Macrophage Depletion Diminishes Lesion Size and Severity in Experimental Choroidal Neovascularization

Diego G. Espinosa-Heidmann,1 Ivan J. Suner,1 Eleut P. Hernandez,1 Dagoberto Monroy,1 Karl G. Csaky,2 and Scott W. Cousins1

PURPOSE. Macrophage recruitment to the choroid has been proposed to contribute to the pathogenesis of choroidal neovascularization (CNV) in AMD. The study was conducted to determine whether treatment with clodronate liposomes (CL2MDP-lip), which cause depletion of blood monocytes and lymph node macrophages, diminishes the severity of neovascularization in a mouse model of laser-induced CNV.

METHODS. Laser-induced CNV was performed in female 16-month-old C57BL/6 mice. Macrophages were depleted by use of CL2MDP-lip intraperitoneally and subcutaneously 72 and 24 hours before and every 2 to 3 days after laser injury. Control mice received injections of either PBS alone or PBS liposomes. Blood monocyte and choroidal macrophage depletion were documented by flow cytometry and choroidal flatmount preparation analysis, respectively. Two weeks after laser injury, mice were injected intravenously with fluoresceinated dextran. The right eyes were removed and prepared for flatmount analysis of CNV surface area (in relative disc areas or DA), vascularity (relative fluorescence), and cellularity (propidium iodide stain). The mice were then perfused with 10% formaldehyde, and the left eyes were removed for histopathology. The means of the various parameters for four CNV lesions per eye were calculated. Fluorescein angiography was also performed.

RESULTS. Flow cytometry of circulating monocytes and immunohistochemical analysis of choroidal macrophage density confirmed the effective depletion of blood monocytes and choroidal macrophages respectively in CL2MDP-lip–treated mice. Compared with the control, flatmount analysis of macrophage depleted mice demonstrated a significant reduction in size of the CNV area (2.8 ± 0.5 DA vs. 1.1 ± 0.1 DA; P < 0.043). The treated group also revealed less vascularity (1.6 ± 0.1 units vs. 1.1 ± 0.0 units; P < 0.0092) and cellularity of CNV lesions (3.3 ± 0.6 DA vs. 1.7 ± 0.1 DA, P < 0.04). Histopathology revealed that, in the macrophage-depleted group, CNV was smaller in diameter (1270 ± 73 pixels vs. 770 ± 82 pixels, P < 0.0006) and thickness (120 ± 7 pixels vs. 96 ± 7 pixels, P < 0.019).

CONCLUSIONS. Macrophage depletion using CL2MDP-lip reduces size, cellularity, and vascularity of CNV. This observation supports the hypothesis that macrophages contribute to the severity of CNV lesions. (Invest Ophtalmol Vis Sci. 2003;44: 3586–3592) DOI:10.1167/iovs.03-0038

Choroidal neovascularization (CNV) is the major vision-threatening complication associated with several common retinal degenerative or inflammatory diseases, especially age-related macular degeneration (AMD).1–3 The pathogenesis of neovascular AMD is clearly multifactorial, with age, systemic health, genetic, and environmental risk factors playing roles in onset and progression.4–8 Recently, however, inflammatory mechanisms and immune activation have been hypothesized to play a role in the pathogenesis of this disease.9 Several studies have identified the presence of macrophages within CNV,10–12 but no experimental confirmation has been provided to confirm the pathogenic involvement of these cells to formation or severity of CNV.

Selective depletion of macrophages in vivo can be achieved with dichloromethylene diphosphonate-liposomes (CL2MDP-lip).13 The liposomes are ingested by the macrophages, which are then destroyed after phospholipase-mediated disruption of the liposome and intracellular release of CL2MDP. The exact mechanism of macrophage depletion by intracellular accumulation of CL2MDP-lip is unknown, but it is believed that the intralysosomal accumulation of CL2MDP generates signals to induce macrophage apoptosis.14 The purpose of this study was to determine whether macrophage depletion by CL2MDP diminishes the severity of experimental CNV. Our results indicate that macrophage depletion in aged-mice decreased the severity of CNV, with the decrease defined as smaller vascular and cellular surface area and less vascularity, in an experimental model of laser CNV.

MATERIALS AND METHODS

Mice

Mice used in this study were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-four female, normal C57BL/6 mice aged 16 months (n = 22, weight, 25–30 g), at the onset of the study, were purchased from the National Institute on Aging (Bethesda, MD). Also, seven BALB/c retired breeders were used for the immunostaining protocol, because uveal tract wholemount preparations are more informative when applied to albino animals in which uveal tissues are sufficiently thin and transparent to allow transillumination and resolution of individual stained cells.

Preparation of CL2MDP-lip

CL2MDP-lip was prepared by dissolving 1,2 dioleolyl-sn-glycero-3-phosphocholine and cholesterol (both from Avanti Polar Lipids, Alabaster, AL) in a mixture of methanol and chloroform (1:1). After low-vacuum rotary evaporation at 37°C to remove the organic phase, the lipids were mixed with clodronic acid (1.9 g) dissolved in phosphate-buffered saline (0.6 M). They were allowed to sit at room temperature for 2 hours in a nitrogen-purged chamber to induce liposome
swelling. Resuspension of liposomes was achieved by water sonication at room temperature for 5 minutes. The resultant liposomes were washed at 10,000 × g in ultracentrifuge twice for 30 minutes at 16°C. The milky liposomes were removed gently with a pipette, resuspended in 4 mL of sterilized PBS, and stored in a nitrogen chamber for up to 1 week. For the PBS-liposomes the lipids were mixed with sterilized PBS.

Macrophage Depletion

Mice were anesthetized with an intramuscular administration of ketamine hydrochloride (42.8 mg/kg), xylazine (8.5 mg/kg), and acepromazine (1.4 mg/kg). Splenic and systemic macrophage depletions (with PBS or PBS-liposomes) were performed with CL_2-MDP-lip (200 µL = 1 mg) by intraperitoneal (IP) administration 4 days and 24 hours before the laser procedure and afterward every 2 to 3 days for 2 weeks. Macrophage depletion from draining lymph nodes located at the level of the submandibular, axillary, and inguinal regions was performed bilaterally by injecting subcutaneous (SC) CL_2-MDP-lip (50 µL = 0.25 mg) 24 hours before laser application and afterward every 2 to 3 days for 2 weeks. Control groups (C57BL/6 mice, n = 8) each received IP and SC administration of PBS alone or PBS-liposomes injections.

Choroidal Flatmount Preparation and Immunostaining Protocol

Briefly, white blood cells were separated from EDTA-anticoagulated whole blood by gradient separation technique.19 For flow cytometry, the monocytes were labeled with a rat anti-mouse F4/80 antigen-FITC conjugate (10 µg/mL; MCA497F; Serotec, Raleigh, NC) were used, followed by incubation with a secondary biotinylated antirat antibody (ABC Kit; Vector Laboratories, Burlingame, CA). A peroxidase substrate (Vector VIP, Vector Laboratories) was used. The micrograph was developed to the desired intensity. Macrophage density morphometry was performed after digitizing the choroidal flatmount preparations with a light microscope (×400 magnification) connected to a color video camera and a frame grabber. Three representative sections were analyzed at high-power magnification per piece of choroidal tissue, and the results were averaged with those in the remainder of the pieces to obtain the density of macrophages per eye. This was compared between macrophage-depleted animals (BALB/c, n = 3) and the control (BALB/c, n = 4; PBS or PBS-liposomes).

Monocyte Isolation and Flow Cytometric Evaluation

Briefly, blood white cells were separated from EDTA-anticoagulated whole blood by gradient separation technique.19 For flow cytometry, the monocytes were labeled with a rat anti-mouse F4/80 antigen-FITC conjugate (10 µg/mL; MCA497F; Serotec). Cells were prepared for analysis in flow cytometer using digitonin (1 mg/mL) and propidium iodide (PI, 500 µg/mL) modified from previous publications.20

Laser treatment, fluorescein angiography, histology, and flatmount preparation and analysis were performed as described in an earlier published study.1

Statistical Analysis

Morphometric data for different lesions in each eye were averaged to provide one value per eye. The mean ± SD of these measures for each group was calculated and probabilities (t-test) were obtained on computer (Prism, ver. 3.0; GraphPad, San Diego, CA). P < 0.05 was considered statistically significant in all forms of statistical analysis used.

RESULTS

First, we confirmed that CL_2-MDP-lip treatment adequately depleted circulating monocytes and choroidal macrophages. Flow cytometry was used to determine the degree of depletion of circulating monocytes. The total leukocyte population isolated from whole blood was stained for F4/80, a mouse macrophage-specific marker. Because monocytes constitute only 2% to 8% of total blood leukocytes, we enriched the sample for monocytes by using flow cytometry to identify the subset of large leukocytes (consisting of monocytes and large granular lymphocytes). Within this subset, leukocytes from mice treated with PBS or PBS-liposomes typically demonstrated approximately 60% positivity for F4/80. In contrast, CL_2-MDP-lip treatment reduced the percentage of F4/80-positive cells to 15%, indicating a reduction of approximately 75% (Fig. 1).

Treatment with CL_2-MDP-lip also depleted choroidal macrophages in normal eyes before laser-induced CNV (Fig. 2). Choroidal flatmount preparations were immunostained with anti-F4/80. CL_2-MDP-lip–treated mice showed a 46% reduction in the density of choroidal macrophages compared with control mice (9.6 ± 0.4 vs. 5.2 ± 1.8 macrophages per high-power field with P < 0.028).

Comparisons of fluorescein angiograms between macrophage-depleted and control mice revealed that treated mice had a smaller area of early hyperfluorescence and a much smaller area and intensity of late fluorescein leakage, indicating that the formation of CNV was less severe in treated animals (Fig. 3). However, the frequency of individual lesions with evident fluorescein leakage was equivalent in both groups, indicating that macrophage treatment did not prevent formation of CNV.

Histopathology confirmed that CNV in control mice revealed a much larger diameter and thicker center, which was confirmed with quantitative analysis (Fig. 4). Morphologic features of CNV between macrophage depleted and nondepleted mice were similar.

Flatmount analysis better demonstrated the decreased size, vascularity, and cellularity in CNV from mice depleted of macrophages (Fig. 5). CNV (defined as a surface area >0.5 disc areas) was observed in 84 (100%) of the lesions induced in 16-month-old animals of any group studied. As reported previously, CNV lesions reached maximum size within 14 days. CNV in mice that served as the control were large, with irregular borders and extensions. As shown previously, in aged (i.e., 16 month old) macrophage-intact mice, CNV from different laser spots often became confluent. (Figs. 5A, 5B). In contrast, macrophage-depleted mice demonstrated much smaller lesions, typically maintaining four small discrete circular lesions with poorly defined margins (Figs. 5C, 5D). A reduction of surface area was observed when compared with the PBS (by 50.8%) and PBS-liposome–treated groups (by 33.75%).

The relative vascularity of CNV was calculated by measuring the ratio of FITC fluorescence in the lesion, compared with the background choroid (Fig. 6). The margins of the lesion outlined by propidium fluorescence (i.e., cell nuclei) were slightly larger than the margins by FITC, suggesting the presence of more avascular cellular growth at the margins of the CNV (Figs. 6A, 6C). The data showed a significantly lesser vascular luminosity index in mice that were macrophage depleted (Fig. 6B).

We also evaluated the cell density in CNV by measuring the ratio of fluorescence luminosity from the PI nuclear staining in the lesion compared with the background choroid. This cellular density index was not significantly different in the macrophage-depleted mice, in part because the cells involved in CNV formation in both control and macrophage-depleted groups may represent different cell types. More specifically, these cells...
could represent pericytes, fibroblasts, and endothelial cells that were not affected by the depletion of macrophages (Fig. 6D).

**DISCUSSION**

Macrophages in tissues are derived from circulating blood monocytes. Within tissue they undergo maturation, adaptation to their local microenvironment, and differentiation into various types of cells that perform specific housekeeping, trophic, and immunologic functions. Macrophages play an important role in pathogen recognition and clearance, as well as scavenging of senescent and dying cells. They also function as antigen-presenting cells for T lymphocytes and as inflammatory effector cells. Several different stages of metabolic and functional activities, which in turn, represent different programs of gene activation are often described, including scavenging and activated macrophages. Activated macrophages are most efficient at synthesizing and releasing mediators to amplify inflammation and can mediate chronic injury leading to processes such as fibrosis, wound repair, extracellular matrix turnover, and angiogenesis.

In studies using rats, macrophage subpopulations are better delineated by monoclonal antibodies. The ED-1 positive, blood-derived macrophage population represents short-lived cells with high turnover that are recent recruits to tissues. These cells are thought to serve inflammatory and scavenging functions. In contrast, the ED-2 tissue-resident subset comprises relatively long-lived cells with low turnover and long duration in tissues. These cells are thought to serve trophic and tissue-maintenance functions. Dendritic cells are specialized noninflammatory monocytes that present antigen to naïve T cells.

The normal choroid is richly invested with various subsets of monocytes and macrophages, including blood-derived mac-

**FIGURE 1.** Flow cytometry of white blood cells obtained from mouse blood. (A) Dual light-scatter characteristics for the gated population of macrophages and large lymphocytes. Acquisition gates were set to detect only the PI fluorescence-positive events from a cell suspension in which no antibody staining was used. (B-D) Positive staining of blood monocytes in the gated population after labeling them with the macrophage marker F4/80 after treatment with PBS (B), PBS-liposomes (C), or CL$_2$MDP-lip (D). CL$_2$MDP-lip-treated mouse showed depletion of monocytes to 15%.

**FIGURE 2.** Choroidal flatmount preparations from BALB/c mouse for detection of choroidal macrophages using anti-F4/80 staining. (A) Choroidal macrophage density in a control mouse compared with the diminished density of macrophages (B) in which the animals received treatment with CL$_2$MDP-lip for 2 weeks. Magnification, ×400.
Although corticosteroids inhibit angiogenic factors released by macrophages from the choroid, these drugs have the potential to influence cellular permeability and downregulate the expression of inflammatory markers. Taken together, these observations imply a potential pathogenic role for cytokines, inflammatory cell infiltration in AMD, and Sarks et al.32 demonstrated significant macrophage infiltration in specimens from surgically excised CNV and that these cells appear to be synthesizing various cytokines and growth factors. In a primate model for laser-induced experimental subretinal neovascularization, Ishibashi et al.37 showed that macrophages migrate into the laser wounds and seemed to contribute to the neovascular response. Finally, ocular corticosteroid therapy has been used in the treatment of CNV. Although corticosteroids inhibit many cellular processes they are potent modulators of macrophage functions. These studies suggest that more research is needed to explore the therapeutic potential of non-specific anti-inflammatory therapy in AMD. Specifically, triamcinolone acetate has been used to treat CNV lesions, based on research in which these drugs have the potential to influence cellular permeability and downregulate the expression of inflammatory markers.

In this study, we evaluated the effect of macrophage depletion on the severity of experimental CNV in aged (16 months) old mice. Aging has been shown to increase the severity of CNV lesions in mice.1 We found that CNV in macrophage-depleted mice were smaller, thinner, less cellular, and less vascularized than nondepleted mice but did not decrease the incidence of CNV. However, we did not observe a difference in the cell density or frequency of actively leaking lesions between compared groups. Nevertheless, the total cellular component of the depleted animals was less because of the decrease in size of the CNV.

The effective depletion of macrophages was confirmed by flow cytometry of circulating monocytes and microscopic examination of choroidal flatmount preparations for choroidal macrophages. Different percentages of depletion were determined with the two analyses. The level of depletion of blood monocytes achieved in our study was consistent with published results (70%–95%).17,43 In other studies of nonocular tissues,

![Figure 3](image1.png)

**Figure 3.** Fluorescein angiograms of eyes obtained 2 weeks after diode laser photocoagulation to induce CNV. (A–C) Angiogram of a control (PBS treated) animal. (A) A pointlike hyperfluorescent area that greatly increased in size and intensity, as shown in (B) and (C). There was intense leakage in the late phases (1- and 5-minute frames, respectively corresponding to intermediate [B] and late [C] phases) of the angiogram which shows the severity of the CNV lesion. (D–F) Fluorescein angiogram of a treated (CL2MDLP) animal. A similar angiographic pattern can be seen but the intensity and the size of the hyperfluorescent lesion is smaller. (D) Depicts the initial phase of the angiogram in which a pointlike hyperfluorescence is evident, which grows moderately in size and intensity during intermediate (E) and late (F) phases of the angiogram.

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**Figure 4.** Quantitative analysis of histopathology sections. (A) Maximum lesion diameter (measured in pixels) in which the CL2MDP-lip treated group shows significantly smaller diameter than the control group ($P < 0.0006$). (B) Maximum lesion thickness (measured in pixels) in which the CL2MDP-liposomes treated group shows a significantly lesser lesion thickness than the control group ($P < 0.03$). *Statistically significant difference.
depletion of tissue macrophages by clodronate has been more variable. The ED-1–positive, blood-derived macrophage population appears more susceptible to depletion by clodronate than do the ED-2–positive tissue-resident macrophages.44,45 Our findings seem to concur, because circulating monocytes were 75% depleted, but choroidal macrophages were only reduced by 46%. We presume that the residual choroidal macrophages represent mostly tissue-resident macrophages. If true, then our results suggest that the blood derived circulating monocytes are more likely to contribute to CNV pathogenesis.

**FIGURE 5.** Flatmount preparations of the posterior pole of a mouse eye using FITC-dextran and PI stains 2 weeks after diode laser photocoagulation. (A, B) Vascularity and cellularity of the lesions of the control group. CNV lesions were large and with irregular borders. (C, D) Small discrete circular lesions (dotted circles) of the CL2MDP-lip–treated animals. The size, vascularity, and cellularity of the lesions were less in the macrophage-depleted mice (Fig. 6). D, optic disc.

**FIGURE 6.** Quantitative analysis of flatmount specimens, showing that CL2MDP-lip–treated animals had significantly less severe CNV lesions when measured with FITC and PI staining. (A) Vascular margins (as measured in disc areas) of the lesions in both the control and the treated mice. Compared with control animals, analysis of flatmounted retinas of macrophage depleted mice demonstrated significant reduction in size of CNV lesions (2.8 ± 0.5 DA vs. 1.4 ± 0.1 DA, P < 0.045). (B) Vascular luminosity index. The treated group also revealed less vascularity of CNV lesions (1.6 ± 0.1 units vs. 1.1 ± 0.0 units, P < 0.0092). (C) Cellular margins (size), measured in DAs in which the treated group also showed a decrease in size compared with the nontreated one (3.3 ± 0.6 DA vs. 1.7 ± 0.1 DA, P < 0.04). (D) Cell density was reported as the cell-density index, which was not significantly different between groups. Nevertheless, the total cellular component of the depleted animals was less due to the decrease in size of the CNV. *Statistically significant difference.
PBS alone and PBS-liposome-treated animals were used as control subjects for completeness. Empty liposomes, in common with other particulate compounds, may influence macrophage biology and must be used as a specificity control when liposomes are used as a drug delivery method for specific agents. However, as stated by investigators who developed this procedure, the best control for studies in which general macrophage depletion is required (as in our study) are PBS sham injections. This control would represent normal, healthy, non-blocked, nonsuppressed, and nonstimulated macrophages.13

Recent data from several groups suggest that innate immune mechanisms and localized choroidal inflammation may generally contribute to the pathogenesis of AMD. In addition to macrophages, other relevant innate immune mechanisms involved in AMD pathogenesis include injurious stimuli (oxidants or infectious agents) and amplification cascades (such as complement, mediators systems, and cytokines). For example, Anderson et al.14 have identified complement and immune complexes in association with nodular drusen, which are potentially at high risk for CNV formation.15 Perhaps immune complexes are stimuli for the recruitment of macrophages or monocytes in human AMD.

References


