristics of study subjects

A total of 14 allopurinol-treated Japanese patients, who were diagnosed with definite SJS/TEN were recruited for the whole-genome association study (IDs 1–14 in Table 1). Patients, IDs 1, 2, 3, 9, 10, 13 and 14 were reported in our previous paper.[10] After the GWAS, an additional four allopurinol-treated Japanese SJS/TEN patients were recruited for *HLA* typing (IDs 15–18). Therefore, a total of 18 allopurinol-treated Japanese SJS/TEN patients participated in the study (Table 1). In all, 12 of 18 patients were male and 6 were female, and the average age was 72.3 ± 10.0 (mean $\pm$ s.d.) years. In all, 12 of 18 cases showed systemic complications of liver and/or renal dysfunction, and most patients had high fever. The average period of SJS/TEN onset after allopurinol treatment was 21.7 ± 11.9 days. Drug-induced lymphocyte stimulation tests were examined in 13 of 18 patients to determine the causative agent; however, in these tests, only two cases (IDs 1 and 5) were positive for allopurinol and only one (ID 16) was positive for oxipurinol, a metabolite of allopurinol. The patient (ID 1) who was positive for the drug-induced lymphocyte stimulation test for allopurinol was also positive for other co-administrated drugs (Table 1). On the other hand, patients who received a patch test showed positive reactions for allopurinol although only two patients were examined (ID 4, 10). The patient who was patch test positive for allopurinol (ID 4) was also patch test positive for other co-administrated drugs (Table 1). Four patients (ID 1, 2, 4 and 14) were co-administrated non-steroidal anti-inflammatory drugs, four (ID 7, 8, 11 and 15) were co-administrated angiotensin II receptor antagonists and three (ID 4, 7 and 17) were co-administrated statin anti-hyperlipemic agents.

Whole-genome association study of major determinants for allopurinol-related SJS/TEN

A total of 14 allopurinol-related SJS/TEN patients (IDs 1–14), who were diagnosed with definite SJS/TEN, and 991 ethnically matched controls, were genotyped with the use of the Illumina Human 1M-Duo BeadChip containing 11 63 218 SNPs. A series of quality-control steps resulted in the elimination of 2 72 897 polymorphisms. For each SNP, Fisher's exact tests were performed to compare the dominant genotype distributions and minor allelic frequencies in the allopurinol-related SJS/TEN patients (the case group) versus those in the ethnically matched healthy control group. The resulting *P*-values were adjusted with the Bonferroni's correction ($P<5.62\times10^{-8}$). The distribution of *P*-values from the Fisher's exact tests (dominant genotype mode) along each chromosome indicated that 21 SNPs were significantly associated with the cases, all of which were located on the chromosome 6: 6p21.3, 6p22.1 and 6p21.1 (Figures 1a and b). The quantile–quantile (Q–Q) plot for the distribution of *P*-values showed that observed *P*-values matched the expected *P*-values over the range of $0<-\log_{10}(p)<4.0$ (Figure 2). A departure was observed at the extreme tail ($-\log_{10}(p)>4.0$) of the distribution of test statistics for the allopurinol-related Japanese SJS/TEN, suggesting that the identified associations are likely due to true variants rather than potential biases such as genotyping error. These SNPs, with their associated genes, are described in Table 2. As is observed in all SNPs in Table 2, minor allele frequencies in the controls were quite small, ranging around 0.5–0.6%. The genotypic distributions of the case and control groups are identical among groups with the same *P*-value, suggesting that these SNPs might be linked. These SNPs also have ORs that are much higher than the ORs of SNPs commonly observed in sporadic cancer and other complex diseases, suggesting they are of higher penetrance. For example, the most significant SNPs (rs2734583, rs3094011 and GA005234) had an OR of 66.8 (95% confidence interval, 19.8–225.0), and the twentieth most significant SNPs (rs9263827 and rs1634776) had an OR of 60.9 (95% confidence interval, 18.3–202.5). Most SNPs in Table 2 are associated with known or predicted genes; of these, 13 are in known genes. Three SNPs (rs17190526, rs9263726 and rs2233945) were found in *PSORS1C1* (psoriasis susceptibility 1 candidate 1), which is considered as one of the potential psoriasis genes.[17–19] The *CCHCR1* (coiled coil α helical rod protein 1), which is a regulator of keratinocyte proliferation or differentiation and is over-expressed in keratinocytes in psoriatic lesions,[20–23] contained four SNPs (rs9263745, rs130077, rs9263781 and rs9263785). *HCP5* (HLA complex P5), which is involved in hypersensitivity to abacavir,[24–26] had three SNPs (rs3094011, rs3099844 and rs31431643). *TCF19* (transcription factor 19), which is a potential trans-activating factor that might play an important role in the transcription of genes required for the later stages of cell cycle progression,[27] contained two SNPs (rs9263794 and rs10448701). Two SNPs (rs9263796 and rs9263800) were also found in *POU5F1* (POU class 5 homeobox; alternative names for Oct4). *BAT1* (HLA-B

Table 1 Summary of clinical characteristics of Japanese patients with allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis

								Co-administered drugs	
Patient ID[a]	*ADR type*	*Sex/age (years)*	*Highest BT (°C)*	*Total area of blistering skin (%)*	*Systemic complications*	*DLST to allopurinol (PT)*	*Period of onset (days) by allopurinol*	*Drug name*	*DLST result/period of onset*
1	SJS	F/53	38.1	0.5	liver dysfunction renal dysfunction	+	26	loxoprofen clarithromycin	+/9 days +/26 days
2	TEN	M/58	37.1	15	neutropenia liver dysfunction	–	ca 10 days	loxoprofen levofloxacin	–/1 day –/1 day
3	SJS	M/77	unknown	unknown	none	not tested	16	none	
4	TEN	F/72	>37	20	none	–(PT+)	16	pitavastatin lansoprazole salicylamide, acetaminophen, caffeine, promethazine, methylenedisalicylate serrapeptase loxoprofen acetaminophen	–/16 days –/179 days –(PT+)/8 days –/1 day –/8 days (PT+)/8 days
5	TEN	M/82	39	35	none	+	52	none	
6	SJS	M/67	1	1	liver dysfunction	not tested	14	none	
7	SJS	M/76	38.8	unknown	GI tract disturbance liver dysfunction renal dysfunction	not tested	<26 days	losartan furosemide carbon atorvastatin	not tested/8 days not tested/3 days not tested/7 days not tested/8 days
8	SJS	M/83	>38	10	renal dysfunction	–	20	amlodipine olmesartan medoxomil	not tested/very long not tested/very long
9	TEN	M/75	>38	20	neutropenia liver dysfunction renal dysfunction	–	6	none	
10	SJS	M/75	38.4	6	neutropenia liver dysfunction renal dysfunction	–(PT+)	14	none	
11	SJS	M/74	37.8	8	neutropenia liver dysfunction renal dysfunction	–	38	cefazolin Furosemide Sodium polystyrene sulfonate olmesartan medoxomil	not tested/1 day not tested/53 day not tested/51 day not tested/59 day
12	SJS	M/67	38.9	2	liver dysfunction	not tested	17	none	
13	SJS	F/81	39.2	0.5	renal dysfunction	–	28	spironolactone	–/24 days
14	SJS	M/83	39	0	respiratory involvement	–	29	diclofenac	–/1 day
15	TEN	F/73	38	10	liver dysfunction renal dysfunction	–	27	valsartan epoetin β	–/18 days –/2 days
16	SJS	M/53	40	5	liver dysfunction	–(oxipurinol +)	19	none	
17	SJS	F/86	38	0	liver dysfunction renal dysfunction	–	30	rosuvastatin	–/43 days
18	TEN	F/66	37.8	15	none	not tested	2	none	

Abbreviations: ADR, adverse drug reaction; BT, body temperature; DLST; drug-induced lymphocyte stimulation test; F, female; M, male; PT, patch test; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis.

[a]Patients ID 1–14 were applied for whole genome analysis. ID 1–18 were for the *HLA* typing and the analysis of linkage disequilibrium.

Patients IDs 1, 2, 3, 9, 10, 13, and 14 were reported in our previous paper.[10]

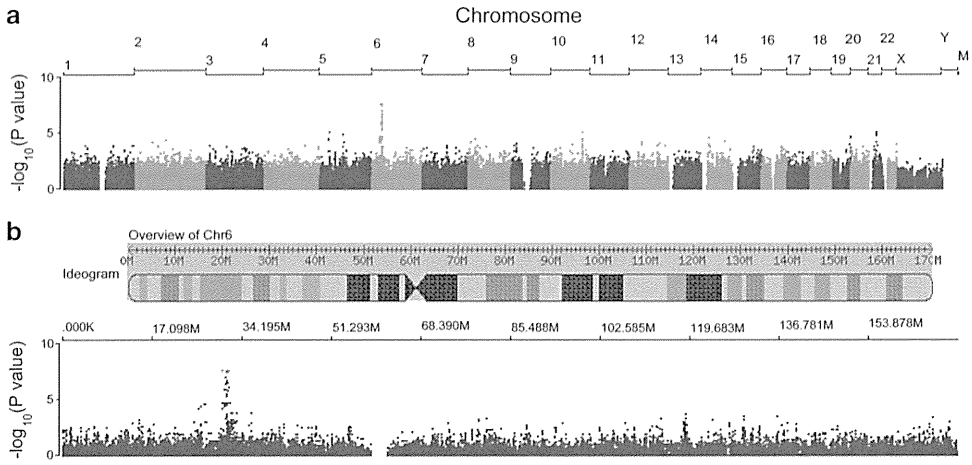

Figure 1 Genome-wide association study of allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis. Each dot represents a single nucleotide polymorphism (SNP). The *x* axis: the position of the SNP on chromosomes. The *y* axis: the $-\log_{10}$ of Fisher's exact test *P*-values (dominant genotype mode) of the SNP in the case–control association study. SNPs with *P*-values $<5.62\times10^{-8}$ are highlighted in red. (**a**) Whole genome. (**b**) Chromosome 6.

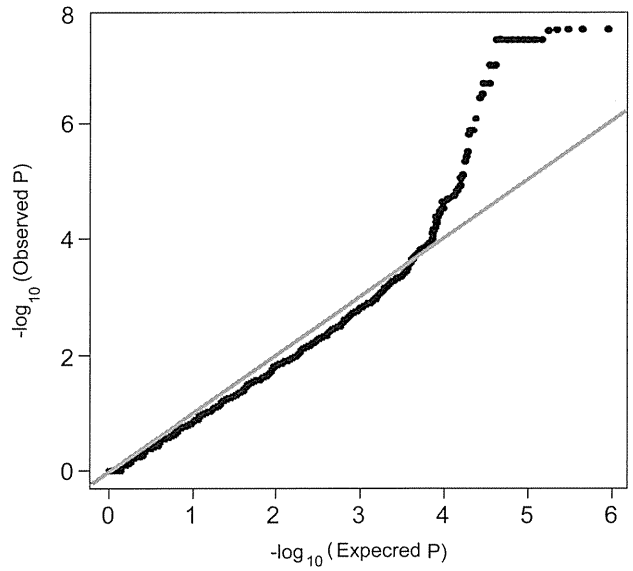

Figure 2 Quantile–quantile plot of Fisher's exact test statistics obtained from the genome-wide association study for allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis under dominant genotype mode. The solid red line represents the null model where observed Fisher's exact test values match the expected values. The dots represent observed versus the expected values from the case–control study.

associated transcript 1) and *PSORS1C3* each carried one SNP (rs2734583 and rs9263827). The SNPs, rs1634776 and rs4084090, were located in more than 10 kb away from the *HLA-B* and *HLA-C* genes, respectively. Two pseudo genes, *MICC* (major histocompatibility complex class I polypeptide-related sequence) and *PPIAP9* (peptidylprolyl isomerase A (cyclophilin A) pseudogene 9), had one SNP each (GA005234 and rs9267445). Previous report using Han-Chinese patients with allopurinol-induced SCAR indicated rs3117583 of *BAT3*, rs1150793 of *MSH5* and rs2855804 of *MICB*, which are located in *HLA* region, showed significant *P*-values ($P<1\times10^{-7}$).[7] In this study using Japanese patients, both rs3117583 and rs1150793 showed $P=6.34\times10^{-3}$ (allele frequency mode) and $P=6.14\times10^{-3}$ (dominant genotype mode). There was no data of rs2855804 in the Illumina Human 1M-Duo BeadChip.

HLA types of allopurinol-related SJS/TEN patients

Classical class I *HLA* types (*A*, *B* and *Cw*) of allopurinol-related SJS/TEN patients were determined because the *HLA-B*5801* type is associated with allopurinol-related SCARs in Han Chinese,[7] Caucasians[9] and Japanese[10] (Table 3). In this analysis, four patients with allopurinol-related SJS/TEN (IDs 15–18), who were recruited after BeadChip analysis, joined the case group (total of 18 allopurinol-related SJS/TEN patients). Eight cases of *HLA-A*3303* (allele frequency = 22.2%), 10 cases of *HLA-B*5801* (allele frequency = 27.8%) and 10 cases of *HLA-Cw*0302* (allele frequency = 27.8%) were found in 18 allopurinol-related SJS/TEN patients (Table 3). By comparison, the allelic frequencies of *HLA-A*3303*, *HLA-B*5801* and *HLA-Cw*0302* were 7.9%, 0.6% and 0%, respectively in Japanese general population (Tables 4a–c). The OR of *HLA-A*3303* was calculated as 3.32 (Table 4a). The OR of *HLA-B*5801* was calculated as 62.8 (Table 4b), which was a little larger than the previously reported OR in Japanese patients.[10] *HLA-Cw*0302* also showed significant association with allopurinol-related SJS/TEN (Table 4c). *HLA-A*3303* and *HLA-Cw*0302* are in LD with *HLA-B*5801* in the Japanese although the general frequency of *HLA-A*3303* is higher than other two types. Other *HLA-A*, *B* and *Cw* types, which were not listed in Tables 4a–c, showed very low frequencies in the general Japanese population, or were not found in 18 allopurinol-related SJS/TEN patients.