EXTENDED REPORT

The relative contributions of each subset of ocular infiltrated cells in experimental choroidal neovascularisation

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Aim: Choroidal neovascularisation (CNV) is a major cause of blindness in adults. The aim of this study was to investigate the role of infiltrating cells in the development of experimental CNV.

Methods: CNV was induced in C57BL/6 (B6) mice by laser photocoagulation (PC). After PC, the numbers of each subset of infiltrated cells were analysed by flow cytometry at multiple time points. Each subset (except for macrophages) was depleted by the specific antibodies in vivo. Thereafter, the area of CNV was compared between the control B6 mice and the specific antibody treated mice 7 days after PC. The CNV formation in neutrophil depleted CC chemokine receptor-2 (CCR2) knockout mice was also examined to minimise the effects of macrophages.

Results: In the early phase of CNV formation, a large number of neutrophils and macrophages infiltrated to the eyes. Natural killer (NK) cells and T lymphocytes were barely detected while no B lymphocytes were detected. The CNV areas did not significantly change compared between the control B6 mice and the specific antibody treated mice. However, the neutrophil depleted CCR2KO mice resulted in a reduction of CNV.

Conclusion: Although lymphocytes and NK cells had little effect on CNV formation, neutrophils partially contributed to CNV in the absence of macrophages.

Choroidal neovascularisation (CNV) is known to cause a severe visual loss in the patients, such as age related macular degeneration (AMD).^{1,2} While the driving force of retinal angiogenesis has been considered to be hypoxia, the primary stimulus for CNV is still not clear. One of the contributors to the development of CNV has been hypothesised to be "inflammation."^{3–5} We have recently described the role of macrophages in the development of experimental CNV.⁶ However, the types and the kinetics of ocular infiltrating cells other than macrophages in experimental CNV have not yet been analysed.

More importantly, the relative roles of ocular infiltrated cells other than macrophages are still largely unknown. The relation between inflammatory cells and angiogenesis had been mainly studied in the fields of tumour, RA, and vasculitis.^{7–9} Benelli *et al* demonstrated neutrophils to be a key cellular target for tumour angiogenesis.¹⁰ Moreover, some reports have demonstrated that natural killer (NK) cells have an important role in tumour angiogenesis.^{9,11} T cells have been reported to have the ability to promote angiogenesis in immune inflammation,¹² in a corneal micropocket assay system,¹³ and in a tumour model.^{14,15}

The purpose of this study was to investigate whether ocular infiltrating cells other than macrophages (such as neutrophils, NK cells, and lymphocytes) contribute to the development of CNV or not. We used a PC induced CNV model and examined the area of CNV in each subset depleted mice using a specific antibody in vivo.

MATERIALS AND METHODS

Mice

Female 8 week old mice were used in all experiments. C57BL/6 (B6) mice were purchased from Japan SLC (Shizuoka, Japan) and kept under specific pathogen free conditions at Kyushu University. CCR2 knockout (KO) mice and wild type (WT) CCR2 (+/+) mice were generated by mating

homozygous mice of the same genetic background (hybrid of C57BL/6 and 129/Sv).¹⁶ All treatments of the animals conformed to the ARVO statement for the use of animals in ophthalmic and vision research.

Antibodies and reagents

The following reagents were used for the flow cytometric studies. Cy-chrome conjugated anti-mouse CD45 mAb (clone name; 30-F11), FITC conjugated anti-mouse Ly-6G (Gr-1) mAb (clone name; RB6-8C5), Cy-chrome conjugated anti-mouse TCR β chain mAb (clone name; H57-597) and biotin conjugated anti-mouse NK1.1 mAb (clone name; PK136) were purchased from BD PharMingen (San Diego, CA, USA). FITC conjugated anti-mouse CD19 mAb (clone name; MB19-1) was purchased from eBioscience (San Diego, CA, USA). Biotin conjugated anti-F4/80 mAb (clone name; A3-1) was purchased from Caltag Lab (Burlingame, CA, USA). Streptavidin/R-PE was purchased from Molecular Probes (Oregon, USA).

The Abs used for isotype controls were as follows: Cy-chrome conjugated Rat IgG2b (clone name; A95-1) for anti-mouse CD45 mAb, FITC conjugated Rat IgG2b (clone name; A95-1), for anti-mouse Ly-6G (Gr-1) mAb, Cy-chrome conjugated Hamster IgG group2 (clone name; Ha4/8) for anti-mouse TCR β chain mAb, biotin conjugated Mouse IgG2a (clone name; G155–178) for anti-mouse NK1.1 mAb and FITC conjugated Mouse IgA (clone name; M18–254) for anti-mouse CD19 mAb were purchased from BD PharMingen (San Diego, CA, USA). Biotin conjugated rat IgG2b (clone name; R2b15) for anti-F4/80 mAb was purchased from Caltag Lab (Burlingame, CA, USA).

The antibody preparations for in vivo were as follows: anti-mouse-granulocyte antibody, anti-Gr-1 mAb is a rat

Abbreviations: AMD, age related macular degeneration; CNV, choroidal neovascularisation; NK, natural killer cells; PC, photocoagulation; RPE, retinal pigmented epithelium

immunoglobulin G2b monoclonal antibody specific for murine neutrophilic and eosinophilic granulocytes.^{17, 18} Five week old female SCID mice were given intraperitoneal injections of pristane purchased from Sigma (St. Louis, MO). Ten days later, the mice received intraperitoneal inoculations of hybridoma cells (RB6-8C5) which was kindly provided by Dr F Sendo (Yamagata University, Yamagata, Japan). Two weeks after the inoculation, anti-mouse Gr-1 mAb were purified from ascites of SCID mice using ammonium sulphate treatment independently. Anti-mouse CD4 mAb were harvested from hybridoma cells (GK. 1. 5) and for anti-mouse CD8 mAb, we used hybridoma cells (53–6. 72) in the same methods. Rabbit anti-asialo GM1 Ab was purchased from Wako Pure Chemicals (Osaka, Japan).

Antibody treatment

To deplete neutrophils, anti-Gr-1 mAb (400 µg per mouse in each inoculation) was injected intraperitoneally 1 day before treatment and 1, 3, and 5 days after treatment. To examine the effect of anti-Gr-1 mAb administration on circulating neutrophils, the total number of peripheral white blood cells (WBC) was counted after staining with May-Grünwald Giemsa staining. For CD4+ or CD8+ T cell depletion, the mixture of each 100 µg of anti-mouse CD4 mAb and anti-mouse CD8 mAb were inoculated intraperitoneally into mice 2 days before treatment and 0 and 3 and 6 days after treatment. To deplete NK cells, the mice were injected with anti-asialo GM 1 Ab intraperitoneally 1 day before treatment and 2 and 5 days after treatment. The depletion of these cells was confirmed in the splenocytes by flow cytometry, 2 days after treatment, and 95% of the appropriate subsets were constantly depleted.

Induction of CNV

PC induced CNV was generated by a previously described technique with some modifications.¹⁹ Briefly, the mice were anaesthetised with ketamine hydrochloride (100 mg/kg body weight), and the pupils were dilated with 1% tropicamide. Krypton multicolour laser photocoagulation (wavelength 630 nm, 0.1 second, spot size 100 µm, power 150 mW) was performed around the disc of the retina through a slit lamp delivery system using a photocoagulator (MC7000; NIDEK, Aichi, Japan). The posterior pole of the retina was thus burned while a hand held cover slide was used as a contact lens. Only lesions in which a subretinal bubble or focal serous detachment of the retina developed were used for the experiments.

Isolation and counting of the ocular infiltrating inflammatory cells

To examine the ocular infiltrating cells in the retina and choroid, single cells were prepared from mouse eyes according to the procedure used to isolate of hepatic lymphocytes, with some modifications.^{20, 21} To collect a sufficient number of ocular infiltrating cells, 40–50 burns were delivered to the eyes of the mice to induce panretinal photocoagulation. After PC, the eyes were enucleated and then the anterior segment (cornea, iris, and lens) was taken out. The posterior segment of the eye including the sclera, choroid, retina was disrupted with scissors and then shaken in medium supplemented with 0. 5 mg/ml of Collagenase type D (Boehringer Mannheim, Germany) at 37°C for 40 minutes. As the basic medium, we used RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, 5 mg/ml HEPES buffer. The supernatants were collected, passed through a metal mesh, washed three times, and viable cells were thus obtained.

Flow cytometry

Intraocular infiltrating cells were adjusted to the designated concentrations and then were stained with the following reagents and colours respectively. For identification of neutrophils, cells were stained with Cy-chrome conjugated anti-mouse CD45 mAb (30-F11) and FITC conjugated anti-mouse Ly-6G(Gr-1) mAb (RB6-8C5) and for macrophage identification, cells were stained with Cy-Chrome conjugated anti-CD45 mAb and biotin conjugated anti-F4/80 mAb and then were counterstained by Streptavidin/R-PE were used. Cells were stained with Cy-chrome conjugated anti-mouse TCRβmAb (H57-597) for T lymphocyte identification and were stained with FITC conjugated anti-mouse CD19 mAb (MB19-1) for B lymphocytes identification. NK cells were identification by staining with biotin conjugated anti-mouse NK1. 1 mAb (PK136) and then were counterstained by Streptavidin/R-PE. Flow cytometry was performed with EPICS XL (Beckman Coulter, Mannheim, Germany). The number of ocular infiltrating cells was calculated from the percentage of each population in the gate of the precounted total number of viable cells using trypan blue dye exclusion.

Choroidal flat mount preparation

In a mouse experimental CNV model, neovascularisation can be detected within 1 week.¹⁹ To study the effect of macrophage infiltration on the development of CNV, we evaluated the early phase of CNV on day 7 using the flat mount technique as previously described.²² To individually evaluate the size of the CNV lesions, four burns were performed while leaving a space (3, 6, 9, 12 o'clock positions around the optic disc). The mice were anaesthetised and perfused with 1 ml of phosphate buffered saline containing 50 mg/ml of fluorescent labelled dextran (25 000 average molecular weight; Sigma, St Louis, MO, USA) and the eyes were removed and fixed for 30 minutes in 4% paraformaldehyde. The cornea and lens were removed and then the entire retina was carefully dissected from the eye cup. Radial cuts (average 8) were made from the edge of the eye cup to the equator and then the eyecup was flat mounted in an aqua-mount with the sclera facing down and the choroid facing up. Flat mounts were examined by fluorescence microscopy and the images were digitised using a three charge coupled device colour video camera and a frame grabber. To measure the total area of hyperfluorescence associated with each burn corresponding to the total number of fibrovascular scars, the MacScope (version 2. 3; Mitani, Fukui, Japan) was used.

Statistics

We used the statistical software StatView version 4.5 (Abacus Concepts Inc, Berkeley, CA, USA). A one way analysis of variance (ANOVA) was used to evaluate any differences in the numbers of ocular infiltrating inflammatory cells per eye. Multiple comparisons between each possible pair of groups were also evaluated by the Bonferroni's methods. A two sided p value less than 0.05 was considered statistically significant. The areas of CNVs were analysed to identify any significant differences among the experimental groups by Student's *t* test. A value of $p \leq 0.05$ was considered to be significant.

RESULTS

The kinetics of each subset of ocular infiltrating cells after photocoagulation

PC caused a disruption of the retinal pigment epithelium layer, Bruch's membrane, and choroidal vessels. Thereafter, many inflammatory cells were accumulated at the site of PC.²³ To analyse the phenotype of ocular infiltrating cells, single cells were prepared from the PC injured eyes by the treatment of collagenase as described in Materials and methods. The cells were analysed by flow cytometry and

Figure 1 A representative FACS analysis of ocular infiltrating cells before v after PC. A flow cytometric analysis was carried out on each ocular infiltration inflammatory cells. Left panel: The discrimination of ocular infiltrating cells by forward and side scattered gate. Only viable cells were analysed in the live gate. Typical neutrophil and macrophage gate and NK cell and lymphocyte gate were indicated. Right panel: Neutrophil and macrophage: The rectangular gates indicated neutrophils and macrophages. Neutrophils were stained with FITC conjugated anti-Gr-1 mAb and Cy-chrome conjugated anti-CD45 mAb. Macrophages were stained with biotin conjugated anti-F4/80 monoclonal antibody (mAb) counterstained by Streptavidin/R-PE and Cy-chrome conjugated anti-CD45 mAb. Lymphocyte: The quadrant gate indicated T cells and B cells. Cells were stained with Cy-chrome conjugated anti-mouse TCR β chain mAb (T cells) and FITC conjugated anti-mouse CD19 mAb (B cells). NK cell: NK cells were indicated by the histogram. Cells were stained with and biotin conjugated anti-mouse NK1.1 mAb counter stained by Streptavidin/R-PE.

only viable cells (excluding dead cells) were gated for further investigations (fig 1, left panel). The discrimination of each subset of ocular infiltrating cells was primarily performed based on forward and side scattered images (fig 1, left panel) and then confirmed by the specific antibodies as described in materials and methods. Representative pattern of each subset (neutrophils, macrophages, lymphocytes and NK cells) were shown in figure 1, right panel. Neutrophil and macrophage gate and NK cell and lymphocyte gate were indicated. Specific gates were set up based on the staining pattern of isotype control antibodies.

In the neutrophils and macrophages gate, it contained large amounts of CD45 negative cells (fig 1, right panel). In

the NK cells and lymphocytes gate, most of the cells were also CD45 negative cells (data not shown). We thought CD45 negative cells were not the bone marrow derived inflammatory cells and must be viable resident cells (including retinal pigment epithelial cells, glia cells, rod cells, cone cells and so on) dispersed by the collagenase treatment. In fact, these CD45 negative cells were observed even in the eye before PC (fig 1, right panel).

After PC, as shown in figure 2, a large number of neutrophils and macrophages were infiltrated in the eye in early phase, peaked at 24 hours and 48 hours, respectively. A small amount of T cell and NK cell infiltration were detected. In this model, no B cells were detected throughout the entire

Figure 2 The kinetics of each subset of ocular infiltrating inflammatory cells after PC. The harvested cells from the murine eyes were analysed by flow cytometry at 12 hours, 24 hours, 2 days, 5 days, and 7 days after PC. The numbers of ocular infiltrating inflammatory cells per eye were calculated by the percentage of each population in the live gate and the precounted total number of viable cells using trypan blue dye exclusion. The gate of each antibody positive cell was set up according to the staining pattern of isotype control. These are the data of a representative series. We used 45 eyes in a series of kinetics experiments, and nine eyes were examined in individual time point ($n=3$, three eyes were pooled to obtain the designated cell numbers). We repeated this series three times and confirmed the similar results. The bars show the means (SEM) of three independent experiments for each time point ($n=3$ per time point for each individual experiment). * $p<0.01$

disease course. Neutrophils were the major ocular infiltrating cells and, in fact, the amount of the infiltration of neutrophils was significantly larger than those of other cells at 12 hours, 24 hours, and 48 hours after PC. All of the bone marrow derived inflammatory cells were diminished until 7 days after PC.

The effect of neutrophil depletion in CNV

We have recently shown the role of macrophages in CNV formation.⁶ In this study, we found that not only macrophages but also a large number of neutrophils accumulated in the eye after PC. To examine the effect of neutrophils in the development of CNV, we thus depleted neutrophils in B6 mice by using anti-mouse Gr-1 Ab. Thereafter, the area of CNV was compared between the control B6 mice and antibody treated mice 7 days after PC. Although the area of CNV tended to decrease in Gr-1 mAb treated mice, no significant difference was observed compared to the non-treated control B6 mice (fig 3).

The effect of either T lymphocyte or NK cell depletion in CNV formation

In general, it is known that both T cells and NK cells have an important role in the initiation of inflammation.^{24–25} We thus first examined the effects of T lymphocytes in CNV formation. T lymphocytes were depleted by the combination of anti-mouse CD4 mAb (GK 1.5) and anti-mouse CD8 mAb (53–6.72) in B6 mice. One day after mAbs treatment, PC was performed, and then the areas of CNVs were evaluated 7 days post-PC. Only slight differences were observed between the antibody treated group and the non-treated control group (fig 4A). This result suggested that the existence of T cells had no effect on CNV formation.

We next examined the role of NK cells in this model. We depleted NK cells using anti-asialoGM1 Ab in B6 mice. There was no significant difference between anti-asialo GM1 Ab-treated B6 mice and non-treated control mice (fig 4B). We

Figure 3 The effect of depletion of neutrophils in CNV formation. (A) The representative CNV lesions of choroidal flat mounts. Seven days after PC, the mice were perfused with fluorescein labelled dextran and the eyes were removed to make choroidal flat mounts. CNV was detected as a hyperfluorescence vascular structure. The area of CNV was evaluated by a fluorescence microscope as described in Materials and methods. The photograph on the left shows typical CNV 7 days after PC in C57BL/6 mice, and the photograph on the right shows CNV in C57BL/6 mice treated with an intraperitoneal inoculation of anti-Gr-1 mAb. (B) The area of CNV per eye 7 days after PC in C57BL/6 mice treated with an intraperitoneal inoculation of anti-Gr-1 mAb ($n=13$) and control C57BL/6 mice ($n=22$) were compared. The bars show the mean (SEM). NS, not significant.

thus concluded that the NK cells had little effect on CNV development.

The effect of depletion of neutrophils in CCR2 KO mice
CCR2 is the main receptor of C-C chemokine MCP-1, and it is strongly associated with macrophage migration to the particular lesions.^{26–27} Therefore, macrophages could not migrate into any inflammatory lesions in CCR2 KO mice.^{28–30} We have previously observed a marked reduction in the number of ocular infiltrating macrophages and an impairment of CNV formation in CCR2 KO mice.⁶

Neutrophils have been reported to produce various angiogenic factors in several experimental models and they are thus considered to have a crucial role in angiogenesis associated disease.³¹ In addition, our result showed that the area of CNV in Gr-1 mAb treated mice had tendency to decrease compared to the non-treated control B6 mice (fig 3). We thus hypothesised that the contribution of macrophages was so strong that it and masked the angiogenic capacity of neutrophils in our model.

To confirm this hypothesis, we used CCR2KO mice and compared the CNV formation between anti-Gr-1 Ab treated mice and control CCR2KO mice. On day 7 after PC, a marked reduction in the CNV area was observed in CCR2KO mice compared to CCR2WT mice (C57BL/6 × 129Sv mice). Interestingly, the neutrophil depleted CCR2 KO mice significantly decreased the CNV area compared to the CCR2WT mice 7 days after PC (fig 5A,B). We thus concluded that although the macrophages are the main effector cells in CNV development, the neutrophils actually had an

Figure 4 The effect of depletion of either T lymphocytes or NK cells in CNV formation. (A) The effect of T lymphocytes depletion in CNV formation. The area of CNV per eye in T lymphocytes depleted C57BL/6 mice treated with an intraperitoneal inoculation of anti-CD4 and CD8 mAb ($n=14$) and control C57BL/6 mice ($n=12$). The bars show the mean (SEM). (B) Comparison of the area of CNV in C57BL/6 mice treated with an intraperitoneal inoculation of anti-asialo GM-1 mAb ($n=8$) and control C57BL/6 mice ($n=10$).

angiogenic capacity and thereby partially contributed to CNV formation.

DISCUSSION

In this report, we examined the kinetics and relative contributions of various ocular infiltrated cells other than macrophages in the experimental CNV. We found that the NK cells and the lymphocytes were nothing to do with and the neutrophils partially contributed to CNV formation. Although it was shown that both NK cells and lymphocytes have a critical role in the initiation of the angiogenesis process in the tumour model,^{9–11} we think PC injured ocular tissues may strongly recruit neutrophils and macrophages, which participate in an innate immune responses, without the aid of T cells, B cells, and NK cells for CNV formation.

It is important to note that the angiogenic capacity of neutrophils actually existed but it was masked in the presence of macrophages, which are the dominant angiogenic promoter in this model.⁶ Our data indicated that neutrophil dependent choroidal angiogenesis could be dominant under special conditions that may impair the macrophage functions. Some investigators have reported that macrophages have a critical role on experimental CNV formation and they have suggested the therapeutic potential of macrophage depletion.^{32–33} However, our data implied that macrophage depletion might induce the neutrophil dependent CNV.

In contrast with our data, several reports have shown that neutrophils and macrophages are equally contributed to angiogenesis in the other models.³¹ For instance, in a tumour³⁴ and skin wound healing model,³⁵ neutrophils have been shown to be one of the main effector cells for angiogenesis. We assumed that the relative roles and dynamics of the local infiltrated cells could be altered

Figure 5 The effect of neutrophil depletion in CNV formation of CCR2 KO mice. (A) The representative CNV lesions of choroidal flat mounts. The area of CNV was evaluated as described in Materials and methods. The photograph on the left shows typical CNV 7 days after PC in CCR2KO mice, and the photograph on the right shows CNV in CCR2KO mice treated with an intraperitoneal inoculation of anti-Gr-1 mAb. (B) The area of CNV per eye were compared among CCR2WT mice (background: C57BL/6 $\times$ 129Sv) ($n=18$), no treated CCR2KO mice ($n=28$) and Gr-1 treated CCR2KO mice ($n=23$) 7 days after PC. The bars show the mean (SEM), * $p<0.01$.

depending on differences in the organs, tissue, and site. Especially, the immune inflammatory cell dynamics in the eye are unique, and also demonstrate tissue peculiarity because the eye is considered to be an immune privileged organ.^{36–38} Whether our results depend on tissue specificity or not, further study is still required.

We need to discuss the fact that partial CNV formation was observed even after the removal of neutrophils in CCR2 KO mice (fig 4). As a result, not only bone marrow derived ocular infiltrated cells but also resident cells such as retinal pigmented epithelium (RPE); fibroblasts and microglia are thus suggested to contribute to CNV. Some reports have shown that resident RPE cells directly produced various angiogenic factors and thus contribute to the pathogenesis of age related macular degeneration (AMD).^{39–40} Ocular infiltrated inflammatory cells may have a role in CNV by amplifying the angiogenic abilities of resident cells.

There was the discrepancy between the results of CNV size in figures 3 and 4 and figure 5. In figures 3 and 4, CNV size in antibody no treated B6 mice ranged from 0.09–0.12 mm² and in figure 5, CNV size in antibody no treated CCR2WT mice was 0.2 mm². We considered this size discrepancy must be the result of the strain difference. The background of CCR2KO mice is the hybrid of C57BL/6 $\times$ 129Sv mice, the CNV size of C57BL/6 $\times$ 129Sv mice (CCR2WT mice) is constantly larger than that of C57BL/6 mice.

The process of CNV observed in the patients is not simple and must combine various steps and cellular events.^{41–42} In addition, PC induced experimental CNV model has been thought to reflect merely the early phase of human CNV formation.⁴³ However, the inflammation is extremely important in the initial phase of CNV⁵ and we believe that our data

may thus help to eventually prevent CNV formation in human disease in the future.

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