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Corneal Endothelial Expansion Promoted by Human Bone Marrow Mesenchymal Stem Cell-Derived Conditioned Medium

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Abstract

Healthy corneal endothelium is essential for maintaining corneal clarity, as the damage of corneal endothelial cells and loss of cell count causes severe visual impairment. Corneal transplantation is currently the only therapy for severe corneal disorders. The greatly limited proliferative ability of human corneal endothelial cells (HCECs), even *in vitro*, has challenged researchers to establish efficient techniques for the cultivating HCECs, a pivotal issue for clinical applications. The aim of this study was to evaluate conditioned medium (CM) obtained from human bone marrow-derived mesenchymal stem cells (MSCs) (MSC-CM) for use as a consistent expansion protocol of HCECs. When HCECs were maintained in the presence of MSC-CM, cell morphology assumed a hexagonal shape similar to corneal endothelial cells *in vivo*, as opposed to the irregular cell shape observed in control cultures in the absence of MSC-CM. They also maintained the functional protein phenotypes; ZO-1 and Na^+/K^+-ATPase were localized at the intercellular adherent junctions and pump proteins of corneal endothelium were accordingly expressed. In comparison to the proliferative potential observed in the control cultures, HCECs maintained in MSC-CM were found to have more than twice as many Ki67-positive cells and a greatly increased incorporation of BrdU into DNA. MSC-CM further facilitated the cell migration of HCECs. Lastly, the mechanism of cell proliferation mediated by MSC-CM was investigated, and phosphorylation of Akt and ERK1/2 was observed in HCECs after exposure to MSC-CM. The inhibitor to PI 3-kinase maintained the level of $p27^{Kip1}$ for up to 24 hours and greatly blocked the expression of cyclin D1 and D3 during the early G1 phase, leading to the reduction of cell density. These findings indicate that MSC-CM not only stimulates the proliferation of HCECs by regulating the G1 proteins of the cell cycle but also maintains the characteristic differentiated phenotypes necessary for the endothelial functions.

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Competing Interests: Please note that Makiko Nakahara, Naoki Okumura, Shigeru Kinoshita, and Noriko Koizumi have financial competing interests (patents currently pending, "culture medium including human bone marrow mesenchymal stem cell-derived conditioned medium for corneal endothelial expansion" patent application in Japan; 2012-196725, application date; September 7, 2012). EunDuck P. Kay has no conflicts of interest. Michio Hagiya, Kiwamu Imagawa, and Yuuki Hosoda are paid employees of JCR Pharmaceuticals Co., Ltd., Ashiya, Japan. Please also note that this statement does not alter the authors' adherence to all of the PLOS ONE policies on sharing data and materials.

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Introduction

Human corneal endothelium is known to play a critical role in maintaining corneal transparency by regulating corneal hydration [1]. The proliferative ability of human corneal endothelial cells (HCECs) is severely limited *in vivo* [2], therefore, cell loss due to the aging process or pathological conditions causes a concurrent compensatory migration of the existing cells and the enlargement of those cells to achieve a contact-inhibited monolayer. Maintenance of such a phenotype is necessary for functional integrity and corneal deturgescence [3], [4]. If the density of corneal endothelial cells (CECs) is below 500 cells/mm^2, Na^+/K^+-ATPase pump and barrier functions are not compensated by residual CECs. The failure of endothelial functions is known to subsequently cause stromal and epithelial edema, as well as loss of corneal clarity and visual acuity. In addition, irreversible corneal haziness is often observed in corneal endothelial disorders such as Fuchs' corneal dystrophy, pseudophakic bullous keratopathy, or trauma-related injuries [5], [6].

The treatment of severe corneal disorders requires either full-thickness corneal transplantation or endothelial keratoplasty to restore clear vision. Recently, highly effective surgical techniques for the treatment of corneal disorders, i.e., Descemet's stripping automated endothelial keratoplasty (DSAEK) and Descemet's membrane endothelial keratoplasty (DMEK), have been developed, with these procedures being aimed at replacing penetrating keratoplasty [7–10]. However, the worldwide shortage of transplantable donor corneas, the continual cell damage after transplantation, and primary graft failure are issues that remain to be resolved [11], [12]. To overcome these problems, our group, as well as several other groups, have explored new treatment

protocols for corneal endothelial dysfunctions through the use of tissue engineering techniques [13–17]. Among researchers worldwide, the common goal is to establish optimum experimental protocols for the *in vitro* expansion of HCECs for clinical application.

HCECs are arrested at the G1 phase of the cell cycle [2], [18], and this characteristic property of HCECs indicates that HCECs have the potential to proliferate in response to growth stimulation factors. Recently, we reported that Y-27632, a specific inhibitor of the Rho-associated coiled-coil forming kinases (ROCKs), promoted the adhesion and proliferation of monkey CECs [17], [19]. It has also been reported that FGF-2 stimulates the cell proliferation of HCECs through degradation of $p27^{Kip1}$ (p27) [20]. The findings from these studies confirm that the proliferative potential of HCECs can be resumed and that such cells can be cultivated for clinical transplantation into the eye, thus replacing the endothelial keratoplasty. Although a variety of methods to expand HCECs *in vitro* have been explored, protocols for the expansion of HCECs for tissue engineering therapy have yet to be established [21], [22]. We recently reported that the use of conditioned medium (CM) obtained from NIH-3T3 (NIH-3T3-CM) resulted in efficient cultures of HCECs [23]. However, the use of NIH-3T3-CM faces the obstacle that CM derived from mouse cells contains a xenoantigen for human cells [24], [25]. To overcome this difficult obstacle, CM obtained from human bone marrow (BM)-derived mesenchymal stem cells (MCSs) (MSC-CM) was investigated in this present study, as BM-derived stem cells reportedly promote tissue repair by the secretion of cytokines and growth factors that enhance regeneration of injured cells, thus stimulating the proliferation and differentiation of endogenous stem-like progenitors found in most tissues [26–28].

In the present study, we provide evidence suggesting that CM obtained from BM-MSCs stimulates cell proliferation and motility of HCECs, while maintaining the contact-inhibited monolayer with functional adherent junctions and pump functions. Our findings show that the proliferative action of MSC-CM is facilitated via the downregulation of p27 and the upregulation of cyclin D through phosphatidylinositol 3-kinase (PI 3-kinase) and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways. These results indicate that MSC-CM provides a feasible means by which to expand proliferative and functional HCECs for use as a subsequent clinical intervention for corneal endothelial dysfunction.

Materials and Methods

Ethics statement

The human tissue used in this study was handled in accordance with the tenets set forth in the Declaration of Helsinki. Informed written consent was obtained from the next of kin of all deceased donors in regard to eye donation for research. Human donor corneas were obtained from SightLife™ (http://www.sightlife.org/, Seattle, WA). All tissue was recovered under the tenants of the Uniform Anatomical Gift Act (UAGA) of the particular state in which the donor consent was obtained and the tissue was recovered.

Cell cultures

All human corneas had been stored at 4°C in storage medium (Optisol; Chiron Vision, Irvine, CA) for less than 14 days prior to the use of the associated HCECs in the culture. Donor age ranged from 51 to 68 years. The culture medium was prepared according to published protocols, but with some modifications [23], [29]. The Descemet's membrane/corneal endothelium complex was stripped and digested with 1 mg/mL collagenase A (Roche Applied Science, Penzberg, Germany) at 37°C for 2 hours, followed by washing with OptiMEM-I (Life Technologies, Carlsbad, CA). HCECs obtained from the individual donor corneas were resuspended in basal growth medium (OptiMEM-I, 8% fetal bovine serum (FBS), 5 ng/mL epidermal growth factor (EGF), 20 μg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO), 200 mg/L calcium chloride, 0.08% chondroitin sulfate (Sigma-Aldrich), and 50 μg/mL gentamicin (Life Technologies)) and plated into 2 wells of a 12-well plate coated with FNC Coating Mix® (Athena Environmental Sciences, Inc., Baltimore, MD). The HCECs were maintained in a humidified atmosphere at 37°C in 5% CO_2, and the culture medium was replaced with fresh media every 2 days. When the cells reached confluency in 14 to 28 days, they were rinsed in Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS), trypsinized with 0.05% Trypsin-EDTA (Life Technologies) for 5 minutes at 37°C, and passaged at a 1:2 ratio. U0126 (10 μM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and LY294002 (10 μM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used to inhibit MEK and PI 3-kinase, respectively.

Preparation of NIH-3T3-CM

Inactivation of the 3T3 fibroblasts was performed as described previously [30], [31]. Briefly, confluent 3T3 fibroblasts were incubated with 4 μg/mL mitomycin C (MMC) (Kyowa Hakkko Kirin Co., Ltd., Tokyo, Japan) for 2 hours, and then seeded onto plastic dishes at a cell density of 2×10^4 cells/cm^2. Next, the attached cells were washed 3 times with PBS, and the medium was replaced with basal culture medium containing OptiMEM-I, 8% FBS, 5 ng/mL EGF, 20 μg/mL ascorbic acid, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, and 50 μg/mL of gentamicin. The NIH-3T3 was maintained for an additional 24 hours. The medium was collected and centrifuged at 2000 xg for 10 minutes, and the supernatant was filtered through a 0.22- μm filtration unit (EMD Millipore Corporation, Billerica, MA) and used as NIH-3T3-CM.

Preparation of MSC-CM

BM-MSCs were obtained from JCR Pharmaceuticals Co., Ltd. (Kobe, Japan). BM-MSCs passaged 3 times were used for the experiments. The BM-MSCs were plated at a cell density of 1.3×10^4 cells/cm^2 and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, and were then maintained for 1 day. The attached cells were washed 3 times with PBS, and the medium was replaced with basal growth medium. The BM-MSCs were then maintained for an additional 24 hours. The medium was collected and centrifuged at 2000 xg for 10 minutes, and the supernatant was filtered through a 0.22-μm filtration unit (EMD Millipore Corporation) and used as MSC-CM.

Total RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

HCECs after 5 passages were seeded at a cell density of 1.6×10^4 cells/cm^2 and maintained for 1 day, and the medium was replaced with either MSC-CM or NIH-3T3-CM. The cultures were maintained for 8 days. Total RNA was isolated by use of the RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. The quality of the RNA preparations was measured by use of the NanoDrop® (Thermo Fisher Scientific Inc., Waltham, MA) spectrophotometer. First-strand cDNA was synthesized with 1 μg of total RNA by use of the ReverTra Ace® (Toyobo Corporation, Osaka, Japan) reverse transcriptase kit. The

cDNA samples were subjected to PCR with specific primers as listed in Table 1; genes involved in the transport of the corneal endothelium were analyzed in comparison with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. PCR reactions were then performed with Extaq DNA polymerase (Takara Bio Inc., Otsu, Japan) as follows: denaturation at 94°C for 30 seconds, 33 cycles of annealing at 54°C for 30 seconds, and elongation at 72°C for 30 seconds. The PCR products were separated by electrophoresis on 1.5% agarose gels, followed by ethidium bromide staining and detection under ultraviolet illumination.

Cell proliferation assay

HCECs were cultured at the density of 5000 cells/well in a 96-well plate in the presence or absence of CM derived from NIH-3T3 or BM-MSC. DNA synthesis was detected as incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA by use of the Cell Proliferation Biotrak ELISA system, version 2 (GE Healthcare Life Sciences, Buckinghamshire, UK) according to the manufacturer's instructions. Briefly, HCECs were incubated with 10 μM BrdU for 24 hours at 37°C and 5% CO_2 in a humidified atmosphere. Cultured cells were incubated with fixation solution for 2 hours and incubated with 100 μl of monoclonal antibody against BrdU for 30 minutes. The BrdU absorbance was measured directly using a spectrophotometric microplate reader at a test wavelength of 450 nm.

Immunofluorescent staining

Cultured HCECs on a 48-well cell culture plate were fixed in 4% paraformaldehyde for 10 minutes at room temperature and then incubated for 30 minutes with 1% bovine serum albumin (BSA). Immunocytochemical analyses of ZO-1 (Zymed Laboratories, South San Francisco, CA) and Na^+/K^+-ATPase (Upstate Biotec, Lake Placid, NY) were respectively performed with a 1:200 dilution of ZO-1 polyclonal antibody and a 1:200 dilution of Na^+/K^+-ATPase monoclonal antibody. Either Alexa Fluor® 488-conjugated goat anti-mouse (Life Technologies) or Alexa Fluor® 594-conjugated goat anti-rabbit IgG (Life Technologies) was used for the secondary antibody with a 1:1000 dilution. Nuclei were stained with DAPI (Vector Laboratories, Burlingame, CA).The cells were then examined by fluorescence microscopy (BZ-9000; Keyence, Osaka, Japan).

Scratch-induced directional migration assay

HCECs were cultured in 6-well plates in basal growth media. When the cells reached confluence, they were maintained in either control basal growth medium, NIH-3T3-CM, or MSC-CM for an additional 7 days. Scrape-wounding of the cells was performed using a plastic pipette tip. Following scraping, the medium containing detached cells was removed and replaced with basal growth medium, NIH-3T3-CM, or MSC-CM; cells were further maintained for 20 hours until the monolayer was restored. Cell migration and the recovery to a cell monolayer were determined by phase contrast microscopy. The width of the wound area was measured using ImageJ software (U.S. National Institutes of Health, Bethesda, MD), and three fields from each well were measured.

Western blot analysis

The HCECs were washed with ice-cold PBS and then lysed with ice-cold RIPA buffer (Bio-Rad Laboratories, Hercules, CA) containing Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich) and Protease Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan). The lysates were centrifuged at 15,000 rpm for 10 minutes at 4°C to sediment debris. The supernatant representing total proteins was collected and the protein concentration of the sample was assessed by use of the BCA™ Protein Assay Kit (Takara Bio). An equal amount of protein was fractionated by SDS-PAGE; proteins were transferred to PVDF membranes. The membranes were then blocked with 3% non-fat dry milk (Cell Signaling Technology, Inc., Danvers, MA) in TBS-T buffer (50 mM Tris, pH 7.5, 150 mM $NaCl_2$, and 0.1% Tween20) for 1 hour at room temperature, followed by overnight incubation at 4°C with the following primary antibodies: Na^+/K^+-ATPase (1:1000; Merck Millipore), ZO-1 (1:1000; Zymed Laboratories), GAPDH (1:3000; Abcam, Cambridge, UK), Akt1 (1:2000; Cell Signaling Technology), phosphorylated Akt (1:2000; Cell Signaling Technology), ERK1/2 (BD, Franklin Lakes, NJ), and phosphorylated ERK1/2 (BD). The blots were washed, and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000: anti-rabbit IgG, anti-mouse IgG; Cell Signaling Technology). The blots were then developed with luminal for enhanced chemiluminescence (ECL) using the ECL Advance Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ), documented by LAS4000S (Fuji Film, Tokyo, Japan), and analyzed with Image Gauge (Fuji Film).

Concentration of MSC-CM

Supernatants of MSC cultured in OptiMEM-I supplemented with gentamicin were collected after 24 hours. After centrifugation at 1000 rpm for 10 minutes to remove cell debris, cell-free supernatant was concentrated 17-fold by centrifugation at 2500 xg

Table 1. Oligonucleotide sequences for RT-PCR.

Gene	Sense primer	Anti-sense primer	Size (bp)
keratin 12	5′-GGCCTACATGAAGAAGAACCAC-3′	5′-CTCGATCTCCAGGTTCTGAAAG-3′	295
CLCN3	5′-GAGTTTTGCCTTTCTTGCAGTT-3′	5′-GAAAAGATATTTCCGCAGCAAC-3′	203
VDAC3	5′-ATAAGTTGGCTGAAGGGTTGAA-3′	5′-TTCTGTGACAGTTTGGATTTGG-3′	235
SLC4A4	5′-GCTTGCAGATTACTACCCCATC-3′	5′-TTGAACACTCCTTCTTCGACAA-3′	209
p-120	5′-AGGATCCAGCAAACGATACAGT-3′	5′-AGGTCAGCTATGGCAGAAAGAG-3′	244
ZO-1	5′-TTCTGAGGCCTGTAACCATTTT-3′	5′-AATTGGATACCACTGGGCATAG-3′	245
Na^+/K^+-ATPase	5′-ACGGCAGTGATCTAAAGGACAT-3′	5′-GAAGAATCATGTCAGCAGCTTG-3′	255
GAPDH	5′-GAGATCCCTCCAAAATCAAGTG-3′	5′-GAGTCCTTCCACGATACCAAAG-3′	245

doi:10.1371/journal.pone.0069009.t001

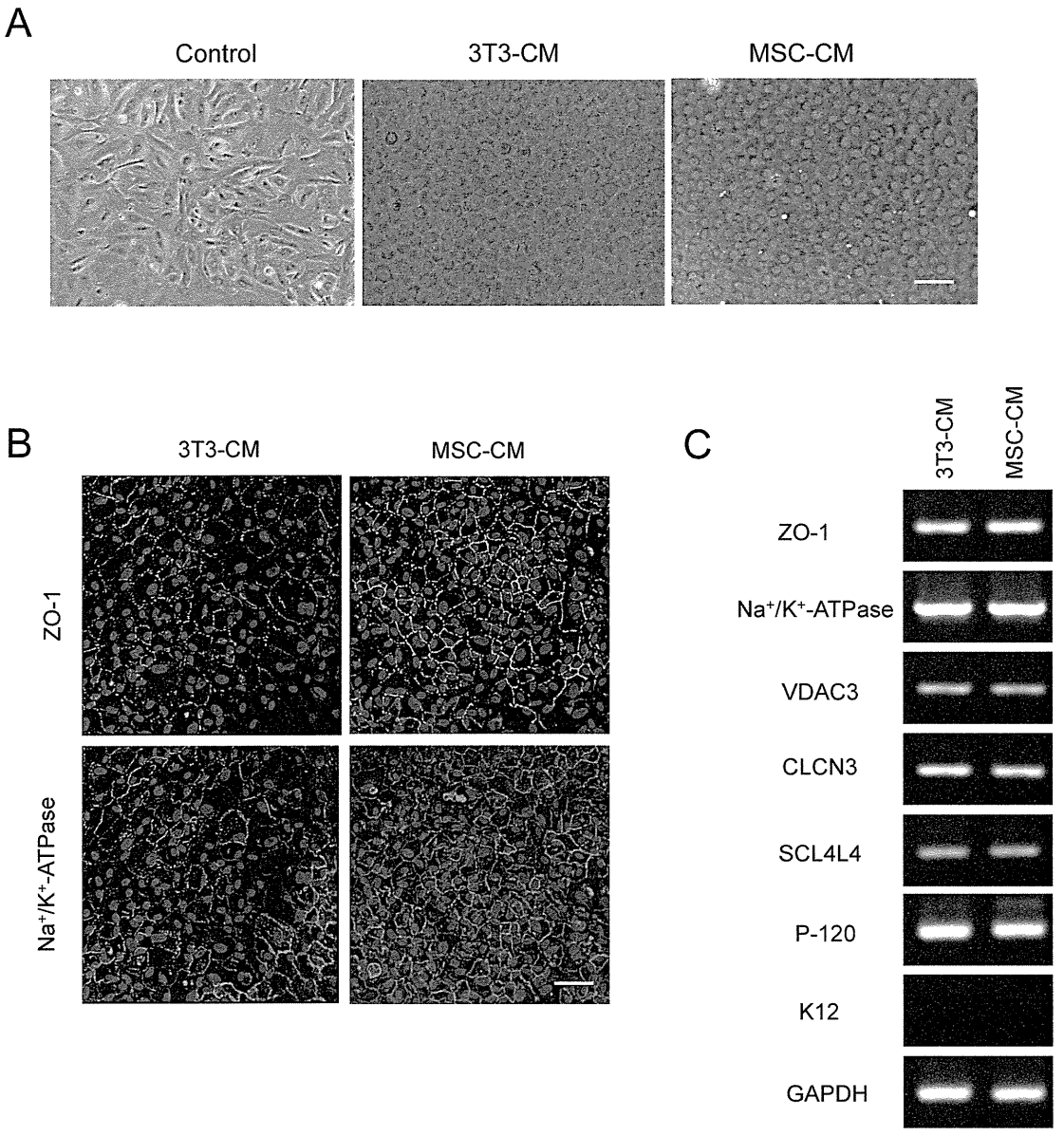

Figure 1. MSC-CM and NIH3T3-CM maintain corneal endothelial phenotype *in vitro* expansion. (A) Effect of MSC-CM on morphology of primary cultures of HCECs. Representative phase-contrast images of primary culture from different CMs. Cultured HCECs were maintained in basal growth medium, MSC-CM, or NIH3T3-CM for 30 days. Scale bar: 200 μm. (B) HCECs cultured in either MSC-CM or NIH3T3-CM for 14 days expressed ZO-1 and Na^+/K^+-ATPase. The pictures are representative of 2 independent experiments. (C) Expression of genes involved in the active transmembrane transporter activity in HCECs cultured with both NIH3T3-CM and MSC-CM was assessed by RT-PCR. The experiments were performed in duplicate.
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for 3 hours using Ultra-PL 3 ultrafiltration units (Amicon; EMD Millipore) with a 3-kDa molecular weight cutoff.

Statistical analysis

The statistical significance (*P*-value) in mean values of the two-sample comparison was determined with the Student's t-test. The statistical significance in the comparison of multiple sample sets was analyzed with Dunnett's multiple-comparisons test. Results were expressed as mean ± SEM.

Results

MSC-CM and NIH-3T3-CM maintain corneal endothelial phenotype in vitro expansion

Current isolation and cultivation methods to establish HCECs *in vitro* face an unexpected obstacle due to spontaneous morphological fibroblastic change and severely limited proliferative ability. Therefore, we tested the CM obtained from human BM-MSCs in this study. HCECs were maintained in basal growth medium, NIH-3T3-CM, or MSC-CM for 30 days. The control cells maintained in basal growth medium showed loss of the

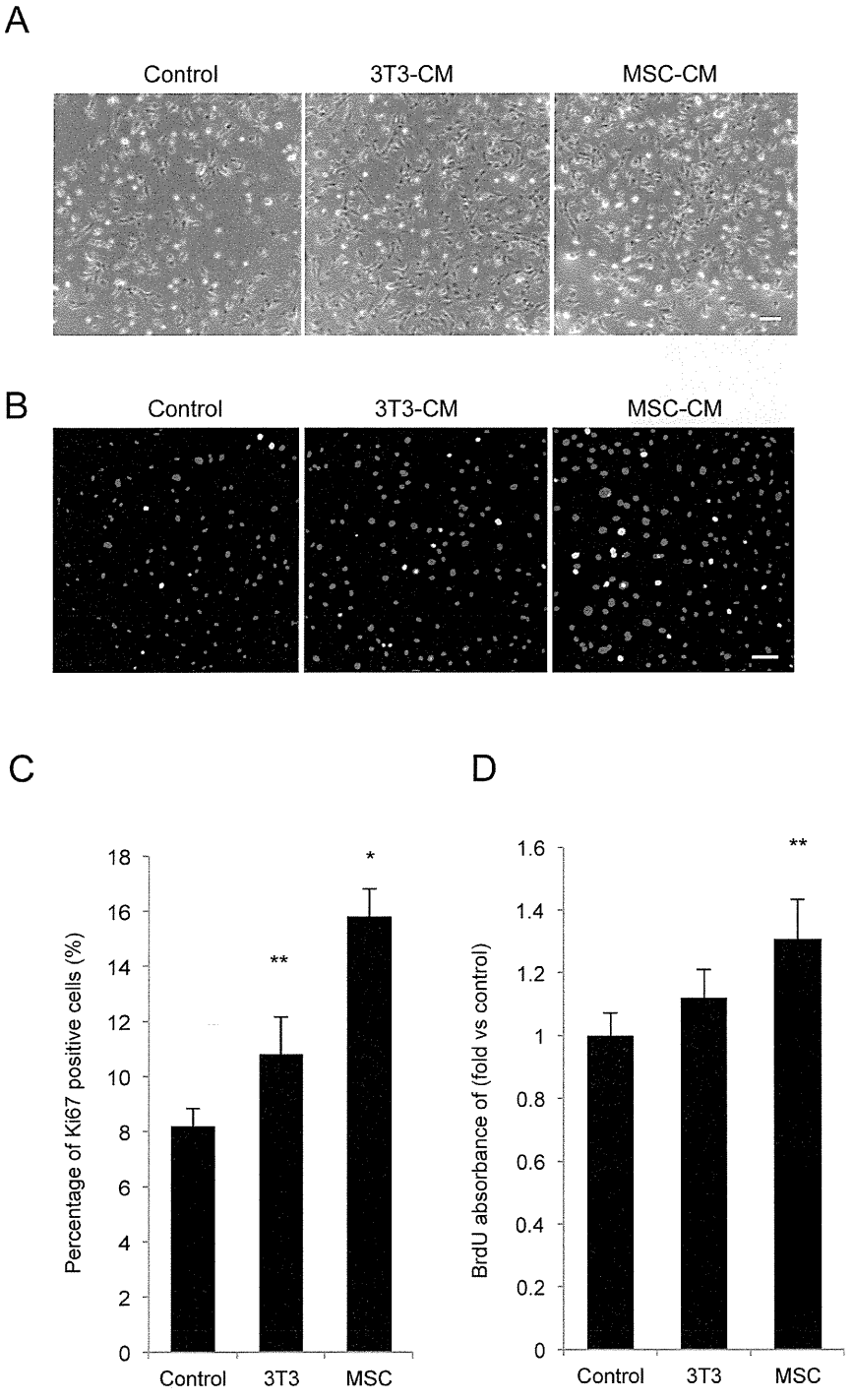
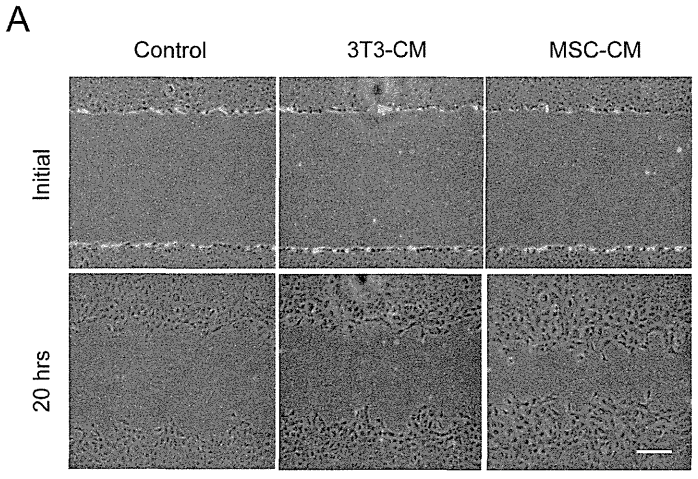
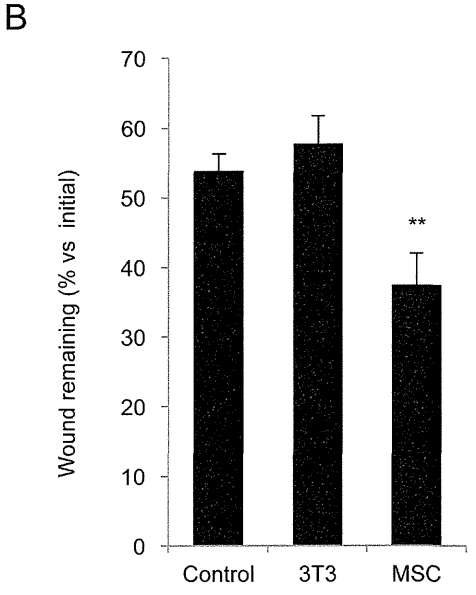


Figure 2. MSC-CM enhances the proliferation of HCECs. (A) Phase-contrast images of HCECs cultured with MCS-CM. HCECs were seeded and cultured with MSC-CM, NIH3T3-CM, or basal growth medium (control) for 5 days. Scale bar: 200 μm. (B+C) To test proliferative potential, HCECs maintained for 5 days under the experimental conditions were immunostained with the cell-cycle-progression population marker Ki67, and the percentages of Ki-67 positive cells were then evaluated. The experiment was performed in duplicate. Scale bar: 200 μm. (D) HCECs were cultured in basal growth medium (control), NIH3T3-CM, or MSC-CM. Proliferation of HCECs was evaluated by BrdU incorporation assay after 5 days of incubation. The experiment was performed in triplicate. * $p<0.01$, ** $p<0.05$.
doi:10.1371/journal.pone.0069009.g002

characteristic polygonal cell morphology, whereas HCECs maintained in either CM demonstrated a contact-inhibited monolayer of hexagonal cells (Figure 1A). Immunostaining of ZO-1 and Na^+/K^+-ATPase was clearly outlined at the intercellular adherent junction in HCECs maintained with either MSC-CM or NIH-3T3-CM (Figure 1B), similar to the previous findings [32]. Expression of genes involved in the active transmembrane transporter activity was assessed by RT-PCR (Figure 1C). The transcripts of ZO-1, Na^+/K^+-ATPase, voltage dependent anion channel3 (VDAC3), chloride channel protein 3 (CLCN3), sodium





C

	Control	3T3-CM	MSC-CM
Speed (μm/min)	0.26±0.09	0.19±0.04	0.37±0.07

Figure 3. MSC-CM promotes cell motility in an *in vitro* wound model. (A+B) HCECs were cultured with basal growth medium (control), NIH3T3-CM, or MSC-CM for 40 days, and the monolayer cells were then wounded by scratching. After 20 hours, the remaining wound area was quantified by Image J software. ** $p<0.05$. Scale bar: 200 μm. (C) The speed of cell motility was measured from the image. The experiments were performed in triplicate.
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bicarbonate co-transporter member4 (SLC4A4), and p-120 were expressed in HCECs, while keratin 12 (K12) was not expressed.

Effect of MSC-CM on the proliferation of HCECs

HCECs were cultured in basal growth medium, MSC-CM, or NIH-3T3-CM (Figure 2A), and the proliferative potential was then assessed using two respective methods: Ki67 staining and BrdU incorporation into the newly synthesized DNA. HCECs maintained for 5 days under the experimental conditions were immunostained with the cell cycle progression population marker Ki67 (Figure 2B). The control cells showed 8.2% Ki67-positive cells, whereas HCECs treated with MSC-CM showed 15.8% Ki67-positive cells (Figure 2C). When incorporation of BrdU into the newly synthesized DNA was measured, HCECs maintained in MSC-CM showed a much higher incorporation of BrdU into DNA than did the control cells (Figure 2D). Of interest, HCECs maintained in NIH-3T3-CM demonstrated lower proliferative potential when compared to HCECs maintained in MSC-CM

Effect of MSC-CM on the wound closure of HCECs

Scratch-induced directional migration assay was employed to compare the wound closure in HCECs maintained in MSC-CM to those of control cells and the NIH-3T3-CM-treated cells (Figure 3A, B). The wound was introduced to the confluent cultures and wound closure was measured 20 hours after the initial wounding. Cells maintained in MSC-CM demonstrated the fastest healing rate; 63% of the initial wound area was covered with cells. On the other hand, both the control cells and the cells maintained in NIH-3T3-CM showed that much less area of the initial wound was recovered by cells. When wound healing over a 20-hour

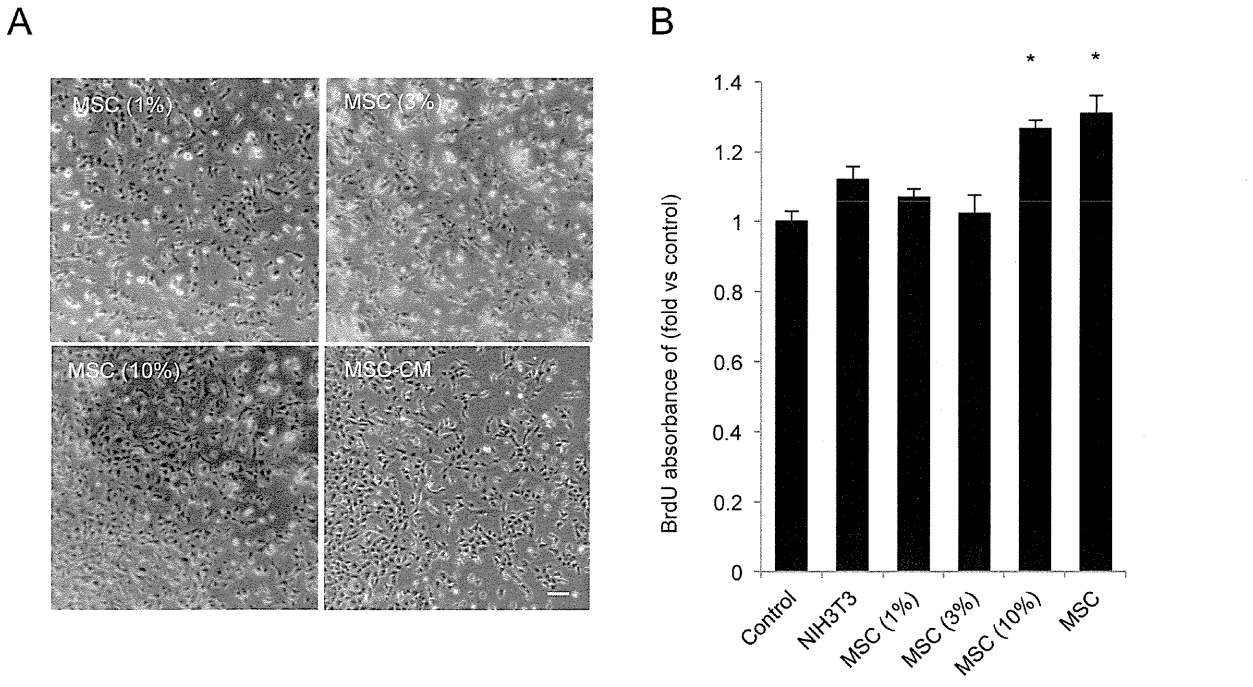

Figure 4. MSC-CM-derived factors enhance HCEC proliferation. (A) HCECs were maintained with basal growth medium supplemented with 1, 3, or 10% of concentrated MSC-CM or full strength MSC-CM. (B) The effect of soluble factors from MSC-CM on the proliferation of HCECs was evaluated by BrdU incorporation assay after 4 days of incubation. * $p<0.01$. The experiments were performed in duplicate. Scale bar: 200 μm.
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period was converted into the migration rate of HCECs, the MSC-CM-treated HCECs demonstrated 0.37 μm/min, the control cells showed 0.26 μm/min, and the NIH-3T3-CM-treated cells showed 0.19 μm/min (Figure 3C), similar to the earlier findings [33].

Effect of MSC-CM-derived factors on CEC proliferation

The fact that the full strength of MSC-CM exerted proliferative activity led us to examine whether or not there is a dilution-dependent activity of MSC-CM on the proliferation of HCECs. To test the dilution effect, MSC-CM was concentrated and added to basal growth medium at the final concentration of 1%, 3%, or 10%. Then, the proliferative activity of the concentrated MSC-CM was compared to that of the full-strength CM (Figure 4A, B). Cells maintained in 10%-strength MSC-CM showed BrdU incorporation into DNA similar to the level achieved with the full-strength CM. On the other hand, MSC-CM at the strength of 1% and 3% produced no proliferative activity. These findings indicated that the soluble factors derived from MSC promote proliferation of HCECs, and also that the effect is dose-dependent.

Involvement of PI 3-kinase and ERK1/2 in the proliferation of HCECs in response to MSC-CM stimulation

It has been known that CECs, regardless of the species, utilize PI 3-kinase and ERK1/2 pathways for cell proliferation mediated by FGF-2 [20], [34]. Therefore, we tested whether or not MSC-CM activated the PI 3-kinase and ERK1/2 pathways. When serum-starved cells were treated with MSC-CM for 15, 30, 60, or 180 minutes, phosphorylation of Akt was greatly induced from 15 minutes following treatment of the cells with CM. Such enhancement on the phosphorylation of Akt sustained for 60 minutes, after which the phosphorylation of Akt was greatly reduced (Figure 5A). The control cells showed faint levels of phosphorylated Akt. Phosphorylation of ERK1/2 was also enhanced 15 minutes following treating the cells with MSC-CM, and such phosphorylation attenuated up to 180 minutes (Figure 5B). To test whether or not the cell proliferation was induced by either PI 3-kinase or MEK, cell density was measured in the presence of the respective inhibitors to PI 3-kinase and ERK1/2; both LY294002 (PI 3-kinase inhibitor) and U0126 (MEK inhibitor) were found to block cell proliferation (Figure 5C). The cells treated with either inhibitor showed an enlarged cell shape due to the lesser cell numbers (Figure 5D). Cell proliferation observed in CECs was linked to the degradation of p27, the potent inhibitor of the G1 phase of the cell cycle [20], [34]. Therefore, we examined the amount of p27 in the absence or presence of LY294002 at the early G1 phase (8 hours) or the late G1 phase (24 hours). p27 appeared to be maintained at a low level regardless of the G1 stage in the presence of MSC-CM (Figure 5E), whereas p27 level was greatly increased in the presence of LY294002 during the late G1 phase of the cell cycle. On the other hand, cyclin D1 and cyclin D3 expressed in the presence of MSC-CM were greatly reduced by the action of LY294002 during the early G1 phase (8 hours) of the cell cycle (Figure 5E). These findings indicated that MSC-CM may employ PI 3-kinase signaling to regulate cell cycle progression through the action on p27 and cyclin D.

Discussion

Human corneal endothelium is a physiologically important monolayer of the cornea, as the simple but crucial role of the endothelium is to maintain cornea clarity. In order to keep the entire cornea transparent, it is essential for corneal endothelium to

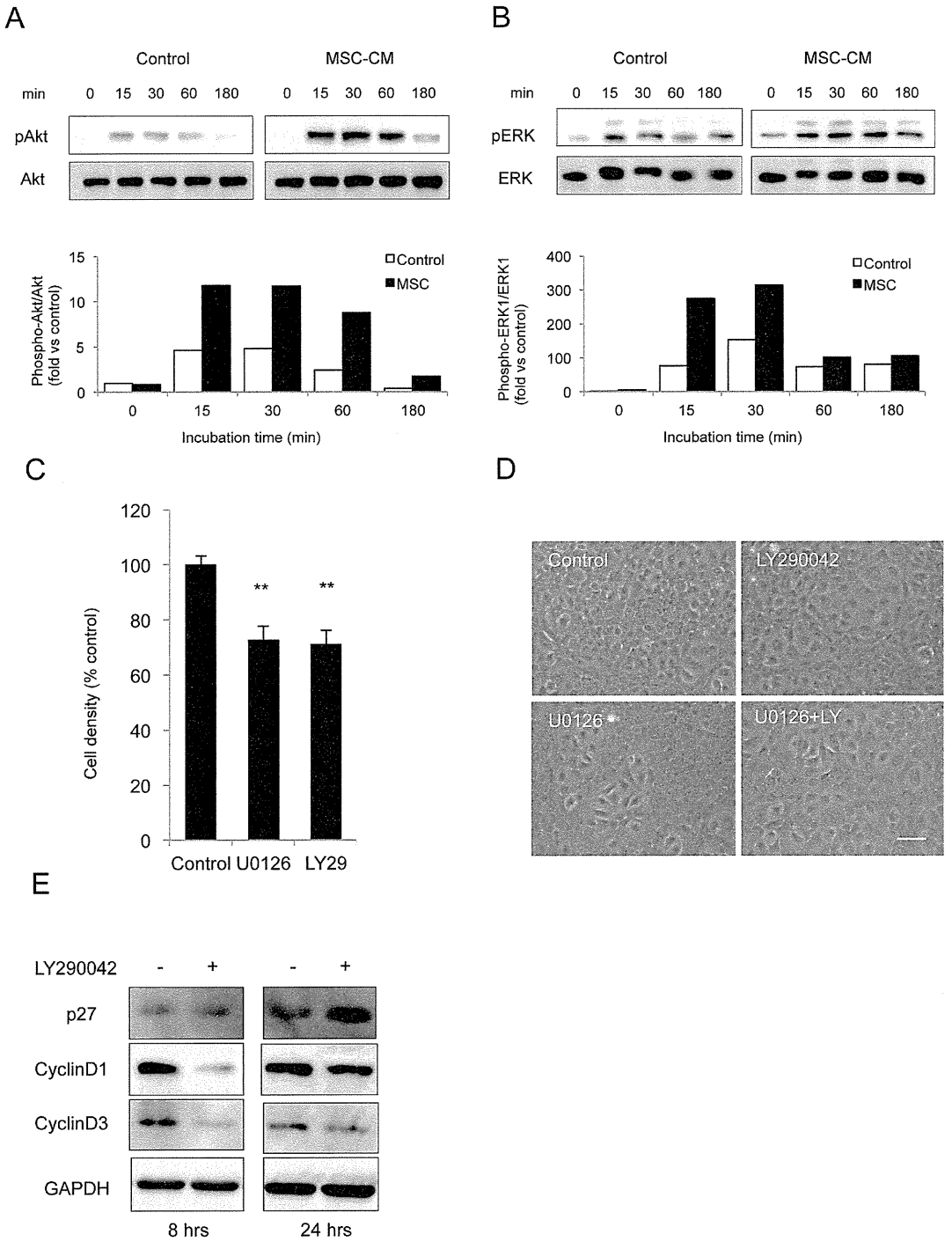

Figure 5. Involvement of PI 3-kinase and ERK1/2 in the proliferation of HCECs in response to MSC-CM stimulation. (A+B) HCECs were cultured without serum for 24 hours followed by treatment with MSC-CM for 15, 30, 60, or 180 minutes. Phosphorylation of Akt and ERK1/2 was evaluated by Western blot analysis. The experiments were performed in duplicate. (C+D) HCECs were cultured in the presence of the PI 3-kinase inhibitor (LY294002) or MEK inhibitor (U0126). Cell density was evaluated via the use of phase contrast microscopy. The experiments were performed in duplicate. Scale bar: 200 μm. (E) HCECs were cultured without serum for 24 hours, and then treated with MSC-CM in the absence or presence of LY294002. Expression of p27, cyclin D1, and cyclin D3 was evaluated by Western blot analysis, both at the early G1 phase (8 hours) and the late G1 phase (24 hours).
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retain the unique contact-inhibited monolayer, through which the tissue operates active pump and barrier functions. Decompensation of the corneal endothelium resulting from various causes ultimately leads to its inability to efficiently pump fluid out of the stroma, thus leading to corneal edema, loss of visual acuity, and cornea-related blindness. The function of the endothelium is compromised if the cell density falls below a critical threshold of 500 cells/mm^2. In the United States, over 40,000 corneal

transplantations were performed in 2011 [35]. Since corneal endothelial dysfunction is the major indication for performing corneal transplantations, endothelial keratoplasty represented over 40% of all corneal grafts performed in both 2009 and 2010 [36].

Various methods have been attempted to treat endothelial dysfunction, and in the most recent years, DSAEK and DMEK have been extensively employed [7–9], [37]. However, these relatively new procedures still face some obstacles, such as the worldwide shortage of transplantable donor corneas, continuing cell loss after transplantation, technical difficulty, and primary graft failure [11], [12]. In order to address the obstacle produced by the worldwide shortage of donor corneas, the idea of one donor cornea treating one patient has been challenged, and the concept of using one donor cornea for treating multiple patients has been widely accepted. Such a timely goal prompts researchers to establish optimum technologies for isolation and cultivation of HCECs, with the cultivated cells then being used for transplantation as a new clinical intervention for corneal endothelial dysfunction.

To achieve such a goal, our group demonstrated that transplantation of cultivated CECs in combination with a ROCK inhibitor enables the injection of the cells into the anterior chamber to regenerate corneal endothelium as a functional monolayer in an animal model [16], [17]; and this technique has the potential to be applied in the clinical setting if developed properly. However, this treatment pathway faces practical difficulties, as HCECs are arrested at the G1 phase of the cell cycle *in vivo* [2], [18] and they do not readily proliferate *in vitro*. Worse yet, HCECs naturally exhibit massive fibroblastic change with loss of pump and barrier functions during *in vitro* cultivation. To overcome such undesired events, our group has successfully used CM obtained from NIH-3T3 fibroblasts, which maintain endothelial phenotypes [23]. Similarly, mouse ESC-CM was used to enhance cell proliferation and survival of HCECs in culture [38]. Nonetheless, the use of NIH-3T3-CM or mouse ESC-CM faces a major obstacle in that the CM of the mouse cell cultures contains a xenoantigen for human cells [24], [25]. To overcome this issue, we tested the effect of CM obtained from GMP-grade human BM-derived MSCs for application in the clinical setting.

In this present study, we demonstrated findings critical for the successful cultivation protocols of HCECs that may be used for transplantation in the clinical setting. When HCECs were maintained in the presence of MSC-CM, cell morphology assumed a hexagonal shape similar to the corneal endothelial cells *in vivo*. The HCECs also maintained the functional phenotypes; ZO-1 and Na^+/K^+-ATPase were localized at the intercellular adherent junctions and major pump proteins (VDAC3, CLCN3, SLC4A4, and p120) present in HCECs were accordingly expressed. MSC-CM facilitated cell motility of HCECs. Of importance, MSC-CM was found to greatly stimulate the proliferation of HCECs through the PI 3-kinase and ERK1/2 pathways by degrading p27, similar to the findings reported previously [20]. In addition to its action on p27, MSC-CM was found to upregulate the expression of cyclin D1 during the early G1 phase of the cell cycle, which is another crucial step for G1/S progression.

Taken together, our findings indicate that MSC-CM not only stimulates the proliferation of HCECs by regulating the G1 proteins of the cell cycle, but also maintains the characteristic differentiated phenotypes necessary for the endothelial functions. Such dual cellular activities (proliferation and differentiation), in opposite nature, are employed for the self-renewal of stem cells/progenitors in adult tissues. Our findings suggest that HCECs maintained in MSC-CM acquire the stem-cell-like properties, which subsequently regenerate HCECs into functional corneal endothelium. These findings are the first evidence to show that when treated with MSC-CM, HCECs retain the required proliferative potential with the capacity to be fully differentiated. Therefore, the findings of this study may provide a feasible means by which to bolster the current concerted efforts to establish functioning HCECs with high growth potential [22], [39]. Thus, a combination of tissue-engineered human corneal endothelium coupled with surgical procedures presents a possible roadmap by which to treat endothelial dysfunctions.

Author Contributions

Conceived and designed the experiments: MN NO EPK SK NK. Performed the experiments: MN NO. Analyzed the data: MN NO EPK SK NK. Contributed reagents/materials/analysis tools: NO MH KI YH SK NK. Wrote the paper: MN NO EPK NK.

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Prevalence of Dry Eye Disease and its Risk Factors in Visual Display Terminal Users: The Osaka Study

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• PURPOSE: To investigate tear function and prevalence of dry eye disease (DED) in visual display terminal (VDT) users.
• DESIGN: Cross-sectional study.
• METHODS: Six hundred and seventy-two young and middle-aged Japanese office workers who used VDT completed questionnaires and underwent dry eye testing. We estimated the prevalence of DED using logistic regression analysis to examine associations between DED and possible risk factors. The ocular surface feature, prevalence of DED, and risk factors were evaluated.
• RESULTS: Of the 672 workers, 561 (83.5%, mean age: 43.3 ± 9.1 years) completed the questionnaire. The percentage of women with a composite outcome of definite DED or probable DED was 76.5%, which was higher than that among men (60.2%; odds ratio [OR] = 2.00; 95% confidence interval [CI], 1.29-3.10, P = .002). Workers over 30 years of age had a higher risk of DED (OR = 2.22; 95% CI, 1.06-4.66), as did workers using a VDT > 8 hours per day (OR = 1.94; 95% CI, 1.22-3.09). Average Schirmer value was 18.7 ± 11.7 mm and tear break-up time (TBUT) was 4.0 ± 2.5 seconds (78.6% of study participants had TBUT ≤5 seconds).
• CONCLUSIONS: DED is prevalent among young to middle-aged Japanese VDT users. Ophthalmic findings revealed short TBUT and corneal staining accompanied by normal Schirmer test values. Increased risk for DED was noted for women aged over 30 years and prolonged VDT use. Measures to modify the adverse impact of VDT use on the ocular surface may provide a positive impact on public health and quality of life for office workers using VDTs. (Am J Ophthalmol 2013;156: 759–766. © 2013 by Elsevier Inc. All rights reserved.)

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DRY EYE DISEASE (DED) IS AN IMPORTANT PUBLIC health problem causing ocular discomfort, fatigue, and visual disturbance that may interfere with daily activities.[1] Dry eye is one of the most prevalent eye diseases and reasons for seeking eye care.[2] Based on data from the largest epidemiologic studies of DED—the Women's Health Study and the Physicians' Health Study—it has been estimated that about 7.8% or 3.23 million American women and 4.7% or 1.6 million men over 50 years of age have DED.[3,4] Other studies have evaluated the prevalence of DED in different parts of the world.[5,6] It is thought that many people have less severe symptoms and probably a more episodic manifestation of DED that is noticed only during exposure to certain contributory factors such as visual display terminals (VDTs).[7,8]

Home use of computers and portable information terminals has risen steadily, and VDT exposure is increasingly common not only in VDT workers but also in the general population because of widespread use of mobile devices or smart phones among both young and old.

We previously reported the prevalence of DED in VDT users using a questionnaire-based assessment, and found that clinical diagnosis or severe symptoms were prevalent among young and middle-aged Japanese office workers.[9] Although there have been a number of largely anecdotal reports relating DED symptoms to VDT use, only a few studies have investigated tear function and estimation of the magnitude of the problem or risk factors that might be amenable to modification.[10,11] The authors therefore set out to investigate tear function and to estimate the prevalence of DED among Japanese office workers who use VDTs, and tried to identify key risk factors based on clinical examinations by dry eye specialists and implementing the latest version of the Japanese dry eye diagnostic criteria. The aims of this study were to study tear function, to estimate the prevalence of and factors associated with DED, and to evaluate its ocular manifestations in Japanese men and women who use VDTs.

METHODS

• STUDY POPULATION: Under the supervision of the Japanese Dry Eye Society, the authors arbitrarily selected 2

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large companies listed on the Japanese stock market and sent a letter to the industrial physician of the health management section of each company to explain the purpose of the study and to request their participation. The only company in the pharmaceutical sector that responded to our letter and consented to participate was enrolled in this study. Following an internal review of the protocol and the consequences of the study, permission was granted to conduct the study in employees who were willing to participate. Subjects were invited by e-mail to answer the questionnaires and to attend a general ophthalmic check-up. A maximum of 2 e-mail reminders were sent. Subjects who reported a history of refractive surgery were excluded from the study protocol. This cross-sectional research followed the tenets of the Declaration of Helsinki, and the protocol was approved prospectively by the Institutional Review Board of Ryogoku Eye Clinic Tokyo, Japan.

• QUESTIONNAIRE: We administered a dry eye questionnaire widely used in Japan.[12] Briefly, the questionnaire includes 12 questions pertaining to the diagnostic symptoms of DED.[12] Possible answers to questions regarding symptoms included "constantly," "often," "sometimes," and "never." Subjects who responded to more than 1 of the 12 questions by "constantly" or "often" were considered positive for subjective symptoms of DED. Information on age, sex, and smoking (current smoker or not) was also obtained. Based on our previous studies,[9,13] we defined the duration of VDT use (stratified, none to over 10 hours in 1-hour categories) and contact lens (CL) use (yes or no). Past/current history of certain common systemic diseases (hypertension [HT], diabetes mellitus [DM]) was determined by asking participants whether they had ever been told of these conditions by their physician. We defined systemic medication use as medication prescribed only by a doctor, and not over-the-counter supplements.

• CLINICAL EVALUATION: *Tear function tests and ocular surface evaluation.* Ophthalmic examinations included assessment of conjunctival and corneal vital staining with lissamine green and fluorescein, tear break-up time (TBUT), and Schirmer test. The condition of the ocular surface was evaluated as reported previously.[14] Briefly, corneal and conjunctival epithelial damage was evaluated by the double vital staining method. Two microliters of a preservative-free combination of 1% lissamine green and 1% sodium fluorescein were instilled into the conjunctival sac by micropipette. To evaluate keratoconjunctival epithelial damage, the cornea and conjunctiva were assessed by fluorescein and lissamine green staining, respectively. The eye was categorized into 3 equal compartments representing nasal conjunctiva, cornea, and temporal conjunctiva, and the maximum staining score for each area was 3 points. Overall epithelial damage was scored on a scale of 0-9 points.

Tear stability and quantity were assessed by 2 different methods: TBUT and Schirmer test. To determine TBUT, fluorescein vital staining was performed; patients were requested to blink 3 times to ensure adequate mixing of the fluorescein dye with tears. The time interval between the last complete blink and the appearance of the first corneal dark spot was measured by stopwatch, with the mean of 3 measurements regarded as TBUT in this study. The Schirmer test was performed without topical anesthesia, following all other examinations. Strips (Whatman No.41; Showa, Tokyo, Japan) were placed for 5 minutes at the outer one third of the temporal lower conjunctival fornix. The strips were then removed, and the length of filter paper that had been wetted (in mm) was recorded. To avoid the influence of conjunctivocorneal staining on the Schirmer test, we proceeded with that test after a 10-minute interval. The grading of meibomian gland dysfunction (MGD) was performed according to modified Bron's classification,[15] as follows: grade 0, no glandular dropout and easy meibum expressibility with clear transparent meibomian secretion; grade 1, glandular dropout in one third of the eyelid length, with acinar cluster visibility in the remaining eyelid, granular secretion, difficult expressibility, turbid, nonsticky secretion; grade 2, glandular dropout in one third of the eyelid with loss of acinar cluster visibility but with observable yellow stripes, meibomian secretion not easily expressible, opaque, white granular secretion; grade 3, meibomian seborrhea with increased sticky secretion. All ophthalmic examinations were performed by ophthalmologists specializing in DED. To avoid the influence of air conditioning on TBUT and other dry eye tests, we did not use any air conditioner in the examination room. The room temperature was maintained at 25.0°C-26.5°C during examinations, with 60%-65% humidity. Subjects were not allowed to use a VDT for 1 hour prior to their examinations.

Diagnosis of dry eye disease. Diagnosis was established according to the Japanese dry eye diagnostic criteria, as follows: (1) presence of dry eye symptoms; (2) presence of qualitative or quantitative disturbance of the tear film in 1 or both eyes (Schirmer test ≤5mm or TBUT ≤5 seconds); and (3) presence of conjunctivocorneal epithelial damage (total staining score ≥3 points) in 1 or both eyes. The presence of all 3 criteria was necessary for a definite diagnosis of DED. Subjects showing the presence of 2 of the 3 criteria were diagnosed with probable DED, while those with 1 or no positive criteria were diagnosed as non-DED[16] (Table 1). With the aim of comparing differences between dry eye diagnosis in our study group and Japanese and international diagnostic criteria, we also implemented the Dry Eye WorkShop (DEWS) severity grading system[17] to assess differences in diagnosis.

• STATISTICAL ANALYSIS: To compare differences in the Schirmer test, TBUT, epithelial staining, and MGD

TABLE 1. The Dry Eye Diagnostic Criteria in Japan

Symptoms of dry eye[a]	+	−	+	+	+	−	−	−
Tear abnormality[b]	+	+	−	+	−	+	−	−
Epithelial damage[c]	+	+	+	−	−	−	+	−
Dry eye diagnosis	Definite dry eye disease	Probable dry eye disease			Non–dry eye disease			

+ = yes; − = no.

[a]At least 1 positive sign of dry eye questions.

[b]The presence of at least 1 positive criterion is essential: (1) Schirmer test-I ≤5 mm; or (2) TBUT ≤5 seconds.

[c]The presence of at least 1 positive criterion is essential: (1) total score of fluorescein ≥3 points; or (2) lisamine green staining ≥3 points.

TABLE 2. Characteristics of the Study Population: Dry Eye Disease Among Visual Display Terminal Users

Variables	Men (n = 374) n (%)	Women (n = 187) n (%)
Age (y)		
22-29	14 (3.7)	20 (10.7)
30-39	87 (23.3)	68 (36.4)
40-49	150 (40.1)	81 (43.3)
50-65	123 (32.9)	18 (9.6)
Current smoker	105 (28.1)	5 (2.7)
VDT use (hours)		
0-4	55 (14.7)	18 (9.5)
4-8	246 (65.7)	110 (58.8)
>8	73 (19.5)	59 (31.5)
Contact lens user	77 (20.6)	93 (49.7)
Past/current history of certain common systemic diseases		
Hypertension	24 (6.4)	3 (1.6)
Diabetes mellitus	6 (1.6)	0 (0.0)

VDT = visual display terminal.

grading between sexes, we used Student *t* test. The prevalence of DED was calculated and the corresponding 95% confidence interval (CI) estimated. Using a logistic regression model, we calculated odds ratios (ORs) and 95% CIs of DED for sex, demographic, lifestyle, and medical factors. First, we carried out univariate analyses of the associations between each factor and definite and probable DED. Then, mutual adjustment for all associated factors identified in univariate analyses ($P < .2$) was performed. Although the age category was not associated with diagnosis of DED in univariate analysis, we collapsed age into 2 categories (≤30 and >30 years) and included this variable in the multivariable models, since previous studies found age to be an important risk factor for DED.[18] We categorized prolonged VDT working hours as >8 hours, since the average was 7.9 hours for all subjects. *P* values of <.05 were considered to indicate statistically significant differences. All statistical analyses were performed using SAS software, version 9.2 (SAS Inc, Cary, North Carolina, USA).

RESULTS

OF THE 672 OFFICE WORKERS APPROACHED, 561 (83.5%) participated in this study: 374 male (66.7%) and 187 female participants (33.3%) aged between 22 and 65 years (Table 2). Participants ranging in age from 30-49 years accounted for 68.8% of the study population. The mean duration of VDT use was 7.7 ± 2.1 hours among men and 8.3 ± 2.3 hours among women. Most workers were healthy, with only 14.4% reporting any type of systemic disease and 24.2% reporting the use of systemic medication.

The results of ocular findings are shown in Table 3. The average Schirmer test value was 16.7 ± 11.5 mm in men and 22.6 ± 11.2 mm in women, and 83.1% of all subjects had a Schirmer score >5 mm. Mean TBUT was 4.3 ± 2.7 seconds in men and 3.4 ± 1.9 seconds in women, and 78.6% of subjects showed a TBUT of ≤5 seconds. Most subjects scored low on epithelial staining: 471 of 561 (83.9%) under 3 points and only 1 patient (0.2%) with 7 points or over. Meibomian gland scoring showed that 279 of 374 (74.6%) in men and 154 of 187 (82.3%) in women were grade 0, with no statistically significant difference by sex ($P = .08$).

The prevalence of DED by sex is shown in Table 4. The proportion of women with a composite outcome of definite DED (18.7%) was higher than that of men (8.0%). Most instances of definite DED were seen among subjects who met the criteria of symptoms, epithelial damage, and TBUT ≤5 seconds. One hundred and ninety-five of 374 men (52.1%) and 108 of 187 women (57.8%) were diagnosed as having probable DED ($P = .24$). The majority of subjects with probable DED (70.3% of men and 85.2% of women) had symptoms and TBUT ≤5 seconds. The next most frequent group consisted of subjects with symptoms, TBUT ≤5 seconds, and an abnormal Schirmer value. The non-DED group was composed of 149 men (39.8%) and 44 women (23.5%), and 39.3% of this group had TBUT ≤5 seconds, followed by a group of subjects with symptoms only (26.9%).

The results of univariate and multivariate analysis of the associations between demographic, lifestyle, and medical factors and DED are shown in Table 5. According to the multivariate-adjusted model, factors associated at $P < .2$ with the composite outcome of definite or probable DED were female sex (OR = 2.00, 95% CI = 1.29-3.10, $P = .002$), age over 30 years (vs <30 years, OR = 2.22, 95%

TABLE 3. Detailed Ocular Finding Results in Visual Display Terminal Users

	Men (n = 374)	Women (n = 187)	Total (n = 561)	*P* Value
Schirmer (mm)				
>5	296 (79.1%)	170 (90.9%)	466 (83.1%)	.0001
≤5	78 (20.9%)	17 (9.1%)	95 (16.9%)	
Mean ± SD	16.7 ± 11.5	22.6 ± 11.2	18.7 ± 11.7	
TBUT (seconds)				
>5	96 (25.7%)	24 (12.8%)	120 (21.4%)	.0005
≤5	278 (74.3%)	163 (87.2%)	441 (78.6%)	
Mean ± SD	4.3 ± 2.7	3.4 ± 1.9	4.0 ± 2.5	
Epithelial staining (points)				
0-2	327 (87.5%)	144 (77.0%)	471 (84.0%)	.001
3-6	46 (12.3%)	43 (23.0%)	89 (15.8%)	
7-9	1 (0.2%)	0 (0.0%)	1 (0.2%)	
Mean ± SD	1.0 ± 1.3	1.4 ± 1.4	1.1 ± 1.3	
MGD (grade)				
0	279 (74.6%)	154 (82.3%)	433 (77.2%)	.084
I	69 (18.4%)	19 (10.2%)	88 (15.7%)	
II	16 (4.3%)	9 (4.8%)	25 (4.4%)	
III	10 (2.7%)	5 (2.7%)	15 (2.7%)	

MGD = meibomian gland dysfunction; TBUT = tear break-up time.

CI = 1.06-4.66, $P = .04$), and prolonged VDT use >8 hours (vs short VDT use, ie, <8 hours, OR = 1.94, 95% CI = 1.22-3.09, $P = .005$). Although CL users comprised 30% of all subjects, we did not observe a significant association between CL use and the risk of DED in the multivariate model (OR = 1.13, 95% CI = 0.74-1.72, $P = .57$).

DISCUSSION

DESPITE THE FREQUENCY WITH WHICH VDT WORKERS HAVE reported eye problems, large-scale studies delineating the risk factors associated with visual problems and symptoms of DED among workers who underwent clinical dry eye examination have not been published previously. This cross-sectional prevalence study was carried out to examine the prevalence of DED according to Japanese diagnostic criteria, with the chief aims of evaluating tear function, ocular surface status, and potential risk factors in office workers using VDT. The prevalence of definite DED was 8.0% in men and 18.7% in women based on clinical evaluation, while that of probable DED in men and women was 52.1% and 57.8%, respectively. Inter-sex differences were statistically significant, as well as age >30 years, and we further identified that workers using VDTs for ≥8 hours per day had a significantly increased risk of DED.

In the present study we used the Japanese criteria and performed both questionnaire-based assessment of symptoms and clinical dry eye examinations on all subjects, and surprisingly found the prevalence of definite and probable DED in those who had sought treatment as being twice that recorded in our previous work on VDT users, where the assessment of DED was based on questionnaire data only.[9] We believe that the dissimilarity in DED prevalence between these 2 study groups is likely attributable to differences between diagnostic criteria, since only 9.3% of subjects had symptoms of DED alone. These results imply that conducting clinical dry eye examinations in addition to questionnaires led to an increased prevalence of probable DED in young Japanese VDT workers compared with our previous estimate, which involved a dry eye questionnaire only.

This study also revealed that most subjects using VDTs had normal lacrimal function, according to Schirmer test results. These findings differ from those reported by Nakamura and associates, which suggested that cumulative years of VDT use might result in aqueous deficiency.[19] The present study did not measure the cumulative effect of prolonged VDT work, which may explain the differences between these observations. In regard to tear abnormalities, only a minority of subjects had a stable tear film: 74.3% of men and 87.2% of women had short TBUT values. In the DEWS report 2 subtypes of DED are noted, with evaporative DED, which are most commonly from MGD, as an integral part of the disease.[17] Recent paper has pointed out the high prevalence of MGD, in surveys of patients with DED and in general population.[20]

It has been hypothesized that excessive evaporation of tear fluid attributable to prolonged blinking intervals while gazing is a causative factor in VDT-associated dry eye.[21,22] Moreover, previous studies showed that VDT use increased

TABLE 4. Prevalence of Dry Eye Disease by Sex in Visual Display Terminal Users

	Men (n = 374)		Women (n = 187)	
	No. of DED	Prevalence (95% CI)	No. of DED	Prevalence (95% CI)
Definite dry eye disease	30	8.0 (5.5-11.3)	35	18.7 (13.4-25.1)
Symptom + epithelial damage + TBUT≤5 + Schirmer ≤5	9	2.4 (1.1-4.5)	6	3.2 (1.2-6.9)
Symptom + epithelial damage + TBUT≤5	21	5.6 (3.5-8.5)	29	15.5 (10.6-21.5)
Probable dry eye disease	195	52.1 (46.9-57.3)	108	57.8 (50.3-64.9)
Symptom + Schirmer ≤5	15	4.0 (2.3-6.5)	0	0 (0-2.0)
Symptom + TBUT ≤5	137	36.6 (31.7-41.7)	92	49.2 (41.8-56.6)
Symptom + TBUT ≤5 + Schirmer ≤5	27	7.2 (4.8-10.3)	8	4.3 (1.9-8.3)
Epithelial damage + Schirmer ≤5	2	0.5 (0.1-1.9)	0	0 (0-2.0)
Epithelial damage + TBUT ≤5	8	2.1 (0.9-4.2)	7	3.7 (1.5-7.6)
Epithelial damage + TBUT ≤5 + Schirmer ≤5	3	0.8 (0.2-2.3)	1	0.5 (0-2.9)
Symptom + Epithelial damage	3	0.8 (0.2-2.3)	0	0 (0-2.0)
Non–dry eye disease	149	39.8 (34.8-45.0)	44	23.5 (17.6-30.3)
Symptom only	37	9.9 (7.1-13.4)	15	8.0 (4.6-12.9)
Schirmer ≤5 only	7	1.9 (0.8-3.8)	0	0 (0-2.0)
TBUT ≤5 only	58	15.5 (12.0-19.6)	189	6 (5.8-14.8)
Schirmer ≤5 and TBUT ≤5 (normal in epithelial damage and symptom)	15	4.0 (2.3-6.5)	2	1.1 (0.1-3.8)
Epithelial damage only	1	0.3 (0-1.5)	0	0 (0-2.0)
Normal in 3 categories	31	8.3 (5.7-11.6)	9	4.8 (2.2-8.9)

CI = confidence interval; DED = dry eye disease; TBUT = tear break-up time.

the proportion of incomplete blinks and accelerated evaporation of the tear film.[22,23] Although we did not measure average and incomplete blinking rates, it is suggested by our findings that tear function in VDT users may be affected by blink frequency and patterns inducing tear film instability, with a short TBUT as the probable cause of VDT-associated DED. Taking into consideration the distribution of tear function and symptoms in normal subjects, one may postulate that DED in VDT workers starts with a shortened TBUT, leading to the development of subjective symptoms. This hypothesis can be supported by the observation that the majority of subjects in this study with probable DED had a combination of DED symptoms and shortened TBUT.

Lacrimal gland function has been reported to decrease gradually with aging,[24] resulting in reduced tear secretion and DED in the elderly; some studies showed a higher prevalence of DED in elderly populations.[25–27] However, in this study no age-related trend was observed in relation to the prevalence of DED in either sex, though the subjects were younger than in most other studies. This is likely attributable to our study population's consisting of relatively young individuals and few subjects over 60 years.

It has also been reported in previous studies that medication use in HT is a risk factor for DED,[3,26,27] and that HT can be a predictor of DED. One recent study found a marginally increased risk of DED associated with the use of diuretics but a decreased risk with the use of ACE inhibitors.[27] Although we failed to identify such an association among this group of younger male VDT users, since few subjects had HT, the power to detect such an association was low. The sex-related difference found in the prevalence of DED may be attributable to differences in the way that hormonal profiles change with age between men and women.[28] Although previous studies showed an association between depression and DED, in this study we did not observe any subjects who had been diagnosed with depression.[29,30]

In this study we employed corneal fluorescein staining, TBUT, and Schirmer testing to evaluate the ocular surface and tear function, since these are the essential procedures for diagnosis of DED in Japan. It should be noted that corneal fluorescein staining has been reported to have poor sensitivity and may be absent in approximately 40%-50% of mild to moderate DED cases.[31,32] In addition, TBUT and Schirmer tests have also been suggested as having poor specificity[31] in regard to the diagnosis of DED. In order to avoid these problems as far as possible in our study, we implemented the Japanese diagnostic criteria, which require the results of not only tear abnormality but also epithelial damage and symptom assessment. If the results of the current study are viewed according to the DEWS severity grading based on Schirmer, TBUT, and vital staining, we can claim that 58.1% of the subjects in our study were diagnosed with grade 1 DED severity, 8.3% grade 2, and 4.6% grade 3. A previous study showed work productivity loss from DED.[33] Our results showed that the office workers who spend long hours viewing

TABLE 5. The Associations Between Demographic, Lifestyle, and Medical Factors and Dry Eye Disease

Variables	Prevalence (%)	Crude OR (95% CI)	*P* Value	Multi-adjusted OR (95% CI)[a]	*P* Value
Sex					
Men	60.2 (225/374)	1.00	-	1.00	-
Women	76.5	2.15 (1.45-3.20)	.001	2.00 (1.29-3.10)	.002
Age (y)					
22-29	55.9 (19/34)	1.00	-	1.00	-
≥30	66.2 (349/527)	1.55 (0.77-3.12)	.22	2.22 (1.06-4.66)	.04
Current smoker (no or yes)					
No	67.4 (304/451)	1.00	-	1.00	-
Yes	58.2 (64/110)	0.67 (0.44-1.03)	.07	0.86 (0.54-1.35)	.50
VDT use (hours)					
0-8	62.0	1.00	-	1.00	-
≥8	77.3	2.08 (1.33-3.27)	.001	1.94 (1.22-3.09)	.005
Contact lens use (no or yes)					
No	63.4	1.00	-	1.00	-
Yes	70.6	1.38 (0.94-2.04)	.10	1.13 (0.74-1.72)	.57
Past/current history of certain common systemic diseases (no or yes)					
Systemic disease					
No	65.2	1.00	-	-	-
Yes	67.9 (55/ 81)	1.13 (0.68-1.87)	.64	-	-
Hypertension					
No	65.9	1.00	-	-	-
Yes	59.3 (16/27)	0.75 (0.34-1.65)	.48	-	-

CI = confidence interval; OR = odds ratio; VDT = visual display terminal.
[a]Adjusted for age and all of the associated factors identified in the univariate analyses ($P < .2$).

VDT develop DED. Therefore, one may suggest that there is a necessity of improving the ergonomics of the work environment to prevent the development of DED.

We would like to emphasize the potential limitations of our study, which are as follows: First, the study was based on a self-administered questionnaire, which might have introduced bias regarding misunderstandings about the answers relating to medication use and subjects' health status. Second, the current study lacked information about the percentage of pre- and postmenopausal participants, status of menopause, and use of hormone replacement therapy, which have been widely accepted as being related to DED; and about the use of tear supplements such as artificial tears. It should also be noted that the use of such medications may reduce DED symptoms, and therefore their use may not have reflected subjects' level of symptoms. A third possible limitation is that a break of only 1 hour was allowed in VDT usage prior to clinical examinations. Because of the brief nature of the timing relating to the VDT task, we may not have measured the actual impact of VDT in a clinical setting, which might be permanent or task related. Fourth, information on confounding factors for DED reported in previous studies, such as passive smoking, work environment parameters, lifestyle, and continued or intermittent VDT use, was not obtained in this study. Such limitations should be carefully considered during the carrying out of epidemiologic studies such as this. Since the average VDT work span is around 8 hours, we could not evaluate the appropriate time span of VDT usage that would not induce DED. Therefore, we need further study focusing on subjects working fewer hours on VDTs. Although symptoms have been considered to be an important component of DED, recent studies have reported a high prevalence of subjects with objective evidence of DED who are asymptomatic employing commonly used instruments to assess distress.[34,35] In fact, 1 paper has reported that over 40% of subjects with DED may be asymptomatic.[34] So the requirement of symptoms in Japanese diagnostic criteria to diagnose DED might have underestimated the prevalence of DED significantly, which is another limitation of the current study.

Finally, since this was a cross-sectional study, further studies are required to determine the temporal association between individual factors and DED, as well as to further elucidate the mechanism of short TBUT in VDT users. It would be of interest to determine whether modifications in the work environment, such as VDT usage duration, temperature, humidity, and work tasks, might alter the prevalence of DED and the risk of development of short TBUT.

In summary, this epidemiologic study in Japan showed a high prevalence of definite and probable dry eye in relatively young VDT users using clinical dry eye evaluations,

with the majority of subjects having short TBUT without abnormal tear secretion or obvious ocular surface staining. Female sex, age, and VDT usage of over 8 hours were identified as risk factors for definite and probable DED. We hope these data from our study will provide an enhanced understanding of DED to clinicians and researchers and help develop more targeted interventions for patients with DED.

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Biosketch

Dr Miki Uchino graduated from Yamanashi University School of Medicine, Yamanashi, Japan in 2000. Afterward, she worked as a resident in Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan. Dr Uchino's primary interests are dry eyes especially severe dry eyes associated with graft-versus-host disease and epidemiology of dry eye disease.