Preventing Stem Cell Incorporation into Choroidal Neovascularization by Targeting Homing and Attachment Factors

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PURPOSE. The primary cause of vision loss in people more than 50 years of age is age-related macular degeneration (ARMD). The wet form of ARMD is characterized by choroidal neovascularization (CNV). A prior study has shown that adult hematopoietic stem cells (HSCs) contribute to approximately 50% of newly formed vasculature in CNV. Stromal-derived factor (SDF)-1 is involved with homing of HSCs from bone marrow to target tissue. Vascular endothelial cadherin (VE-cadherin, or CD144) is involved in endothelial cell adhesion. Preventing homing and/or adhesion of progenitor cells to damaged choroid could reduce CNV.

METHODS. Adult C57BL/6j mice were lethally irradiated, and then received a transplant of purified c-kit+/Sca-1+ HSCs from the bone marrow of green fluorescent protein (gfp) homozygous donor mice. Bruch’s membrane rupture by laser photocoagulation was used to induce CNV. Animals were injected subretinally with anti-SDF-1, anti-CD144, or control, before or after laser photocoagulation. The eyes were enucleated, and the neural retinas were separated from the RPE/choroid/sclera complex. All tissues were flatmounted and qualitatively and quantitatively assessed by fluorescence microscopy.

RESULTS. CNV lesions from eyes treated with anti-CD144 showed significantly less incorporation of gfp+ cells compared with those treated with anti-SDF-1. Antibody treatment generally reduced the degree of gfp+ stem cell recruitment and incorporation into the CNV lesions, compared with the control. Treatment with either antibody also significantly reduced the size of the CNV lesions.

CONCLUSIONS. These results indicate that homing and adhesion of progenitor cells to CNV may be targeted differentially or in combination to prevent CNV. (Invest Ophthalmol Vis Sci. 2005;46:343–348) DOI:10.1167/iovs.04-0153

In the Western world, age-related macular degeneration (ARMD) is the primary cause of vision loss in people more than 50 years of age, affecting 28% of people older than 75 years.1–5 Of the two forms of ARMD, the dry form is by far the most common, accounting for nearly 90% of reported cases. However, 90% of all vision loss occurring from ARMD results from the wet, or exudative, form, which is characterized by choroidal neovascularization (CNV).4

A putative role for endothelial precursor cell (EPC) involvement in postnatal vasculogenesis was postulated as early as 1997.6 Further work in this area confirmed the bone marrow origin of these circulating EPCs.6,7 The importance of EPC recruitment and participation in pathophysiological angiogenesis has been shown in myocardial ischemia8 and infarction,9 as well as tumor infiltration10 and ocular neovascularization.11–13

The mechanisms involved in homing, recruitment, and migration of cells to areas of NV remain to be fully elucidated. Factors such as chemokines are thought to be involved in this process. One such factor is stromal-derived factor (SDF)-1, which has been shown to be involved with stem cells’ homing and recruitment from the bone marrow to target tissue.14–16 and especially recruitment of EPCs.17–19 Yamaguchi et al.20 have recently shown that local SDF-1 increases vasculogenesis by increasing EPC recruitment in damaged tissue.

In addition to homing and recruitment of EPCs, the mechanisms underlying their ultimate incorporation into NV involve attachment. Vascular-endothelial cadherin (VE-cadherin, CD144) is expressed on the surface of endothelial cells21 and has been shown to be involved with endothelial migration and adhesion.22–24 An antibody against CD144 was effective at abrogating formation of endothelial tubes in an in vitro assay.25

In a previous report, we showed in a mouse model that EPCs derived from adult bone marrow participate in CNV resulting from the rupture of Bruch’s membrane,11 and our observation was confirmed independently.13 Inhibiting the migration of EPCs and/or blocking the attachment of such cells could prevent aberrant blood vessels from forming in the choroid. We postulated that blockade of homing and/or adhesion factors such as SDF-1 and CD144 could decrease the contribution of EPC to CNV. We tested this hypothesis by expanding on our established mouse model of ruptured Bruch’s membrane by administering exogenous agents to reduce the extent of CNV.

METHODS

Animals

All animals were treated in accordance with The Guiding Principles in the Care and Use of Animals (DHSAW Publication, NIH 80-23), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6j mice (wild type, WT) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in the institutional animal care facilities. Transgenic mice homozygous for green fluorescent protein (gfp) were obtained from a breeding colony established in the institutional animal care facilities. There were two groups of experimental animals, the first consisting of chimeric mice (as described later) and the other with WT mice (Table 1).
Table 1. Number of Animals Allocated to Each Treatment Group for the Two Quantitative Evaluations

<table>
<thead>
<tr>
<th>Experiment 1: EPC Contribution to CNV</th>
<th>Chimeric Mice</th>
<th>D − 7</th>
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<th>D + 1</th>
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<td>Anti-CD144</td>
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<table>
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<th>D − 1</th>
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Transplantation Procedure

To generate the C57BL/6 gfp chimeric mice for the first experiment, femoral and tibial bone marrow was harvested from gfp + transgenic (homozygous) mice. Antibodies to c-kit (CD117) and Sca-1 (both from BD PharMingen, San Diego, CA) were used to deplete the total bone marrow population of differentiated cells and to separate the stem cells, using flow cytometry (FACStar Plus; BD Immunocytometry Systems, San Jose, CA). The purity of this remaining cell population was then assessed by flow cytometry and typically resulted in a 95% hemangioblast cell population. These cells were then injected into lethally irradiated adult C57BL/6 mice in the retro-orbital sinus. After at least 1 month, successful engraftment into these animals was confirmed by flow cytometry analysis of their blood. The blood characterization was performed as previously described.11

Evaluation of EPC Contribution to CNV

Four groups of animals were used for this experiment, all consisting of C57BL/6 gfp chimeric mice. All four groups were treated with laser photocoagulation in a manner similar to that used by Ryan26 and previously described,11 in which each injured eye received three laser burns to rupture Bruch’s membrane. Only burns in which a bubble was produced, indicating rupture of Bruch’s membrane, were included in the study. Of these four groups, the first group was not injected subretinally at any time. The second, third, and fourth groups were injected with 1 μL PBS, SDF-1 antibody (0.5 mg/mL; R&D Systems, Minneapolis, MN), or CD144 antibody (0.5 mg/mL; BD PharMingen) subretinally at three time points in relation to laser injury: 7 days before, 1 day before, and 1 day after laser injury (Table 1). In all four groups of animals, only one eye was injured (and injected, if applicable). All animals were euthanatized 3 weeks after laser injury. At the time of euthanasia, the mice were perfused with 4% paraformaldehyde. The eyes were enucleated and incubated in 4% paraformaldehyde for 30 minutes and then in PBS for at least 50 minutes. The neural retina was dissected from the posterior cup (retinal pigment epithelium [RPE])/choroid/sclera complex. Both the neural retinas and posterior cups were permeabilized and then reacted with rhodamine-conjugated agglutinin (Vector Laboratories, Burlingame, CA) and anti-gfp (Chemicon, Temecula, CA) as previously described.11 Both the neural retinas and the posterior cups were flattened with four to seven radial cuts.27 The tissue was then examined quantitatively by fluorescence microscopy, as previously described.11

Quantitative Evaluation of Lesion Vascular Area

To assess the vascular area of the lesions, four experimental groups of animals were used in which all were C57BL/6 gfp mice. All four groups were injured with laser photocoagulation in one eye as was previously described. There were uninjected animals, as well as animals that were injected subretinally with 1 μL of an irrelevant antibody (0.5 mg/mL mouse IgG, Sigma-Aldrich, St. Louis, MO), SDF-1 antibody (0.5 mg/mL), or CD144 antibody (0.5 mg/mL) at the same three time points in relation to laser injury, as described in the previous section (Table 1). All animals were euthanatized 3 weeks after laser injury. At the time of euthanasia, the mice were perfused and eyes were fixed, dissected, and reacted with rhodamine-conjugated agglutinin, as described earlier. Flatmounted posterior cups were imaged with an epifluorescence microscope (Axioplan 2; Carl Zeiss Meditec, Oberkochen, Germany) connected to a charge-coupled device (CCD), high-resolution camera (RGB Spot; Diagnostic Imaging, Sterling Heights, MI).

Captured digital images were evaluated with ImageJ software (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Images were split into separate RGB channels for analysis of the red channel as follows: (1) A calibration for the specific objective and microscope was applied to set the pixel-to-length ratio; (2) a threshold was applied using the Otsu algorithm; (3) images were made binary; (4) a region-of-interest (ROI) was outlined to include the entire lesion area; and (5) a particle analysis was performed to quantify the pixel area above the threshold level within the ROI.

Statistical Analysis

To quantify the EPC contribution to CNV, first, relative fluorescence units (RFU) for green (gfp + cells), red (vascular cells), and yellow (colocalized) fluorescence were determined.11 Second, the percentage of gfp-expressing vascular cells within the total vascular lesion was calculated (colocalized RFU divided by vascular RFU). Data for lesions from individual animals were averaged. The resultant values were then analyzed (SPSS software; SPSS, Chicago, IL) by t-test for independent-sample means, assuming unequal variance among treatment groups, and reported as the mean ± SEM.

Similarly, the vascular area of all lesions within an animal were averaged, and those resultant values were analyzed on computer, as described earlier (SPSS) and reported as the mean lesion area ± SEM for each treatment group.

In both analyses, P < 0.05 was considered significant when comparing a treatment group to the uninjected control animals. All animals that displayed a CNV lesion were included in the analyses. Table 1 describes the number of animals allotted to each treatment group.

Results

Laser burns from uninjected eyes were used as a baseline for comparison to the various treatments. Figure 1 shows a hematoxylin and eosin-stained, 10-µm section of a lesion between the posterior cup and the neural retina.

Figure 2 shows representative fluorescence micrographs of a posterior cup from an untreated (no laser, no injection) eye (Fig. 2A) and a neural retina from a uninjected eye that received laser rupture of Bruch’s membrane (Fig. 2B), showing a diffuse infiltration of gfp + cells at sites corresponding to the positions of the laser burns. The posterior cup of the same eye whose neural retina is shown in Figure 2B is depicted in Figure 2C, and clearly demonstrates colocalization of CNV with gfp + cells. Figure 2D is the green channel of the image depicted in Figure 2C, and shows the computer-generated outline of the laser burns used in the quantitative analysis of fluorescence intensity.

Figure 3 depicts representative fluorescence micrographs showing qualitative reductions in gfp + -cell involvement in laser-induced CNV. Figure 3A shows the posterior cup from an eye injected subretinally with PBS. Figures 3B and 3C show posterior cups from eyes injected subretinally with anti-SDF-1 and anti-CD144, respectively. All eyes depicted were from animals injected 1 day before laser injury. Note the considerable decrease in gfp + cells within the lesions in Figures 3B

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(anti-SDF-1) and 3C (anti-CD144) compared with Figure 3A (PBS) and Figure 2C (no injection).

Figure 4 shows the effect of antibody injection on the incorporation of gfp\(^+\) cells (Fig. 4A), total vascular staining (Fig. 4B), and the percentage of gfp\(^+\) vascular cells (Fig. 4C) within each lesion. Figure 4D shows the effect of the antibodies on lesion area, as measured by rhodamine-agglutinin staining. In all four panels of Figure 4, the uninjected eyes were statistically identical to the eyes that were injected with PBS or IgG as a control.

As depicted in Figure 4A, both antibody treatments were effective at decreasing the total bone marrow-derived cells in the lesion (shown by decreased green fluorescence) when injected before the laser injury. There was no significant difference between the two treatments at these two time points. When injected 1 day after the laser injury, only anti-CD144 was effective at reducing the number of gfp\(^+\) cells within the lesion.

When the total vascular staining of the lesions were measured, both treatments were effective at all three time points tested (Fig. 4B). When injected 7 days before laser injury, anti-SDF-1 was statistically more effective than anti-CD144 at reducing the vascularity within the lesions.

Figure 4C shows the percent contribution of the gfp\(^+\) vascular cells (those that showed colocalized red and green fluorescence) to the total vascular lesion. The results were
similar to those shown in Figure 4A, which suggests that most of the gfp\(^+\) cells in the CNV lesion at the time examined were EPC derived. Both antibodies were moderately effective at decreasing EPC incorporation when injected 7 days before laser injury. The most dramatic inhibitory effect was seen when either antibody was injected 1 day before laser injury. When injected 1 day after laser injury, anti-CD144 inhibited the incorporation of gfp\(^+\) cells, but anti-SDF-1 did not.

Finally, the data represented in Figure 4D show that both antibodies were equally effective at reducing the total vascular area of CNV lesions, by as much as two thirds of the area seen in uninjected controls. Injection of an irrelevant antibody did not significantly affect lesion size, compared with uninjected animals.

**DISCUSSION**

SDF-1 is a member of the CXC chemokine subfamily that was initially identified from a signal sequence trap cloning strategy\(^{28}\) as a bone marrow stromal cell–derived chemottractant for hematopoietic progenitor (CD34\(^+\)) cells.\(^{29}\) A role for SDF-1 in blood vessel formation has been clearly established from analysis of mice whose genes for SDF-1 and/or its receptor CXCR4 were disrupted.\(^{30}\) Mice deficient in either of these genes display defective vasculature formation—most notably, an absence of large vessels within the developing gastrointestinal tract. CXCR4 expression has been identified on endothelial cells of the developing brain and heart as well as on EPCs. These latter cells migrate in response to SDF-1.\(^{31}\) In an in vivo animal model of ischemic neovascularization SDF-1 augmented EPC recruitment.\(^{31}\) Increased homing of CD34\(^+\) progenitor cells to the liver after a stressful insult is also dependent on SDF-1.\(^{32}\)

In addition to chemokines, leukocyte migration is also coordinated by the presence of junctions.\(^{33}\) There are three main types of junctions between endothelial cells: tight junctions, gap junctions, and adherens junctions.\(^{34}\) Adherens-type junctions are formed by Ca\(^{2+}\)-dependent transmembrane adhesion proteins belonging to a class of molecules including cadherins.\(^{35}\) There are several members in the cadherin superfamily; VE-cadherin (CD144) is found only at endothelial cell junctions.\(^{34,36}\) CD144 has been shown to be involved in cell differentiation, growth, and migration.\(^{37}\) In a knockout study, CD144\(^-/\) mice died before embryonic day 10. The causes of death were shown to be vascular insufficiency, endothelial disconnection, and impairment of remodeling of the primitive vascular network into a mature vascular network.\(^{38}\) CD144 is a maturation marker exclusively expressed by endothelial cells and endothelial progenitors (CD34\(^+\) cells).\(^{39}\) Circulating endothelial precursors mobilized in response to vascular trauma were positive for CD34 and CD133. These cells were also positive for CD144.\(^{40}\) Gaps in CD144 offer one method for leukocyte trafficking through vessels.\(^{34}\)

Thus, a potential strategy for limiting the involvement of EPCs in pathologic neovascularization may rely on interfering with both homing (through SDF-1) and attachment and remodeling (through CD144). We have shown previously that adult bone marrow–derived stem cells contribute significantly to areas of neovascularization in the damaged choroid. In this study, we demonstrated that blockade of either homing signals (through antibody to SDF-1) or endothelial-specific attachment factors (through antibody to CD144) can successfully reduce the contribution of these stem cells to the neovascular lesion. Treatment with either of these antibodies proved efficient at reducing the degree of EPC involvement in the CNV lesion, as well as in decreasing the vascular area of the lesion. It is conceivable that the pro-migratory signal of SDF-1 is quenched by virtue of the antibody preventing SDF-1’s ability to interact with CXCR4 on EPC. Interfering with attachment of EPCs using antibody to CD144 was more effective than interfering with homing and recruitment of these cells with an antibody to SDF-1. Antibody binding to CD144 on the surface of EPC may prevent the proper formation of the adherens junctions necessary for endothelial cell migration, thus limiting EPC translocation.
It remains to be seen why adherence (via CD144) may be more important than homing (via SDF-1) in relation to EPC involvement in NV. Possible mechanisms involving CD144 include interference with downstream signaling or vascular remodeling. These functions may be more vital to the neovascular process than recruitment of progenitor cells. The timing of administration of either treatment was also critical to the efficacy of treatment. Exposure to either of the antibodies 1 day before inducing the lesion was more effective than exposure 7 days before or 1 day after injury to Bruch’s membrane. Administration of the SDF-1 antibody or the CD144 antibody was equally effective at reducing the CNV lesion area at all three time points tested.

These findings provide a mechanism for EPC recruitment and incorporation into areas of CNV and suggest potential therapies for the mitigation of damage caused by aberrant angiogenesis. In future studies, we will determine the effectiveness with dose–response measurements as well as the duration of presence and or effectiveness of antibodies injected into the subretinal matrix.

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References


