EXTENDED REPORT

Identification of resident and inflammatory bone marrow derived cells in the sclera by bone marrow and haematopoietic stem cell transplantation

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Aims: To characterise bone marrow derived cells in the sclera under normal and inflammatory conditions, we examined their differentiation after transplantation from two different sources, bone marrow and haematopoietic stem cells (HSC).

Methods: Bone marrow and HSC from green fluorescent protein (GFP) transgenic mice were transplanted into irradiated wild-type mice. At 1 month after transplantation, mice were sacrificed and their sclera examined by histology, immunohistochemistry (CD11b, CD11c, CD45), and transmission and scanning electron microscopy. To investigate bone marrow derived cell recruitment under inflammatory conditions, experimental autoimmune uveitis (EAU) was induced in transplanted mice.

Results: GFP positive cells were distributed in the entire sclera and comprised 22.4 (2.8)% (bone marrow) and 28.4 (10.9)% (HSC) of the total cells in the limbal zone and 18.1 (6.7)% (bone marrow) and 26.3 (3.4)% (HSC) in the peripapillary zone. Immunohistochemistry showed that GFP (+) CD11c (+), GFP (+) CD11b (+) cells migrated in the sclera after bone marrow and HSC transplantation. Transmission and scanning electron microscopy revealed antigen presenting cells among the scleral fibroblasts. In EAU mice, vast infiltration of GFP (+) cells developed into the sclera.

Conclusion: We have provided direct and novel evidence for the migration of bone marrow and HSC cells into the sclera differentiating into macrophages and dendritic cells. Vast infiltration of bone marrow and HSC cells was found to be part of the inflammatory process in EAU.

Bone marrow derived cells fulfil a variety of functions, including antigen presentation, phagocytosis and inflammatory cytokine excretion. Macrophages and dendritic cells are bone marrow derived antigen presenting cells (APC) which serve in the first step of the acquired immune responses. Recent studies have demonstrated the tremendous plasticity of bone marrow derived stem cells as they can differentiate into epithelial types¹ and cells with visceral mesoderm, neuroectoderm and endoderm characteristics.^{1–5} These findings suggest the potential of bone marrow derived stem cells to transdifferentiate into a variety of organ tissues, including those of the eye.

Recently, the presence of APCs such as macrophages and dendritic cells was reported in the cornea.^{6–14} According to the MHC class II expression, normal central cornea was thought to be devoid of APCs.^{15 16} The CD45+/CD11b+/CD11c+ dendritic cells distributed in corneal epithelium⁸ and corneal stroma,^{9 17} and CD11b+/CD11c- macrophages in the corneal stroma.^{7 9}

The sclera develops from the mesoderm simultaneously with the cornea, and forms the outer structure of the eye, contributing to the preservation of the ocular shape and serving as a functional barrier.^{18 19} There are many ocular inflammatory diseases that involve the sclera, including infections and inflammatory conditions.^{20–23} Sclera is made up of dense collagenous fibre lamellae and contains a paucity of cells, mainly fibroblasts.¹⁸ Both macrophages and dendritic cells may play important roles in these processes but little is known about bone marrow derived cells transdifferentiating into scleral cells.

We performed transplantation of bone marrow from green fluorescent protein (GFP) transgenic mice into wild-type

animals and reported the migration and differentiation of bone marrow derived cells into the cornea,^{10 11} vitreous²⁴ and the subretinal space after retinal detachment.²⁵ Using a similar approach, investigators have also demonstrated the distribution of cells of myeloid lineage in the cornea.^{26 27}

The purpose of this study was to investigate the characterisation of bone marrow derived cells in the sclera under both normal and inflammatory conditions in experimental autoimmune uveitis (EAU) by transplanting two different cellular sources, bone marrow cells and haematopoietic stem cells (HSC) from GFP mice, using our protocol.^{10 11 28 29} As these two different cellular sources differ in the maturity, purity and subpopulation as cellular sources, one might expect them to show differences in distribution, differentiation and responses to induced inflammation *in vivo*. This study is an essential step towards understanding the physiological and immunological responses of bone marrow and HSC derived cells in the sclera.

MATERIALS AND METHODS

Experimental animals

Animals were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and with the experimental procedure approved by the Committee for Animal Research. Adult (total n = 26) and newborn (n = 16) C57BL/6J

Abbreviations: APC, antigen presenting cells; EAU, experimental autoimmune uveitis; GFP, green fluorescent protein; HSC, haematopoietic stem cells; PBS, phosphate buffered saline; SEM, scanning electron microscopy; TEM, transmission electron microscopy

Figure 1 Schematic diagram of bone marrow (BM) and haematopoietic stem cell (HSC) transplantation (A) and representative images of BM (B) and HSC (C) by transmission electron microscopy. BM cells encompass all subtypes of haematopoietic cells of various developmental stages (B). HSC show a round, indented nucleus with a high nucleus-cytoplasm ratio and peripheral chromatin condensation (C). Original magnification B $\times 2200$, bar 2 μm ; C $\times 4800$, bar 1 μm .

mice were used as normal control recipients for bone marrow transplantation and HSC transplantation, respectively. Bone marrow and HSC were obtained from enhanced GFP transgenic mice (from Dr Masaru Okabe, Osaka University, Japan).^{30, 31}

Bone marrow transplantation

To investigate the participation of bone marrow cells in the sclera, we performed bone marrow transplantation (fig 1A, B). Female GFP mice (8–10 weeks) were killed under deep ether anaesthesia by cervical dislocation. Bone marrow cells were obtained by flushing the femurs with sterile phosphate buffered saline (PBS), washed several times in sterile PBS, filtered twice through a nylon mesh of 70 μm pore size, counted and resuspended in PBS at a concentration of 5×10^7 cells/ml.^{10, 24} Transmission electron microscopy (TEM) showed that the bone marrow contained all haematopoietic subtypes at various developmental stages (fig 1B). The GFP positive bone marrow cells were transplanted by tail vein injection into C57BL/6J mice ($n = 20$) after irradiation with 9 Gy of x rays with lead covers on both eyes. A successful bone marrow transplantation was confirmed by identification of GFP positive cells in the peripheral blood of transplanted animals 2 weeks after irradiation.^{10, 24}

Haematopoietic stem cell transplantation

To characterise bone marrow derived stem cells in the mouse sclera, we examined HSC transplantation.^{10, 11, 28, 29} To deplete mature haematopoietic cells, bone marrow cells were incubated with lineage specific antibodies (B220, CD3, Gr-1, Mac-1 and TER 119) for 30 min at 4°C. After washing with PBS containing 2% fetal bovine serum, the cells were incubated with sheep anti-rat immunomagnetic beads (Dynabeads M-450 coupled to sheep anti-rat IgG; Dynal, Great Neck, New York, USA). Cells not bound to immunobeads were further purified for Sca-1 (+) cells. After negative and positive selection, the purity for Lin(-) Sca1(+) cells of all GFP+ cells exceeded 95%.^{10, 28, 29} TEM demonstrated that HSC had round indented nuclei with a high nucleus-cytoplasm ratio and peripheral chromatin condensation, as previously reported (fig 1C).³² Ten thousand Lin(-) Sca-1 (+) cells (fig 1A, C) were transplanted into newborn

C57BL/6J mice (within 2 days of birth, $n = 16$). One month after HSC transplantation, the mice were used for histological and immunohistochemical study.

Tissue preparation

To examine the posterior eye, eyes were harvested 2 weeks ($n = 3$, bone marrow) and 1 month ($n = 6$ bone marrow and $n = 6$ HSC) after transplantation, episcleral tissue was carefully removed and the eyes observed by fluorescent biomicroscopy (total $n = 9$ bone marrow and $n = 6$ HSC). The eyes were fixed in paraformaldehyde followed by acetone fixation and embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany). Thick sections (4 μm) were cut from the blocks, stained by propidium iodide for nuclear visualisation (Molecular Probes, Eugene, Oregon, USA) and observed under a fluorescent microscope. The number of GFP positive (GFP+) cells and propidium positive (PI+) cells were counted on five randomly selected sections. The ratio of GFP+/PI+ was generated and expressed as mean (SD). The whole sclera was divided into three equal zones, a limbal zone, equatorial zone and peripapillary zone.

Immunohistochemistry

Eyes were harvested ($n = 6$, bone marrow, HSC, and normal mice), fixed in paraformaldehyde and 10 μm thick cryosections were made. The primary antibodies (all from PharMingen, San Diego, California, USA) were anti-mouse CD11c (clone HL3), anti-mouse CD45 (clone 30-F11) and anti-mouse CD11b (clone M1/70). Cy5 conjugated anti-rat IgG (Zymed Laboratories, San Francisco, California, USA) and goat anti-hamster IgG (PharMingen) were used as secondary antibodies at a dilution of 1:200 for 20 min.

TEM and SEM

The eyes were fixed in 1% glutaraldehyde and 1% paraformaldehyde in PBS ($n = 3$, bone marrow and HSC), postfixed in veronal acetate buffer osmium tetroxide (2%) and embedded in Epon. The specimens were observed with a JEM 100CX TEM (Jeol, Tokyo, Japan).^{33, 34} For scanning electron microscopy (SEM), the eyes were placed in PBS buffered trypsin solution at

Figure 2 Fluorescent biomicroscopic images of bone marrow transplanted mouse eyes 2 weeks after transplantation. Green fluorescent protein (GFP) positive donor derived cells accumulated in the limbal zone, the boarder between the sclera and cornea (A, B). In the posterior segment, a small number of GFP positive cells were noted in the sclera, especially around the optic nerve head (C, D). Original magnification A $\times 3$; B $\times 8$; C $\times 6$; D $\times 10$.

37°C for 30 min followed by fixation ($n = 3$, bone marrow and HSC), post-fixed, saturated in t-butyl alcohol and processed for critical point drying (Eiko, Tokyo). The tissue was sputtered with Au by argon plasma coater (Eiko). The scleral surface was studied by a JEM 840 SEM (Jeol).^{25 35}

Experimental autoimmune uveitis

EAU was induced in C57BL/6J mice by immunising with human IRBP peptide 1–20 sequence (GPTHLFQPSLVLDMAKVLLD).^{36–38} One month after transplantation of bone marrow or HSC, mice were immunised subcutaneously in one footpad and the base of the tail with the peptide in 0.2 ml of emulsion in complete Freund's adjuvant (1/1, v/v), supplemented with *Mycobacterium tubercu-*

losis strain H37RA to 2.5 mg/ml, and then inoculated intraperitoneally with 1.5 μ g of pertussis toxin ($n = 4$ bone marrow and HSC).

Fundusoscopic examination of mice was performed after 9 days and the presence of uveitis was confirmed. As the autoimmune inflammation peaks 2 weeks after immunisation,³⁸ the eyes were harvested on day 15 ($n = 2$ bone marrow and HSC) and day 19 ($n = 2$ bone marrow and HSC) and then processed for fluorescent micrography and TEM analysis.

RESULTS

Migration of bone marrow and HSC cells into the sclera

Immediately after bone marrow transplantation, there were no GFP positive cells in the recipient mouse sclera. Within 2 weeks

Figure 3 Fluorescent biomicroscopic images of haematopoietic stem cell transplanted mouse eyes 1 month after transplantation. Green fluorescent protein (GFP) positive donor cells were noted throughout the sclera. GFP positive cells accumulated in the limbal and peripapillary zone. Original magnification A $\times 3$; B $\times 8$; C $\times 4$; D $\times 10$.

Figure 4 Fluorescent micrographs of green fluorescent protein (GFP) labelled bone marrow cells and haematopoietic stem cells (HSC) in the sclera. Technovit embedded sections clearly showed the distribution of GFP positive cells (green) in bone marrow transplanted mice (A limbal zone; B equatorial zone; C peripapillary zone) and in HSC transplanted mice (D limbal zone; E equatorial zone; F peripapillary zone). Schematic diagrams show the zone of the sclera (A–C, upper right). GFP labelled cells were spindle shaped and had elongated ramified slender cytoplasmic processes. GFP positive cells were noted in vessels and choroid. Original magnification $\times 400$.

after transplantation, some GFP positive donor derived cells appeared in the limbal zone, the border between the sclera and cornea (fig 2A, B). GFP positive cells gradually increased in the limbal zone following transplantation. In the posterior segment, a few GFP positive cells were noted in the sclera, especially around the optic nerve head (fig 2C, D). In the HSC transplanted mice, a large number of GFP positive cells were noted throughout the entire sclera 1 month after transplantation (fig 3A), particularly in the limbal zone (fig 3B) and

peripapillary zone (fig 3C). GFP positive cells were also observed in the cornea^{10 11} and in the optic nerve.

Distribution of bone marrow and HSC in the sclera

In cross sections 1 month after transplantation, GFP positive cells were distributed in the dense collagen fibres and the GFP+ cells/PI+ total cells ratio was 22.4 (2.8)% in the limbal zone (fig 4A), 14.1 (3.3)% in the equatorial zone (fig 4B) and 18.1 (6.7)% in the peripapillary zone (fig 4C). The ratio of GFP

Figure 5 Representative images of immunophenotypic analysis of bone marrow cells. Immunohistochemistry demonstrates CD45 positive cells (green) in the sclera, in the limbal zone (A) and equatorial zone (B). Green fluorescent protein positive cells (green) were mainly CD11b positive macrophages (C, arrows, red) and CD11c positive dendritic cells (D, arrowheads, red). Original magnification A $\times 200$; B–D $\times 400$.

Table 1 Immunohistochemistry results of the cellular surface markers CD45, CD11b and CD11c

	Limbal zone		Equatorial zone		Peripapillary zone	
CD45	Normal	22.0 (5.0)	Normal	18.0 (4.9)	Normal	16.8 (4.7)
	BM	21.4 (7.5)	BM	12.7 (2.4)	BM	17.0 (7.4)
	HSC	22.3 (9.5)	HSC	18.6 (7.5)	HSC	17.2 (2.5)
CD11b	Normal	11.7 (3.1)	Normal	9.5 (1.8)	Normal	8.1 (1.9)
	BM	8.7 (3.3)	BM	5.2 (2.5)	BM	7.6 (1.5)
	HSC	10.4 (1.9)	HSC	10.4 (2.7)	HSC	9.9 (5.3)
CD11c	Normal	7.0 (2.5)	Normal	5.4 (0.8)	Normal	5.5 (2.9)
	BM	5.2 (2.0)	BM	2.7 (2.8)	BM	3.3 (1.4)
	HSC	8.7 (2.5)	HSC	7.5 (2.6)	HSC	8.3 (4.6)

BM, bone marrow cells; HSC, haematopoietic stem cells.

The ratio of marker positive cells/total cells was generated and expressed as mean (SD). The whole sclera was divided into three equal zones, a limbal zone, equatorial zone and peripapillary zone.

positive cells was higher in both the limbal and the peripapillary zones compared with that in the equatorial zone. GFP positive cells were also found in large numbers in choroidal tissue and vessels (fig 4B). In HSC transplanted mice, the distribution of HSC derived GFP cells in the sclera was similar to that of bone marrow transplanted mice. GFP positive cells comprised 28.4 (10.9)% in the limbal zone (fig 4D), 25.1 (9.7)% in the equatorial zone (fig 4E) and 26.3 (3.7)% in the peripapillary zone (fig 4F) of the total cells counted.

Immunophenotypic analysis of bone marrow and HSC derived cells

To characterise bone marrow derived GFP positive cells in scleral tissue, we performed immunohistochemistry for leucocyte markers (myeloid cell marker; CD45, macrophage marker; and CD11b, dendritic cell marker; CD11c). In bone marrow transplanted mice, GFP positive cells were also CD45 positive in the sclera. CD45 positive cells have round to slender shape in the limbal zone (fig 5A) and peripapillary zone, and linear shape in the equatorial zone (fig 5B). In the limbal zone, GFP positive cells were CD11b positive macrophages (38.2 (10.3)% of GFP positive cells, fig 5C) and CD11c positive dendritic cells (23.6 (10.4)%). In the peripapillary zone, GFP positive cells were positive for CD11b (44.0 (10.2)%), and CD11c (20.4

(10.5)%, fig 5D). In HSC transplanted mice, immunophenotypic analysis revealed CD11b (41.2 (18.3)%) and CD11c (37.5 (28.3)%) in the limbal zone, and CD11b (38.8 (24.1)%) and CD11c (33.2 (21.2)%) in the peripapillary zone, respectively. The results of immunohistochemistry of the cellular markers (CD45, CD11b, CD11c) are listed in table 1. Immunohistochemistry of normal eyes also showed a similar population of CD45, CD11b and CD11c positive cells in the sclera. We thus believe transplantation itself maintained normal conditions at the sclera and did not cause unexpected inflammation. Negative control sections, incubated with normal rat and hamster IgG, and without a primary antibody, exhibited no discernible specific immunoreactivity over the entire region.

TEM and SEM

TEM demonstrated a typical fibrous connective tissue in the sclera, predominantly consisting of collagen and fibroblasts. TEM revealed that the scleral cells were divided broadly into two categories. One category was scleral fibroblasts which resided between the collagen fibre bundle lamellae and showed flattened spindle shapes and ramified cytoplasmic processes in contact with neighbouring fibroblasts (fig 6A, B, arrows). The other category was cells with active organella rich cytosols, exhibited no discernible specific immunoreactivity over the entire region.

Figure 6 Transmission and scanning electron microscopy of the scleral cells. The sclera was composed of fibrous collagen bundle layers. The ultrastructural studies revealed that the scleral cells were divided broadly into two categories. The first category was the scleral fibroblast, typically demonstrated as having a flattened spindle shape, translucent cytosol, peripherally condensed chromatin in the large elongated nucleus and ramified cytoplasmic processes contacting neighbouring fibroblasts (A, peripapillary zone; B, limbal zone, arrows). The other cell type had active organella rich cytosol with indented nucleus with condensed chromatin (A and B, arrowheads). In scanning electron microscopy, the tryptic digestion clearly demonstrated the scleral cells residing in the collagen fibre bundle lamellae. The scleral fibroblasts demonstrated a flattened and polygonal cellular shape, contacting each other by slender cytoplasmic processes (C, arrows). In contrast, the other cell type had a more irregular cellular shape, and abundant ramified cytoplasmic processes (D, arrowheads). Original magnification A–C $\times 3300$; D $\times 4000$.

Figure 7 Fluorescent and transmission electron micrographs of bone marrow derived cells in experimental autoimmune uveitis (EAU). Green fluorescent protein positive cells infiltrated abundantly into the retina, choroid and vitreous, especially around the retinal vessels (A), in the sclera (B, equatorial zone; C, peripapillary zone). Transmission electron microscopy demonstrated the myeloid lineage cells (arrows) and fibroblasts (arrowheads) in sclera after induction of EAU (D). The burst infiltration of bone marrow derived cells in the sclera was observed especially in the peripapillary zone. Original magnification A×200; B, C 400; D×3300.

smooth endoplasmic reticulum, multivesicular body-like vacuoles and indented nuclei with condensed chromatin, a pattern typical of myeloid lineage cells.

In SEM, the scleral fibroblasts had flattened and polygonal cellular shapes, connecting via slender cytoplasmic processes with one another (fig 6C, arrows). In contrast, the cells of the second category had more irregular shapes, and abundant ramified cytoplasmic processes (fig 6D, arrowheads).

Experimental autoimmune uveitis

EAU was also assessed by histopathology, 15 and 19 days after immunisation. In bone marrow transplanted mice, GFP positive cells infiltrated abundantly into the retina, choroid and vitreous, especially around the retinal vessels on day 15 (fig 7A). GFP positive cells infiltrated the sclera mainly in the equatorial zone (fig 7B) and peripapillary zone (fig 7C, arrows). In the HSC transplanted mice, GFP positive cells were also found in the sclera around the optic nerve. TEM demonstrated abundant infiltration of bone marrow cells in the retina, choroid and vitreous, especially around the retinal vessels. TEM also revealed massive infiltration of bone marrow derived cells in the sclera (fig 7D, arrows).

DISCUSSION

Despite the important functions of the sclera for preservation of ocular shape and serving as a functional barrier, little is known about bone marrow derived cells in the sclera. In this study we demonstrated that bone marrow derived APCs turned over in the normal sclera and a large number of bone marrow derived cells were recruited during EAU, contributing to the ocular immune response. To our knowledge, this is the first quantitative study investigating bone marrow derived APC migration into the sclera under normal and inflammatory conditions.

In our immunological studies, we demonstrated that most of bone marrow and HSC derived GFP positive cells expressed the leucocyte common antigen CD45 and some of them also developed cell surface markers for APCs (CD11b, CD11c). Our ultrastructural studies showed two types of scleral cells, one is

the scleral fibroblasts similar to previous reports in the cornea^{18 39 40} and the other cells showing macrophage/dendritic cell-like characters (figs 1B, 1C, 6A, 6B), as described in the cornea.⁹ Taken together, these results indicate that the grafted bone marrow and HSC cells differentiated into mature dendritic cells or macrophages, and distributed in the sclera as resident APCs in the normal sclera.

In the present study, we grafted GFP labelled bone marrow and HSC and found that both cell types migrated in the sclera in a similar time course and distribution. We showed that bone marrow derived cells could differentiate into APC lineage cells, namely the resident macrophages and dendritic cells in the sclera. Our immunological experiments suggest that transplanted HSC first home to bone marrow and engraft in the recipient mice, and then provide mature bone marrow derived cells in the sclera. HSC transplanted mice showed a relatively higher rate of GFP positive cells than bone marrow transplanted mice but the difference was not significant. Further studies are needed to clarify the differentiation of these two cell sources.

In our study, GFP positive cells infiltrated the sclera 2 weeks after transplantation in the limbal and peripapillary zones. The transplanted GFP positive cells migrated into the sclera from 2 weeks and accounted for 14~22% and 25~28% of sclera cells after 1 month in both bone marrow and HSC transplantation, respectively. These data suggest that these cells infiltrated from limbal vessels and optic nerve vessels and then migrated into the equatorial zone. It means that transplanted cells replaced host cells and turned over. Previously, we also reported turnover of corneal cells¹⁰ and hyalocyte²⁴ with our bone marrow transplantation models.

In our EAU experiments, bone marrow and HSC transplanted mice demonstrated vast cellular infiltration not only in the retina, choroid and vitreous but also in the sclera. As the EAU immunisation was obtained with the retinal antigen, namely IRBP, the cells were expected to infiltrate the retina. In the time course of the GFP positive cell infiltration, GFP positive cells developed in the sclera on day 15 at the same time as the choroid. EAU immunisation causes inflammation not merely at

the retina/choroids but also influences the sclera. Bone marrow derived cells might play some role in augmenting and/or maintaining experimental uveitis. These results may further our understanding of scleral pathology, such as during uveitis and scleritis.^{20–23 41}

In conclusion, we presented direct evidence for migration of GFP labelled bone marrow and HSC derived cells into the sclera, differentiating mainly in dendritic cells and macrophages. The sclera has a constitutive population of bone marrow derived cells under normal condition and vast cellular infiltration in the experimental pathological processes of EAU.

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