Neurobiology

Activation of Bone Marrow-Derived Microglia Promotes Photoreceptor Survival in Inherited Retinal Degeneration

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The role of microglia in neurodegeneration is controversial, although microglial activation in the retina has been shown to provide an early response against infection, injury, ischemia, and degeneration. Here we show that endogenous bone marrow (BM)-derived microglia play a protective role in vascular and neural degeneration in the retinitis pigmentosa model of inherited retinal degeneration. BM-derived cells were recruited to the degenerating retina where they differentiated into microglia and subsequently localized to the degenerating vessels and neurons. Inhibition of stromal-derived factor-1 in the retina reduced the number of BM-derived microglia and accelerated the rate of neurovascular degeneration. Systemic depletion of myeloid progenitors also accelerated the degenerative process. Conversely, activation of BM-derived myeloid progenitors by systemic administration of both granulocyte colony-stimulating factor and erythropoietin resulted in the deceleration of retinal degeneration and the promotion of cone cell survival. These data indicate that BM-derived microglia may play a protective role in retinitis pigmentosa. Functional activation of BM-derived myeloid progenitors by cytokine therapy may provide a novel strategy for the treatment of inherited retinal degeneration and other neurodegenerative diseases, regardless of the underlying genetic defect. (*Am J Pathol* 2008, 172:1693–1703; DOI: 10.2353/ajpath.2008.080024)

Inherited degeneration of the retina affects as many as 1 in 3500 individuals, for a total of more than 1 million throughout the world, and is characterized by progressive night blindness, visual field loss, optic nerve atrophy, vessel attenuation, altered vascular hyperpermeability,

and loss of central vision, often progressing to complete blindness.¹ Molecular genetic analysis has identified mutations involving more than 110 different genes, accounting for only a relatively small percentage of the known affected individuals.² Recent advances in gene therapy have led to successful reversal of retinal degeneration, slow (*rds*)³ and retinal degeneration (*rd*)⁴ phenotypes in mice, and the RPE65 phenotype in dogs.⁵ The development of other neuroprotective approaches that affect secondary biochemical pathways would be advantageous because they would not depend on the underlying disease-causing mutation. The potential use of calcium channel blockers,⁶ trophic factors,⁷ and dietary supplements⁸ has been explored.

Recently, intravitreally injected, lineage-negative (Lin[−]) hematopoietic stem cells (HSCs) have been reported to rescue retinal degeneration in *rd1* and *rd10* mice.⁹ In the study, exogenous Lin[−] HSCs prevented retinal vascular degeneration and this vascular rescue correlated with neuronal rescue.⁹ Although this approach showed a dramatic rescue effect, there was a limitation in that intravitreally injected bone marrow (BM)-derived stem cells were effectively incorporated into the retina only during an early, postnatal developmental stage, but not in adult mice. Directly injected exogenous Lin[−] HSCs only target activated astrocytes that are observed in neonatal mice or an injury-induced model in the adult.¹⁰

In the current study, we have investigated the role of endogenous circulating BM-derived stem cells in inherited retinal degeneration and explored a new therapeutic potential. Recruited endogenous BM-derived stem cells in the retina contain a variety of progenitor cells, including those capable of becoming vascular endothelial cells,^{10,11} mural cells,¹² epithelial cells (including retinal pigment epithelium),¹³ and microglia/

Supported by the Ministry of Education, Science, Sports, and Culture, Japan (grant-in-aid no. 17689045).

Accepted for publication March 12, 2008.

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macrophages.¹⁴ The role of BM-derived stem cells has recently been suggested in several neural degenerative diseases,^{15,16} including Alzheimer's disease¹⁷ and amyotrophic lateral sclerosis.¹⁸ However, the relationship between BM-derived stem cells and inherited retinal degeneration remains elusive.

Materials and Methods

Mouse Strains

All animals were handled in accordance with the standards of the Association for Research in Vision and Ophthalmology for the use of animals in ophthalmic and vision research. All experimental procedures were approved by the Institutional Review Board at Kyoto University Graduate School of Medicine. C57BL/6, C3H/HeJ (*rd1/rd1*), and C57BL/6-Tg (CAG-EGFP) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). B6.CXB1-*Pde6b*^{rd10}/J (*rd10*) and B6(A)-*Rpe65*^{rd12}/J (*rd12*) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C3H/HeJ is homozygous for the *rd1* mutation, which causes rapid early onset retinal degeneration. This mutation is located in exon 7 of the *Pde6b* gene that encodes the rod photoreceptor cGMP phosphodiesterase β subunit. Mutations in this gene have been found in human patients with autosomal recessive retinitis pigmentosa (RP).¹⁹ Retinal degeneration in *rd10* mice is caused by a mutation in exon 13 of *Pde6b* gene. This is also a clinically relevant RP model with later onset and milder retinal degeneration than *rd1/rd1*.²⁰ Retinal degeneration in *rd12* mice is caused by a mutation in exon 3 of the *Rpe65* gene, and this strain is a model for human Leber congenital amaurosis. This mutation causes slower onset and slower progression of retinal degeneration than *rd1/rd1* or *rd10*.²¹

BM Transplantation

The tibias and femurs were dissected and the BM was extracted by slowly flushing medium (RPMI 1640 containing 2.5% HEPES and 1% gentamicin) inside the diaphyseal channel with a 27-gauge needle. The BM that was obtained was homogenized, filtered (70 μ m nylon filter; BD Biosciences, Tokyo, Japan), centrifuged, and resuspended in the medium, as described earlier. Recipient mice were lethally irradiated (950 cGy) using a Gammacell 40 exactor (Nordion, Vancouver, Canada) and 1×10^7 nonpurified BM cells were administered intravenously.

Counting the Number of Infiltrated GFP⁺ Cells into the Retina

Eyes were nucleated after fixation with 4% paraformaldehyde and the retinas were laid flat with radial relaxing incisions to obtain a whole-mount preparation. For quantification of GFP⁺ cells in the retina, fluorescent microscopy (AxioVision; Zeiss, Oberkochen, Germany) was

used and four independent fields ($\times 40$ objective lens) were chosen randomly from the mid-peripheral area between the optic disk and the retina-ciliary body border. GFP⁺ cells in all of the retinal layers were summed up in each field and the mean number of the four fields was calculated for each eye.

Immunohistochemistry

Retinas were harvested at various time points and fixed with 4% paraformaldehyde and methanol, followed by blocking in 50% fetal bovine serum/20% normal goat serum for 1 hour at room temperature. To stain retinal vasculature, anti-CD31 (BD Biosciences Pharmingen, San Diego, CA) and anti-collagen type IV (Chemicon, Temecula, CA) were used. To confirm microglial phenotype, anti-Iba1 (Wako, Osaka, Japan), anti-F4/80 (Serotec, Oxford, UK), anti-CD11b (BD Biosciences), and CD68 (Serotec) were used. To confirm other cellular surface markers of astrocytes, mural cells, and neurons, we used anti-GFAP Cy3-conjugated (Sigma, St. Louis, MO), anti- α smooth muscle actin Cy3-conjugated (Sigma), and anti-NeuN antibodies (Chemicon), respectively. For secondary antibodies, Alexa 488- or 594-conjugated antibodies (Invitrogen, Eugene, OR) were used. The retinas were laid flat with radial relaxing incisions to obtain a whole-mount preparation and the images were obtained by confocal microscopy (LSM 5 Pascal, Zeiss). For a sectional view, cryostat sections (10 μ m) were counterstained by DAPI (Sigma) and the images were taken by fluorescence microscopy (AxioVision, Zeiss).

Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total mRNA was prepared from freshly dissected whole retinas of mice, using a RNeasy kit (Qiagen, Tokyo, Japan). The mRNA was transcribed using a First-Strand cDNA synthesis kit (Applied Biosystems, Tokyo, Japan), and real-time PCR was performed using TaqMan PreAmp master mix (Applied Biosystems). Primers for stromal-derived factor (SDF)-1 were purchased from Applied Biosystems (assay ID: Mm00445552_m1). All samples were normalized to β -actin (Applied Biosystems). Real-time PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems) for 40 cycles.

Intravitreal Injection

Mice were anesthetized, and an anti-SDF-1 antibody (MAB310; R&D Systems, Minneapolis, MN) or saline control was injected intravitreally using 33-Gauge needle (Hamilton, Reno, NV). A total volume of 0.5 μ l was administered to achieve a final effective concentration of 100 μ g/ml of antibody in the vitreous.

Quantification of Retinal Vascular Length

We quantified the retinal vessel length following a previously described method.⁹ Briefly, images of vasculature in intermediate and deep vascular plexuses were obtained using a confocal microscope (LSM 5 Pascal, Zeiss) with an attached software. For quantification of the vasculature, four independent fields ($461\ \mu\text{m} \times 461\ \mu\text{m}$) were chosen randomly from the midportion of the intermediate and deep vascular layer, and the total length of the vasculature was measured using Angiogenesis Image Analyzer software (Kurabo, Osaka, Japan).

Counting the Number of Neurons in the Outer Nuclear Layer

Mice were transcardially perfused with 4% paraformaldehyde, and the eyes were enucleated, fixed, embedded in paraffin, and processed for standard hematoxylin and eosin (H&E) staining. A single section ($4\ \mu\text{m}$) containing the superior pole, inferior pole, optic nerve head, and the entire peripheral retina was visualized with a light microscope (AxioVision, Zeiss) with a $\times 20$ objective lens. The number of nuclei located in the outer nuclear layer in the mid-peripheral area of two different fields (1000 pixels in Photoshop) within one section were counted and averaged for analysis.

Measurement of Cone Cell Density

We measured cone cell density following a previously described method,²² with a slight modification. Briefly, eyecups were fixed in 4% paraformaldehyde for 30 minutes and then the entire retina was carefully dissected away from the retinal pigment epithelium. Retinas were placed in Alexa Fluor 594-conjugated lectin PNA (Molecular Probes, Tokyo, Japan) at a concentration of $5\ \mu\text{g}/\text{ml}$ in phosphate-buffered saline (PBS) with 20% normal goat serum for 2 hours at 4°C . The flat-mounted retinas were examined using a confocal microscope (LSM 5 Pascal, Zeiss) with a $\times 20$ objective lens. The number of cones present within four $230 \times 230\ \mu\text{m}$ (512×512 pixels) squares located $500\ \mu\text{m}$ away from the center of the optic disk was counted and each of four independent fields was averaged.

Colony Forming Unit-Granulocyte Macrophage (CFU-GM) Assay

BM cells obtained from tibias and femurs were homogenized, filtered, and corrected by light-density gradient centrifugation (Histopaque 1083; Amersham Biosciences, Buckinghamshire, UK). A total of 2×10^3 cells per well was seeded into MethoCult medium (GF M3534; StemCell Technologies, UK) and after 7 days in culture, colonies (>50 cells) in three wells were scored by two independent investigators.

Systemic Depletion of Circulating Microglial Progenitors

Clodronate-loaded liposomes were provided by N. van Rooijen (Vrije Universiteit, Amsterdam, The Netherlands) and were injected intraperitoneally, with appropriate dilution for each experiment, at 0.1 ml volume per day.

Systemic Administration of Granulocyte Colony-Stimulating Factor (G-CSF) and Erythropoietin (Epo)

Human recombinant G-CSF ($300\ \mu\text{g}/\text{kg}$) and Epo ($1000\ \text{IU}/\text{kg}$) (Kirin Pharma, Tokyo, Japan) were co-administered once daily in 0.1 ml of saline.

Recording of Electroretinograms (ERGs)

After overnight dark adaptation, mice were anesthetized by intraperitoneal injection of $50\ \mu\text{g}/\text{kg}$ of ketamine and $10\ \mu\text{g}/\text{kg}$ of xylazine. ERGs were recorded from the corneal surface of each eye after pupil dilation (0.4% tropicamide and 0.5% phenylephrine hydrochloride) using a gold loop corneal electrode with a light-emitting diode (Mayo Corp., Inazawa, Japan). A reference electrode was placed in the mouth and a ground electrode was inserted into the tail. Stimuli were produced with a light-emitting diode stimulator (Mayo Corp.). The ERG response signals were amplified, digitized at 10 kHz with a band-pass filter of 0.3 to 500 Hz, and computer-analyzed (PowerLab 2/25; AD Instruments, New South Wales, Australia). Rod-cone maximal combined responses were recorded by a white flash at an intensity of $30\ \text{cd}'\text{s}/\text{m}^2$. After 10 minutes of light adaptation at an intensity of $25.1\ \text{cd}/\text{m}^2$, cone responses were estimated by a white stimulus of $30\ \text{cd}'\text{s}/\text{m}^2$. The stimulus frequency was 1 Hz and 32 photopic measurements were averaged.

Statistical Analysis

All results for continuous variables are expressed as the mean $\pm$ SEM. Comparisons between two groups were analyzed by Student's *t*-test for paired and unpaired analyses. A *P* value less than 0.05 was considered statistically significant. All analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL).

Results

BM-Derived Cells Are Recruited into the Degenerating Retina Where They Differentiate into Microglia, and Localize to the Site of Degenerating Vessels and Neurons

The relationship between BM-derived cells and inherited retinal degeneration is unknown. To clarify this, we first developed a bone marrow transplantation (BMT) model. BM obtained from donor C57BL/6-Tg (CAG-EGFP) mice

Figure 1. BM-derived cells were recruited into the degenerating retina. **A–F:** Retinal flatmount after BMT with GFP donor mice. Seven days after BMT in wild-type (WT) mice (**A**) and *rd1/rd1* mice (**B**). Eleven days after BMT in WT mice (**C**) and *rd1/rd1* mice (**D**). Fifteen days after BMT in WT mice (**E**) and *rd1/rd1* mice (**F**). **G:** GFP⁺ cell counts in the retina of WT mice and *rd1/rd1* mice (*n* = 6). **H:** GFP⁺ cell percentage of BM in WT mice and *rd1/rd1* mice (*n* = 6). NS, not significant. Retinal flatmount after GFP labeling and BMT in other degeneration models, *rd10* (**I**) and *rd12* (**J**). BMT was performed at P15 in *rd10*, and at P30 in *rd12*. The images were collected 15 days after BMT in *rd10* and 60 days after BMT in *rd12*. Scale bars = 100 μ m.

was transplanted into recipient *rd1* mice or wild-type (WT) C57BL/6 mice on postnatal (P) day 15. Seven days after BMT, both *rd1* and controls showed few GFP⁺ cells in the retina (Figure 1, A and B). On day 11, however, numerous GFP⁺ cells had been recruited in the retina of *rd1* but not WT mice (Figure 1, C and D). By day 15, the number of GFP⁺ cells detected in the *rd1* retina was 43-fold greater than in controls (Figure 1, E–G). The number of GFP⁺ cells detected in the BM of transplanted mice was not different between the two groups (Figure 1H). This recruitment of BM-derived cells into the degenerating retina was also seen in the other strains of mice with retinal degeneration [ie, *rd10* (B6.CXB1-*Pde6b*^{rd10}/J) and *rd12* (B6(A)-*Rpe65*^{rd12}/J); Figure 1, I and J]. This suggests that the recruitment of BM-derived cells into the degenerating retina does not depend on the background of the recipient strain or to the specific type of gene mutation, but rather to retinal degeneration itself.

Next, we wanted to determine the localization of transplanted BM-derived cells in the degenerating retina and whether they differentiated (Figure 2). We noted that few GFP⁺ cells were incorporated into the capillary vessels, indicating that the cells originated from circulating endothelial progenitor cells (Figure 2A). However, the vast majority of the GFP⁺ cells were classified into three types by morphology (Figure 2, B–D), as follows: 1) the spindle shape along the retinal vessels (Figure 2B), 2) the amorphous/amoeboid shape around the relatively superficial retinal vessels (Figure 2C), and 3) the ramified morphology in the deeper layer of the retina (Figure 2D). Subsequently, we performed immunohistochemistry using several cellular surface markers, including vascular endothelium, mural cells, astrocytes, microglia, and neurons, and found that all three types of cells expressed microglial markers (ie, Iba-1, F4/80, and CD11b; Figure 2, E–M). We found very few CD31⁺ endothelial pheno-

type (Figure 2A) and no GFAP⁺ astrocytes (Figure 2N), α -smooth muscle actin⁺ mural cells (Figure 2, O and P), or Neu-N⁺ neurons (data not shown). These BM-derived microglia were especially condensed around the optic disk and retinal vascular trunk (Figure 2Q). In addition, 4 months after BMT, most of the retinal microglia (more than 80%) were shown to be of BM origin and the remainder were tissue resident microglia (Figure 2R). These results may indicate that microglia in the degenerating retina may give rise to a turnover by infiltration of BM-derived microglia. These recruited cells were demonstrated, both in *rd1* and *rd10* mice, to migrate toward the outer nuclear layer or subretinal spaces where no microglia exist in physiological conditions (Figure 2, S–U).²³ In summary, the degenerating retina recruits the BM-derived cells and most of these BM-derived cells differentiate into cells with a microglial phenotype. In addition, BM-derived microglia migrate toward the regressing vessels and dying neurons in the retina probably respond to some signals originating from degenerating vessels and neurons, and this phenomenon may suggest their roles in neurovascular degeneration.

SDF-1 Plays a Role in the Recruitment of Circulating Microglial Progenitors into the Degenerating Retina and Inhibition of SDF-1 Accelerates Neurovascular Degeneration

We next investigated the mechanism underlying the recruitment of BM-derived stem cells into the degenerating retina. We focused on SDF-1, which is known as an entrapment factor of BM-derived stem cells to the lesion sites.²⁴ Quantitative real-time polymerase chain reaction showed that the expression patterns of SDF-1 mRNA

Figure 2. Retinal images after BMT. **A:** Incorporated BM-derived cells (green) in the retinal vasculature (CD31, red) in *rd1* mice. **B–D:** Three types of morphologies of BM-derived cells (green) and association with retinal vasculature (collagen IV, red). **B:** Spindle-shaped BM-derived cells were around the superficial retinal vasculature. **C:** Amoeboid BM-derived cells were in the intermediate retinal vasculature. **D:** Ramified BM-derived cells were in the deep retinal vasculature. **E–M:** Morphological and immunohistochemical analysis of BM-derived cells (green) in the degenerating retina for Iba-1 (**E–G**), F4/80 (**H–J**), and CD11b (**K–M**) (red). **N:** BM-derived cells (green) are found in the astrocyte network (GFAP, red). **O:** No transplanted BM-derived cells (green) were found around the retinal vessels (α -smooth muscle actin, SMA; red) in WT retina. **P:** Many transplanted BM-derived cells were found around retinal vessels (α -SMA, red) in *rd1* retina. **Q:** Numerous BM-derived cells (green) were found around the optic disk (**D**) expressing CD11b (red). **R:** Four months after transplantation in *rd1* mice, >80% of CD11b⁺ microglia (red) are BM-derived (green). **S–U:** Sectional view of the retina of *rd1* mice at P30 (15 days after BMT). **S:** BM-derived cells (green) and DAPI (blue). **T:** CD11b (red). **U:** Merged image. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars: 50 μ m (**A–D**, **N–P**); 20 μ m (**E–M**); 100 μ m (**Q**); 200 μ m (**R**).

were similar in both WT C57BL/6 and *rd1* mice (Figure 3A). The expression of SDF-1 mRNA was stable from P6 to P12, elevated at P14, and highest on P18. SDF-1 mRNA was significantly higher in *rd10* mice than the controls at P16 and P18. This peak of SDF-1 mRNA expression was later than the reported peak of rod photoreceptor cell degeneration (approximately P14 to P16)²³ and the peak of expression of other degeneration-related cytokines, such as MCP-1, RANTES, and tumor necrosis factor- α (approximately P12).²³

To investigate whether SDF-1 plays a role in the recruitment of BM-derived cells into the degenerating retina, we injected an anti-SDF-1 neutralizing antibody into the vitreous of *rd1* mice (EGFP⁺ BM cell-transplanted). We found a marked decrease in the number of BM-derived cells (GFP⁺ cells) in the anti-SDF-1-injected retinas compared to the saline-injected retinas (Figure 3, B–D), suggesting that SDF-1 plays a role in the recruitment of BM-derived cells into the degenerating retina.

The retinas of anti-SDF-1-injected *rd1* eyes were significantly thinner than those of control *rd1* eyes (data not

shown). Because it is possible that anti-SDF-1 influences normal vascular development of the retina,²⁵ we used *rd10* mice to investigate whether the inhibition of SDF-1 truly affected retinal degeneration, including both neurons and vessels (Figure 3, E–N). We injected the anti-SDF-1 antibody on P20, when normal vascular development is nearly complete and when degeneration begins in *rd10* mice. In WT mice (C57BL/6), injection of anti-SDF-1 antibody did not change the vasculature or neurons (Figure 3, E, F, I, and J). In contrast, anti-SDF-1 injection in *rd10* mice accelerated vascular and neural degenerations (Figure 3, G, H, and K–N), similar to *rd1* mice.

In addition, we investigated whether BM-derived microglia play a role in cone photoreceptor cell degeneration. In *rd1* mice, rod photoreceptor degeneration begins on P10 and is almost complete by P21, whereas cone cell numbers are essentially normal at P21 but are reduced to undetectable levels by 2 to 4 months of age.²⁶ We selectively stained cone cells with PNA lectin in a flatmount retina²⁶ and found a marked decrease in the number of cone cells in anti-SDF-1-injected eyes at P21 in *rd1* mice (Figure 4, A–C). We also

Figure 3. SDF-1 plays a crucial role for the retention of BM-derived cells in the degenerating retina and inhibition of SDF-1 accelerates retinal degeneration. **A:** Real-time PCR analysis for SDF-1 in the retina of WT mice and *rd1/rd1* mice ($n = 6$ per group). **B** and **C:** Retinal flatmount of saline-injected eye and anti-SDF-1 neutralization antibody-injected eye (injected at P8) 15 days after GFP donor BMT. **D:** Marked reduction of BM-derived cells in the anti-SDF-1-treated eyes ($n = 8$). **E–H:** Deep vasculature (P25) of saline- and anti-SDF-1-injected eyes in WT (**E, F**) and *rd10* mice (**G, H**). No change in anti-SDF-1-injected eyes of WT mice (**F**), in contrast, loss of microvasculature occurred in anti-SDF-1-injected eyes of *rd10* mice (**H**), compared to saline-injected eyes (**G**). **I–L:** Sectional images (P25) of saline- and anti-SDF-1-injected eyes in WT (**I, J**) and *rd10* mice (**K, L**). No change was observed in WT mice (**I, J**); however, there was a marked loss of photoreceptor cells in the outer nuclear layer (ONL) in anti-SDF-1-injected eyes of *rd10* mice (**K, L**). GCL, ganglion cell layer; INL, inner nuclear layer. **M:** Vascular length of deep vasculature (P25) was reduced in anti-SDF-1-injected eyes of *rd10* mice ($n = 8$). **N:** Number of photoreceptor cells in ONL (P25) was reduced in anti-SDF-1-injected eyes of *rd10* mice ($n = 8$). * $P < 0.05$, ** $P < 0.01$. Scale bars = 100 μm .

found a strong positive correlation between the number of microglia and the density of cone cells (Figure 4D). Thus, depletion of BM-derived microglial cells by SDF-1, resulting in an accelerated degeneration of the retina suggested a protective role of these cells in inherited neurovascular degeneration, especially for secondary cone cell loss after primary rod cell degeneration.

Systemic Depletion of Circulating Microglial Progenitors Accelerates Inherited Neurovascular Degeneration

To further confirm the role of BM-derived microglia in retinal degeneration, clodronate liposome (CL-lip), which

Figure 4. Inhibition of SDF-1 accelerates cone photoreceptor degeneration. **A** and **B:** Cone density and number of microglia were (P21) markedly decreased in anti-SDF-1-injected eyes of *rd1* mice (injected on P8). Inner and outer segments of cone photoreceptor were stained by lectin PNA (red). Microglia were stained by CD11b (green). **C:** Quantitative analysis of cone density in *rd1* mice ($n = 6$). **D:** Cone density and the number of microglia have a strong positive correlation ($n = 12$). ** $P < 0.01$. Scale bars = 50 μm .

Figure 5. Systemic depletion of microglial progenitors accelerates inherited retinal degeneration. **A:** GFP⁺ cells recruited into the degenerating *rd1* retina (P25) were significantly reduced in the clodronate liposome (CL-lip)-injected group compared to the vehicle (PBS-lip)-injected group ($n = 6$). **B:** Retina resident microglia positive for Iba-1 was almost at the same level between the groups ($n = 6$). **C:** Microglial progenitors in BM reduced to almost zero in the CL-lip-treated group ($n = 6$). CFU-GM, colony forming unit-granulocyte macrophage. **D–G:** Deep vasculature of PBS-lip- or CL-lip-treated eyes (P25) in WT (**D**, **E**) and *rd10* mice (**F**, **G**). No change was seen in WT mice. In contrast, in *rd10* mice, a marked loss of vasculature was observed in the CL-lip-treated group. **H–K:** Sectional images of the PBS-lip- and CL-lip-treated eyes (P25) in WT (**H**, **I**) and *rd10* mice (**J**, **K**). No change was observed in WT mice, however, in *rd10* mice, slightly reduced photoreceptor cells were seen in the CL-lip-treated group. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. **L:** The vascular length of the deep vasculature was significantly reduced in the CL-lip-treated group of *rd10* mice ($n = 8$). **M:** The number of photoreceptor cells in ONL was significantly reduced in the CL-lip-treated group of *rd10* mice ($n = 8$). * $P < 0.05$, ** $P < 0.01$. NS, not significant. Scale bars = 100 μ m.

is well known to have the powerful effect of microglia/macrophage depletion²⁷ using its phagocytosis capability, was used. Inhibition of the BM-derived GFP⁺ cells recruited to the retina was observed in the CL-lip-injected group [BMT on P15 and CL-lip injection from P20 to P24 (25 mg per day)] compared to PBS-lip-injected controls in *rd1* mice (Figure 5A). In addition, although CL-lip did not influence the number of tissue resident microglia in control mice (Figure 5B), the colony-forming unit of granulocyte-macrophage (CFU-GM) was markedly reduced in the CL-lip-treated mice (Figure 5C). These data indicated that CL-lip treatment can reduce the recruitment of BM-derived microglia into the degenerating retina and this treatment did not influence the number of tissue-resident microglia. CL-lip was shown to have a significant power for systemic reduction of circulating microglial precursors.

We then determined whether this reduction of circulating microglial precursors could influence retinal neurovascular degeneration (Figure 5, D–M). CL-lip was sys-

temically injected into *rd10* mice from P20 to P24 (25 mg per day) to avoid the effect of CL-lip on retinal vascular development.^{25,28} In WT mice (C57BL/6), no effect on the vasculature or neurons was shown, either in the CL-lip- or in the PBS-lip-injected groups (Figure 5, D, E, H, and I). However, in *rd10* mice, severe vascular damage and regression were seen in the CL-lip-injected group. In intermediate and deep vascular plexuses, vessels were significantly regressed in the CL-lip-injected group (Figure 5, F, G, and L). Moreover, in the superficial vascular layer, marked attenuation and bending of the vessels were seen in the CL-lip-injected group (data not shown). In addition, as in the SDF-1 experiments, the number of photoreceptor cells was significantly reduced in the CL-lip-injected group compared to the PBS-lip-injected controls (Figure 5, J, K, and M). These data strengthen the possibility that BM-derived microglia are recruited into the retina and have a protective role for inherited retinal neurovascular degeneration.

Figure 6. Functional activation of microglial progenitors using systemic injection of G-CSF and Epo decelerates inherited retinal degeneration. **A:** CFU-GM in BM was markedly increased by systemic G-CSF and Epo combined treatment compared to saline-injected controls, and this activation was negated by the additional intraperitoneal injection of clodronate liposome (CL-lip). **B–D:** Deep vasculature of each group on P30 in *rd10* mice. **E:** Vessel loss was significantly protected by G-CSF and Epo treatment, and this protection was neutralized by additional CL-lip treatment ($n = 10$). **F–H:** Sectional image of the retina on P30 in *rd10* mice. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. **I:** The number of residual photoreceptor cells in the ONL was significantly increased in the G-CSF- and Epo-treated group, and this protective effect was negated by additional CL-lip treatment ($n = 10$). **J–L:** Bright-flash ERG was recorded in each group on P30 in *rd10* mice. Highly preserved response in G-CSF- and Epo-treated group. **M–O:** Cone stained by lectin PNA (red) and CD11b⁺ microglia (green) on the same planes were photographed on P40 of *rd10* mice. Cone inner segments were well-preserved in G-CSF and Epo treatment and this protective effect was completely negated by additional CL-lip treatment. **P:** Cone density was counted on P40 ($n = 6$). **Q–S:** Cone ERG was recorded on P30. **T:** B-wave amplitude of cone ERG was markedly preserved in G-CSF and Epo treatment eyes and this protective effect was negated by additional CL-lip treatment ($n = 6$). NS, not significant. Scale bars: 100 μ m (**A–E**); 50 μ m (**M–O**).

Activation of Circulating BM-Derived Stem Cells Decelerates the Progression of Inherited Neurovascular Degeneration

Based on the results that showed blocking BM-derived microglia resulted in an acceleration of retinal photoreceptor degeneration, it follows that an increase in the number or activation of those cells into the retina could rescue the photoreceptors. To investigate this hypothesis, the systemic administration of G-CSF and Epo was tested. G-CSF is a well known mobilization factor of circulating HSCs and Epo has been reported to have a mobilizing capacity of HSCs in BM into the circulation.²⁹ We found that although systemic administration of G-CSF (300 μ g/kg) or Epo (1000 IU/kg) for 5 days exhibited a mild increase of CFU-GM, simultaneous injection of both G-CSF and Epo showed an approximate threefold increase of CFU-GM compared to saline-injected controls (Figure 6A). We also found that additional CL-lip injection

(5 mg/day) could neutralize this CFU-GM increase back to the normal level (Figure 6A), indicating this increase in CFU-GM is mediated by macrophages/microglial cells. Subsequently, we injected G-CSF and Epo systemically into *rd10* mice from P20 to P29 and observed vascular and neuronal degeneration on P30. Effectively, both vascular and neuronal degeneration clearly delayed the G-CSF- and Epo-injected groups compared to the controls (Figure 6, B, C, E, F, G, and I). Because both G-CSF and Epo have been reported to have a direct protective effect on neural cells,^{30,31} we additionally administered CL-lip (5 mg/day) to neutralize the CFU-GM activation effect by G-CSF and Epo (Figure 6A) to clarify that this rescue effect is derived from direct or indirect processes. Under these conditions, vascular and neural rescue were also reduced, but not to the levels of controls, suggesting that the combined systemic administration of G-CSF and Epo may have both direct and indirect rescuing effects for retinal degeneration (Figure 6, D, E,

H, and I). A bright-flash ERG showed functional rescue of G-CSF and Epo treatment, and this rescue effect was negated by plus CL-lip treatment (Figure 6, J–L). Judging from these data, indirect rescue via BM-derived microglia may be a major mechanism for this vascular and neuronal rescue.

Furthermore, we investigated how this rescue effect contributed to cone photoreceptor degeneration (Figure 6, M–T). We found that cone density was markedly preserved in the G-CSF- and Epo-treated group (Figure 6, M, N, and P) and that this rescue effect was almost abrogated by CL-lip treatment (Figure 6, O and P). Similarly, this combined treatment with G-CSF and Epo dramatically rescued the cone photoreceptor function (Figure 6, Q–T). We also observed cone density preservation in *rd1* mice, but the effect was not as marked as in *rd10*, probably because the degeneration speed is higher in *rd1* (data not shown). These results suggest that activation of BM-derived microglial cells has a significant rescue effect against the inherited retinal degeneration and the cone rescue effect was characteristic in this strategy. In addition, the possibility of a new cytokine therapy against inherited retinal degeneration, including RP, was suggested.

Discussion

The physiological and pathological roles of microglia in the retina have been under investigation.^{14,23,25,28,32,33} Microglial activation in the retina provides an early response against infection, injury, ischemia, and degeneration.^{33,34} In retinal degeneration, activated microglia migrate into the deeper retina with the expression of tumor necrosis factor- α before the onset of photoreceptor cell death,²³ suggesting that microglial activation may trigger neuronal cell death. On the other hand, microglia secrete neurotrophic factors and promote photoreceptor survival in a light-induced retinal degeneration model,³² and promote vascular repair in an oxygen-induced retinopathy model.²⁸ Although contributions of both tissue resident and BM-derived microglia have been suggested in previous reports, such roles were ambiguous. In the current study, we demonstrated the protective functions of BM-derived microglia in both vascular and neural degeneration. We also proposed that activating the protective functions of BM-derived microglia by systemic cytokine therapy may provide a novel strategy for delaying retinal degeneration. On the other hand, the role of BM-derived microglia in ocular inflammation remains unclear. We did not perform mRNA quantification or immunohistochemistry of proinflammatory or inflammatory cytokines produced by BM-derived microglia in this study. Furthermore, the role in inflammation of tissue resident microglia and the difference of the roles between tissue-resident and BM-derived are also unknown.

Many cytokines are probably involved in the recruitment of BM-derived cells into the degenerating retina. In this study, we investigated the role of SDF-1, which is known as a recruitment factor in several retinal diseases such as diabetic retinopathy³⁵ and age-related macular

degeneration.³⁶ We measured mRNA expression level of SDF-1 in *rd1* mice and found a significantly higher expression level on P16 and P18 compared to WT mice. Intravitreal injection of anti-SDF-1 neutralizing antibodies reduced the number of BM-derived cells in the retina. In this study, BMT was performed on P15, so we could not determine whether the higher expression of SDF-1 on P16 to P18 is really related to the recruitment of BM-derived cells to the retina. The reconstitution of BM by transplantation needs time as shown in Figure 1. BMT in newborn mice may overcome this problem.

The precise mechanisms of retinal protection by BM-derived microglia remain elusive. One hypothesis is that microglia phagocytose cellular debris and clear the degenerative environment. We observed phagocytosed photoreceptor cells inside CD11b⁺ microglia (data not shown). A recent report on Alzheimer's disease demonstrated that BM-derived microglia can eliminate amyloid deposits by a cell-specific phagocytic mechanism.¹⁷ Another possible mechanism is that microglia secrete neurotrophic factors to promote residual cell survival. In the light-induced retinal degeneration model, microglia secrete nerve growth factor or ciliary neurotrophic factor and modulate secondary neurotrophic factor expression in Muller glia, contributing to the protection of photoreceptor cells.³² Muller glia are known to be activated and to express GFAP in retinal degeneration models. In a laser-induced choroidal neovascularization model, BM-derived cells activate Muller cells to express pERK.¹⁴ Activated Muller cells together with BM-derived microglia may possibly play a role in the degenerative process. In addition, the endogenous BM-derived cells also condensed around the optic disk (Figure 2Q) and retinal vessels (Figure 2, B, N, and P), where activated astrocytes exist. It is possible that in addition to the direct effect, those glia-glia interactions may provide a novel protective system for retinal neurovascular degeneration.

It is interesting that a marked protective effect occurred by BM-derived microglia to cone photoreceptor cells in the models of inherited rod photoreceptor cell degeneration (Figure 4, A–D; and Figure 6, M–T). The mechanisms underlying secondary cone degeneration are still not well understood, and some researchers have suggested oxidative stress as causal.²² Our observation has offered new insight for the function of BM-derived microglia and the mechanism underlying secondary cone loss. Interestingly, a similar cone rescue effect through vascular rescue achieved by direct injection of Lin[−] HSCs into the eye was reported.⁹

G-CSF and Epo are commonly used drugs in hematopoietic diseases because they induce the BM stem cells into the circulation, including HSCs. In addition to the hematopoietic effects, both drugs recently reported having neuroprotective effects.^{30,31} Several possible mechanisms of G-CSF-mediated neuroprotection have been suggested, including a direct effect for the neurons and a BM-derived cell-mediated indirect effect. In the current study, we have found that the combined administration of G-CSF and Epo induced a synergistic effect in myeloid progenitor activation (ie, an increase of CFU-GM) and promoted a dramatic rescue of retinal degeneration. Al-

though the single use of G-CSF or Epo also had a retinal protective effect (data not shown), combined therapy showed a synergetic rescue effect, especially for cones. Our data suggested that an indirect effect via activation of microglial progenitors is more potent on the rescue event.

There are several possible advantages for possible cytokine therapy, the first of which would be wide-use regardless of the underlining genetic defect. Also, because the neuroprotective effect of activated BM-derived microglia may be a physiological response to neural degeneration, it is possibly applicable for all types of retinal degeneration. This is a great advantage because there are so many mutations known in human patients. A powerful cone photoreceptor rescue effect is another potential benefit. Cone photoreceptor survival is key to maintain the patient's visual acuity and quality of life. We are hoping this approach will be clinically applicable in the near future because both cytokines are already widely used in human diseases. Of course, there are problems including the dose and undesired side effects that must be addressed for both drugs; systemic activation of microglial progenitors can be a novel therapy for delaying the progression of the disease and maybe other neurodegenerative diseases in the central nervous system. Other drugs that can activate BM-derived microglia are also candidates that should be tested.

Acknowledgments

We thank our colleagues and laboratory members, Kayo Nishida, Hiroko Hizaki, Norimoto Gotoh, He Zou, Tetsushi Kimura, Masafumi Kurimoto, Naoaki Kawagoe, Kaori Asamoto, Junko Nakamura, Megumi Sugimoto, Mayumi Yoshida, and Chiharu Sengoku, for their helpful advice and invaluable technical support; and Yoshinobu Toda and Hisami Taniguchi for their technical support in the histological sectioning of the mouse eyes.

References

- Hartong DT, Berson EL, Dryja TP: Retinitis pigmentosa. *Lancet* 2006, 368:1795–1809
- Humphries P, Kenna P, Farrar GJ: On the molecular genetics of retinitis pigmentosa. *Science* 1992, 256:804–808
- Ali RR, Sarra GM, Stephens C, Alwis MD, Bainbridge JW, Munro PM, Fauser S, Reichel MB, Kinnon C, Hunt DM, Bhattacharya SS, Thrasher AJ: Restoration of photoreceptor ultrastructure and function in retinal degeneration slow mice by gene therapy. *Nat Genet* 2000, 25:306–310
- Takahashi M, Miyoshi H, Verma IM, Gage FH: Rescue from photoreceptor degeneration in the rd mouse by human immunodeficiency virus vector-mediated gene transfer. *J Virol* 1999, 73:7812–7816
- Acland GM, Aguirre GD, Ray J, Zhang Q, Aleman TS, Cideciyan AV, Pearce-Kelling SE, Anand V, Zeng Y, Maguire AM, Jacobson SG, Hauswirth WW, Bennett J: Gene therapy restores vision in a canine model of childhood blindness. *Nat Genet* 2001, 28:92–95
- Frasson M, Sahel JA, Fabre M, Simonutti M, Dreyfus H, Picaud S: Retinitis pigmentosa: rod photoreceptor rescue by a calcium-channel blocker in the rd mouse. *Nat Med* 1999, 5:1183–1187
- Frasson M, Picaud S, Leveillard T, Simonutti M, Mohand-Said S, Dreyfus H, Hicks D, Sabel J: Glial cell line-derived neurotrophic factor induces histologic and functional protection of rod photoreceptors in the rd/rd mouse. *Invest Ophthalmol Vis Sci* 1999, 40:2724–2734
- Berson EL, Rosner B, Sandberg MA, Hayes KC, Nicholson BW, Weigel-DiFranco C, Willett W: A randomized trial of vitamin A and vitamin E supplementation for retinitis pigmentosa. *Arch Ophthalmol* 1993, 111:761–772
- Otani A, Dorrell MI, Kinder K, Moreno SK, Nusinowitz S, Banin E, Heckenlively J, Friedlander M: Rescue of retinal degeneration by intravitreally injected adult bone marrow-derived lineage-negative hematopoietic stem cells. *J Clin Invest* 2004, 114:765–774
- Otani A, Kinder K, Ewalt K, Otero FJ, Schimmel P, Friedlander M: Bone marrow-derived stem cells target retinal astrocytes and can promote or inhibit retinal angiogenesis. *Nat Med* 2002, 8:1004–1010
- Grant MB, May WS, Caballero S, Brown GA, Guthrie SM, Mames RN, Byrne BJ, Vaught T, Spoerri PE, Peck AB, Scott EW: Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. *Nat Med* 2002, 8:607–612
- Espinosa-Heidmann DG, Reinoso MA, Pina Y, Csaky KG, Caicedo A, Cousins SW: Quantitative enumeration of vascular smooth muscle cells and endothelial cells derived from bone marrow precursors in experimental choroidal neovascularization. *Exp Eye Res* 2005, 80:369–378
- Chan-Ling T, Baxter L, Afzal A, Sengupta N, Caballero S, Rosinova E, Grant MB: Hematopoietic stem cells provide repair functions after laser-induced Bruch's membrane rupture model of choroidal neovascularization. *Am J Pathol* 2006, 168:1031–1044
- Caicedo A, Espinosa-Heidmann DG, Pina Y, Hernandez EP, Cousins SW: Blood-derived macrophages infiltrate the retina and activate Muller glial cells under experimental choroidal neovascularization. *Exp Eye Res* 2005, 81:38–47
- Glezer I, Simard AR, Rivest S: Neuroprotective role of the innate immune system by microglia. *Neuroscience* 2007, 147:867–883
- Block ML, Zecca L, Hong JS: Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat Rev Neurosci* 2007, 8:57–69
- Simard AR, Soulet D, Gowing G, Julien JP, Rivest S: Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. *Neuron* 2006, 49:489–502
- Corti S, Locatelli F, Donadoni C, Guglieri M, Papadimitriou D, Strazzer S, Del Bo R, Comi GP: Wild-type bone marrow cells ameliorate the phenotype of SOD1-G93A ALS mice and contribute to CNS, heart and skeletal muscle tissues. *Brain* 2004, 127:2518–2532
- Chang B, Hawes NL, Hurd RE, Davisson MT, Nusinowitz S, Heckenlively JR: Retinal degeneration mutants in the mouse. *Vis Res* 2002, 42:517–525
- Gargini C, Terzibasi E, Mazzoni F, Strettoi E: Retinal organization in the retinal degeneration 10 (rd10) mutant mouse: a morphological and ERG study. *J Comp Neurol* 2007, 500:222–238
- Pang JJ, Chang B, Hawes NL, Hurd RE, Davisson MT, Li J, Noorwez SM, Malhotra R, McDowell JH, Kaushal S, Hauswirth WW, Nusinowitz S, Thompson DA, Heckenlively JR: Retinal degeneration 12 (rd12): a new, spontaneously arising mouse model for human Leber congenital amaurosis (LCA). *Mol Vis* 2005, 11:152–162
- Komeima K, Rogers BS, Lu L, Campochiaro PA: Antioxidants reduce cone cell death in a model of retinitis pigmentosa. *Proc Natl Acad Sci USA* 2006, 103:11300–11305
- Zeng HY, Zhu XA, Zhang C, Yang LP, Wu LM, Tso MO: Identification of sequential events and factors associated with microglial activation, migration, and cytotoxicity in retinal degeneration in rd mice. *Invest Ophthalmol Vis Sci* 2005, 46:2992–2999
- Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, Chimenti S, Landsman L, Abramovitch R, Keshet E: VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 2006, 124:175–189
- Checchin D, Sennlaub F, Levavasseur E, Leduc M, Chemtob S: Potential role of microglia in retinal blood vessel formation. *Invest Ophthalmol Vis Sci* 2006, 47:3595–3602
- Komeima K, Rogers BS, Campochiaro PA: Antioxidants slow photoreceptor cell death in mouse models of retinitis pigmentosa. *J Cell Physiol* 2007, 213:809–815
- van Rooijen N, Sanders A, van den Berg TK: Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. *J Immunol Methods* 1996, 193:93–99
- Ritter MR, Banin E, Moreno SK, Aguilar E, Dorrell MI, Friedlander M:

- Myeloid progenitors differentiate into microglia and promote vascular repair in a model of ischemic retinopathy. *J Clin Invest* 2006, 116:3266–3276
29. Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S: Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood* 2003, 102:1340–1346
 30. Schneider A, Kruger C, Steigleder T, Weber D, Pitzer C, Laage R, Aronowski J, Maurer MH, Gassler N, Mier W, Hasselblatt M, Kollmar R, Schwab S, Sommer C, Bach A, Kuhn HG, Schabitz WR: The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *J Clin Invest* 2005, 115:2083–2098
 31. Grimm C, Wenzel A, Groszer M, Mayser H, Seeliger M, Samardzija M, Bauer C, Gassmann M, Reme CE: HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. *Nat Med* 2002, 8:718–724
 32. Harada T, Harada C, Kohsaka S, Wada E, Yoshida K, Ohno S, Mamada H, Tanaka K, Parada LF, Wada K: Microglia-Muller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration. *J Neurosci* 2002, 22:9228–9236
 33. Langmann T: Microglia activation in retinal degeneration. *J Leukoc Biol* 2007, 81:1345–1351
 34. Schuetz E, Thanos S: Microglia-targeted pharmacotherapy in retinal neurodegenerative diseases. *Curr Drug Targets* 2004, 5:619–627
 35. Butler JM, Guthrie SM, Koc M, Afzal A, Caballero S, Brooks HL, Mames RN, Segal MS, Grant MB, Scott EW: SDF-1 is both necessary and sufficient to promote proliferative retinopathy. *J Clin Invest* 2005, 115:86–93
 36. Bhutto IA, McLeod DS, Merges C, Hasegawa T, Luttly GA: Localisation of SDF-1 and its receptor CXCR4 in retina and choroid of aged human eyes and in eyes with age related macular degeneration. *Br J Ophthalmol* 2006, 90:906–910