The Role of Adult Bone Marrow–Derived Stem Cells in Choroidal Neovascularization

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PURPOSE. Age-related macular degeneration (ARMD) is the primary cause of blindness in people aged of 50 years or more. The wet form leads to severe loss of central vision. Recent evidence supports that adult hematopoietic stem cells (HSCs) contribute to preretal neovascularization. In the current study, it was determined whether HSCs, by producing both blood and blood vessels, provide functional endothelial activity during choroidal neovascularization (CNV) in mice.

METHODS. GFP chimeric mice were developed by bone marrow ablation of C57BL/6J mice and reconstitution with donor tissue from GFP/+/ transgenic mice. GFP chimeric mice underwent laser rupture of Bruch’s membrane and were killed and eyes enucleated at 1, 2, 3, and 4 weeks after laser injury. CNV was examined by confocal microscopy of retinal flatmounts. Because endothelial progenitor cells (EPCs) derive from HSCs, immunocytochemistry was used to quantify relative the EPC contribution to CNV.

RESULTS. Laser injury alone was sufficient to induce stem cell recruitment and subsequent CNV. GFP+ cells formed part of the functional vasculature in the choroid as early as 1 week after injury and were present for the duration of the study. The relative EPC contribution to CNV remained fairly constant throughout the study and constituted almost 50% of the total vasculature.

CONCLUSIONS. Adult stem cells are recruited to the choroid in a model of CNV, where they contribute to forming aberrant new vessels. This observation suggests that targeting stem cell recruitment to the eye may offer a novel therapeutic strategy for ARMD. (Invest Ophthalmol Vis Sci. 2003;44:4908–4913) DOI: 10.1167/iovs.03-0342

The primary cause of blindness in persons 50 or more years of age in developed nations is age-related macular degeneration (ARMD). It will affect an increasing number of people in the coming decades as our population ages.1 Currently, the disease affects approximately 28% of people over the age of 75.1,2 There are approximately 1051 new cases of ARMD per year.1,2 The wet form leads to severe loss of central vision. When the macula sustains damage, central vision is limited. There are two forms of ARMD: nonexudative (dry) and exudative (wet). The exudative form accounts for approximately 90% of the vision loss associated with ARMD.3 The exudative form is characterized by choroidal neovascularization (CNV) that often leads to hemorrhage, retinal pigment epithelial (RPE) detachment, scarring, and occasional RPE tears.1,5 The early visual distortion that is associated with ARMD is caused by leakage or hemorrhage from the choroidal neovascular membrane under the RPE cell layer.1 The main reason for the vision loss associated with ARMD is from the scarring associated with the new vessels.6

Current therapies for ARMD are laser photocoagulation and photodynamic therapy with verteporfin.1,3 Some problems associated with these therapies include endothelial cell damage, photoreceptor damage, and vascular occlusion in the eye.7 Most CNV membranes are untreatable because subretinal fluid, blood, and lipids obscure the view. Early diagnosis and treatment are necessary to delay progression of the disease. Not all patients are eligible for laser photocoagulation treatment. The effectiveness of laser therapy depends on the initial visual acuity and lesion size in patients with CNV, and most patients are not treatable. Furthermore, the treatment itself may damage the retina and result in immediate vision loss.8–12 There is also a high recurrence rate of CNV after laser photocoagulation and photodynamic therapy.1 Because of these problems, treatments for this disease have not been very successful.

Adult bone marrow–derived hematopoietic stem cells (HSCs) are partially defined by the ability to transdifferentiate and form multiple types of tissue from one cell. Recent work by Asahara et al.13 demonstrated the presence of circulating endothelial precursor cells (EPCs) derived from HSCs. This finding suggests that new blood vessel formation involves recruitment of undifferentiated cells from the circulation.

We have shown that recruitment and subsequent differentiation of EPCs to sites of mechanical injury in the retina contributes to retinal neovascularization in a murine model.14 In the current study, we examined whether HSCs contribute to CNV, in a murine model of laser rupture of Bruch’s membrane. If so, this would further support the capacity of the HSCs to transdifferentiate and heighten their importance as a possible therapeutic target in managing vascular retinopathies. Alternatively, the realization that these cells are recruited to specific sites of ocular neovascularization suggests their potential role as vehicles for targeted gene delivery.

METHODS

Animals

All animals were treated in accordance with the principles described in The Guiding Principles in the Care and Use of Animals (NIH), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and housed in our institutional animal care facilities.
Transgenic mice homozygous for the green fluorescent protein (gfp) were obtained from a breeding colony established in the institutional animal care facilities.

**Transplantation Procedure**

To generate the C57BL/6j.gfp chimeric mice, femoral bone marrow was harvested from gfp transgenic (homozygous) mice. From the total bone marrow cell population, B and T cells, granulocytes, and erythrocytes were depleted with magnetic beads coated with antibodies against B220, CD3, Gr-1, and Ter119 (all from BD Biosciences, San Jose, CA). This left only the Lin− (lineage negative) cells. This cell population was then enriched for hemangioblasts by using magnetic beads coated with antibody against Sca-1 (Sca-1 is a standard marker for HSCs). The purity of this remaining cell population was then assessed by flow cytometry and typically resulted in 95% hemangioblasts that were then injected into lethally irradiated adult C57BL/6j mice in the retro-orbital sinus. After 1 month, successful engraftment in these animals was confirmed by flow cytometry analysis of their blood.

**Blood Characterization**

Blood was obtained from C57BL/6j mice, gfp homozygous mice, and C57BL/6j.gfp chimeric mice by tail venipuncture. Leukocytes were isolated by single-density gradient (Ficoll; Amersham Pharmacia Biotech, Piscataway, NJ) centrifugation, and then reacted with antibody to the endothelial cell marker CD31 (Dako, Glostrup, Denmark), followed by rhodamine-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO). After they were washed, the cells were analyzed for two-color fluorescence (FACSCalibur; BD Biosciences). The percentage of cells both expressing gfp and reacting with anti-CD31 was analyzed from the dot plots by setting appropriate gates in the cytometry-analysis software (WinMDI FACS; Scripps Institute, La Jolla, CA).

**Qualitative Evaluation**

In the first experiment, the C57BL/6j.gfp chimeric mice were treated with laser photocoagulation in a manner similar to that described by Ryan. A variable indirect laser ophthalmoscope with a 78-D lens (argon green wavelength, power of 910–1030 mW for 0.05 second) was adjusted to yield a small spot size focused in the RPE level. Three burns were produced in three quadrants of the choroid. One disc area in diameter and one disc area from the optic nerve. This fixed distance allowed the burns to be both reproducible and isolated from each other. The laser produced a bubble in most of the cases, which is indicative of a Bruch’s membrane rupture. In less than 5% of the animals, bleeding was noted after treatment. The mice were killed at 1, 2, 3, and 4 weeks after receiving laser photocoagulation (n = 4 per time point). At the time of euthanasia, the mice were perfused with rhodamine-labeled dextran to visualize the vasculature. The eyes were enucleated and incubated in 4% formaldehyde and then in PBS. The neural retina was dissected from the RPE-choroid-sclera complex. Both parts were flat-mounted with four to seven radial cuts and were examined and photographed separately using confocal microscopy (MRC-1024 Confocal Laser Scanning System; Bio-Rad, Hercules, CA).

**Immunocytochemistry**

Eight adult C57BL/6j.gfp mice and four non-transplant recipients were also treated to the same laser photocoagulation methods as in the first experiment. The mice were killed at 1, 2, 3, and 4 weeks after receiving laser photocoagulation (n = 2 transplant recipients—i.e., six lesions, at each time point; n = 1 non-transplant recipient, i.e., three lesions, at each time point). Whole retinal flatmounts were permeabilized in 0.2% Triton X-100 in Tris-buffered saline for 24 hours at room temperature. Retinas were then washed into HEPES-buffered saline containing 1:500 monoclonal anti-gfp antibody (Chemicon, Temecula, CA) and 1:1000 rhodamine-conjugated Ritcinus communis agglutinin (Vector Laboratories, Burlington, CA) for another 24 hours at room temperature. The retinas were then exposed to 1:50 FITC-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) in TBS for 24 hours at room temperature. After each incubation step, the retinas were washed in TBS for 24 hours at room temperature.

**Quantitative Evaluation**

The retinas used for immunocytochemistry were then evaluated for quantitative changes in fluorescence, by a modification of a method previously described by Mori et al. Each retina was imaged with an RGB CCD camera (Spot; Diagnostic Instruments, Sterling Heights, MI) coupled to a fluorescence microscope (Axioplan 2; Carl Zeiss Meditec, Thornwood, NY) with filters for FITC and rhodamine as well as bright-field microscopy. Retinas in which three distinct burns were not visible were rejected from further analysis. For weeks 1 to 3, six burns qualified for evaluation at each week; for week 4, three lesions qualified. Statistical software (MCID Basic; Imaging Research, Inc., St. Catharines, Ontario, Canada) was then used for mathematical quantification of the levels of green fluorescence (gfp), red fluorescence (vasculature), and colocalization of green and red fluorescence within each retinal lesion. Colocalized fluorescence intensity is thus a measure of donor-derived cells that have fully differentiated and incorporated into the vascular lesion. The percent contribution of donor-derived gfp-expressing EPCs to the vasculature was calculated by dividing the colocalized fluorescence intensity by the total red intensity. These values were averaged for the burns for the duration of the study.

**Results**

Figure 1 is a schematic depicting the number of animals used, the segregation into treatment groups, and a flow chart of the temporal evaluation.

**Blood Characterization**

Figure 2 shows representative dot plots from a flow cytometry analysis of blood obtained from a C57BL/6j mouse (Fig. 2A), a gfp+/− mouse (Fig. 2B), and a chimeric C57BL/6j.gfp mouse (Fig. 2C). Figure 2D tabulates the mean number of circulating cells identified as EPCs by gfp and CD31 coexpression. In the control C57BL/6j mouse, a very small percentage of cells (5.7%) appeared to fluoresce in the green channel and also reacted with anti-CD31. The percentage of gfp+ circulating EPCs in the chimeric animals is essentially identical with that seen in gfp homozygous animals (37.1% vs. 39.2%).

**Qualitative Evaluation**

When the flat-mounted eyes were examined with confocal microscopy, there were obvious burns in the expected locations in the laser-treated eyes. Gfp+ cells were not seen in either the untreated eyes or in the neural retinas of the treated eyes (Figs. 3A–C). When the posterior cups of the treated eyes were examined, the burn areas had numerous green cells at sites of Bruch’s membrane rupture (Fig. 3D). At higher magnification of the posterior cups, there was evidence of gfp+ cell incorporation into choroidal vasculature (Figs. 3E, 3F).

**Immunocytochemistry and Quantitative Evaluation**

Figure 4 shows the red (Fig. 4A), green (Fig. 4B), and colocalized (Fig. 4C) fluorescence in the three lesions of a posterior cup at 2 weeks after treatment. The bright fluorescence between the radial incisions of the flatmount results from non-specific autofluorescence of the suspensory ligaments. There was no evidence of anti-gfp antibody binding in the lesions in retinas that did not receive a transplant. Figure 4D is a plot of the means and standard deviations of the percent contribution of donor-derived gfp-expressing EPCs to the vasculature (as
determined by the calculation described in the Methods section. The relative contribution of the EPCs to the total vascular lesion remained constant at a level of between 40% and 45% throughout the course of study. Two-sample F-tests for variances were performed between each of the four time points. All time points differed significantly ($P < 0.05$) except for weeks 2 and 3.

**DISCUSSION**

The development of more effective treatments for ARMD requires a more thorough understanding of the physiological processes involved in the neovascular response, possibly including choroidal endothelial cell senescence. The primary stimulus for progression of ARMD is unknown but may occur from the accumulation of abnormal extracellular matrix (ECM), which then leads to thickening of Bruch's membrane. Other causes may include the changed metabolism of the ECM or altered ECM of RPE cells which in turn cause increased secretion of angiogenic growth factors that could lead to the growth of CNV. The laser injury induced in the murine eye, although not mimicking ECM changes, may very well result in the release of angiogenic growth factors, which would be expected to lead to neovascularization. This in fact is what was observed with this model, because laser injury alone was sufficient to induce measurable CNV without the administration of exogenous growth factors.

The term hemangioblast is used to describe a primitive cell that can form endothelium as well as blood cells. Hemangioblasts have been shown to have hemangioblast activity. Stem cells can differentiate into an unlimited source of progenitors for treatment of degenerative retinal disorders. EPC may play a role in angiogenesis and can be used to deliver pro- or antiangiogenic agents to blood vessels. Conversely, in NV diseases such as:

**FIGURE 1.** A schematic and flow chart shows the number and type of animals for each phase of the study. Normal (C57BL/6J) mice were used as controls for the immunocytochemical analysis. Eyes from reconstituted chimeric (C57BL/6J.gfp) animals were used for qualitative assessment of CNV by confocal microscopy, as well as for immunocytochemistry. Blood from both these types of animals, as well as from homozygous gfp transgenic animals, was analyzed by flow cytometry to determine the number of circulating endothelial precursor cells.

**FIGURE 2.** Flow cytometry analysis of mouse blood revealed the percentage of gfp$^+$ (i.e., bone-marrow-derived) circulating EPCs. These cells may be recruited to sites of ischemic injury and participate in neovascularization. (A–C) Dot plots showing the number of EPCs (with increasing tetramethylrhodamine isothiocyanate [TRITC] labeling) that also expressed gfp (with increasing gfp detection). (D) Percentage of circulating EPCs that also expressed gfp in each mouse type. The small percentage of putative gfp expressers in the normal mouse was due to signal noise.
E-selectin mediates SDF-1–induced migration of cells. Vascular endothelial growth factor (VEGF) induces E-selectin expression by cells in culture, suggesting that VEGF may mediate SDF-1 activity. Thus, it is highly likely that VEGF release, which is associated with compensatory neovascularization in response to ischemia, can ultimately result in the recruitment of circulating EPCs to the choroid.

When the retina is damaged, any hemorrhaging may increase the inflammatory response. Also, the process of healing that is elicited when the injury is created may cause a release of activating factors from macrophages. Future studies may involve studying the homing signals, such as vascular cell adhesion molecule (VCAM)-1 or intercellular adhesion molecule (ICAM)-1, which cause recruitment of the stem cells to areas of injury. The realization that EPCs in the circulation have the potential to participate in ocular NV suggests a possible target for developing alternative therapies to treat ARMD. The chimeric C57BL/6j.gfp mouse provides an ideal model system for examining the potential role of bone marrow-derived stem cells in CNV. Using this model system, we showed the recruitment and functional differentiation of donor-derived HSCs to sites of ischemic injury in the choroid.

Our finding confirmed the expectation that the percentage of cells both gfp− and reacting with anti-CD31 (thereby implying gfp− circulating EPCs) would be similar in the transplant-recipient animals and the gfp−/+ animals. These results also confirmed durable reconstitution of the transplant recipients before laser treatment. In addition, it was anticipated that the percentage of gfp+ circulating EPCs in the non-transplant-recipient C57BL/6j mouse would be zero. However, we noted a very small percentage of cells that appeared to express gfp and reacted with anti-CD31 (Fig. 2D). This can be attributed to signal noise.

When the flatmounted eyes were examined by confocal microscopy, there were numerous green cells in the expected locations in the posterior cups of the laser-treated eyes (Fig. 3D), but not in the untreated eyes or in the neural retinas of the laser-treated eyes (Figs. 3A–C). This indicates that gfp+, bone marrow–derived cells are recruited to sites of Bruch’s membrane rupture. At higher magnifications, there is evidence of gfp+ cell incorporation into vasculature (Figs. 3E, 3F), confirming that precursor cells are indeed involved in forming choroidal neovascular tissue. In a previous study, we confirmed by immunocytochemistry, that the donor gfp+ stem cells differentiate into endothelium and form functional preretinal vasculature in response to laser-induced branch retinal vein occlusion. Thus, it would not be unreasonable to postulate that a similar process occurs in the choroid in response to the rupture of Bruch’s membrane.

From Figures 4A–C, it becomes clear that the rhodamine-conjugated agglutinin activity is highest in the vasculature within the lesions. The gfp expression is confined to the lesions and colocalizes with the rhodamine-conjugated agglutinin. This indicates that donor-derived precursor cells are being recruited specifically to the site of laser injury. Colocalization implies differentiation and incorporation of these cells into NV. The data presented in Figure 4D support the high contribution of donor cells to CNV, at a level almost equivalent to that of resident cells. The contribution of EPCs to the total vascular lesion remains between 40% and 45% throughout the course of this 4-week study; implying that the resident cells were responsible for the remainder of the NV, approximately 55% to 60%. It is also important to note that even in the gfp−/+ donor animals, significantly less than 100% of the cells express gfp (as seen in Fig. 2B), perhaps due to cell-cycle–dependent expression of gfp. Therefore, we may conclude that our results represent an underestimation of the potential contribution of stem cells to NV.
It has been thought that in wet ARMD, the resident endothelial cells were responsible for the aberrant neovascularization process. In addition to EPC involvement in preretinal neovascularization, \(^1\text{4}\) we now show that EPCs may also be recruited to additional sites in the eye where they play a vital role in angiogenesis. Whereas this work was performed in a murine model, the original description for rupture of Bruch’s membrane was performed with primates. \(^1\text{5}\) Species-dependent response to injury may warrant additional investigation to support the importance of EPC recruitment. Regardless of the potential species differences and their varied physiological responses, the realization that resident endothelial cell proliferation is but one component of ocular neovascularization is an important step in devising new treatment modalities. One avenue for future ARMD treatments may involve targeting cells from the bone marrow, thus inhibiting disease progression at an earlier stage.

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References


