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Vascular Biology, Atherosclerosis, and Endothelium Biology

Topical Simvastatin Accelerates Wound Healing in Diabetes by Enhancing Angiogenesis and Lymphangiogenesis

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Impaired wound healing is a major complication of diabetes. Recent studies have reported reduced lymphangiogenesis and angiogenesis during diabetic wound healing, which are thought to be new therapeutic targets. Statins have effects beyond cholesterol reduction and can stimulate angiogenesis when used systemically. However, the effects of topically applied statins on wound healing have not been well investigated. The present study tested the hypothesis that topical application of simvastatin would promote lymphangiogenesis and angiogenesis during wound healing in genetically diabetic mice. A full-thickness skin wound was generated on the back of the diabetic mice and treated with simvastatin or vehicle topically. Simvastatin administration resulted in significant acceleration of wound recovery, which was notable for increases in both angiogenesis and lymphangiogenesis. Furthermore, simvastatin promoted infiltration of macrophages, which produced vascular endothelial growth factor C in granulation tissues. *In vitro*, simvastatin directly promoted capillary morphogenesis and exerted an antiapoptotic effect on lymphatic endothelial cells. These results suggest that the favorable effects of simvastatin on lymphangiogenesis are due to both a direct influence on lymphatics and indirect effects via macrophages homing to the wound. In conclusion, a simple strategy of topically applied simvastatin may have significant therapeutic potential for enhanced wound healing in patients with impaired microcirculation such as that in diabetes. *(Am J Pathol 2012, 181:2217–2224; http://dx.doi.org/10.1016/j.ajpath.2012.08.023)*

Delayed wound healing is a major complication of diabetes and is caused by increased apoptosis, delayed cellular infiltration, reduced angiogenesis, and decreased formation and organization of collagen fibers.[1–3] Impaired lymphangiogenesis has also recently been established as a major factor in diabetic refractory wound healing.[4,5] The functions of lymphatic vessels in wounds are to drain the protein-rich lymph from the extracellular space, to maintain normal tissue pressure, and to mediate the immune response.[6,7] Delayed wound healing, such as that seen in infections, appears to result from persistent edema and delayed removal of debris and inflammatory cells due to reduced lymphatic development.[8]

Statins are HMG-CoA (5-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitors that are primarily used to lower circulating cholesterol levels. In addition, statins have been found to protect against ischemic injury and stimulate angiogenesis in normocholesterolemic animals.[9–11] This angiogenic effect is partially mediated by direct regulation of proliferation of endothelial cells and capillary morphogenesis via the Akt/PI3K pathway.[11] Simvastatin has been found to enhance vascular endothelial growth factor (VEGF) production and improve wound healing in an experimental model of diabetes,[12] and nitropravastatin stimulates reparative neovascularization and improves recovery from limb ischemia in type 1 diabetic mice.[13] However, systemic administration at an extremely high dose was used to obtain angiogenic effects in

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these studies, and this is inapplicable for clinical use as an angiogenic drug in patients with ischemic disorders. However, topical application of statins with avoidance of systemic adverse effects may be useful for cutaneous wound healing, in which angiogenesis plays a pivotal role.[14] The lymphangiogenic effects of statins have not been widely investigated. In this study, we evaluated the effects of topical simvastatin on angiogenesis and lymphangiogenesis in a mouse model of impaired diabetic wound healing.

Materials and Methods

Animals

Genetically diabetic C57BLKS/J-m+/+*Lepr*^{db} mice (db/db mice) were obtained from Clea Japan, Inc. (Tokyo, Japan). All procedures were performed in accordance with the guidelines of the Animal Care and Use Committees of Kyoto Prefectural University of Medicine.

Creation of Wounds

Mice were between 6 and 10 weeks old at the time of the study. Wounds were generated as described previously.[15–17] In brief, after induction of deep anesthesia by i.p. injection of sodium pentobarbital (160 mg/kg), full-thickness, excisional skin wounds using 8-mm skin biopsy punches were made on the backs of mice, with one wound generated in each mouse. Each wound was covered with a semipermeable polyurethane dressing (OpSite; Smith and Nephew, Massillon, OH) after topical application of simvastatin (Calbiochem, La Jolla, CA) in petroleum jelly (a mixture of 5 mg of simvastatin and 995 mg of jelly) or vehicle (petroleum jelly alone). Simvastatin in petroleum jelly (10 mg of the mixture containing 50 μg of simvastatin) or vehicle were applied to the wound on days 0, 4, 7, and 10 after creation of the wound.

Monitoring of Wound Healing

A total of 5 db/db mice were used at each time point. Wound healing was monitored by taking pictures with a digital camera (Nikon Coolpix 995; Nikon, Tokyo, Japan) on days 0, 4, 7, and 14 after wound creation. Images were analyzed using ImageJ software version 1.46 (NIH, Bethesda, MD)[18] by tracing the wound margin with a high-resolution computer mouse and calculating the pixel area. Wound closure was calculated as follows: Percentage Closed = [(Area on Day 0 − Open Area on Final Day)/Area on Day 0] × 100, as described previously.[15] The areas of the wounds were compared with Student's *t*-test with $P < 0.05$ taken to indicate a significant difference.

Histologic Score

A histologic score was assigned in a masked manner as described previously.[15] Briefly, each specimen received a score of 1 to 12 as follows: 1 to 3, none to minimal cell accumulation and granulation tissue or epithelial migration; 4 to 6, thin, immature granulation dominated by inflammatory cells but with few fibroblasts, capillaries, or collagen deposition and minimal epithelial migration; 7 to 9, moderately thick granulation tissue, ranging from mainly inflammatory cells to more fibroblasts and collagen deposition; and 10 to 12, thick, vascular granulation tissue dominated by fibroblasts and extensive collagen deposition.

Evaluation of Wound Angiogenesis and Lymphangiogenesis

Sections were stained with rat anti-CD31 antibody (1:100) (BD Biosciences, San Jose, CA) or rabbit anti-LYVE-1 antibody (Upstate, Lake Placid, NY). Green fluorescence was generated by labeling with fluorescein isothiocyanate (FITC)–streptavidin (Vector Laboratories, Burlingame, CA) and biotinylated anti-rat or anti-rabbit antibody (both Vector Laboratories). Wound angiogenesis or lymphangiogenesis was analyzed by calculating the percentage of fluorescent area.[16,19] Briefly, for each slide, an image of the granulation tissue at the wound margin was captured. ImageJ software was used to quantitate the fluorescence intensity. The mean percentage of fluorescent pixels of five images served as an index of the angiogenic or lymphangiogenic response.

Evaluation of Macrophage Number, Phenotype, and VEGF-C Expression in Granulation Tissue

Sections of wounds were stained with rat anti-F4/80 antibody (Invitrogen, Carlsbad, CA). Labeling with F4/80 was visualized with Cy3-conjugated anti-rat antibody (Vector Laboratories). Ten granulation tissue fields (two sections from each animal) were selected, and F4/80-positive cells were counted.[16] VEGF-C expression was evaluated using goat anti-VEGF-C antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and FITC-conjugated anti-goat antibody (Vector Laboratories). To determine the phenotype of infiltrating macrophages, IL-13 and tumor necrosis factor (TNF) α expression was evaluated using goat anti-IL-13 antibody and goat anti-TNF-α antibody (Santa Cruz Biotechnology), respectively, and FITC-conjugated anti-goat antibody. F4/80-positive TNF-α–positive cells were defined as an M1 phenotype and F4/80-positive IL-13–positive cells as an M2 phenotype. In each slide, F4/80-positive cells, F4/80-positive TNF-α–positive cells, and F4/80-positive IL-13–positive cells were counted, and percentages of TNF-α–positive macrophages and IL-13–positive macrophages were evaluated. The mean percentages of TNF-α–positive macrophages and IL-13–positive macrophages in five images were used as indexes of the M1 and M2 phenotypes, respectively.

RNA Isolation, cDNA Synthesis, and Quantitative RT-PCR

Tissue sections obtained in RNAlater (Ambion, Paisley, UK) were processed for RNA isolation, cDNA synthesis, and quantitative RT-PCR.[16] VEGF-C, fibroblast growth factor 2, endothelial nitric oxide synthase, stromal cell-derived factor 1α, and platelet-derived growth factor β gene expression levels were normalized based on the level of an internal

reference gene, 18S. The primers used in the study were obtained from QIAGEN (Dűsseldorf, Germany).

Cell Culture

Primary human lymphatic endothelial cells (LECs) were collected as previously described.[20] LECs were cultured at 37°C in 5% CO_2 in endothelial cell basal medium 2 (Lonza, Walkersville, MD) supplemented with 5% fetal bovine serum, human VEGF-A, human fibroblast growth factor 2, human epidermal growth factor, insulin-like growth factor 1, and ascorbic acid. Each experiment was conducted at least three times, with similar results. A representative experiment is shown.

Western Blot Analysis

Cells were lysed with RIPA buffer (Invitrogen) and sonicated. After sonication, cell lysates were centrifuged at 15,400 × *g* for 20 minutes at 4°C, and the supernatants were collected into fresh tubes. Then 4× SDS sample buffer with 0.1 mol/L dithiothreitol was added to samples. Samples were boiled for 5 minutes at 95°C, and 20-μg extracts were separated by 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes for 2 hours at 180 mA. The membranes were incubated with rabbit anti-human Akt (pan) (C67E7) monoclonal antibody (Cell Signaling Technology, Danvers, MA), rabbit anti-human phospho-Akt (Ser473) (D9E) monoclonal antibody (Cell Signaling Technology), or mouse anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology) and detected with horseradish peroxidase–conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA) or horseradish peroxidase–conjugated goat anti-mouse IgG (Bio-Rad). Immunoblots were visualized using an ECL Plus Western Blotting Detection Reagents Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer protocol.

Chord Formation Assay

LECs were used in a chord formation assay.[21] An aliquot (100 μL) of growth factor–depleted Matrigel (Becton Dickinson, Bedford, MA) was added to a 24-well dish and allowed to gel for 30 minutes at 37°C. LECs were seeded at 5×10^4 cells/mL in 500 μL of endothelial cell basal medium 2 containing 3% fetal bovine serum. Cells were cultured in the absence or presence of various doses of simvastatin (Calbiochem, Darmstadt, Germany) with or without pretreatment with a PI3 kinase inhibitor, LY294002 (50 μmol/L) (ENZO Life Sciences, Plymouth Meeting, PA), the mTOR/raptor inhibitor rapamycin (100 nmol/L) (Merck Millipore, Darmstadt, Germany), or the PI3K/mTOR inhibitor wortmannin-rapamycin (100 nmol/L) (Cayman Chemical, Ann Arbor, MI) for 30 minutes. Chord formation was monitored for 24 hours. Digital pictures were taken using a spot image analysis system, and the total length of the chord-like structures at 12 hours was outlined and measured using ImageJ software.

Proliferation Assay

The proliferative activity of cells treated with simvastatin was examined using a CellTiter 96 nonradioactive cell proliferation assay (Promega, Madison, WI). Briefly, subconfluent cells (5000 cells per well) were reseeded on 96-well, flat-bottomed plates with 100 μL of growth media. The cells were treated with simvastatin and incubated for 48 hours at 37°C. Absorbance at 570 nm was recorded using a 96-well enzyme-linked immunosorbent assay (ELISA) plate reader.

Apoptosis Assay

An apoptosis assay was performed using a DeadEnd Fluorometric TUNEL System (Promega). Briefly, LECs were plated on chamber slides and placed in medium. Cells were stimulated by simvastatin and incubated for 24 hours with medium containing 400 μmol/L H_2O_2. To quantify apoptosis, 400 nuclei from random microscopic fields were analyzed by an observer masked to the treatment groups. The number of apoptotic cells was expressed as a percentage of the total cell count.

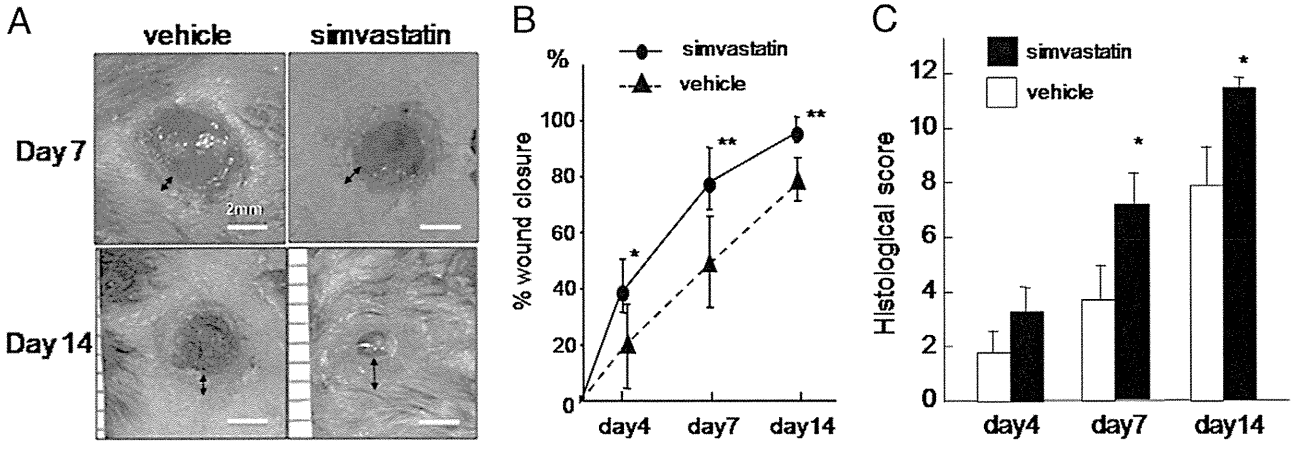

Figure 1. Effects of topical simvastatin on wound closure and histologic score in db/db mice. **A:** Representative macroscopic views of wounds after different treatments and periods. Scale bar = 2 mm. **Arrows** indicate the epithelialized range. **B:** Wound closure was measured on days 4, 7, and 14. $^*P < 0.05$, $^{**}P < 0.001$ versus vehicle ($n = 5$ in each group). **C:** Histologic scores for days 4, 7, and 14, quantified as described in *Materials and Methods*. Higher histologic scores indicate a greater extent of wound healing. $^*P < 0.05$ versus vehicle ($n = 5$ in each group).

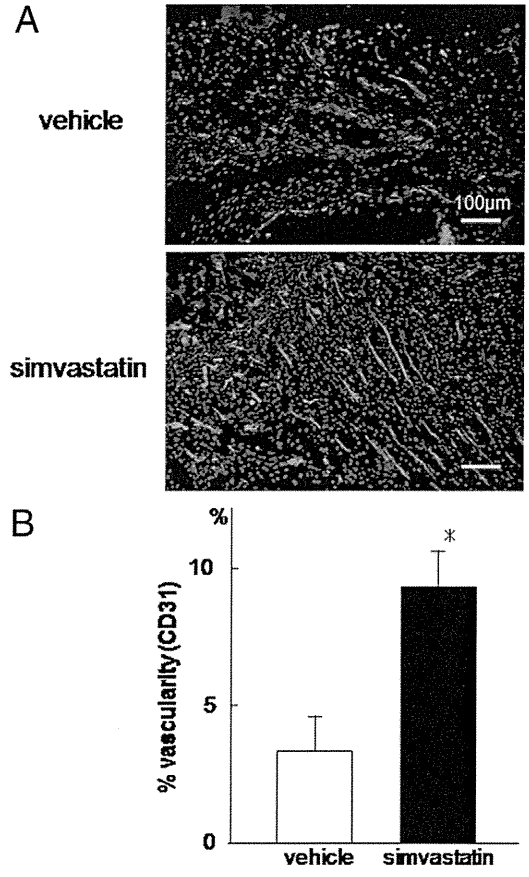

Figure 2. Effects of simvastatin on vascularity in granulation tissues at the wound margin in db/db mice. **A:** Neovascularization at the wound margin in simvastatin- or vehicle-treated diabetic mice after 14 days. Original magnification, ×100. Scale bar = 100 μm. Green and blue fluorescence corresponds to CD31-positive newly formed blood vessels and DAPI-labeled nuclei, respectively. **B:** Percentage of vascularity, quantified as described in *Materials and Methods*. *$P < 0.001$ versus vehicle ($n = 5$ in each group).

Statistical Analysis

All results are presented as mean ± SEM. Statistical comparisons between two groups were performed by Student's *t*-test. Multiple groups were analyzed by one-way analysis of variance followed by appropriate post hoc tests to determine statistical significance. $P < 0.05$ was considered significant. All *in vitro* experiments were performed at least in triplicate.

Results

Simvastatin Accelerates Wound Healing in Diabetic Mice

Wound areas on days 7 and 14 in simvastatin- or vehicle-treated diabetic mice are shown in Figure 1A. On day 14, simvastatin-treated wounds had more than 90% epithelialization, whereas <80% of the wound was epithelialized in the vehicle-treated group (Figure 1B). Simvastatin treatment resulted in significantly smaller wound areas after 4, 7, and 14 days. The difference in percentage of wound closure reached a maximum on day 7 (simvastatin versus control: 79.26% ± 11.09% versus 52.45% ± 16.81%; $P < 0.001$). The histologic score reflects the degree of maturation of granulation tissue, including inflammation, collagen deposition, and reepithelialization, in addition to neovascularization; therefore, higher histologic scores reflect a greater extent of wound healing. The histologic scores for wounds treated with simvastatin were significantly higher than those in the vehicle-treated group (day 4: 3.6 ± 0.70 versus 1.9 ± 0.73; day 7: 7.3 ± 0.94 versus 3.7 ± 0.94, $P < 0.01$; day 14: 11.6 ± 0.51 versus 8.0 ± 1.15, $P < 0.01$)(Figure 1C).

Simvastatin Promotes Both Angiogenesis and Lymphangiogenesis

Wound angiogenesis was analyzed by immunostaining of an endothelial cell–specific marker, CD31, in 10-μm frozen sections to visualize neovascularization. Figure 2A shows neovascularization at the margin in simvastatin- or vehicle-treated wounds in diabetic mice on day 14. A few small vessels were seen at the wound margin in the vehicle-treated group, whereas large numbers of vessels were growing toward the center of the wound in the

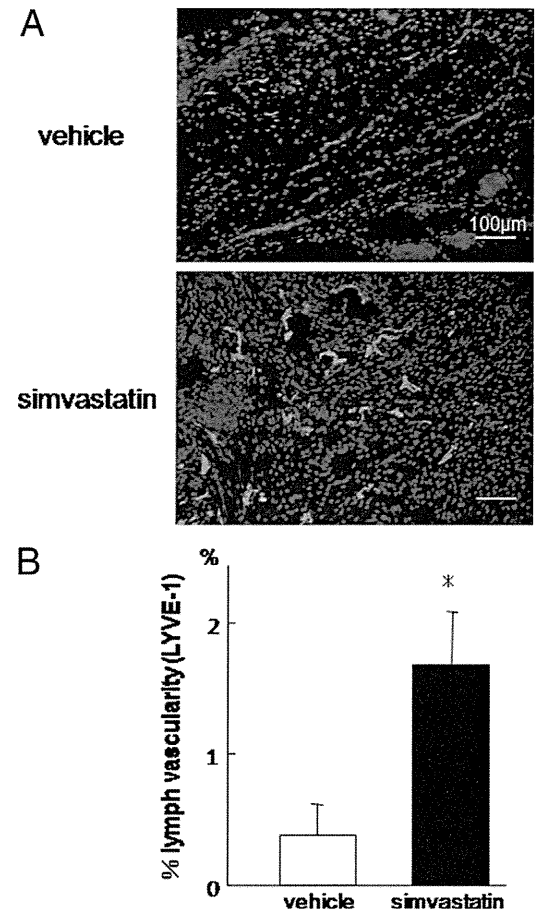

Figure 3. Effects of simvastatin on lymphangiogenesis in granulation tissues at the wound margin in db/db mice. **A:** Lymphangiogenesis at the wound margin in simvastatin- or vehicle-treated diabetic mice after 14 days. Original magnification, ×100. Scale bar =100 μm. Green and blue fluorescence corresponds to LYVE-1–positive newly formed lymphatic vessels and DAPI-labeled nuclei, respectively. **B:** Percentage of lymphatic vascularity, quantified as described in *Materials and Methods*. *$P < 0.001$ versus vehicle ($n = 5$ in each group).

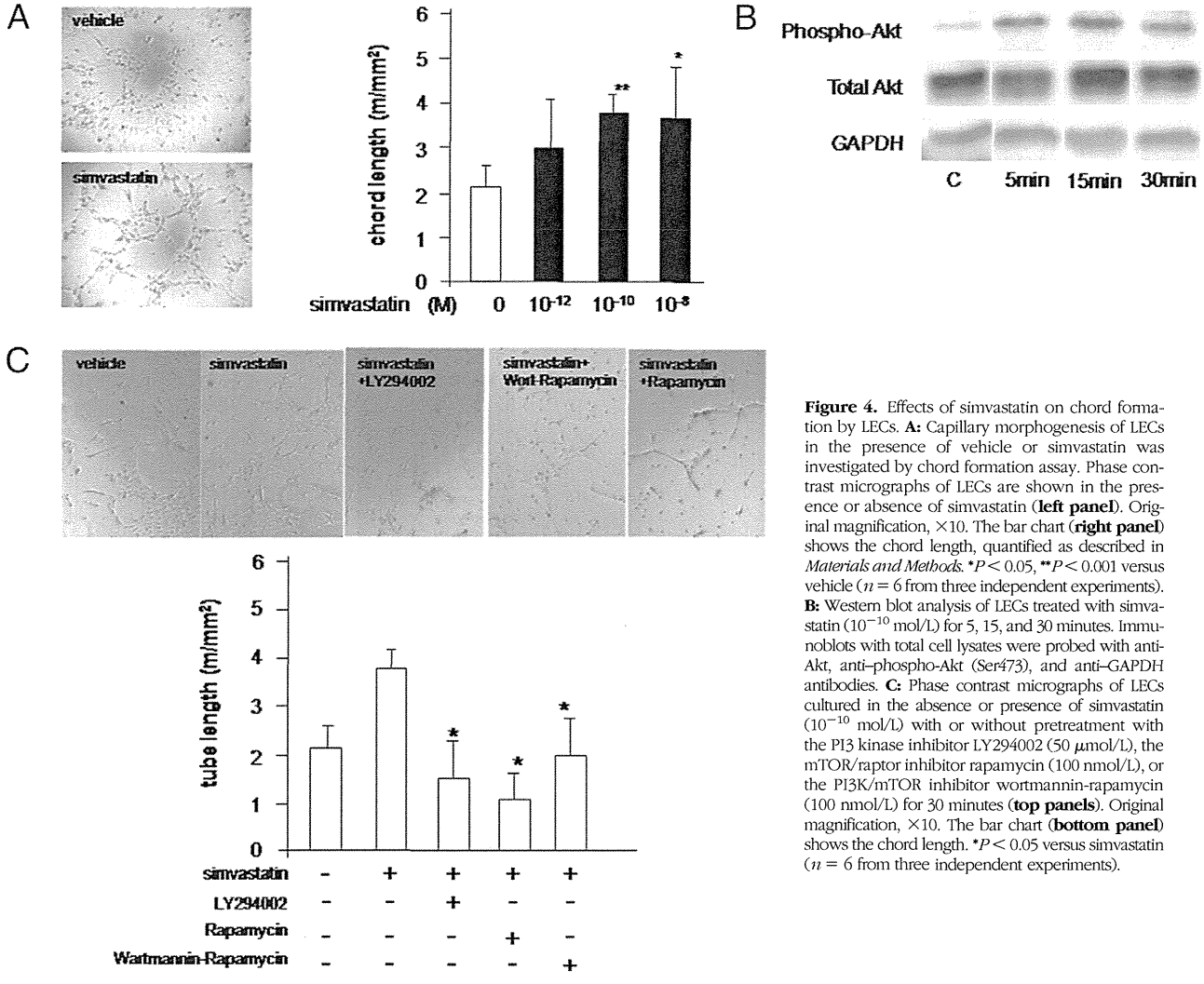

Figure 4. Effects of simvastatin on chord formation by LECs. **A:** Capillary morphogenesis of LECs in the presence of vehicle or simvastatin was investigated by chord formation assay. Phase contrast micrographs of LECs are shown in the presence or absence of simvastatin (**left panel**). Original magnification, ×10. The bar chart (**right panel**) shows the chord length, quantified as described in *Materials and Methods*. $^{*}P < 0.05$, $^{**}P < 0.001$ versus vehicle ($n = 6$ from three independent experiments). **B:** Western blot analysis of LECs treated with simvastatin (10^{-10} mol/L) for 5, 15, and 30 minutes. Immunoblots with total cell lysates were probed with anti-Akt, anti–phospho-Akt (Ser473), and anti–GAPDH antibodies. **C:** Phase contrast micrographs of LECs cultured in the absence or presence of simvastatin (10^{-10} mol/L) with or without pretreatment with the PI3 kinase inhibitor LY294002 (50 μmol/L), the mTOR/raptor inhibitor rapamycin (100 nmol/L), or the PI3K/mTOR inhibitor wortmannin-rapamycin (100 nmol/L) for 30 minutes (**top panels**). Original magnification, ×10. The bar chart (**bottom panel**) shows the chord length. $^{*}P < 0.05$ versus simvastatin ($n = 6$ from three independent experiments).

simvastatin group. Simvastatin significantly enhanced wound vascularity based on image analysis of the percentage of the fluorescent area (9.29% ± 1.29% versus 3.25% ± 1.33%; $P < 0.001$)(Figure 2B). Wound lymphangiogenesis was analyzed by immunostaining of a LEC-specific marker, LYVE-1, in 10-μm frozen sections. Figure 3A shows new lymphatic vessels at the margin of simvastatin- or vehicle-treated wounds in diabetic mice on day 14. Wound lymphatic vascularity was significantly enhanced by simvastatin (percentage of fluorescent area: 1.72% ± 0.460% versus 0.395% ± 0.260%; $P < 0.001$) (Figure 3B). New vessels and lymphatics in granulation tissue in both groups were not covered with α-smooth muscle actin–positive mural cells (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).

Simvastatin Induces Capillary Morphogenesis of LECs and Has an Antiapoptotic Effect but Does Not Induce Proliferation

To characterize the effects of simvastatin on lymphangiogenesis, we performed a chord formation assay in primary human LECs *in vitro*. Treatment with simvastatin promoted LEC chord formation in a dose-dependent manner (Figure 4A). This effect was significantly blocked by the PI3 kinase inhibitor LY294002, the mTOR inhibitor rapamycin, and the PI3/mTOR inhibitor wortmannin-rapamycin ($P < 0.05$)(Figure 4C). The proliferative and antiapoptotic effects of simvastatin on LECs were also examined because these are major effects of simvastatin in vascular endothelial cells. Simvastatin did not promote LEC proliferation, even at higher concentrations, and seemed to be slightly cytotoxic at 10^{-6} mol/L and 10^{-5} mol/L (Figure 5A). However, simvastatin treatment resulted in significant inhibition of H_2O_2-induced apoptosis compared with controls (Figure 5B).

Simvastatin Promotes Macrophage Infiltration and VEGF-C Production in Wounds

The number of macrophages in granulation tissues was evaluated in wounds on day 7. This timing was chosen because reepithelialization was almost complete on day 14 in simvastatin-treated wounds, and inflammatory cells had already diminished. The number of macrophages in simvastatin-treated wounds on day 7 was significantly

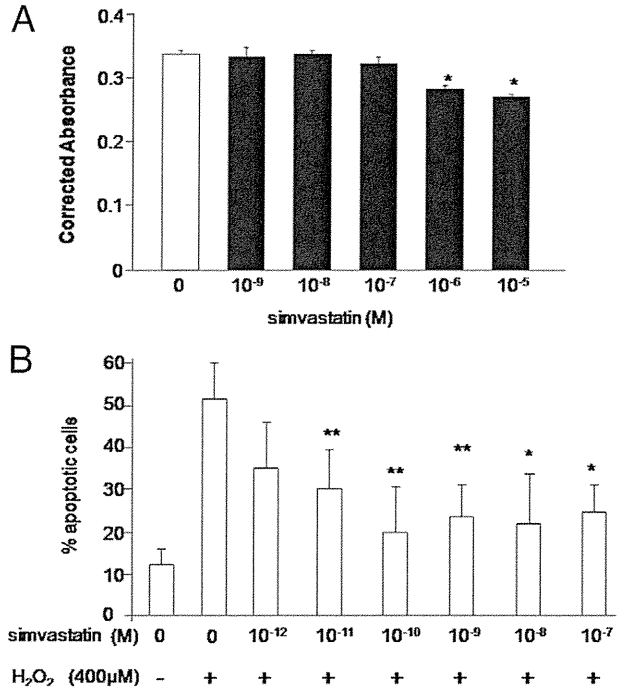

Figure 5. Effects of simvastatin on proliferation and apoptosis of LECs. **A:** Cell proliferation of LECs was investigated by MTS assay. Subconfluent cells (5000 cells per well) were reseeded on 96-well, flat-bottomed plates with 100 μL of growth media. The cells were treated with simvastatin and incubated for 48 hours at 37°C. Absorbance at 570 nm was recorded using a 96-well ELSIA plate reader. Quantification was performed as described in *Materials and Methods*. $^*P < 0.05$ versus vehicle ($n = 8$ from three independent experiments). **B:** Cell apoptosis in LECs was investigated by TUNEL assay. LECs were plated on chamber slides and placed in medium. Cells were stimulated by simvastatin and incubated for 24 hours with medium containing 400 μmol/L H_2O_2. Quantification of apoptotic cells was performed as described in *Materials and Methods*. $^*P < 0.05$, $^{**}P < 0.01$ versus H_2O_2 treatment ($n = 3$ from three independent experiments).

greater than that in controls (Figure 6, A and B). Most of the macrophages in the simvastatin-treated group expressed the M2 marker, IL-13, rather than the M1 marker, TNF-α, whereas most macrophages in the vehicle-treated group expressed TNF-α rather than IL-13 (Figure 6, C–F). The macrophages in the simvastatin-treated group produced VEGF-C (Figure 7A), and VEGF-C expression was significantly up-regulated in simvastatin-treated wounds compared with controls (Figure 7B). Other proangiogenic mediators in wound granulation tissue were evaluated by real-time PCR. Platelet-derived growth factor β, endothelial nitric oxide synthase, and fibroblast growth factor 2 were significantly up-regulated by simvastatin stimulation (see Supplemental Figure S2 at *http://ajp.amjpathol.org*).

Discussion

In this study, we found that topical application of simvastatin accelerated diabetic wound healing via promotion of angiogenesis and lymphangiogenesis. Many studies have reported that statins, including simvastatin, have strong angiogenic effects on vascular endothelial cells or placental stem cells and that these effects are mainly mediated by the PI3-kinase/Akt pathway,[11,22,23] although we note that other findings have also been reported[24] Consistent with these reports, abundant neovascularization and proangiogenic growth factors were observed in wounds treated with topical simvastatin in our *in vivo* study. Statins were originally introduced as systemic antihyperlipidemic drugs; however, a recent study has shown the value of topical simvastatin.[14] An advantage of topical application is that a suitable concentration of simvastatin can be applied without a risk of serious systemic adverse effects, such as rhabdomyolysis. Our results suggest that topical application of simvastatin could be a

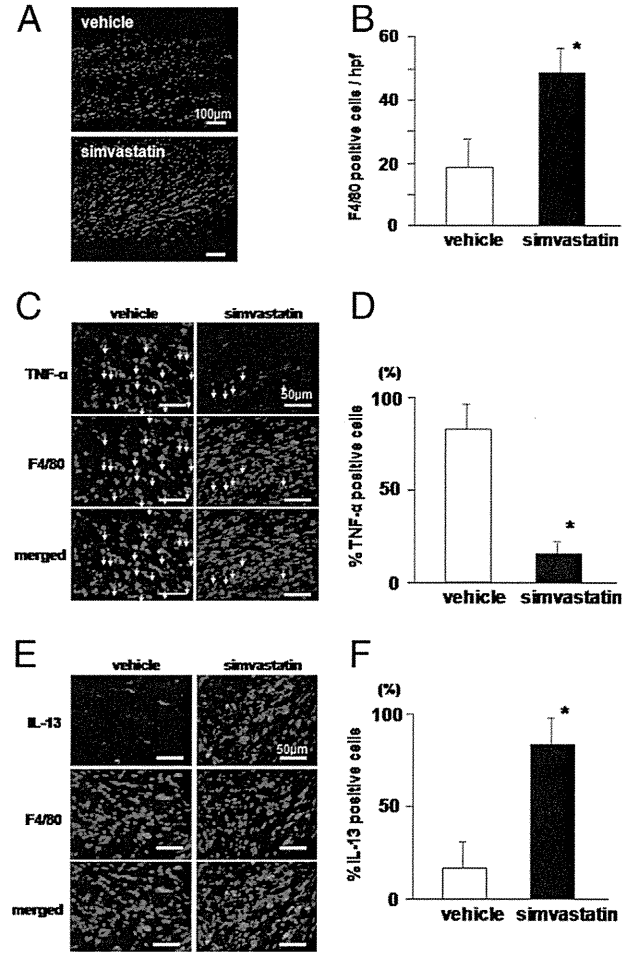

Figure 6. Effects of simvastatin on macrophage infiltration and phenotype in granulation tissue. **A:** Representative photomicrographs of the immunostained wound edge at 7 days after wound creation. Red fluorescence corresponds to F4/80-positive macrophages. Scale bar = 100 μm. **B:** The macrophage count, quantified as described in *Materials and Methods*. $^*P < 0.05$ versus vehicle ($n = 5$). **C:** Representative photomicrographs of the immunodetection of TNF-α and F4/80 in histologic sections from vehicle- or simvastatin-treated wounds (original magnification ×400). Scale bar = 50 μm. Green and red fluorescence corresponds to TNF-α–positive cells and F4/80-positive macrophages, respectively. Yellow indicates TNF-α–producing M1 phenotype macrophages (**white arrows**). **D:** Quantification of percentage of TNF-α–positive macrophages, as described in *Materials and Methods*. $^*P < 0.001$ versus vehicle ($n = 5$ in each group). **E:** Representative photomicrographs of immunodetection of IL-13 and F4/80 in histologic sections from vehicle- or simvastatin-treated wounds (original magnification ×400). Scale bar = 50 μm. Green and red fluorescence correspond to IL-13–positive cells and F4/80-positive macrophages, respectively. Yellow indicates IL-13–producing M2 phenotype macrophages. **F:** Quantification of percentage of IL-13–positive macrophages, as described in *Materials and Methods*. $^*P < 0.001$ versus vehicle ($n = 5$ in each group).

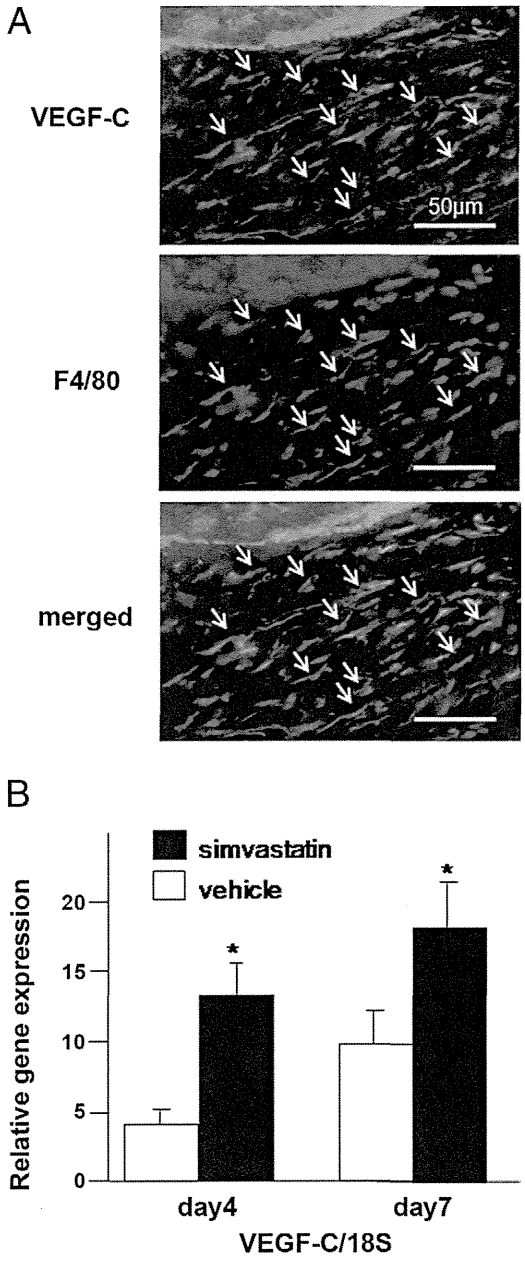

Figure 7. VEGF-C expression in granulation tissue. **A:** Representative photomicrographs of the immunostained wound edge treated with simvastatin at 7 days after wound creation. Green and red fluorescence indicates VEGF-C expression and F4/80-positive macrophages, respectively. Original magnification, ×400. **Arrows** indicate double-positive cells. Scale bar = 50 μm. **B:** Quantitative RT-PCR of VEGF-C in wound granulation tissue. Gene expression levels were normalized based on the level of an internal reference gene, 18S. $^*P < 0.05$ versus vehicle ($n = 5$).

new therapeutic strategy for treatment of local ischemic conditions, such as those in patients with diabetic ulcers.

Lymphangiogenesis is a major factor in diabetic refractory wound healing.[4,5] Therefore, we focused on the effects of simvastatin on wound lymphangiogenesis. Recent studies have suggested that several biological functions of LECs are partially regulated by the AKT/PI3K/mTOR pathway.[25,26] Consistent with these observations, capillary morphogenesis of LECs was significantly stimulated by simvastatin as an effect on vascular endothelial cells that was, at least in part, regulated by the AKT/PI3K/mTOR pathway. Our results suggest that the mechanisms underlying the lymphangiogenic effects of simvastatin in LECs might be similar to those for angiogenic effects. These mechanisms include antiapoptosis and promotion of capillary morphogenesis because LECs develop from a vascular network in an embryonic stage,[27] and these cells have a similar lineage. However, contrary to our expectation, simvastatin did not promote proliferation of LECs *in vitro*. During the wound healing process, new lymphatics are formed in newly generated granulation tissue, indicating that proliferation of preexisting lymphatic vessels is needed.

Because simvastatin did not promote the proliferation of LECs, we evaluated other possible sources of lymphangiogenic factors. Several reports suggest that infiltrating macrophages contribute to lymphangiogenesis as the major producer of VEGF-C in cutaneous wound healing,[4,5] and therefore we evaluated the effects of simvastatin on macrophages. Macrophages carry VEGF receptor 3, in addition to producing VEGF-C, and thus act as both autocrine and paracrine factors. We have previously reported that healing impairment in diabetes involves reduced lymphangiogenesis and suppressed macrophage function, such as recruitment to inflammatory sites and secretion of growth factors.[5] In this study, the number of infiltrating macrophages in granulation tissue was significantly increased by topical application of simvastatin, and most of these macrophages produced VEGF-C. These observations suggest that simvastatin recovers lymphangiogenic function that is impaired in macrophages under diabetic conditions.

Increased apoptosis is a major concern in wound healing in a diabetic state.[3,28–31] Hyperglycemia induces proinflammatory cytokines, such as TNF-α, and oxidative stress, which result in increased apoptosis in diabetes. Our study found that most infiltrating macrophages in diabetic wounds had an M1 proinflammatory phenotype producing abundant TNF-α. Simvastatin decreased H_2O_2-induced apoptosis in LECs *in vitro* and increased M2 anti-inflammatory phenotype macrophages in granulation tissue *in vivo*. We suggest that this anti-apoptotic effect of simvastatin also plays an important role, in addition to promotion of angiogenesis and lymphangiogenesis.

Increased infiltration of macrophages induced by simvastatin may have further benefits because the histologic scores of diabetic wounds were significantly improved by topical application of simvastatin. The histologic score reflects the degree of maturation of granulation tissue, including inflammation, collagen deposition, and reepithelialization, in addition to neovascularization. Macrophages play a central role in all stages of wound healing and orchestrate the wound healing process[32] by exerting proinflammatory functions and facilitating wound healing during the early stage and stimulating proliferation of fibroblasts, keratinocytes, and endothelial cells in the proliferative stage. Because the main focus of this study was lymphangiogenesis, we did not investigate the effects of simvastatin on reepithelialization or formation of extracellular matrix. This will require further experiments in a future study.

In conclusion, regulation of apoptosis and capillary differentiation are essential for development of functional lymphatics during wound healing. The findings of the present study suggest that topical simvastatin can stimulate lymp-

hangiogenesis directly and indirectly via stimulation of macrophages. Vascular remodeling induced by simvastatin might have therapeutic potential in patients with microvascular dysfunction, such as that in diabetic foot ulcer, a major cause of morbidity in the growing population of patients with diabetes. A future investigation is warranted to determine the potential clinical utility of this approach.

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Visual Improvement after Cultivated Oral Mucosal Epithelial Transplantation

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Purpose: To report the effectiveness, disease-specific outcomes, and safety of cultivated oral mucosal epithelial sheet transplantation (COMET), with the primary objective of visual improvement.

Design: Noncomparative, retrospective, interventional case series.

Participants: This study involved 46 eyes in 40 patients with complete limbal stem cell deficiency (LSCD) who underwent COMET for visual improvement. These LSCD disorders fell into the following 4 categories: Stevens-Johnson syndrome (SJS; 21 eyes), ocular cicatricial pemphigoid (OCP; 10 eyes), thermal or chemical injury (7 eyes), or other diseases (8 eyes).

Methods: Best-corrected visual acuity (BCVA) and ocular surface grading score were examined before surgery; at the 4th, 12th, and 24th postoperative week; and at the last follow-up. Data on COMET-related adverse events and postoperative management were collected. The outcomes in each disease category were evaluated separately.

Main Outcome Measures: The primary outcome was the change in median logarithm of the minimum angle of resolution (logMAR) BCVA at the 24th postoperative week. The secondary outcome was the ocular surface grading score.

Results: Median logMAR BCVA at baseline was 2.40 (range, 1.10 to 3.00). In SJS, logMAR BCVA improved significantly during the 24 weeks after surgery. In contrast, the BCVA in OCP was improved significantly only at the 4th postoperative week. In 6 of the 7 thermal or chemical injury cases, logMAR BCVA improved after planned penetrating keratoplasty or deep lamellar keratoplasty. Grading scores of ocular surface abnormalities improved in all categories. Of 31 patients with vision loss (logMAR BCVA, >2) at baseline, COMET produced improvement (logMAR BCVA, ≤2) in 15 patients (48%). Visual improvement was maintained with long-term follow-up (median, 28.7 months). Multivariate stepwise logistic regression analysis showed that corneal neovascularization and symblepharon were correlated significantly with logMAR BCVA improvement at the 24th postoperative week ($P = 0.0023$ and $P = 0.0173$, respectively). Although postoperative persistent epithelial defects and slight to moderate corneal infection occurred in the eyes of 16 and 2 patients, respectively, all were treated successfully with no eye perforation.

Conclusions: Long-term visual improvement was achievable in cases of complete LSCD. Cultivated oral mucosal epithelial sheet transplantation offered substantial visual improvement even for patients with end-stage severe ocular surface disorders accompanying severe tear deficiency. Patients with corneal blindness such as SJS benefited from critical improvement of visual acuity.

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Corneal renewal and repair are mediated by corneal epithelial stem cells situated mainly in the limbus, the narrow region between the cornea and the bulbar conjunctiva.[1] Damage or depletion of the corneal epithelial stem cells, known as limbal stem cell deficiency (LSCD), leads to conjunctival invasion that results in vascularization and scarring of the cornea with an associated profound loss of vision.[1] Limbal stem cell deficiency can be caused by Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid (OCP), and thermal or chemical injury, which are all characterized by the loss of corneal epithelial stem cells. Such LSCD may cause severe ocular surface diseases (OSDs) in which cicatrization resulting from conjunctival fibrosis, symblepharon, and severe dry eye greatly disrupt visual function and can progress gradually with chronic inflammation.[2–4] To date, few effective medical or surgical treatments for severe OSDs have been available.[5–15]

Since 1998, the authors have used amniotic membrane transplantation to treat severe OSDs. Amniotic membrane exhibits an anti-inflammatory effect and also acts as a substrate for epithelialization.[16] The results of previous studies have shown that amniotic membrane transplantation alone[17,18] or amniotic membrane transplantation combined with limbal transplantation[6,19,20] promoted epithelialization, reduced pain, reconstructed the fornix, and minimized inflammation of the ocular surface to a remarkable degree in

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patients with severe OSDs. Based on these promising results, novel methods have been developed for the cultivation of allogeneic corneal[7,8,21] or autologous oral mucosal[22–25] epithelial cells on a denuded amniotic membrane. Immunologic rejection and increased risk of infection or systemic adverse effects associated with the long-term immunosuppressive therapy accompanying allograft transplantation[6] encouraged changing to autologous cultivated oral mucosal epithelial transplantation (COMET) in patients with severe OSDs in 2002.[10,11,23,26]

To clarify the effectiveness, disease-specific outcomes, and safety of COMET, all of the clinical data from all 72 patients that the authors treated with COMET since 2002 were analyzed. The objective of this present study was to summarize the long-term clinical outcomes of 40 of those 72 patients who underwent COMET with the primary objective of visual improvement between June 2002 and December 2008.

Patients and Methods

Patients

Autologous COMET was performed on consecutive patients who were diagnosed with total LSCD based on the complete disappearance of the palisades of Vogt and 360° of conjunctivalization.[1] The COMET treatment protocol was approved by the ethical review board of Kyoto Prefectural University of Medicine, Kyoto, Japan, in 2002. The final decision to perform COMET was made by the university's team of corneal specialists. Before the surgery, written informed consent was obtained from all patients in accordance with the tenets of the Declaration of Helsinki for research involving human subjects. The current retrospective study used an itemized data collection form, and the medical records of all patients who underwent COMET between June 2002 and December 2008 were examined retrospectively. This retrospective study protocol was approved by the ethical review board of Kyoto Prefectural University of Medicine in 2009. In this study, 40 of the 72 patients who underwent COMET were analyzed with the primary objective of visual improvement.

Cell Culture

All of the COMET sheets were prepared at the good manufacturing practices–graded Cell Processing Center at Kyoto Prefectural University of Medicine as previously described.[23,26] Autologous oral mucosal epithelial cells were obtained from a 6-mm–diameter biopsy specimen obtained from each patient's buccal mucosa, and the cells then were cultured on an amniotic membrane spread on the bottom of a culture insert and were cocultured with mitomycin C-inactivated 3T3 fibroblasts (NIH-3T34; RIKEN Cell Bank, Tsukuba, Japan). The cultured cells were submerged in medium for approximately 1 week and then were exposed to air by lowering the medium level (airlifting) for 1 to 2 days. All amniotic membrane was obtained from caesarean sections according to the preparation method described previously.[23] Although fetal bovine serum initially was used as a culture medium, autologous serum was used in later cultures to reduce the risk of transmitting nonhuman pathogens.[26]

Transplantation and Postoperative Management

The surgical procedure (see the Supplemental Video, available at http://aaojournal.org) and postoperative management have been described previously.[24,25] In patients with severe symblepharon or a large area of bare sclera exposed during surgery, amniotic membrane was transplanted onto the bare sclera to reconstruct conjunctival fornices.[18] In patients with a cataract, phacoemulsification and aspiration plus intraocular lens implantation were performed simultaneously with COMET. No penetrating keratoplasty or deep lamellar keratoplasty was performed simultaneously with COMET. For patients with severe corneal stromal opacity, a 2-step surgical approach was planned, with the first step being COMET and the second step being either penetrating or deep lamellar keratoplasty.[25]

Systemic corticosteroid (betamethasone, 1 mg/day) and cyclosporine (2 to 3 mg/kg daily) were administered to prevent postoperative inflammation and immunologic response and then were tapered, depending on the clinical findings. Dexamethasone (0.1%) and antibiotic eye drops were instilled 4 times daily. Dry-eye patients were administered artificial tears. A therapeutic soft contact lens was used for at least 1 month to protect transplanted epithelium from mechanical ablation.

Postoperative Follow-up and Outcomes

Best-corrected visual acuity (BCVA) was converted to the logarithm of the minimum angle of resolution (logMAR). Ocular surface conditions including corneal appearance (epithelial defects, clinical conjunctivalization, neovascularization, opacification, keratinization, and symblepharon) were graded by at least 2 ophthalmologists (C.S., T.I., and T.N.) on a scale from 0 to 3 according to their severity, in accordance with a previously reported grading system.[27] Severe OSDs are characterized by an associated loss of conjunctival stem cells, and the severity of conjunctival involvement affects the visual prognosis. Therefore, findings on upper and lower fornix shortening were added to evaluate the grade of conjunctival appearance. Fornix shortening was graded from 0 to 3 based on the following clinical features: normal depth (grade 0), shortened by less than one quarter (grade 1), shortened by one quarter to one half (grade 2), and shortened by more than one half (grade 3). Upper and lower fornix shortenings were graded separately. The sum of each grading score was defined as the ocular surface grading score (maximum, 24).

Each patients logMAR BCVA, ocular surface grading score, and data on adverse events related to COMET or postoperative management were collected from the medical records at these specific time points: before surgery; at the 4th, 12th, and 24th postoperative weeks; and at the last follow-up examination. The primary outcome was the change in logMAR BCVA at the 24th postoperative week. Because other ocular diseases can affect this visual outcome, a secondary outcome, the ocular surface grading score, also was defined.

Statistical Analysis

The change in BCVA and ocular surface grading score from baseline at each visit, except for the last visit, was analyzed using the Wilcoxon signed-rank test in each disease category (SJS, OCP, thermal or chemical injury) except for other diseases. Multivariate stepwise logistic regression analysis was used to determine the factors influencing visual improvement.

This study defined the critical visual improvement rate as the proportion of patients in whom BCVA at the 24th postoperative week had improved to at least 0.01, as a percentage of the patients with a BCVA of less than 0.01 at baseline. Patients with a visual acuity of 0.01 or more can read and walk using vision aids. Thus, an improvement to at least 0.01 indicates a capacity for independence in daily life. If data were missing from the 24th postoperative week, data from follow-up at the last visit were substituted.

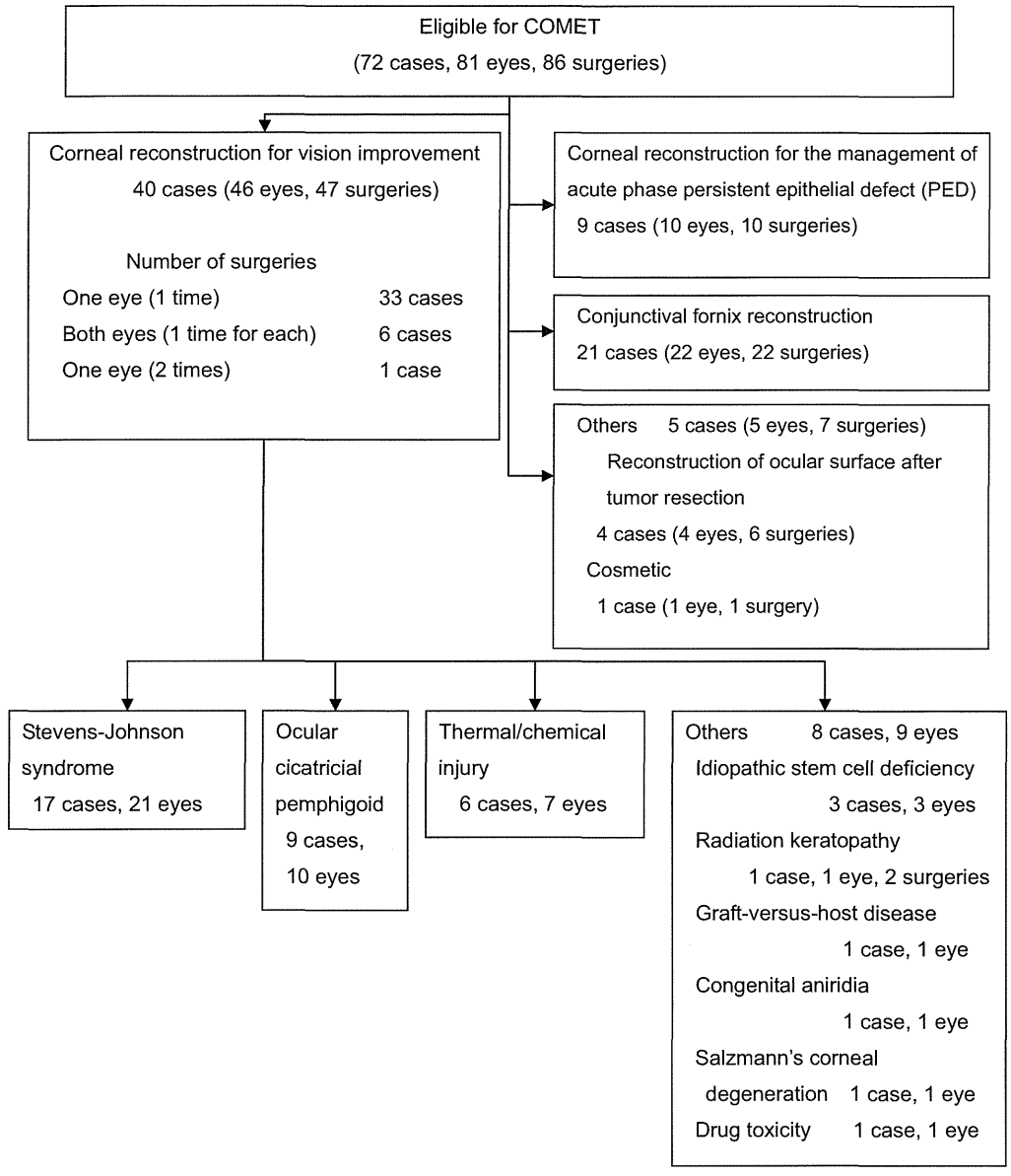

Figure 1. Diagram showing flow of study. Seventy-two patients (81 eyes) underwent cultivated oral mucosal epithelial sheet transplantation (COMET) between June 2002 and December 2008, and 40 patients (46 eyes) were analyzed for visual improvement in this study. Both corneal reconstruction and conjunctival fornix reconstruction were carried out in 3 cases, in the same eye in 1 case, and counted separately.

All statistical analyses were conducted at the Translational Research Informatics Center (Kobe, Japan) with the use of SAS software, version 9.1 (SAS Inc, Cary, NC) or JMP software, version 8.2 (SAS Inc). *P* values of less than 0.05 were considered statistically significant.

Results

Patient Characteristics

Between 2002 and 2008, 47 COMETs (46 eyes in 40 patients) were performed on 21 eyes with SJS, 10 eyes with OCP, 7 eyes with thermal or chemical injury, and 9 eyes with other causes of LSCD (Fig 1). Although 23 eyes (48.9%) previously had been treated with ocular surgery, all of these previous treatments had failed and recurrence of fibrovascular ingrowth on the cornea was observed. Of the 47 surgeries performed, symblepharon and keratinization of the cornea were present in 37 eyes (78.7%) and 10 eyes (21.3%), respectively, thus indicating that most of the eyes were inflicted with end-stage severe OSDs (Table 1).

Outcomes of Cultivated Oral Mucosal Epithelial Sheet Transplantation

Cultivated autologous oral mucosal epithelial sheets were generated successfully from all patients. In all patients, COMET was performed successfully and no epithelial damage was observed during surgery. Cultivated oral mucosal epithelial sheet transplantation was combined with amniotic membrane transplantation in 34 (72%) of the 47 surgeries and with cataract surgery in 11 eyes (23%; Table 2, available at http://aaojournal.org). In 10 patients

Table 1. Baseline Characteristics in Patients Who Underwent Autologous Cultivated Oral Mucosal Epithelial Transplantation

	Total	Stevens-Johnson Syndrome	Ocular Pemphigoid	Thermal/Chemical Injury	Others
No. of COMETs	47	21	10	7	9
Age (yrs)					
Median	57.0	43.0	73.5	50.0	34.0
Range	9–86	14–71	62–86	27–79	9–75
Duration of illness (yrs)					
Median	12.3	17.9	3.5	6.0	5.08
Range	0.3–40.0	3.0–38.0	0.3–15.0	0.5–24.0	0.5–40.0
Prior ocular surgery (%)	23 (48.9)	9 (42.9)	4 (40.0)	3 (42.9)	7 (77.8)
Planned 2-step operations (%)	10 (21.3)	2 (9.5)	0 (0)	6 (85.7)	2 (22.2)
Symblepharon (%)	37 (78.7)	18 (85.7)	10 (100.0)	6 (85.7)	3 (33.3)
Keratinization (%)	10 (21.3)	8 (38.1)	1 (10.0)	0 (0)	1 (11.1)
Preoperative visual acuity*					
Median	2.40	2.4	2.70	2.70	2.40
Range	1.11–3.00	1.40–3.00	1.52–2.70	1.22–2.70	1.10–2.70
Preoperative ocular surface grading score					
Median	14.0	15.0	17.0	13.0	8.0
Range	5.0–21.0	8.0–21.0	10.0–21.0	7.0–17.0	5.0–19.0

COMET = autologous cultivated oral mucosal epithelial transplantation.
*Logarithm of the minimum angle of resolution units.

with severe corneal stromal opacity, a 2-step surgical approach was planned, with COMET followed by penetrating keratoplasty or deep lamellar keratoplasty.[25] Three patients underwent the second surgery before the 24th postoperative week and 5 patients underwent the surgery after the 24th week, but 2 patients did not undergo the second surgery during the study period.

The median preoperative logMAR BCVA was 2.40, and in 31 of the eyes (66%), visual acuity was poorer than 20/2000 (<0.01, logMAR >2). The median preoperative ocular surface grading score was 18.0 (range, 5 to 21). The median patient follow-up period with observation of the primary outcome was 28.7 months after transplantation (range, 6.2 to 85.6 months). Because of heterogeneous etiologic mechanisms, the outcomes in each category are described separately.

Disease-Specific Outcomes

Stevens-Johnson Syndrome. Seventeen patients with SJS underwent COMET (Table 2, available at http://aaojournal.org). The BCVA improved significantly at 4, 12, and 24 weeks after surgery ($P = 0.0005$, $P = 0.0010$, and $P = 0.0117$, respectively; Fig 2A). The ocular surface grading score also improved significantly at 4, 12, and 24 weeks after surgery ($P<0.0001$ for each time point; Fig 2B).

Ocular Cicatricial Pemphigoid. Nine patients (10 eyes) with OCP underwent COMET (Table 1). All 9 patients were older than 60 years, older than many of the patients in this study with other diseases (Table 2, available at http://aaojournal.org). The BCVA was improved significantly at the 4th postoperative week ($P = 0.0156$), but this improvement later disappeared (Fig 2A). In contrast, improvement of the ocular surface grading score was sustained throughout the follow-up period ($P = 0.0020$, $P = 0.0020$, and $P = 0.0078$, respectively; Fig 2B).

Thermal or Chemical Injury. Seven patients (7 eyes) with thermal or chemical injury underwent COMET. Their BCVA did not change until the 24th postoperative week; however, the ocular surface grading score in all 7 patients improved significantly ($P = 0.0156$ for each visit; Fig 2A, B). Although penetrating keratoplasty or deep lamellar keratoplasty surgery was planned for 6 of these 7 patients, only 2 patients underwent this second surgery before the 24th postoperative week visit. Both the BCVA and ocular surface score improved in all 7 patients after the planned surgeries were performed.

Others. Eight other patients underwent COMET: 3 with idiopathic stem cell deficiency, 1 with radiation keratopathy, 1 with graft-versus-host disease, 1 with congenital aniridia, 1 with Salzmanns corneal degeneration, and 1 with drug-toxicity–induced LSCD. In 6 of these 8 patients, BCVA was improved significantly; however, no improvement was seen in 2 of these patients (Table 2, available at http://aaojournal.org; Fig 2A). The 2 patients with no improvement had severe dryness on the ocular surface and had the highest ocular surface grading score in this group. In addition, severe lagophthalmos was present in the 1 patient with radiation keratopathy because of severe lid scarring after irradiation for retinoblastoma. One other patient with graft-versus-host disease had longstanding inflammation on the ocular surface. In both of these 2 cases, keratinization and symblepharon progressed gradually after COMET. Six patients who demonstrated improvement had a low preoperative ocular surface grading score, yet this score was improved considerably in all patients at the 24th postoperative week (Table 2; Fig 2B).

Critical Visual Improvement Rate

The critical visual improvement rate for SJS, OCP, and thermal or chemical injury was 50.0% (7/14), 42.9% (3/7), and 20.0% (1/5), respectively, although the second planned surgery[25] (penetrating or deep lamellar keratoplasty) had yet to be carried out at the 24th postoperative week in 7 of 10 eyes. The clinical observations on both preoperative and postoperative anterior segment slit-lamp photographs are shown in Figure 3 (available at http://aaojournal.org). All patients demonstrated an improvement in their BVCA to 0.01 or more, from a baseline condition of vision loss.

Factors Influencing Visual Improvement

Multivariate stepwise logistic regression analysis was used to estimate the factors influencing postoperative visual acuity after COMET, and the following factors were chosen as variables:

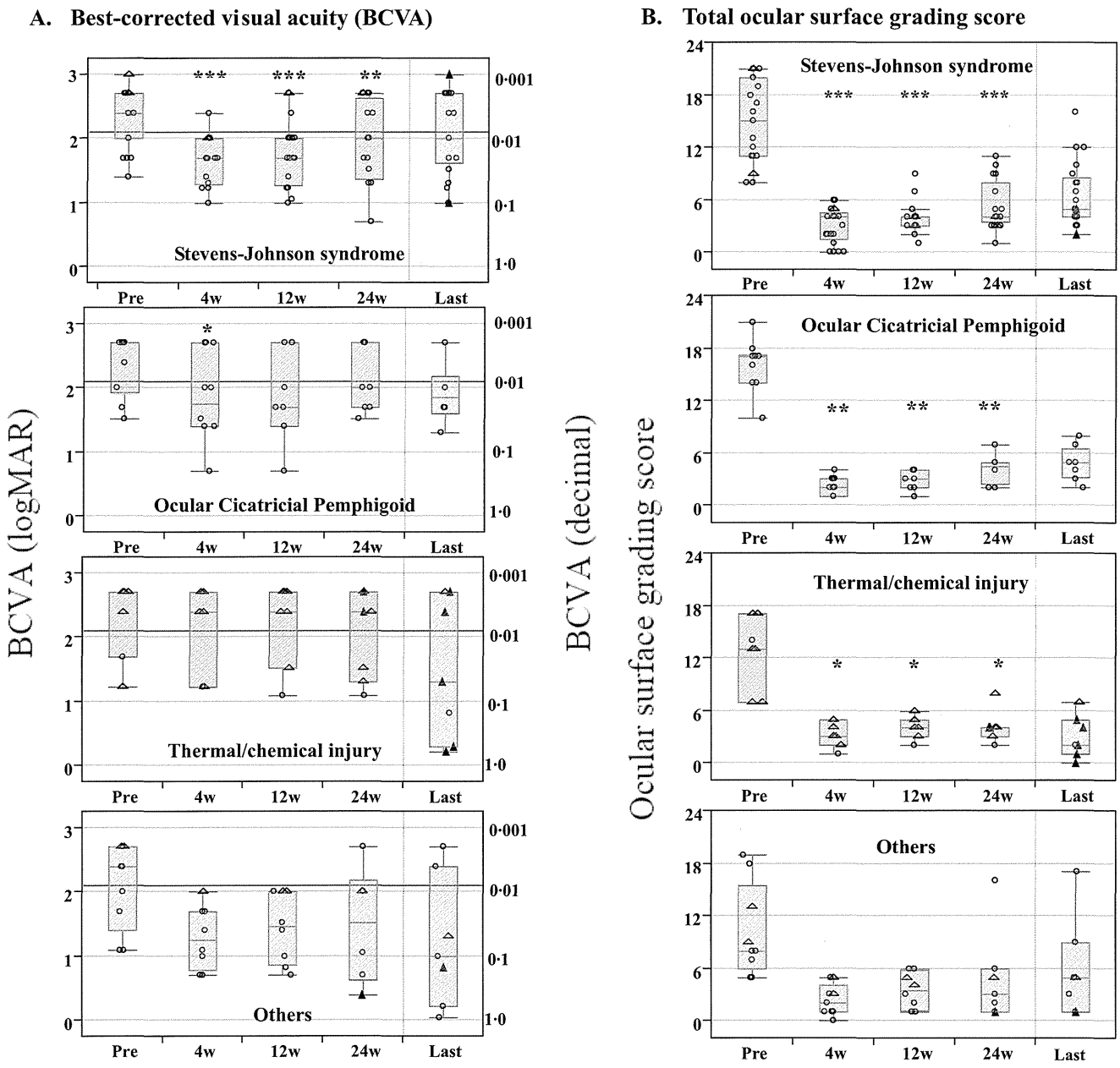

Figure 2. Graphs showing preoperative (Pre) and postoperative clinical outcomes. **A**, Best-corrected visual acuity (BCVA). The BCVA values for each patient are shown grouped according to the cause of corneal dysfunction: Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid (OCP), thermal or chemical injury, and others. The change in BCVA from baseline at each visit, except for the last visit, was analyzed using the Wilcoxon signed-rank test in each disease category (SJS, OCP, thermal or chemical injury) except others. Open circles represent cases treated with autologous cultivated oral mucosal epithelial transplantation (COMET) only. Triangles represent cases treated with a planned 2-step surgical combination of COMET followed by penetrating keratoplasty (PK) or deep lamellar keratoplasty (DLKP). Open triangles are before the second operation, and closed triangles are after the second operation. The horizontal line within each box represents the median value, the bottom and top lines of the box represent the 25th and 75th percentiles, respectively, and the horizontal lines below and above the box represent the lowest and highest values, respectively (or are located 1.5 times the interquartile range away from the box). *$P<0.05$, **$P<0.01$, ***$P<0.001$ (2-sided). **B**, Total ocular surface grading score. Ocular surface grading scores for each patient were calculated and are shown according to each cause of corneal dysfunction: SJS, OCP, thermal or chemical injury, and others. Scores for 8 components of the ocular surface were calculated by the grading system. The total scores before surgery and at the 4th, 12th, and 24th postoperative weeks and at last follow-up examination were calculated. Open circles represent patients treated with COMET only. Triangles represent patients treated with a planned 2-step surgical combination of COMET followed by PK or DLKP. Open triangles are before the second operation, and closed triangles are after the second operation. The change in ocular surface grading score from baseline at each visit, except for the last visit, was analyzed using the Wilcoxon signed-rank test in each disease category (SJS, OCP, thermal or chemical injury) except others. The horizontal line within each box represents the median value, the bottom and top lines of the box represent the 24th and 75th percentiles, respectively, and the horizontal lines below and above the box represent the lowest and highest values, respectively (or are located 1.5 times the interquartile range away from the box). *$P<0.05$, **$P<0.01$, ***$P<0.001$ (2-sided). w = weeks.

Table 3. Summary of Adverse Events in Patients Who Underwent Autologous Cultivated Oral Mucosal Epithelial Transplantation

Event	Total	Disease Category			
		Stevens-Johnson Syndrome	*Ocular Cicatricial Pemphigoid*	*Thermal or Chemical Injury*	*Others*
Hepatic dysfunction	1	1			
Drug-induced allergy	1				1
Persistent epithelial defect	16	10	3	2	1
Corneal stromal melting after the epithelial defect	2		1		1
Ocular infection (keratitis, endophthalmitis)	2	2			
Infiltration	3	2	1		
Elevation of IOP resulting from steroid use	4		1	2	1

IOP = intraocular pressure.
No life-threatening serious adverse events were observed.

disease category, patient age, 2-step surgery, combination with amniotic membrane transplantation, combination with cataract surgery, preoperative logMAR BCVA, and the 8 components of the ocular surface grading system. Corneal neovascularization and symblepharon were found to be correlated significantly with logMAR improvement at the 24th postoperative week ($P = 0.0023$ and $P = 0.0173$, respectively). Visual prognosis was better in the eyes with slight symblepharon than in the eyes with severe symblepharon. In contrast, it was better in the eyes with severe neovascularization than in the eyes with slight neovascularization.

Adverse Events

A summary of the adverse events in the 40 patients who underwent COMET is shown in Table 3. No life-threatening serious adverse events were observed in any of the transplantations. Systemically, moderate liver dysfunction occurred in 1 patient (2.5%; 95% confidential interval [CI], 0.1 to 13.2), but liver function normalized after the discontinuationof systemic drugs.

Postoperative persistent epithelial defects occurred in the eyes of 16 (40.0%) of the 40 patients (95% CI, 24.9 to 56.7), and rather frequently in the SJS eyes (60.0% of SJS patients). Corneal stromal melting after the epithelial defect occurred in 2 patients (5.0%; 95% CI, 0.6 to 16.9), but neither eye became perforated. All of these patients were treated successfully. Slight to moderate corneal infection occurred in 2 patients (5.0%; 95% CI, 0.6 to 16.9); however, both patients healed without scarring. A suspected infection with cell infiltration on the cornea[28] occurred in 3 patients, yet in each patient, it healed within 1 week after receiving a topical instillation of antibiotics. Although a slight elevation of intraocular pressure resulting from steroid use was seen in 4 patients (10.0%; 95% CI, 2.8 to 23.7), this returned to the normal range after reduction of the steroid dose. None of the patients required glaucoma surgery.

Discussion

Severe OSD has proven to be one of the most difficult disorders to treat, and for many patients, vision loss is the end result.[29,31] Keratoprosthesis surgery is one possible way to obtain visual improvement in end-stage severe OSDs; however, serious complications such as endophthalmitis, glaucoma, and tissue melting can arise, especially in SJS or OCP, and can lead to permanent vision loss.[32,33]

At the beginning of 2002, the authors performed ocular surface reconstruction using tissue-engineered autologous oral mucosal epithelial sheets for the first time.[23] In a report of the initial results from the first 12 cases, the successful long-term engraftment of cultivated oral mucosal cells and their transparency was confirmed.[24] Since then, COMET has been used to treat OSD patients, with careful consideration of the surgical indications.[24–26,34] The authors performed 86 COMET operations between 2002 and the end of 2008 for visual improvement, epithelialization of persistent epithelial defects, or conjunctival reconstruction (Fig 1).

In this study, the clinical efficacy and safety of 47 COMETs were evaluated for visual improvement. In 23 eyes (48.9%), previous ocular surgery such as corneal transplantation or amniotic membrane transplantation already had been carried out unsuccessfully at other hospitals. Symblepharon was involved in 37 eyes (78.7%) and keratinization was involved in 10 eyes (21.3%). Symblepharon indicates conjunctival involvement, and pathologic keratinization means that the eye is at the end stage of a severe OSD with chronic inflammation.[3,35] Most of these eyes had severe tear deficiency, which is an important prognostic parameter for surgical outcome.[36] Although such eyes commonly are considered to have contraindications for ocular surface reconstruction, COMET offered substantial visual improvement even for patients with such advanced disease.

In more than half of the eyes, preoperative visual acuity was limited to counting fingers or hand movements. It is striking that such patients were able to come to the hospital without assistance during the 24 weeks after undergoing COMET. For this reason, critical visual improvement rate is proposed as a clear end point for measuring surgical outcome. Considering that most of the eyes in this study were at the end stage of a severe OSD, these results are very favorable and encouraging.

In this study, the preoperative ocular surface grading score was higher (more diseased) in patients with SJS and OCP than in those with thermal or chemical injuries or other

diseases. It should be noted that visual improvement was statistically significant in SJS. In contrast, visual acuity was not improved at the 24th postoperative week in patients with thermal or chemical injury, despite the improvement in total ocular surface grading score. The corneal stroma was damaged severely in most cases of thermal or chemical injury, and such patients obtained visual improvement after undergoing the planned second surgery with penetrating keratoplasty or deep lamellar keratoplasty. In general, the prognosis of penetrating or deep lamellar keratoplasty alone for severe OSDs is very poor.[2] However, the findings of this study show that patients with severe OSDs with corneal stromal opacity can obtain visual improvement after undergoing the surgical combination of COMET and penetrating or deep lamellar keratoplasty.

Best-corrected visual acuity was not improved at the 24th postoperative week in patients with OCP, despite significant improvement of the ocular surface grading score. Because OCP is a progressive autoimmune disease, pathologic keratinization or thickening of the epithelium occurred readily after COMET, thus disrupting visual acuity.

No serious systemic complications occurred in any of the patients. The incidence of postoperative persistent epithelial defects was relatively high, yet still similar to or lower than that reported with other therapies.[6,36–38] Considering that corneal perforation is a common complication after corneal reconstruction in severe OSDs,[38–40] it is noteworthy that no perforation occurred and that none of the eyes demonstrated vision loss after COMET. Ocular surface reconstruction with a combination of COMET and amniotic membrane transplantation was needed to achieve the total replacement of cicatrized tissue. Because cultured epithelial cells on amniotic membrane attach to a basement membrane with hemidesmosomes,[22] these cells can avoid being dropped off and actually survive, regardless of an unstable tear film and the mechanical trauma of blinking. When used as the substrate for oral mucosal cells, amniotic membrane may play a role in protecting the cornea from melting.

Multivariate stepwise logistic regression analysis showed that symblepharon and neovascularization are prognostic factors for visual improvement. Although disease-specific outcomes showed different patterns as described above, disease category was not related to visual prognosis. However, the sample size may be too small to perform such subgroup analyses. Multivariate stepwise logistic regression analysis also was performed for all 86 surgeries to determine the factors influencing persistent epithelial defects. Having SJS and a very low tear meniscus were the prognostic factors for persistent epithelial defects ($P = 0.0204$ and $P = 0.0388$, respectively). Thus, it is likely that both the disease category and dryness of the eye influenced the prognosis.

Long-term ocular surface appearance was examined in 17 of the 72 patients with a follow-up of more than 3 years.[34] No further surgery was carried out in these patients. The ocular surface in each case became stable from 6 months after COMET, with a gradual reduction in corneal neovascularization,[34] as others have reported in similar studies.[40] Moreover, postoperative invasion of conjunctival tissue and symblepharon formation was inhibited significantly for more than 3 years.[34] Deep lamellar or penetrating keratoplasty was performed for the patients with corneal stromal opacity after the stabilization of the ocular surface (as the second step of a 2-step surgical combination), in most cases from 24 weeks after COMET.

After COMET, upper or lower eyelid cicatricial entropion with various degrees of tarsal-plate atrophy sometimes was found. In cases with an eyelid abnormality, eyelid surgery was performed to correct entropion, trichiasis, or lagophthalmos. Eyelid condition is an important factor for maintaining ocular surface stability, as well as for avoiding complications such as infection or persistent epithelial defects.

In conclusion, the findings of this retrospective study showed that long-term visual improvement can be obtained in end-stage severe OSDs with complete LSCD and that COMET offered substantial visual improvement even for patients with severe tear deficiency. The findings also showed that patients with corneal blindness resulting from severe OSDs such as SJS benefited from critical improvement of visual acuity.

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Footnotes and Financial Disclosures

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Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders: case-control study

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ABSTRACT

Objectives: To confirm the downregulation of PTGER4 mRNA in the conjunctiva of Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) and ocular cicatricial pemphigoid (OCP) patients and to examine the expression of its EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders.

Design: Case-control study.

Setting and participants: We performed quantitative reverse transcription-PCR (RT-PCR) analysis of *PTGER4* mRNA in conjunctival tissue sections from patients with SJS/TEN and OCP to confirm the downregulation of *PTGER4* mRNA expression. We also analysed EP4 immunohistologically in other ocular surface disorders. Conjunctival tissues were obtained from patients undergoing surgical reconstruction of the ocular surface due to chemical eye burns, subacute SJS/TEN or chronic SJS/TEN, chronic OCP, severe graft versus host disease (GVHD) and from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva.

Primary and secondary outcome measures: The expression of *PTGER4* mRNA and EP4 protein assessed by quantitative RT-PCR assay and immunohistological methods.

Results: *PTGER4* mRNA was significantly lower in conjunctival tissues from SJS and OCP patients than in the control conjunctivochalasis samples. EP4 protein was detected in conjunctival epithelium from patients with chemical eye burn and in control conjunctival epithelium from patients with conjunctivochalasis. Its expression varied in conjunctival epithelium from patients with Mooren's ulcer. We did not detect EP4 immunoreactivity in conjunctival epithelium from patients with subacute SJS/TEN, severe GVHD, chronic SJS/TEN or OCP.

Conclusions: The strong downregulation of EP4 expression in conjunctival epithelium from patients with OCP or SJS/TEN may be attributable to ocular surface inflammation.

ARTICLE SUMMARY

Article focus

- We previously reported that EP4 protein was down-regulated in devastating ocular surface inflammatory disorders such as chronic Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and chronic ocular cicatricial pemphigoid (OCP). Article focus of this study are to confirm the downregulation of *PTGER4* mRNA, which protein is EP4, in the conjunctiva of SJS/TEN and OCP patients and to examine the expression of its EP4 protein in the conjunctival epithelium of patients with other various ocular surface disorders in addition chronic SJS/TEN and OCP.

Key messages

- EP4 is expressed not only in normal conjunctival epithelium but also in conjunctival epithelium from patients with chemical eye burns and some patients with Mooren's ulcer. On the contrary, it is strongly downregulated in conjunctival epithelium from patients with OCP and chronic SJS/TEN and subacute SJS/TEN.

Strengths and limitations of this study

- The function of EP4 in conjunctival epithelial cells is not elucidated.

INTRODUCTION

The prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 are lipid mediators that form in response to various stimuli. They are released extracellularly immediately after their synthesis and they act by binding to a G protein-coupled rhodopsin-type receptor on the surface of target cells.[1] PGE_2 is produced during inflammatory responses and it suppresses the production of cytokines and chemokines induced by lipopolysaccharide-stimulated macrophages[2 3] and dendritic cells.[4] Elsewhere we reported that PGE_2 modulates the expression of polyI:C-induced proinflammatory genes in human conjunctival epithelial cells.[5]

There are four PGE receptor subtypes, EP1, EP2, EP3 and EP4. The intestinal epithelium has been reported to express EP4 mRNA,[6] and intestinal homeostasis was

maintained and the immune response downregulated by EP4.[7] The ocular surface is also one of the mucosa that is in contact with commensal bacteria like the intestine. Therefore, we focused on the expression of EP4 in human conjunctival epithelium and the difference of its expression between various ocular surface diseases.

We documented that while normal human conjunctival epithelium expressed EP4 protein, it was down-regulated in devastating ocular surface inflammatory disorders such as chronic Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and chronic ocular cicatricial pemphigoid (OCP).[8] Here we examined the mRNA expression of *PTGER4*, which is the gene of EP4 protein, in the conjunctiva of SJS/TEN and OCP patients in the chronic stage to confirm that *PTGER4* mRNA EP4 is down-regulated in their conjunctiva. We also examined the expression of *PTGER4* mRNA protein in the conjunctival epithelium of patients with various ocular surface disorders such as chemical eye burn, Mooren's ulcer, severe graft versus host disease (GVHD) and of patients in the subacute stage of SJS/TEN.

MATERIALS AND METHODS

Human conjunctival tissues

This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experiments were conducted in accordance with the principles set forth in the Helsinki Declaration.

For quantitative reverse transcription-PCR (RT-PCR) the controls were nearly normal conjunctival tissues obtained at surgery for conjunctivochalasis, a disease in which the conjunctiva relaxes due to aging, resulting in a foreign body sensation on the ocular surface. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in four patients in the chronic stage of SJS/TEN and four patients in the chronic stage of OCP.

The controls for immunohistochemical analyses were nearly normal conjunctival tissues obtained during surgery for conjunctivochalasis. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in three patients with chemical (alkali) eye burn (two in the chronic stage and one in the subacute stage), two patients with subacute SJS/TEN, one patient with severe GVHD and from four patients with Mooren's ulcer undergoing resection of inflammatory conjunctiva. SJS/TEN, OCP, Mooren's ulcer, chemical burn and GVHD are all ocular surface inflammatory diseases with persistent inflammation on the ocular surface not only in the acute stage but also in the chronic stage.

Quantitative RT-PCR

Total RNA was isolated from conjunctival tissue sections using the RNeasy mini kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The RT reaction was with the SuperScript preamplification kit (Invitrogen, Carlsbad, California, USA). Quantitative RT-PCR was on an ABI-prism 7700 instrument (Applied Biosystems, Foster City, California, USA). The probes for human PTGER4 and human GAPDH were from Applied Biosystems. For cDNA amplification we performed PCR in a 25 µl total volume that contained a 1 µl cDNA template in 2×TaqMan universal PCR master mix (Applied Biosystems) at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The results were analysed with sequence detection software (Applied Biosystems). The quantification data were normalised to the expression of the housekeeping gene GAPDH.

Immunohistochemistry

For EP4 staining we used rabbit polyclonal antibody to EP4 (Cayman Chemical Co, Ann Arbor, Michigan, USA). The secondary antibody (Biotin-SP-conjugated AffiniPure $F(ab')_2$ fragment donkey antirabbit IgG (H+L), 1:500 dilution; Jackson Immuno Research, Baltimore, Maryland, USA) was applied for 30 min. The VECTASTAIN ABC reagent (Vector Laboratories, Inc, Burlingame, California, USA) was used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector) as a chromogenic substrate.

Data analysis

Data were expressed as the mean±SEM and evaluated by the Student's t test using the Microsoft Excel software program.

RESULTS

We previously documented that EP4 protein expression was down-regulated in conjunctival epithelium of devastating ocular surface inflammatory disorders such as chronic SJS/TEN and chronic OCP.[8] In this study, to confirm the down-regulation of EP4 in the ocular surface of SJS/TEN and OCP patients we examined the expression of *PTGER4* mRNA in control conjunctival tissues from six conjunctival chalasis patients and in conjunctival tissues from four SJS/TEN patients and four OCP patients. Representative findings of EP4 immunoreactivity in each of these groups are shown in figure 1A. Although EP4 protein was detected in the control tissues, conjunctival epithelium from SJS patients and OCP patients did not manifest EP4 immunoreactivity. *PTGER4* mRNA was significantly lower in conjunctival tissues from SJS/TEN and OCP patients than in the control conjunctivochalasis samples (figure 1B).

Moreover, we examined the expression of EP4 protein in the conjunctival epithelium of patients with other various ocular surface disorders. EP4 protein was detected in nearly normal conjunctival epithelium from patients with conjunctivochalasis (figure 2A) and in conjunctival tissues from three patients with chemical eye burn (figure 2B). Its expression varied in conjunctival epithelium from four patients with Mooren's ulcer (figure 2C): in one patient is was similar to the control,