Characterization and Distribution of Bone Marrow–Derived Cells in Mouse Cornea

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Purpose. Bone marrow (BM)–derived stem cells are thought to possess extensive differentiation capacity. The present study was conducted to investigate the characteristics and distribution of these cells in the normal mouse cornea.

Methods. BM cells and BM-derived hematopoietic stem/progenitor cells (HSCs) from enhanced GFP (eGFP) transgenic mice (lin^-, Sca-1^+) were intravenously transplanted into irradiated wild-type C57BL/6 mice. At 4 to 6 months after transplantation, the mice were killed, and their whole corneas examined by histologic and immunohistochemical methods (CD11c, CD11b, and CD45).

Results. At 2 weeks after BM cell transplantation, GFP^+ cells gradually migrated into the cornea from the limbal area. At 2 to 6 months, they were distributed over the entire cornea. In cross sections of whole cornea, GFP^+ cells comprised 27.3% ± 11.1% (BM) and 24.0% ± 8.01% (HSC) of total cells in the peripheral corneal stroma. In the center of the corneal stroma, GFP^+ cells were 7.58% ± 2.63% (BM) and 8.06% ± 1.76% (HSC) of total cells. Immunohistochemistry showed that GFP^+ $CD11c^+$, $CD11b^+$, $CD11c^-$, and $CD11b^-$ cells occupied the entire corneal stroma.

Conclusions. The present study provides direct evidence of the distribution of BM-derived cells in the mouse cornea. Immunohistochemical study showed that some of these cells are BM-derived antigen-presenting cells such as dendritic cells and macrophages. Some elements of BM-derived cells may continue to exist in the corneal stroma. (*Invest Ophthalmol Vis Sci.* 2005;46:497–503) DOI:10.1167/iovs.04-1154

Adult somatic stem cells have been isolated from several tissue sources including neurons,[1,2] retina,[3] corneal limbal epithelium,[4,5] and bone marrow (BM).[6–8] It had been thought that somatic stem cells preferentially generate differentiated cells of the same lineage as their tissue of origin. However, recent studies suggest that tissue-specific stem cells can differentiate into lineages other than their tissue of origin and that, with respect to the developmental potential of different adult cell types, there is far more plasticity than previously thought. Particular attention has been focused on the plasticity of BM-derived stem cells. They are reported to possess extensive differentiation capacities and can differentiate into several epithelial types such as liver, lung, and skin.[9] Furthermore, BM-derived mesenchymal stem cells can differentiate in vitro not only into mesenchymal cells, but also into cells with visceral mesoderm, neuroectoderm, and endoderm characteristics.[10] These findings suggest that BM-derived stem cells may have the ability to transdifferentiate into a variety of tissues, including those of the eye.

Normal corneal tissue is located in the anterior segment of the eye, and it participates in several major functions. It is the gateway into the eye of visual images and plays a critical role in maintaining corneal transparency and avascularity. It is composed of three layers: the corneal epithelium, stroma, and endothelium. Corneal epithelial stem cells exist in the basal cell layer of the limbal region[4,5] and in the transitional zone between the cornea and conjunctiva. They are supported by the limbal vascular arcade. Little is known about stem cells of the corneal stroma and endothelium, and the origin of these cells is not well understood.

From an immunologic point of view, the normal avascular cornea was thought to be an immune-privileged site without functional antigen-presenting cells (APCs) and largely devoid of BM-derived cells. Therefore, higher success rates would be expected with corneal than other organ transplants. This notion has lost favor since the demonstration of large numbers of resident BM-derived cells of different lineages—for example, macrophages and dendritic cells—in both the epithelium and stroma of the normal cornea.[11–13] Until now, indirect evidence obtained by immunohistochemical studies has shown these cells to be present and important questions, such as the original cell type and the physiological and functional significance of these progenitors, remain unanswered.

We are the first to attempt the characterization and clarification of the distribution of BM-derived cells in the normal mouse cornea. In the current study, we sought to acquire a direct demonstration by transplanting BM cells from enhanced green fluorescence protein (eGFP) transgenic mice using our unique protocol.[14–16] We transplanted GFP-labeled BM cells and hematopoietic stem/progenitor cells (HSCs) into syngeneic C57BL/6 (wild-type) mice and found BM-derived cells distributed in the mouse cornea. We then evaluated the characteristics of these BM-derived cells by immunohistochemical studies.

Materials and Methods

Experimental Animals

The mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental

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Supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Health, Labor, and Welfare and the Japanese Ministry of Education, Culture, Sports, Science, and Technology, and research grants from the Kyoto Foundation for the Promotion of Medical Science and the Intramural Research Fund of Kyoto Prefectural University of Medicine.

Submitted for publication September 29, 2004; revised October 26, 2004; accepted November 3, 2004.

Disclosure: **T. Nakamura**, None; **F. Ishikawa**, None; **K.-h. Sonoda**, None; **T. Hisatomi**, None; **H. Qiao**, None; **J. Yamada**, None; **M. Fukata**, None; **T. Ishibashi**, None; **M. Harada**, None; **S. Kinoshita**, None

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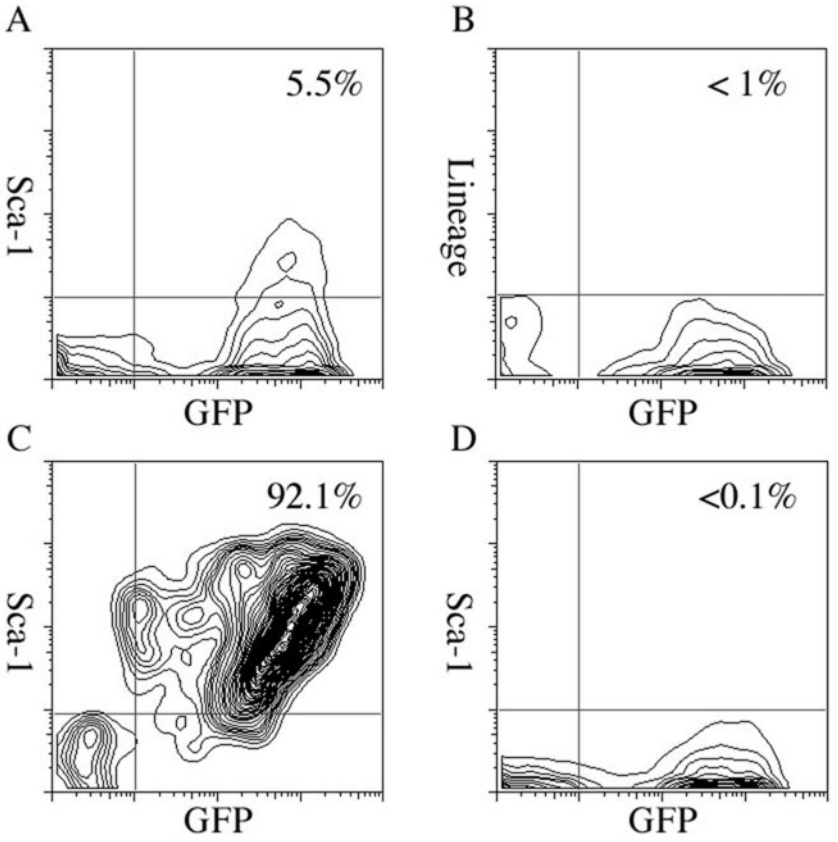

FIGURE 1. Enrichment of hematopoietic stem cells confirmed by flow cytometry. Original BM cells (**A**). After lineage depletion (**B**). Positive (**C**) and negative (**D**) selection for Sca-1$^+$ cells. After negative and positive selection, the lin$^-$ Sca-1$^+$ cell purity of all GFP$^+$ cells exceeded 95%. Each percentage represents the amount of double-positive cells among all nucleated cells.

procedures were approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine and Kyushu University. Adult ($n = 3$) and newborn ($n = 6$) C57BL/6 mice were the recipients of BM cell and HSC transplants, respectively. BM cells were obtained from mice that transgenically express GFP, driven by the chicken β-actin promoter.[17]

Bone Marrow Transplantation

To observe directly the migration of BM-derived cells into the mouse cornea, we used BM cell transplantation.[16] Female eGFP mice (8–10 weeks old) were killed by cervical dislocation while under deep ether anesthesia, and BM cells were obtained by flushing the femurs with sterile phosphate-buffered saline (PBS). The BM cells were washed several times in sterile PBS, filtered twice through a nylon mesh (pore size, 70 μm), counted, and resuspended in PBS at 5×10^7 cells/mL. To generate chimeric mice, all BM cells (6×10^6 to 1×10^7) derived from eGFP transgenic mice were intravenously injected into 8-week-old C57BL/6 recipients that had been lethally x-irradiated with 9 Gy. Their eyes were protected with lead shields to prevent radiation retinopathy. These BM cell transplant recipients were then maintained under special pathogen-free conditions, and successful BM cell transplantation was confirmed by the identification of GFP$^+$ cells in peripheral blood at 2 weeks after transplantation. The corneas of three mice were carefully studied by fluorescence biomicroscopy until 6 months after transplantation. We also used these corneas for histologic and immunohistochemical studies.

Hematopoietic Stem Cell Transplantation

To characterize BM-derived stem/progenitor cells in the mouse cornea, we performed HSC transplantation.[14,15] BM cells were harvested from femurs and tibias of 8- to 12-week-old eGFP mice. Single-cell suspensions of donor cells were prepared by repeated serial passage through a 23-gauge needle. To deplete mature hematopoietic cells, the BM cells were incubated with lineage-specific antibodies (B220, CD3, Gr-1, Mac-1, and TER 119) for 30 minutes at 4°C. After washing with PBS containing 2% fetal bovine serum, the cells were incubated with sheep anti-rat immunomagnetic beads (Dynabeads M-450 coupled to sheep anti-rat IgG; Dynal, Great Neck, NY). Cells not bound to the immunobeads were further purified for Sca-1$^+$ cells. The purity of lineage$^-$ cells was higher than 92% in all experiments. After negative selection of mature hematopoietic and immune cells, positive selection of Sca-1$^+$ cells was performed as just described. After negative and positive selection, the purity of lin$^-$ Sca-1$^+$ cells of all the eGFP$^+$ cells exceeded 95% (Fig. 1).[14,15] To obtain high cell purity, samples were applied twice to columns in each experiment. The resultant 10^4 lin$^-$ Sca-1$^+$ cells were transplanted into C57BL/6 mice within 2 days of their birth. The HSC transplant recipients were maintained under special pathogen-free conditions for 4 weeks. Successful HSC transplantation was confirmed by the identification of GFP$^+$ cells in the peripheral blood at 4 weeks after transplantation. At 4 to 5 months after HSC transplantation, six mice were used for histologic and immunohistochemical studies.

Antibodies

The primary antibodies (all from BD-PharMingen, San Diego, CA) used in this study were purified hamster anti-mouse CD11c (clone HL3), purified rat anti-mouse CD45 (clone 30-F11), and RPE-conjugated rat anti-mouse CD11b (clone M1/70). Secondary antibodies were Cy3-conjugated goat anti-hamster IgG and Cy3-conjugated donkey anti-rat IgG (Vector Laboratories, Inc., Burlingame, CA).

Immunohistochemistry

Immunohistochemical studies of markers for APCs were performed by using a previously reported method[11–13] and a modified version of our method.[18,19] Briefly, freshly excised corneas were fixed for 60 minutes at 4°C in 4% paraformaldehyde, extensively washed with PBS, fast frozen in liquid nitrogen, and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek II; Miles Laboratories, Elkhart, IN).

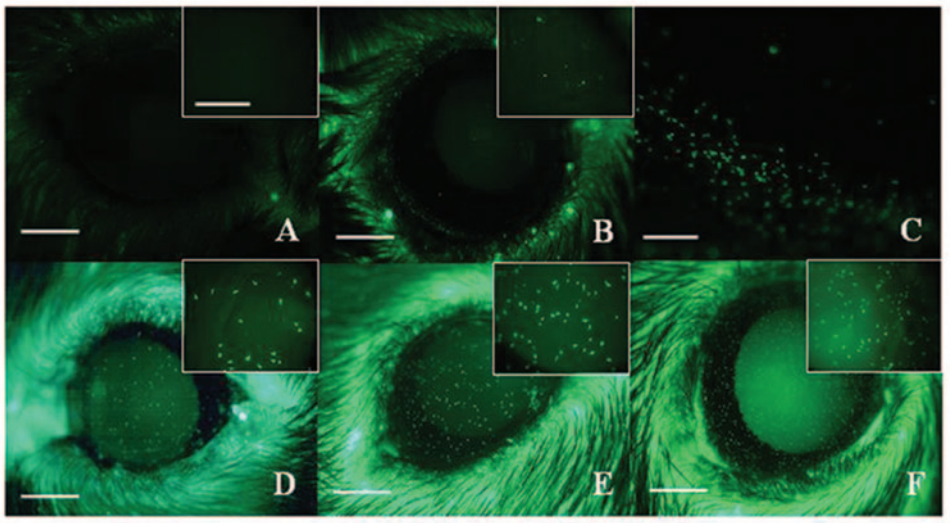

FIGURE 2. Representative time-course slit lamp photographs of murine eyes after BM cell transplantation. (**A**) One week, (**B**) 2 weeks, and (**C**) a high magnification of (**B**) at the limbal area; (**D**) 1, (**E**) 2, and (**F**) 6 months after transplantation. *Boxes* contain data from the center of the cornea. In the early stages (within the first week) after BM cell transplantation, we observed no GFP^+ cells in the recipient mouse cornea (**A**). Within 2 weeks, there was intense staining for GFP^+ cells in the periphery of the cornea (**B**, **C**). Within 2 months, GFP^+ cells appeared to migrate into the center of the cornea. Their numbers increased in both the periphery and center of the cornea (**D**, **E**). Starting at 2 months after BM transplantation, cell density reached a plateau that persisted up to 6 months (**F**). Scale bars: (**A**, **B**, **D**–**F**) 1 mm; (**C** and *insets*) 250 μm.

Cryostat sections (7 μm in thickness) were placed on gelatin-coated slides, air-dried, and rehydrated in PBS at room temperature for 15 minutes. To block nonspecific binding, the tissues were incubated with both anti-Fc receptor mAb (CD16/32; BD PharMingen, San Diego, CA) and 2% bovine serum albumin (BSA) for CD11c and CD11b and with 2% BSA and 10% donkey serum for CD45 at room temperature for 30 minutes. Then the sections were incubated at room temperature for 1 hour with the primary antibody and washed three times in PBS containing 0.15% Triton X-100 (PBST) for 15 minutes. The controls were incubated with the appropriate normal rat and hamster IgG (Dako, Kyoto, Japan) at the same concentration as, but without, the primary antibody. After staining with the primary antibody (CD11c, CD45), the sections were incubated at room temperature for 1 hour with appropriate secondary antibodies, Cy3-conjugated goat anti-hamster IgG, and Cy3-conjugated donkey anti-rat IgG. After several washes with PBS, the sections were coverslipped using antifade mounting medium, with or without propidium iodide (PI; Vectashield; Vector Laboratories) and examined under a confocal microscope (Fluoview; Olympus, Tokyo, Japan).

Quantitative Evaluation

For statistical assessment of corneal cell distribution and characterization, four different fields and six different sections of each cornea were analyzed (24 areas/eye). For analytical purposes, each cornea was divided into central and peripheral areas. The central area was defined as the area within 1 mm of the center and the peripheral area as that within a 1- to 1.5-mm radial distance from the center.

RESULTS

Migration of BM Cells into the Cornea

In the early stages (first week) after BM cell transplantation, there were no GFP^+ cells in the recipient mouse cornea (Fig. 2A). Within 2 weeks of transplantation, some GFP^+ cells appeared in the periphery of the cornea. However, only a small number of GFP^+ cells were present in the center of the cornea (Figs. 2B, 2C). Within 2 months, the number of GFP^+ cells in both the periphery and center of the cornea gradually increased. From 2 months after BM cell transplantation, the cell density reached a relative plateau that persisted up to 6 months (Figs. 2D–F). Our quantitative analysis of GFP^+ cells in the mouse cornea is summarized in Figure 3.

Distribution of BM Cells and HSCs

To determine whether there were BM-derived GFP^+ cells in the recipient cornea, we performed histologic analysis under a dual-channel fluorescence microscope. Cross-sections of recipient corneas showed that most of the GFP^+ cells were distributed in the peripheral corneal stroma and that cell density gradually decreased toward the center (Fig. 4A–D). In the entire corneal epithelium, we noted only a small number of

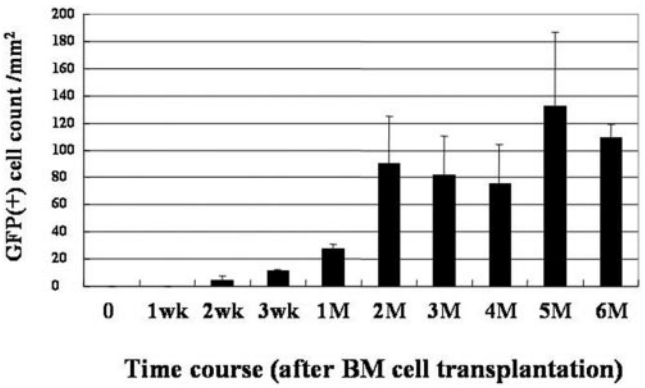

FIGURE 3. Quantitative analysis of GFP^+ cells in the mouse cornea at the indicated times after BM cell transplantation. During the first 2 months, the number of GFP^+ cells gradually increased. Thereafter, cell density reached a relative plateau that persisted up to 6 months after transplantation.

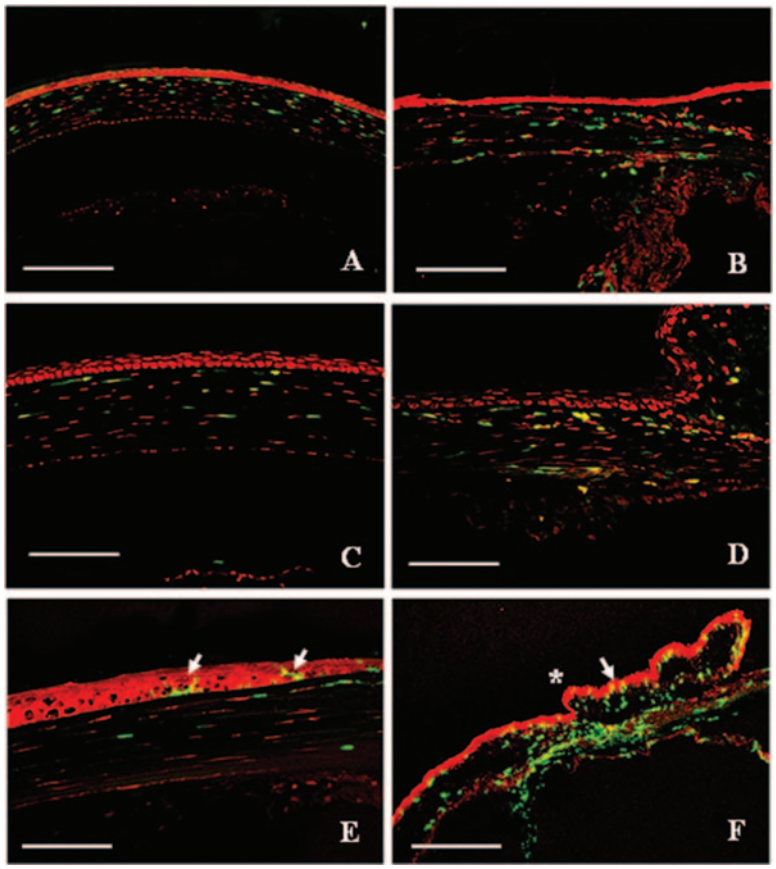

FIGURE 4. Representative cross sections of recipient corneas (**A**, **B**, **E**, BM transplantation; **C**, **D**, **F**, HSC transplantation) show that most of the GFP^+ cells were distributed in the peripheral corneal stroma and that the cell density gradually decreases toward the center (**A**, **C**, central area; **B**, **D**, peripheral area). Only a small number of GFP^+ cells were observed throughout the epithelium (**E**, *arrows*). In contrast, in conjunctival epithelium, several GFP^+ cells were noted (**F**, *arrow*; area to the right of the conjunctiva ✶). Cell nuclei were stained with PI (*red*). Scale bars: (**A**, **B**, **F**) 200 μm; (**C**, **D**, **E**) 100 μm.

these cells (Fig. 4E), whereas in the conjunctival epithelium we observed several GFP^+ cells (Fig. 4F). The percentage of GFP^+ cells per section was calculated as the number of GFP^+ cells divided by the total number of PI^+ cells $\times$ 100. In the peripheral cornea of mice receiving BM cell transplants, GFP^+ cells were 2.03% $\pm$ 1.87% (epithelium) and 27.3% $\pm$ 11.1% (stroma). At the center of the cornea, they were 0.93% $\pm$ 0.65% (epithelium) and 7.58% $\pm$ 2.63% (stroma). By contrast, in the peripheral corneas of mice transplanted with HSC, GFP^+ cells were 0.78% $\pm$ 0.51% (epithelium) and 24.0% $\pm$ 8.01% (stroma). At the center of the cornea, they were 0.58% $\pm$ 0.4% (epithelium) and 8.06% $\pm$ 1.76% (stroma; Fig. 5). The differences between epithelium and stroma in each category were statistically significant (Mann-Whitney test; $P < 0.01$).

Immunohistochemical Analysis

To characterize BM-derived GFP^+ cells in corneal tissue, primarily the corneal stroma, we used fluorescence immunohistology with antibodies to the leukocyte markers CD11c, CD11b, and CD45. Negative control sections, incubated with normal rat and hamster IgG but without the primary antibody, exhibited no discernible specific immunoreactivity over the entire region.

$CD11c^+$ or $CD11b^+$ indicate cells coexpressing GFP and CD11c or GFP and CD11b, respectively. The percentage of $CD11c^+$ or $CD11b^+$ cells was calculated by dividing the respective number of cells by the total number of GFP^+ cells $\times$ 100. In the corneal peripheral stroma of BM cell recipients, we observed 19.4% $\pm$ 9.93% $CD11c^+$ cells and 38.7% $\pm$ 16.3% $CD11b^+$ cells. In the central stroma, 15.3% $\pm$ 8.94% were $CD11c^+$ cells and 48.7% $\pm$ 13.1% were $CD11b^+$ cells. In the corneal peripheral stroma of HSC recipients, there were 35.7% $\pm$ 14.0% $CD11c^+$ cells and 56.7% $\pm$ 22.4% $CD11b^+$ cells. In the central stroma, 41.5% $\pm$ 17.8% were $CD11c^+$ cells and 53.7% $\pm$ 13.9% were $CD11b^+$ cells (Figs. 6 , 7, 8). Most GFP^+ cells in the cornea were immunostained with CD45 in both BM- and HSC-recipients (Fig. 9). Asterisks in Figure 8 indicate statistically significant difference between $CD11c^+$ and $CD11b^+$ (Mann-Whitney test; $^*P < 0.01$, $^{**}P < 0.05$).

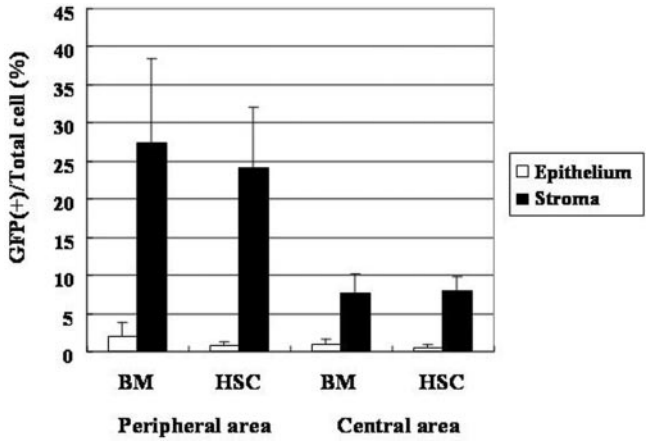

FIGURE 5. Distribution of GFP^+ cells in the cornea of mice transplanted with BM cells or HSCs. The percentage of GFP^+ cells per section was calculated as the number of GFP^+ cells divided by the total number of PI^+ cells plus GFP^+ cells $\times$ 100. Most of the GFP^+ cells were distributed in the peripheral corneal stroma. Cell density gradually decreased toward the center. In the entire area covered by epithelium, there were only a few GFP^+ cells. The differences between epithelium and stroma in each category were statistically significant (Mann-Whitney test; $P < 0.01$).

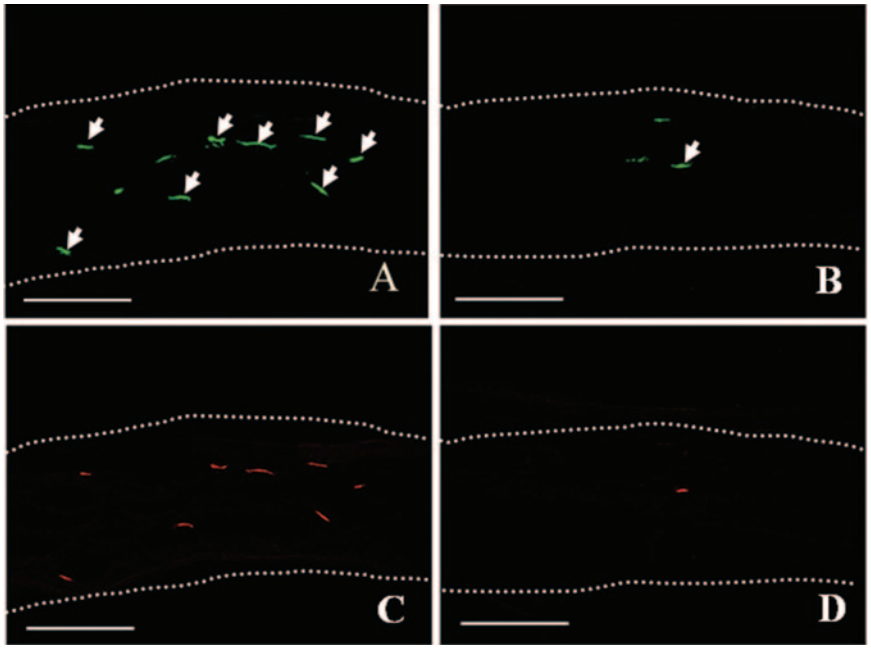

FIGURE 6. Representative immunohistochemical staining for CD11c (*red*) in the peripheral (**A**, **C**) and central (**B**, **D**) cornea of mice receiving HSC transplants. Some GFP^+ cells (*green*) immunostained with CD11c (*arrows*). Note the presence of GFP^+CD11c^- cells throughout the transplant-recipient cornea. GFP (**A**, **B**), CD11c (**C**, **D**). *Dotted lines*: perimeter of whole corneal tissue. Scale bars, 100 μm.

DISCUSSION

The cornea is a transparent, avascular tissue, with integrity maintained by various factors derived from the tear film and aqueous humor. Although the normal cornea does not contain vessels, there is indirect immunohistochemical evidence that it is endowed with a significant number of resident BM-derived APCs.[11–13] Hamrah et al.[11] reported that corneal epithelium contains major histocompatibility complex (MHC) class II-negative Langerhans' cells and corneal stroma a large number of resident BM-derived cells of different lineages. These cells were not only macrophages but also $CD11c^+$ dendritic cells. Brissette-Storkus et al.[12] also documented that the normal murine corneal stroma contains a significant number of $CD45^+$ leukocytes and that most of these cells are monocytes or macrophages. However, to date, there has been no direct demonstration of their existence. BM-derived stem cells, such as hematopoietic- and mesenchymal stem cells, have extensive differentiation capacity.[8,9] We considered two possible mechanisms of BM-derived cell differentiation: One is that BM-derived stem cells that have differentiated into APCs such as Langerhans' cells or macrophages migrate into corneal tissue. Alternatively, BM-derived stem cells transdifferentiate into corneal cells such as corneal keratocytes, and function in the cornea. We examined these possibilities using our unique protocol and found that some BM-derived cells were definitely distributed in the cornea. We also determined that these cells are partially of BM-derived APC lineage, a finding that directly confirms the cell origin of

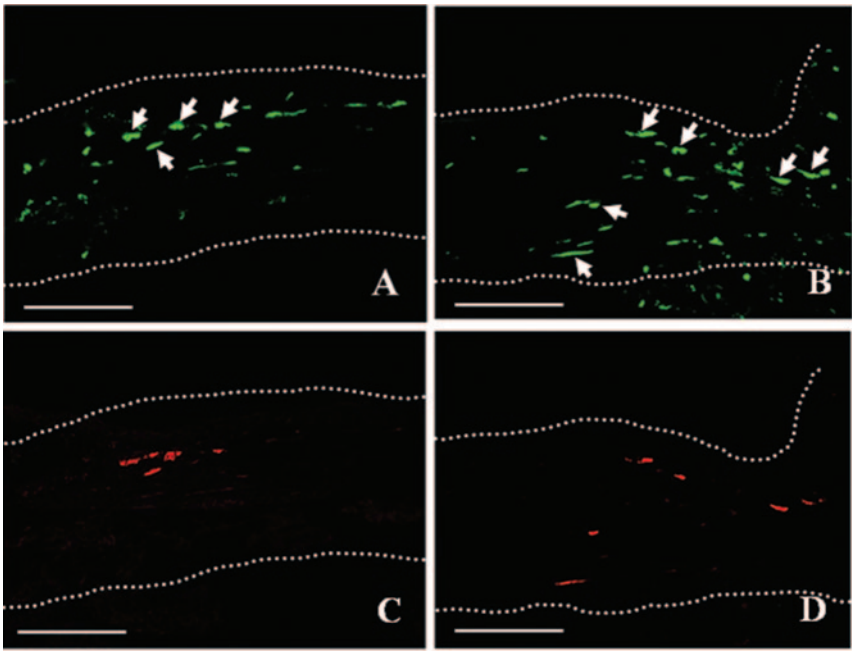

FIGURE 7. Representative immunohistochemical staining for CD11b (*red*) in the cornea of mice receiving BM cell (**A**, **C**) or HSC (**B**, **D**) transplants. Some GFP^+ cells (*green*) immunostained with CD11b (*arrows*). Note the GFP^+CD11b^- cells dispersed throughout the transplant-recipient cornea. GFP (**A**, **B**), CD11b (**C**, **D**). *Dotted lines*: whole corneal tissue. Scale bars, 100 μm.

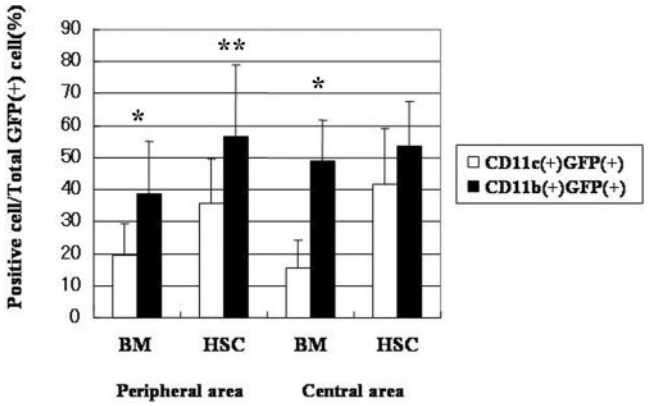

FIGURE 8. Quantitative analysis of the immunohistochemistry for CD11c and CD11b in the cornea of mice receiving transplants of BM cells or HSCs. The percentage of positive cells per section was calculated as the number of cells that coexpressed GFP and CD11c or GFP and CD11b, divided by the total number of GFP^+ cells $\times$ 100. The number of $CD11c^+$ cells in both the peripheral and central areas of the cornea was greater in mice receiving transplants of HSCs than in those receiving BM cells. Approximately 50% of GFP^+ cells were immunostained with CD11b in both BM cell and HSC recipients. There were statistically significant differences between $CD11c^+$ and $CD11b^+$ (Mann-Whitney test; $^*P < 0.01$, $^{**}P < 0.05$).

a significant number of resident dendritic cells in the corneal tissue.

Our observations in corneas receiving GFP-labeled BM cell transplants are of particular interest. Ours is the first report on the time course of the migration of GFP-labeled BM cells into the cornea. Within 2 months after BM cell transplantation, the density of GFP-labeled cells gradually increased; thereafter, cell density was comparatively stable, and finally, at 6 months, it reached a plateau. These findings led us to the interesting hypothesis that BM-derived cells continuously migrate into corneal tissue and contribute to corneal integrity. At present, we do not know the longevity of GFP-labeled BM cells in the mouse cornea. Using other experimental protocols, further cell biological study is needed to clarify this point.

There have been no reports on the distribution of hematopoietic stem/progenitor cells (not bone marrow cells) in the mouse cornea. Although the type of transplantation necessary to obtain these data is very difficult, our group has mastered the technique by using a unique protocol that facilitates our long-term observation of the eyes of transplant-recipient mice.

Our study demonstrates that most of the GFP^+ cells were distributed in the corneal stroma: Approximately 25% were found in the periphery and 7% in the center. In contrast, a small number, approximately 1%, were found in the corneal epithelium. The distribution rates of GFP^+ cells were similar in mice receiving with BM cells and HSCs. These results suggest that cells migrating into the corneal tissue may be definite populations of BM cells, such as HSCs or undifferentiated progenitor cells.

Based on our immunohistochemical results, we divided GFP^+ cells in the corneal tissue into four groups: GFP^+CD11c^+, GFP^+CD11b^+, GFP^+CD11c^-, and GFP^+CD11b^- cells. GFP^+CD11c^+ cells (approximately 40% in the HSC transplantation experiment) are thought to express the dendritic cell phenotype[20–22] and GFP^+CD11b^+ cells (approximately 55% in HSCs) either the dendritic cell or macrophage phenotype.[23] Using a protocol similar to ours, Espinosa-Heidmann et al.[24] found that BM-derived progenitor cells contributed to experimental choroidal neovascularization. When they used the F4/80 antibody (monocyte marker), they observed $GFP^+F4/80^+$ cells in the limbus, ciliary body, and normal choroid and sclera, suggesting a high turnover and recruitment rate of infiltrated macrophages. Based on their findings and our observations, we postulate that some of the GFP^+ cells in the mouse cornea are BM-derived APCs.

Some of the GFP^+ cells were negative for cell-surface markers for APCs (CD11c and CD11b), and their origin is unclear. Corneal stroma is composed of both corneal keratocytes and a variety of extracellular matrices comprising collagen subtypes. In our experience, the morphology of GFP^+ cells in the corneal stroma and of corneal keratocytes is very similar. If BM-derived stem cells terminally transdifferentiate into corneal keratocytes, they can be expected eventually to lose surface CD45 expression. We posit that our immunologic experiment did not detect immature $Sca\text{-}1^+$ cells in the mouse cornea (data not shown), suggesting that transplanted hematopoietic stem/progenitor cells first homed to BM and engrafted in the recipient mice, and then provided mature BM-derived cells in the cornea. Based on our present results we cannot unequivocally claim that BM-derived GFP^+ cells can transdifferentiate into corneal cell phenotypes or neurons. Therefore, morphologic and immunohistochemical studies are under way to examine extracellular matrices and cell-surface markers that are uniquely synthesized by corneal keratocytes.

Several technical and conceptual issues deserve consideration in the interpretation of our results. It is important to note that even in eGFP mice significantly fewer than 100% of the cells express GFP. As this may be due to cell-cycle dependent expression of GFP, we suggest that our results underestimate the potential contribution of BM-derived cells in the mouse cornea. We are currently investigating whether the findings we made with our animal model are applicable to humans. Therefore, we are studying the distribution of BM-derived cells in human corneas.

In conclusion, ours is the first study that presents direct evidence for the migration into the cornea of GFP-labeled BM-derived cells. We provide immunohistochemical evidence that some of the migrating cells were BM-derived cells such as

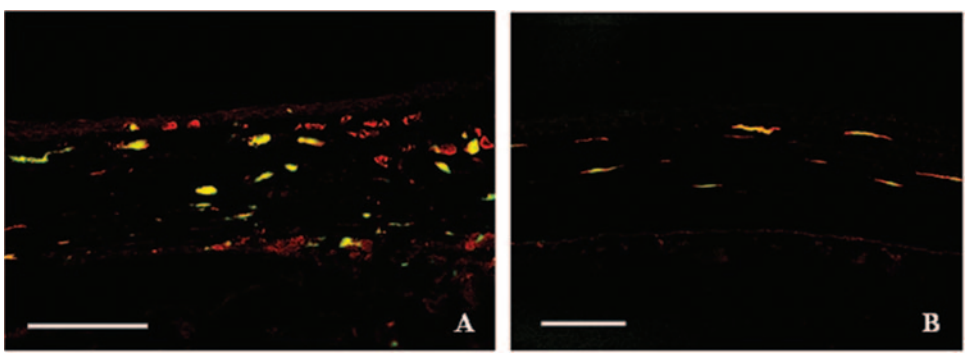

FIGURE 9. Representative immunohistochemical staining for CD45 (*red*) in the cornea of HSC-recipient mice. (**A**) Peripheral, (**B**) central retina. Most GFP^+ cells were immunostained with CD45 (*yellow*). Scale bars: (**A**) 100 μm; (**B**) 50 μm.

dendritic cells and macrophages. Cell biology studies will determine the lineage(s) of the other cells.

Acknowledgments

The authors thank Hisayo Sogabe for assisting in the immunohistochemical studies and quantitative evaluations.

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