Visualization of Intravital Immune Cell Dynamics After Conjunctival Surgery Using Multiphoton Microscopy

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PURPOSE. To visualize intravital immune cell dynamics in the subconjunctival tissue during the wound-healing process using multiphoton microscopy.

METHODS. Gene-targeted mice expressing enhanced green fluorescent protein under the control of the endogenous lysozyme M promoter (LysMeGFP mice) were anesthetized with isoflurane, and injured by a 10-0 nylon conjunctival suture. Vessels were visualized by intravenous injection of 70 kDa rhodamine-conjugated dextran. Using a multiphoton microscope, the three-dimensional images of the subconjunctival tissue were acquired every minute for 20 minutes before and 0.5, 3, 6, and 72 hours after injury. Raw imaging data were processed for four-dimensional images and analyzed for the number and the velocity of the LysMeGFP–positive cells using Imaris software.

RESULTS. The intravital LysMeGFP–positive cells and the red-labeled vessels were successfully visualized using a multiphoton microscope. The conjunctival and scleral collagen fibers were detected as secondary harmonic generation signals, which were colored blue. Compared with mice without injury, the number of LysMeGFP–positive cells in the subconjunctival tissue after conjunctival surgery increased in a time-dependent manner. The cell velocities significantly increased until 3 hours after surgery (5.9 ± 3.2 μm/min; P < 0.0001) and the elevated level was sustained until 72 hours after injury (5.9 ± 3.3 μm/min).

CONCLUSION. This is the first report to visualize and evaluate intravital cellular dynamics during inflammation in the subconjunctival tissue using multiphoton microscopy. This technique may be a useful tool to characterize the molecular mechanisms of the wound-healing process after various ocular injuries, such as glaucoma surgery.

Keywords: multiphoton microscopy, Lysozyme M-eGFP–positive cells, subconjunctival tissue

 Conjunctival inflammation is induced in various situations, such as conjunctival surgery, including phacoemulsification, vitrectomy, and glaucoma surgeries. The wound-healing process in the conjunctiva often has an important impact on the surgical success of glaucoma filtering surgery to lower IOP, where excess wound healing results in scarring of the filtering bleb and reelevation of the IOP.1,2 Our previous studies showed that a history of intraocular surgeries with conjunctival incisions and sutures was a prognostic factor for surgical failure of trabeculectomy, a standard filtering surgery for glaucoma.3–5 Furthermore, bleb formation was dependent on radial conjunctival incisions during fornix-based trabeculectomy and surgical conjunctival scarring from previous trabeculectomies.5 Thus, it may be possible to characterize the molecular mechanisms of conjunctival wound healing and to control the key factors involved in the surgical success of glaucoma filtering surgery. However, it is first necessary to understand the cellular dynamics, such as the behavior of immune cells in the conjunctiva and the subconjunctival tissue, to improve the surgical results of trabeculectomy. Mononuclear cells and granulocytes are reportedly involved in the wound-healing process after trabeculectomy, and neutrophils are one of the first inflammatory cells of the immune system to enter the conjunctival scarring area.7,8 However, because of technical limitations, there have been few reports of intravital imaging of the immune cells after conjunctival surgery.

Compared with conventional confocal microscopy, recent advances in multiphoton microscopy have facilitated three-dimensional (3D) deep tissue imaging over extended periods with minimal phototoxicity. Using this technique, the cellular dynamics in various organs, including the skin, lymph node, thymus gland, brain, lung, pancreas, spleen, liver, intestine, kidney, and bone marrow, have been investigated.9–15 The cellular dynamics of some ocular tissues, including the cornea...
and retina, have been characterized using multiphoton microscopy.\textsuperscript{16–18} However, there have been few reports investigating the cellular dynamics during subconjunctival inflammation, which is an important risk factor for excessive scarring after trabeculectomy.

The aim of this study was to develop a methodology to detect the behavior of cells in the subconjunctival tissue by four-dimensional (4D) imaging using multiphoton microscopy. LysM-positive inflammatory cells play a major role in the immune response, so we characterized the activity of these cells during the wound-healing process in the subconjunctival tissue. Furthermore, we also evaluated the behavior of the intravital immune cells after conjunctival surgery.

METHODS

Mice

All animal experiments were performed according to institutional guidelines under protocols approved by the Animal Experimental Committee of Osaka University. All studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Green-targeted mice expressing the enhanced green fluorescent protein under the control of the endogenous lysozyme M promoter (LysM-eGFP) knockin mice\textsuperscript{19} were used at 8 to 10 weeks of age for imaging.

Multiphoton Intravital Conjunctiva and Sclera Imaging

Intravital microscopy of mouse eyes was performed using protocols modified from a previous study.\textsuperscript{9–12} The LysM-eGFP knockin mice were anesthetized with isoflurane (2.0%, vaporized in 100% oxygen). The facial hair and eyelash around the eyelid were cut. For the surgery model, the conjunctiva was sutured with a 10-0 nylon filament (Mani, Brussels, Belgium) (Figs. 1A, 1B). A handmade eye cup was mounted on the eyelid using glue, and was filled with PBS. The mouse’s head was immobilized in a custom-made stereotactic holder. Vessels were visualized by intravenous injection of 70 kDa rhodamine-conjugated dextran immediately before imaging. The imaging system was composed of a multiphoton microscope (A1-MP; Nikon, Tokyo, Japan) driven by a laser (Chameleon Vision II Ti, Sapphire; Coherent, Santa Clara, CA, USA) tuned to 880 nm, together with an upright microscope equipped with a ×25 water immersion objective (apochromatic aberration correction, 1.1 numerical aperture; Nikon). The microscope was enclosed in an environmental chamber in which the anesthetized mice were warmed by a heater. Image stacks were collected at 5-\(\mu\)m vertical step sizes with an approximate z depth of 100 to 120 \(\mu\)m from the ocular surface. Images were acquired every minute for 20 minutes before, and 0.5, 3, 6, and 72 hours after, the use of the nylon suture. Raw imaging data were analyzed using Imaris image analysis software (Bitplane, Zurich, Switzerland) with a Gaussian filter for noise reduction. The automatic 3D object tracking with Imaris spots was aided by manual corrections to retrieve cell spatial coordinates over time (Supplementary Video S1).

Statistical Analysis

Data analyses were performed using the JMP statistical software package (version 8; SAS, Inc., Cary, NC, USA). The number and the velocity of the cells before and after injury were compared using Dunnett’s test; \(P\) values less than 0.05 were considered statistically significant. Data are expressed as means ± SD.

RESULTS

We successfully obtained images of intravital immune cell dynamics in subconjunctival tissue. Intravital LysM-eGFP-positive cells and red-labeled vessels were visualized using multiphoton microscopy. Conjunctival and scleral collagen fibers were detected as secondary harmonic generation signals, which are visualized in blue (Fig. 2). In addition, the number and the velocity of the cells were statistically evaluated at each time point after injury.

The intact conjunctiva and sclera of LysM-eGFP mice were examined and the behavior of the LysM-eGFP-positive cells in the subconjunctival tissue was observed for 20 minutes (Figs.
The mean (± SD) number of cells was 66.5 ± 29.0 in the visual field (a cube of 512 × 512 × 100 μm) and the mean (± SD) velocity of the cells was 2.3 ± 0.6 μm/min. Following the experiments, the conjunctiva of LysM-eGFP mice was injured by a 10-0 nylon suture, and the adjoining area of the suture site was observed. At 30 minutes after the injury, the number of LysM-eGFP–positive cells was 83.5 ± 0.7 in the visual field, and the mean velocity of the cells was 2.0 ± 0.9 μm/min. This velocity was not significantly different compared with the corresponding values before injury (Figs. 2B, 3B, 4, 5; Supplementary Video S3). Three hours after the injury, the number and the velocity of the LysM-eGFP–positive cells increased compared with those of the animal before injury. The number of cells was 108.5 ± 2.1 in the visual field and the mean velocity of the cells was 5.9 ± 3.2 μm/min (Figs. 2C, 3C, 4, 5; Supplementary Video S4). Moreover, the number and the velocity of the cells increased at 6 hours after injury. The number of cells was 118.5 ± 17.7 in the visual field and the mean velocity of the cells was 6.2 ± 2.8 μm/min (Figs. 2D, 3D, 4, 5; Supplementary Video S5). At this time period, the image presented more LysM-eGFP–positive cells around the vessel wall with time. Seventy-two hours after injury, the LysM-eGFP–positive cells sustained their activity. The cell number was 208.0 ± 38.2 in the visual field, the mean velocity of the cells was 5.9 ± 3.3 μm/min, and the vessels were enlarged (Figs. 2E, 3E, 4, 5; Supplementary Video S6). The number of LysM-eGFP–positive cells in the subconjunctival tissue increased in a time-dependent manner after the injury (Fig. 4). The velocity of the LysM-eGFP–positive cells significantly increased until 3 hours after surgery (P < 0.0001) and the elevated level was sustained until 72 hours after the injury (Fig. 5).

**DISCUSSION**

Steven et al. described the intravital transmigration of immune cells into the lymphatic vessels using a two-photon microscope, and characterized the interactions of immune and subsequent tumor cells during corneal vascularization after inflammation induced by corneal sutures. In addition, Zhang et al. demonstrated the utility of the two-photon microscope for observations of the detailed laminar structure in the mouse cornea. These reports demonstrated the usefulness of multiphoton microscopy for intravital imaging of the ocular surface. In the present study, we successfully visualized the intravital behavior of the immune cells in the subconjunctival tissue using multiphoton microscopy. Past studies have reported that the immune response in the subconjunctival tissue is associated with the wound-healing process after ocular surgery, including trabeculectomy. It is clinically important to clarify its molecular mechanism, because the excess wound healing causes closing of the new aqueous pathway created by trabeculectomy. To the best of our knowledge, this is the first report showing intravital
FIGURE 3. The movements of the LysM-eGFP–positive cells were tracked for 20 minutes. White spheres designate the LysM-eGFP–positive cells and the colored lines show the associated trajectories of the cells. (A) Intact model. (B) Thirty minutes after injury. (C) Three hours after injury. (D) Six hours after injury. (E) Seventy-two hours after injury.

FIGURE 4. The change in the number of the LysM-eGFP–positive cells after the injury. Each bar indicates the total number of LysM-eGFP–positive cells in the visual field at each time point after the injury. The number of cells in the visual field increased in a time-dependent manner after the injury. The error bars represent the SDs.

FIGURE 5. The distributions of the LysM-eGFP–positive cell velocities in the visual field after the injury. The velocity of the cells in the visual field significantly increased 3 hours after the injury and the elevated level was sustained until 72 hours. Data points (n = 152, 225, 372, 331, and 344, respectively) represent individual cells. Each error bar represents the median ± interquartile range. The P values were calculated using the Dunnett’s test.
imaging of the behavior of immune cells after conjunctival surgery, using multiphoton microscopy.

Our studies showed that the number of LysM-eGFP-positive cells in the subconjunctival tissue increased in a time-dependent manner after the injury, indicating the invasion of cells to the injured area. Approximately 6 hours after injury, there was an especially large accumulation of LysM-eGFP-positive cells. In LysM-eGFP mice, eGFP-positive cells were specifically observed in the myelomonocytic lineage, especially in mature neutrophils and granulocytes. Recently, it was reported that LysM-eGFP-positive cells in the blood included both neutrophils (51.3%) and inflammatory monocytes (44.2%). Previous studies have reported an influx of inflammatory cells, including polymorphonuclear leukocytes, lymphocytes, and macrophages, into the injury site during the first few days after wounding. Most importantly, neutrophils are one of the first inflammatory cells of the immune system to enter the wound area, accumulating within 6 hours after injury, and disappearing by the third day after wounding. Another study reported that neutrophils are present within minutes of the injury, and generally peak at 48 hours. These studies evaluated the influx of inflammatory cells using histologic analyses, and a similar pattern of inflammatory cell invasion was seen in our study using intravital imaging. Moreover, the involvement of IL-8 in neutrophil accumulation involved an allergic inflammation at the ocular surface. However, there have been few reports evaluating the associations between some cytokines/chemokines and neutrophil recruitment at the inflammatory subconjunctival tissue. Because the in vivo cell motility cannot be assessed by conventional histologic analysis, the findings in the present study provide a novel insight into the process of wound healing in subconjunctival tissues. Honda et al. evaluated the behavior of liver neutrophils associated with hepatic ischemia-reperfusion injury. Consistent with our results, they reported a gradual increase in the number of neutrophils after injury. In addition, they reported an average neutrophil velocity of approximately 5 μm/min before injury, and a maximum average velocity of 7.5 ± 0.3 μm/min at 2 hours after injury. In our study, the cell velocities were sustained until 72 hours after the conjunctival suture. These results showed that the motility of LysM-eGFP-positive cells is increased at an early stage after injury, and that approximately 6.0 μm/min is the maximum velocity of the LysM-eGFP-positive cells in the inflammatory subconjunctival tissue. Because the in vivo cell motility cannot be assessed by conventional histologic analysis, the findings in the present study provide a novel insight into the process of wound healing in subconjunctival tissues. Honda et al. evaluated the behavior of liver neutrophils associated with hepatic ischemia-reperfusion injury. Consistent with our results, they reported a gradual increase in the number of neutrophils after injury. In addition, they reported an average neutrophil velocity of approximately 5 μm/min before injury, and a maximum average velocity of 7.5 ± 0.3 μm/min at 2 hours after injury. In our study, the cell velocities were sustained until 72 hours after injury. In contrast, the other studies reported a decrease of cell velocities after the peak time point. One of the reasons may involve an insufficient observation period to detect the peak time of the cell velocities. Another possible explanation is that the nylon filament continuously causes an inflammatory response, resulting in an elongated increase in the velocity of the LysM-eGFP-positive cells. Consistent with this possibility, Ueta et al. suggested that inflammatory cells aggregate at the nylon suture in the cornea, and remain in the vicinity of the suture as long as the nylon filament exists.

In the present study, we placed a 1.0 mm nylon suture at the conjunctiva of the mouse as an injury model, because it was simple to perform and produced reproducible results. However, a nylon suture injury is less invasive compared with a trabeculectomy that involves cataracting the scleral surface, making a scleral flap, treating the subconjunctival tissue with antimetabolic drugs such as mitomycin C, creating a fistula in the anterior chamber, and performing peripheral iridectomy in addition to the nylon suture. Although the present study characterized some of the common dynamics after conjunctival injury, the inflammatory response after trabeculectomy may not be exactly the same as the results of the present study. In addition, other proinflammatory cells such as macrophages and fibroblasts are also reported to play critical roles after conjunctival injury and the dynamics of these cells also should be investigated. Our previous study showed that the artificial aqueous outflow pathway started to close at an early stage after trabeculectomy, which correlated with surgical success at a late stage. An understanding of the wound-healing process after trabeculectomy is therefore necessary to improve surgical results, and further studies are required to elucidate the wound-healing process in the subconjunctival tissue after trabeculectomy. Overall, this is the first report to visualize intravital immune cell dynamics in the subconjunctival tissue using multiphoton microscopy. Four-dimensional intravital imaging showed that the number and the velocity of the LysM-eGFP-positive cells increased after injury. Intravital imaging in the conjunctival tissue therefore be a useful tool to further characterize the molecular mechanisms of the wound-healing process after conjunctival surgeries such as trabeculectomy.

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References

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