Effects of Amniotic Membrane–Derived Fibroblast Supernatant on Corneal Epithelium

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PURPOSE. To evaluate the effects of human amniotic membrane–derived fibroblast (AMF) cell supernatant (AMF-sup) on corneal epithelium.

METHODS. The phenotype of AMF cells was analyzed by flow cytometry using cell-surface markers. AMF cells were also induced to form osteoblasts and neural cells, and cell phenotypes were observed by staining and RT-PCR. Cultivated human corneal limbal epithelial sheets generated using AMF-sup were analyzed using immunohistochemistry and colony-forming efficiency, and the wound healing of epithelial defects was observed using a tissue-punch method. The effects of instillation of each supernatant in a rabbit corneal epithelial wound healing model were compared.

RESULTS. Mesenchymal stem cell (CD29, CD44, CD73, and CD90) and neural crest (CD49d and CD56) markers were expressed on the AMF cell surface. Following induction of differentiation, isolated AMF cells showed characteristics of osteoblasts and neural cells. Application of AMF-sup resulted in maintenance of the limbal epithelial phenotype and immature state, and significantly promoted wound healing in cultivated human corneal limbal epithelial sheets (P < 0.05) and rabbit corneal epithelium (P < 0.05) compared with the control.

CONCLUSIONS. These data suggest that AMF cells have multi-differentiation potential, and that AMF-sup is effective in maintaining the limbal epithelial phenotype and promoting corneal epithelial wound healing, which may be of value in ocular surface reconstruction.

Keywords: amniotic membrane fibroblast, supernatant, corneal wound healing

The amniotic membrane (AM) is the innermost fetal membrane. It is an embryonic membrane that consists of epithelial tissue of ectodermal origin and mesenchymal tissue of mesodermal origin. The AM exhibits anti-inflammatory effects and promotes wound healing; it has abundant levels of collagen and lacks blood vessels. The AM is of interest in regenerative medicine as an alternative substrate for many tissues such as skin,1 blood vessels,2 trachea,3 and cornea.4 We transplanted limbal tissue, or cultivated corneal epithelial sheets containing corneal epithelial stem cells that used the AM as a substrate, in patients with severe ocular surface diseases associated with corneal stem cell deficiency, such as Stevens-Johnson syndrome.5,6 We also reported previously that the AM expresses the major histocompatibility complex (MHC) class I gene, HLA-G, expression of which is upregulated in corneal and conjunctival epithelial cells cultured on AM.7 The AM also exhibits anti-inflammatory effects in ocular surface diseases with severe inflammation by trapping inflammatory cells that infiltrate the ocular surface. This process is mediated by interaction between hyaluronic acid (HA) expressed on the AM and CD44 expressed on inflammatory cells.8,9 Although several studies have shown that AM has many beneficial effects, many of the underlying mechanisms remain unknown.

Mesenchymal stem cells (MSCs) exhibit multipotency, differentiating into osteoblasts, chondrocytes, and adipocytes. MSCs have been observed in various tissues, including skin,10 adipose,11 bone marrow,12 muscle,13 and dental pulp.14 MSCs also exhibit tissue regeneration effects such as improved cardiac function,15 revascularization,16 and intestinal tissue repair,17 and are important for tissue homeostasis and wound healing. Recently, it was reported that bone marrow–derived MSCs were transplanted into rabbit and rat models of chemical burns to test the wound healing benefits of MSCs in corneal diseases.18,19 However, MSCs are also found in AM, with the ability to differentiate not only into mesenchymal cells such as osteoblasts, chondrocytes, and adipocytes but also into cardiomyocytes or hepatocytes beyond the germ layer.20,21 AM-derived MSCs have immunosuppressive effects involving secretion of immunosuppressive HLA-G, low expression of MHC class I, and no expression of MHC class II antigens, so they are useful as an allogeneic cell source for regenerative tissues.22,23 It has been reported that the stem cell secretome is a major player in the immune-modulating and regenerative properties of AM-derived MSCs.24

We therefore hypothesized that AM-derived fibroblast (AMF) cell secretome might play a role in wound healing in the cornea. In this study, isolated AMF cells similar to MSCs were analyzed using differentiation methods and flow cytometry. We demonstrated the benefits of AMF supernatant (AMF-sup), representing the AMF cell secretome, in wound healing models of human corneal epithelial sheets and rabbit cornea.
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**Materials and Methods**

**Antibodies**

Mouse monoclonal antibodies specific to bIII-tubulin (TuJ-1), neurofilament medium (NF-09), keratin 15 (K15; LH15), and p63 (4A4) were obtained from R&D Systems (Minneapolis, MN, USA), Abcam (Cambridge, UK), Lab Vision (Fremont, CA, USA), and Santa Cruz Biotechnology (San Diego, CA, USA), respectively. Rabbit polyclonal antibody specific to K12 was obtained from TransGenic, Inc. (Kumamoto, Japan). Isotype mouse IgG2a and rabbit IgG were obtained from Dako Cytomation (Glostrup, Denmark) and Jackson ImmunoResearch Laboratories (West Grove, PA, USA), respectively. Fluorescein isothiocyanate (FITC)-, rhodamine-, and Cy3-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories.

**Preparation of Amniotic Membrane (AM) and AM-Derived Fibroblast (AMF) Cell Isolation**

This study was performed in accordance with the Declaration of Helsinki. AM was supplied by the Tokyo Dental College General Hospital Amniotic Membrane Bank following approval of the experimental protocol by the Tokyo Dental College Ethics Committee, subject to informed consent, and the Japanese Society of Tissue Transplantation. The cryopreserved AM (5 × 5 cm) was washed twice with phosphate-buffered saline (PBS), cut into small pieces, and treated with 2 mg/mL collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 37°C overnight to isolate human AMF cells. Then, the isolated AMF cells were cultured in Advanced Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C in humidified air with 5% CO2 for 2 weeks. The AMF cells were expanded via two or three passages and used in subsequent experiments.

**Flow Cytometry**

AMF cells from passage 2 or 3 were harvested, washed twice with PBS and incubated with TrypLE (Invitrogen, Carlsbad, CA, USA) at 37°C, and collected. The AMF cells were fixed with 4% paraformaldehyde (PFA; Wako Pure Chemical Industries, Ltd.) at 4°C for 5 minutes, washed with 2 mL 0.1% sodium azide (Wako Pure Chemical Industries, Ltd.) at 4°C for 7 minutes, and centrifuged at 2380g for 5 minutes. Suspensions of 1.0 × 10^6 cells/mL were incubated with individual antibodies (Table 1) for 30 minutes on ice and measured using a flow cytometer (EPICS XL; Beckman Coulter, Fullerton, CA, USA). Flow cytometry data from 5000 events were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). In the control samples, isotype antibodies conjugated with FITC or phycoerythrin (Beckman Coulter) were used.

**In Vitro Differentiation**

On reaching semiconfluence, the AMF cells were washed with PBS and incubated with TrypLE (Invitrogen) at 37°C for 7 minutes. The collected cells were seeded at a density of 1.0 × 10^5/cm^2 on four-well chamber slides (LAB-TEK; Nalge Nunc International, Rochester, NY, USA) or at 5.0 × 10^3/cm^2 in six-well plates (Corning, Lowell, MA, USA).

For osteogenic induction, the AMF cells were cultured in Advanced DMEM containing 10% FCS. Once AMF cells reached confluency, they were cultured further in osteogenic induction medium (Lonza, Walkersville, MD, USA) containing dexamethasone, ascorbate, and growth supplement with L-glutamine, β-glycerophosphate, and gentamycin (GA-1000; Lonza). The medium was changed three times per week, and the cultures were analyzed after 2 weeks.

For neurogenic induction, AMF cells were cultured on fibronectin (PromoCell, Heidelberg, Germany)-coated chambers or plates in Advanced DMEM containing 10% FCS until

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they reached confluency. Chambers or plates were coated with 10 μg/mL fibronectin for 10 minutes at room temperature. The AMF cells were cultured further in neurogenic differentiation medium (PromoCell). The control groups were cultured in Advanced DMEM containing 10% FCS. The medium was changed three times a week, and the cultures were analyzed after 2 weeks.

Alkaline Phosphatase Staining

After 2 weeks of culture, the AMF cells were fixed in 0.4% cold PFA, rinsed twice with alkaline phosphatase (ALP) solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 10 mM MgCl₂), and stained with a bromocresol purple solution (Roche, Basel, Switzerland) for 30 minutes at 37°C. Slides were rinsed with deionized water and observed under a microscope.

Alizarin Red S Staining

Cultured AMF cells were fixed in 70% cold ethanol for 10 minutes and rinsed with deionized water. The fixed AMF cells were stained with Alizarin Red S solution (Sigma-Aldrich Corp., St. Louis, MO, USA) for 10 minutes at room temperature. Stained AMF cells were rinsed with deionized water and observed under a microscope.

Immunostaining

Frozen sections of cultivated human corneal limbal epithelial sheets (hCLESs) using supernatant, or chamber slides of cultivated AMF cells in the neurogenic induction were fixed for 10 minutes in 2% PFA or acetone (Wako Pure Chemical Industries, Ltd.) on ice. Both fixed specimens were blocked by incubation with 3% normal donkey serum (Chemicon Int., Inc., Temecula, CA, USA), 1% bovine serum albumin (Sigma-Aldrich Corp.), and 0.3% Triton X-100 (Sigma-Aldrich Corp.) for 1 hour at room temperature to permeabilize the cell membranes. Antibodies were applied for 90 minutes at room temperature, followed by incubation with FITC-, rhodamine-, and/or Cy3-conjugated secondary antibodies. Primary antibodies were substituted with isotype antibodies as negative controls (Table 2). After three washes with PBS, both specimens were incubated with 0.5 μg/mL 4’,6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Tokyo, Japan) at room temperature for 5 minutes. Finally, both specimens were washed three times in PBS, and a coverslip was added using an aqueous mounting medium containing an antifading agent (Fluoromount/Plus;Diagnostic Biosystems, Pleasanton, CA, USA). Images were observed under a fluorescence microscope (Axioplan 2 Imaging; Carl Zeiss, Inc., Thornwood, NY, USA).

Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from AMF cells after osteogenic or neurogenic induction using an SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Complementary DNA was prepared from total RNA using 0.25 M dithiothreitol, 5X reaction buffer, RNase inhibitor, and avian myeloblastosis virus reverse transcriptase (Takara Bio, Inc., Shiga, Japan) by incubating a 25-μL mixture at 41°C for 1 hour. The cDNA was used as a template for polymerase chain reaction (PCR) amplification. Amplification (1 μL cDNA in a total reaction volume of 50 μL) was performed for three cycles of 95°C for 1 second, 52°C for 30 seconds, and 72°C for 20 seconds, followed by 25 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 20 seconds on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Primer sequences, reaction conditions, and product sizes are provided in Table 2. Amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed in the same manner to verify cDNA quality. Amplification products were separated by electrophoresis on 1.5% agarose gels (Takara Bio, Inc.).

Preparation of AM Supernatant (AM-Sup) and AMF-Sup

To eliminate other influences, such as the effect of growth factors, we prepared DMEM/F12 containing 100 U/mL penicillin (Wako Pure Chemical Industries, Ltd.) and 10 mg/mL streptomycin (Wako Pure Chemical Industries, Ltd.) without the culture medium supplements (DF–). DF– was used as a negative control. For preparation of AM-sup, semiconfluent AMF cells were washed with DF-- twice to eliminate the influence of FCS and incubated with DF– at 37°C and 5% CO₂ for 2 days. AM-sup, the cryopreserved AM (5 × 5 cm) was washed with DF-- twice and incubated with DF– at 37°C and 5% CO₂ for 2 days. After 2 days, the supernatants were separated from cells and debris using a 0.22-mm filter (Millipore, Burlington, MA, USA). AM-sup was used as a positive control. These supernatants, which were not concentrated and were without FCS and supplements, were used in the following experiments.

Cultivation of Human Corneal Limbal Epithelial Sheets

Human corneas were obtained from Eye Banks in the United States for research purposes. Limbal rims of corneoscleral tissue were prepared by careful removal of excess sclera, iris, and corneal endothelium. Limbal epithelial cells were isolated as described previously.25 Dispersed epithelial cells (2 × 10⁶/mL) were seeded onto inserts in Advanced DMEM/Ham’s F-12...
medium (F12) containing 20 ng/mL recombinant human epidermal growth factor (Invitrogen), B27 supplement (Invitrogen), 100 U/mL penicillin (Wako Pure Chemical Industries, Ltd.), and 10 mg/mL streptomycin (Wako Pure Chemical Industries, Ltd.)

26 Seeded limbal epithelial cells were submerged in medium until confluence and cultured at the air–liquid interface for 4 days. To compare the effects of the supernatants, the medium used for hCLES culture was replaced with DF/C0, AM-sup, or AMF-sup in the air–liquid interface culture for 4 days.

**Colony-Forming Efficiency (CFE)**

To evaluate the proliferative potential of cells in hCLESs cultured with each supernatant (i.e., DF/C0, AM-sup, or AMF-sup), mitomycin C (MMC; Sigma-Aldrich Corp.) treated with NIH 3T3 fibroblasts was used in a CFE assay as described previously.27–29 NIH 3T3 fibroblasts in DMEM containing 10% FCS were treated with MMC (4 µg/mL) for 2 hours at 37°C. Each dish was seeded with 3 × 10^2 cells. Growth capacity was evaluated on day 14, when cultured cells were stained with rhodamine B (Wako Pure Chemical Industries, Ltd.) for 30 minutes. The number of colonies obtained from each sheet (n = 4) was determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA). CFE (%) was calculated using the following formula: CFE (%) = number of colonies / number of seeded cells (3 × 10^2 cells/dish) × 100.

**In Vitro Wound Healing Model**

Epithelial defects in hCLESs were generated by physically scraping the epithelium with a gill corneal knife (Bausch & Lomb Surgical, Inc., St. Louis, MO, USA) and making a mark with a 6-mm punch biopsy tool (KAI Industries Co., Ltd., Gifu, Japan). The hCLESs were then incubated with DF/C0, AM-sup, or
Amniotic Membrane Fibroblast Supernatant

AMF-sup until wound closure. Wound closures were observed daily using a stereoscopic microscope (Olympus, Tokyo, Japan). Epithelial defect areas \((n=6)\) were analyzed using ImageJ software.

In Vivo Wound Healing Model

All experimental procedures and protocols were approved by the Animal Care and Use Committee of Tokyo Dental College, and conformed with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, institutional guidelines, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female Japanese white rabbits (2.5 kg; Shiraishi Animals Co., Ltd, Saitama, Japan) were anesthetized intravenously with a mixture of medetomidine hydrochloride (0.5 mg/kg; Domitor; Meiji Seika Kaisha, Tokyo, Japan), midazolam hydrochloride (2.0 mg/kg; Dormicaum; Astellas Pharma, Tokyo, Japan), and butorphanol (0.5 mg/kg; Vetorphale; Meiji Seika Kaisha). Corneal epithelial defects were created in the left eye \((n=6)\) by physically scraping the corneal epithelium with a grinder (Kohnan Shoji Co., Osaka, Japan) and making a mark with an 8-mm punch biopsy tool (KAI Industries Co., Ltd.). Following surgery, each supernatant \((DF-, AM-sup, or AMF-sup)\) was transferred to an eye drop bottle, and one drop of approximately 50 \(\mu L\) was applied three times a day until wound closure. Epithelial defect areas were observed by fluorescent imaging and quantified using ImageJ.

Statistical Analysis

Statistical comparisons for the CFE and wound-closure assays were performed by nonpaired Student's \(t\)-test using Excel software (Microsoft, Redmond, WA, USA). \(P < 0.05\) was considered statistically significant.

RESULTS

Analysis of AMF Cell Characteristics

The expression profiles of isolated and expanded AMF cells were analyzed by flow cytometry (Fig. 1). MSC (CD29, CD44, CD73, and CD90) and neural crest (CD49d and CD56) markers, as well as inflammatory substance-degrading enzyme (CD10) and a fibroblast marker (FSP-1), were expressed on the AMF cell surface (Fig. 1). Some cells were negative for other MSCs (CD105, CD146, CD166, and STRO-1) and hematopoietic cell (CD14, CD34, and CD45) and endothelial cell (CD31) markers (Fig. 1). Following osteogenic induction, AMF cells showed ALP and Alizarin Red S-positive staining similar to osteoblasts (Figs. 2A, 2B). Although ALP and osteopontin were expressed only weakly, their expression was upregulated following induction of differentiation (Fig. 2C). AMF cells also exhibited expression of TuJ-1, neurofilament medium, and myelin basic protein following neurogenic induction (Figs. 2D–F).
Effect of AMF-Sup on hCLESs

The hCLESs were cultured with DF−, which is DMEM/F12 but without supplements, AM-sup, or AMF-sup at the air–liquid interface for 4 days. Human CLESs cultured with AM-sup exhibited a corneal limbal phenotype and maintained K15 and p63 expression compared with DF− cultures; this phenotype was similar to the hCLES phenotype observed following culture with AM-sup (Figs. 3A, 3B). Human CLESs cultured with AMF-sup had greater CFE compared with those cultured with DF− (Figs. 4A, 4B; n = 4, P < 0.05).

Effect of AMF-Sup on Wound Healing Models

We observed wound closure following generation of an epithelial defect in hCLESs as an in vitro wound healing model. Human CLESs cultured with AM-sup and AMF-sup promoted wound closure compared with DF− alone after 3 days (Figs. 5A, 5B; n = 6, P < 0.05). We also observed significantly faster wound closure following generation of an epithelial defect in rabbit cornea, which was used as an in vivo

**Figure 3.** Comparison of limbal phenotype immunostaining in human corneal limbal epithelial sheets (hCLESs) cultured with each supernatant (DF−, AM-sup, or AMF-sup). K12 (green) was double staining with K15 (A, red) or p63 (B, red). Nuclei were stained with DAPI. Scale bars: 50 μm.

**Figure 4.** Colony-forming efficiency (CFE) of hCLESs cultured with each supernatant (DF−, AM-sup, or AMF-sup). Each colony was stained with rhodamine B (A). CFE (%) was calculated as the percentage of colonies divided by the number of epithelial cells plated in the dish (B). Asterisks indicate standard deviation (SD; n = 4); P < 0.05.

**Figure 5.** Effect of each supernatant (DF−, AM-sup, or AMF-sup) in the hCLES wound healing model. Photographs of the epithelial defect area from 0 (0 d) to 3 days (3 d) (A). The epithelial defect area was calculated as the ratio of the epithelial defect area to the area at 0 d (B). Asterisks indicate SD (n = 6); P < 0.05.
model of wound healing. Instillation of AMF-sup promoted more rapid rabbit corneal wound closure compared with DF- and AM-sup after 2 days (Figs. 6A, 6B; n = 6, P < 0.05).

**DISCUSSION**

In this study, AMF cells exhibited expression of MSCs (CD29, CD44, CD73, and CD90) and neural crest (CD49d and CD56) markers, as well as a fibroblast (FSP-1) marker. AMF cells also exhibited the ability to differentiate into osteoblasts and neural cells (Figs. 1, 2). Many reports have described the multi-differentiation ability of AM-derived cells including neural cell induction. A neural crest origin is a key component of the ability of MSCs to differentiate into neural cells past germ layers such as dermal papillae, the epidermal bulge area of hair follicles, dental pulp, buccal mucosa, and corneal stroma. Embryonic tissue containing AM contains several different stem cell populations, which show superior properties to those of adult stem cells, such as the potential to differentiate into the three germ layers. These properties imply that AMF cells may be immature cell populations that, like MSCs, may be able to differentiate into neural cells.

Coculture with feeder cells is important for engineering transplantable epithelial sheets. Coculture of 3T3 feeder cells using a separate culture system was shown to be beneficial for epidermal, corneal, and oral mucosal epithelial cell proliferation, differentiation, and stratification. Until recently, as substitutes for xenobiotic mouse embryonic 3T3 feeder cells, bone marrow–, adipose tissue–, or oral mucosa–derived human MSCs were used as feeder cells for corneal epithelial sheets, which maintained a corneal epithelial phenotype containing proliferative immature cells. A supply of soluble factors and extracellular vesicles from feeder cells is likely required to maintain the epithelial phenotype in terms of stratification, differentiation, proliferation, and immature state. Human CLEs cultured with AMF-sup maintained the limbal phenotype longer than under the other conditions tested (Figs. 3, 4), implying that AMF-derived soluble factors and extracellular vesicles may be important for maintaining the limbal phenotype of epithelial sheets.

AM-conditioned medium contains several factors, including epidermal growth factor, nerve growth factor, vascular endothelial growth factor, transforming growth factor (TGF)-β, basic fibroblast growth factor (bFGF), and interleukin (IL)-1β, IL-6, and IL-8. De-epithelialized AM expresses a variety of cytokines such as bFGF, keratinocyte growth factor, TGF-β, and TGF-β1. Basic FGF secreted mainly by fibroblasts plays a role in re-epithelialization and production of the extracellular matrix. Basic FGF has also been shown to promote the proliferation of rabbit keratocytes in vitro and to contribute to corneal epithelial wound healing in rabbits. The anti-inflammatory protein TNF-alpha-stimulated gene/protein 6 (TSG-6), which is produced from bone marrow–derived MSCs in response to injured-tissue signals, decreased the corneal inflammatory response, promoted corneal epithelial wound healing, and activated corneal epithelial stem/progenitor cells in a mouse corneal allotransplantation model and a diabetic mouse model. TSG-6 in conditioned medium from human AM-dermed MSCs was also related to the inflammatory response using LPS-stimulated murine bone marrow–derived neutrophils. Human corneal epithelial cell migration increased significantly with the use of 15% and 30% AM-conditioned medium. We observed that AMF-sup had similar effects to AM-sup in this study (Figs. 3–5). Previous studies reported that AM transplantation promoted corneal or conjunctival epithelial wound healing and partially maintained limbal stem cell function. Therefore, AMF-sup containing secretome may partially explain the effects of AM in a clinical setting. It is possible that these soluble factors in AMF-sup promoted re-epithelialization in the wound healing models used in this study.

Grafted human AM–derived MSCs in rabbit corneal alkali wounds have been shown to enhance epithelial healing and reduce corneal opacification and neovascularization. Human bone marrow–derived MSC secretome, consisting of cell-derived soluble factors and extracellular vesicles delivered within a viscoelastic gel carrier combining HA and chondroitin sulfate, enhanced epithelial cell proliferation and wound healing after injury.
healing following mechanical and chemical injuries to the cornea.33 HA was present at higher levels in the AM stroma, and AM-derived stromal cells produced more HA in medium than did AM epithelial cells and skin-derived fibroblasts.29,64 These data imply that the secretome contained within AMF-sup and HA promoted wound healing in the in vitro and in vivo models used in this study.

The hCLEEs were continuously exposed to each supernatant until wound closure, whereas the rabbit corneal epithelium was transiently exposed to each supernatant via supernatant instillation three times a day. Thus, the differences in wound healing effects in vitro and in vivo may be a result of differences in the exposure times of hCLEEs or the rabbit corneal epithelium to AMF-sup.

A limitation of this study needs to be considered. Although we demonstrated that AMF-sup exhibited wound healing properties, we did not identify specific substances in the supernatant that promoted wound healing.

Although further studies are needed, AMF-sup has multiple differentiation phenotypes, and application of the supernatant resulted in maintenance of the immature corneal limbal epithelial phenotype and promoted wound healing of hCLEEs and rabbit corneal epithelial defects. Thus, AMF-sup may be useful for corneal wound healing in ocular surface reconstruction.

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References


