nonhematopoietic cells was confirmed by several other reports (2–6). Regenerative medicine has currently been accepted as a novel potential therapy in the 21st century (7–9).

Among gastrointestinal disorders, Crohn's disease and ulcerative colitis have necessitated novel therapeutic modalities (10, 11). These inflammatory bowel disorders (IBD) are frequently refractory to various medications, and the patients must restrict their diets. Disturbance of immunological homeostasis due to altered bacterial flora in the gastrointestinal tract may be a part of the potential etiologies for these disorders (12–14). To explore the future therapy for IBD, we analyzed the capacity of human BM- and cord blood (CB)-derived progenitor cells to generate gastrointestinal epithelial cells both in clinical and experimental transplantation settings. In the clinical transplantation setting, three sources for hematopoietic stem/progenitor cells, BM, CB, and mobilized peripheral blood (MPB) were analyzed for their capacity to generate donor-derived epithelial cells. Gastrointestinal specimens derived from pediatric and juvenile recipients of allogeneic sex-mismatched transplantation were analyzed for the presence of donor-derived epithelial cells by performing fluorescence in situ hybridization (FISH) analysis and immunofluorescent studies on the same specimens. The analysis of pediatric and juvenile recipients in the present study excluded the possibility of microchimerism reminiscent of fetal-derived cells. We further examined the potential of "purified" human CB- and BM-derived progenitor cells in a xenogeneic transplantation assay.

Recently, we have developed a novel transplantation assay (15) using newborn NOD/LtSz-*Prkdc*^{scid}/*Prkdc*^{scid}β2*m*^{null} (NOD/SCID/β2M^{null}) mice as recipients in order to characterize the phenotype of human hematopoietic stem cells (16). Since the immature environment of newborn recipients might be tolerant for engraftment of xenogeneic human cells, we applied the xenotransplantation assay to examine the generation of human epithelial cells in the gastrointestinal tract. In the xenotransplantation assay, human CB- and BM-derived CD34$^+$ cells gave rise to epithelial cells in murine gastrointestinal tissues. In addition to quantitative analysis of the incidence of human epithelial cells, xenogeneic transplantation enabled us to examine the possibility of cell fusion as a mechanism for the generation of donor-derived epithelial cells by double FISH analyses using probes specific for murine and human chromosomes. Our data in both clinical and experimental transplantation studies suggest that human hematopoietic tissues contain progenitor cells that can possess the capacity to generate gastrointestinal epithelial cells. The multipotential capacity of human CD34$^+$ cells described in the study would encourage hematopoietic stem cell transplantation to be considered as one of the therapies for intractable bowel disorders in the future.

MATERIALS AND METHODS

Sex-mismatched transplantation

We analyzed 11 clinical specimens derived from 8 female recipients, who underwent hematopoietic stem cell transplantation for hematological disorders (5 leukemias, 2 myelodysplastic disorders, and 1 Epstein-Barr virus infection) from 1998 to 2003. The sources of stem cells were BM for 5 patients, CB for 2 patients, and mobilized peripheral blood for 1 patient. Five out of 8 patients were treated with irradiation-combined regimen before stem cell transplantation. The patients exhibited gastrointestinal dysfunction at 26–202 days posttransplantation, and gastrointestinal fiberscopy was performed for the diagnosis of graft-vs.-

host disease (GVHD). A part of a formalin-fixed specimen was subjected to FISH analyses using a human Y chromosome probe (Vysis, Abbott Park, IL) and immunostaining with anti-cytokeratin antibody (Sigma, St. Louis, MO) for the analysis of donor-derived epithelial cells. All specimens were studied only after informed consent was obtained.

Mice

NOD/LtSz-*Prkdc*^{scid}/*Prkdc*^{scid}*B2m*^{null} (NOD/SCID/β2M^{null}) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained under defined flora with irradiated food and acidified water at the animal facility of Kyushu University. All the experiments were performed according to the guidelines established by the Institutional Animal Committee and Institutional Review Board of Kyushu University.

Experimental xenogeneic transplantation

For the xenogeneic transplantation assay, human CB and BM mononuclear cells (MNCs) were purchased from Cambrex (Baltimore, MD). CB and BM MNCs were depleted of T cells by using mouse anti-human CD3, CD4, and CD8 antibodies (BD Immunocytometry, San Jose, CA). To isolate CD34$^+$ subpopulations, anti-human CD34 microbeads (Miltenyi Biotec, Germany) were used according to the manufacturer's protocol. CD3$^-$CD4$^-$CD8$^-$CD34$^+$ cells (1×10^5) derived from CB MNCs were injected i.v. into 6 newborn NOD/SCID/β2M^{null} mice (within 2 days of birth) after conditioning with 100 cGy total body irradiation. Similarly, 1×10^5 CD3$^-$CD4$^-$CD8$^-$CD34$^+$ cells derived from BM MNCs were injected i.v. into 4 newborn NOD/SCID/β2M^{null} mice. In either CB or BM transplantation, the purity of the isolated CD34$^+$ cells was higher than 92%.

Analysis of hematopoietic chimerism

At 3 months after xenogeneic transplantation, peripheral blood was harvested from the retro-orbital plexus, and BM cells were harvested from the femurs and tibias of recipient mice. Chimerisms of donor-derived cells in peripheral blood and BM were determined by the expression of human CD45 in the xenogeneic assay using a FACSCalibur (Becton Dickinson, San Jose, CA). To analyze the lineage expression of donor-derived cells, we stained peripheral blood cells or BM cells with PE-conjugated mouse anti-human CD34, CD33, CD19, and CD3 monoclonal antibodies (BD Immunocytometry, San Jose, CA).

Histological analysis

After the hematological chimerism was determined, the gastrointestinal tissues derived from 6 recipient mice transplanted with human CB and 4 recipient mice transplanted with human BM were analyzed. Gastrointestinal tissues were fixed in 4% paraformaldehyde for 30 min at room temperature. Fixed tissue was dehydrated with graded alcohol, embedded in paraffin, and sections (4–10 μm) were prepared. To identify intestinal lymphocytes, we stained sections with mouse anti-human CD45 antibody (DAKO, Carpinteria, CA). To distinguish epithelial cells from hematopoietic cells, we used anti-human cytokeratin antibody (Sigma). Each section was incubated with primary antibody for 1 h at room temperature following blocking with 5% normal donkey serum. After washing, sections were incubated with Cy3-conjugated donkey anti-mouse

IgG (Jackson Immunoresearch, West Grove, PA). In xenogeneic or allogeneic transplantations, donor-derived cells were identified with FISH analyses, using a human X or a human Y chromosome probe (Vysis, Abbott Park, IL). A FITC-conjugated murine centromere probe (Cambio, UK) was used to identify the murine cells. Specimens subjected to immunostaining and/or FISH analyses were examined with laser-scanning confocal microscopy (LSM510Meta, Carl Zeiss, Germany). To remove any nonspecific signals in FISH analyses, only the signals with emission wavelength between 515 and 540 for Spectrum Green and between 575 and 590 for Spectrum Orange were identified as positive by confocal microscopy. In addition, to confirm that the positive signal for the FISH probe was located inside of nuclei and to rule out the possibility of cell overlay, serial X-Y images were obtained to reconstruct X-Z image. For detailed morphological analyses, differential interference contrast (DIC) images were obtained. For negative controls, intestinal tissues derived from nontransplanted mice were subjected to FISH analysis using a probe for human X chromosome in xenogeneic transplantation settings. In the allogeneic/clinical setting, intestinal tissues derived from sex-matched female recipients were analyzed with probe for human Y chromosome as a negative control. When the cells exhibited both morphological and phenotypic (cytokeratin-positive) characteristics compatible with epithelial cells, the cells were judged as epithelial cells.

RESULTS

Generation of gastrointestinal epithelial cells in human allogeneic transplantation

We analyzed the clinical cases of CB, BM, and MPB transplantations to examine whether each hematopoietic cell source contained progenitor cells capable of generating epithelial cells in the gastrointestinal tract of allogeneic recipients. After donor-type hematological chimerism was confirmed, we performed FISH analyses using a Y chromosome probe on gastrointestinal specimens derived from female recipients, who underwent hematopoietic cell transplantation from male donors. When intestinal tissue derived from sex-matched female recipient was subjected to FISH analysis using Y chromosome, no signals were obtained (data not shown). First, to answer the criticism of cell-overlay in previous studies using FISH analyses, we performed detailed analysis on signals for human Y chromosome probe with laser-scanning confocal microscopy (Fig. 1). Nomarsky imaging showed the morphology of donor-derived cells and the proper incorporation of donor-derived cells into the sequence of epithelial cells in villi (Fig. 1*A–E*). To clarify the localization of signal for chromosome inside or outside of the nucleus, we obtained the 12 serial images from different levesl at 0.4 μm intervals (Fig. 1*F*). X-Z imaging, reconstructed from serial 12 X-Y images, demonstrated that the FITC-labeled signal for the human Y chromosome was localized at the same level of the nucleus (Fig. 1*G*).

To further identify the type of donor-derived cells, we performed FISH analysis and immunostaining on the same specimen, not using serial sections of the specimen. We obtained four types of information from each specimen: the nature of nuclei, contour of the cells, antigen expression, and donor or recipient origin. As negative staining for CD45 could not determine the cells as epithelial cells, expression of cytokeratin was examined by immunostaining along with FISH analysis (Fig. 2*A–C*). Two of the four Y chromosome$^+$ cells were positively stained with anti-cytokeratin antibody, whereas the other two cells were negatively stained with anti-cytokeratin antibody (Fig. 2*B*). Nomarsky imaging of the specimen provided the information of the contour of the donor-derived cells and the sequence of epithelial cells (Fig. 2*C*). The

complete analysis using FISH and immunostaining for different portions of specimens demonstrated that the incidence of donor-derived epithelial cells was between 0.4% and 1.9%, as shown in Table 1. In the present study, using specimens derived from pediatric and juvenile recipients without the experience of pregnancy, fetal-derived microchimerism cannot be a reason for the presence of Y chromosome$^+$ cells. The three sources of stem cells, CB, BM, and MPB, contained the stem/progenitor cells, which could give rise to epithelial cells in allogeneic recipients.

Generation of gastrointestinal epithelial cells by human CB- and BM-derived CD34$^+$ cells in NOD/SCID/β2M^{null} mice

Next, we examined the capacity of "purified" human progenitor cells in an experimental transplantation setting. We transplanted 1×10^5 human CB- and BM-derived CD34$^+$ progenitor cells into newborn NOD/SCID/β2M^{null} mice, which exhibited extremely low activity of NK cells as well as complete lack of mature B cells and T cells. When newborn NOD/SCID/β2M^{null} recipients reached maturity at 3 months posttransplantation, a high level of engraftment by human CD45$^+$ cells and multilineage reconstitution of human progenitor cells (CD34$^+$), myeloid-lineage cells (CD33$^+$), B-lineage cells (CD19$^+$), and T-lineage cells (CD3$^+$) were observed in xenogeneic BM (Fig. 3). No cytokines were administered to recipients in order to heighten hematological chimerism or to foster the differentiation of epithelial cells. A high level of hematopoietic engraftment by human cells is considered to be essential to support the generation of hematopoietic tissue-derived epithelial cells.

Similar to the analysis of clinical specimens, the gastrointestinal tissues of recipient mice were analyzed for the presence of human cells by performing FISH analysis and immunofluorescent studies on the same specimens. The incidence of human CD34$^+$ cell-derived epithelial cells in xenogeneic intestinal or gastric tissue was 0.23%–0.58% in CB recipients and 0.15%–0.30% in BM recipients (Table 2). When we examined the human cells in mesenteric lymphoid nodes, the vast majority of these cells were dually positive for human X chromosome and human CD45 (Fig. 4*A*), showing that human CD34$^+$ progenitor cells could effectively reconstitute lymphoid tissues of the recipient intestine. No cytokeratin$^+$ epithelial cells were stained with anti-human CD45 antibody. Furthermore, we examined the possibility of cell fusion as a mechanism for the generation of CB- or BM-derived epithelial cells. To this end, we performed double FISH analyses using human and murine chromosome probes and immunostaining for cytokeratin on the same specimen. Nomarsky imaging demonstrated that a human CB-derived cell was identified in organized sequence of epithelial cells in villi (Fig. 4*B*). In Figure 4*C*, a human X chromosome$^+$ cell was positively stained with anti-cytokeratin antibody, suggesting that the human cell was anepithelial cell, not an intestinal lymphocyte.

We tested whether human chromosome$^+$ epithelial cells with donor chromosomes were generated through fusion between donor-derived progenitors and recipient-derived epithelial cells. Although the majority of epithelial cells had murine centromeres, double FISH analyses using species-specific probes demonstrated that the nucleus of the human X chromosome$^+$ cell was not labeled with mouse centromere probe (Fig. 4*D*), indicating that the human chromosome$^+$ epithelial cell was not generated by fusion of human CD34$^+$ cells and murine epithelial cells. X-Z imaging reconstructed from 10 slices of 0.3 μm serial X-Y images confirmed that a dot indicating the presence of human X chromosome was located inside the nucleus of the epithelial

cell (Fig. 4*E*), not the artifact due to cell overlay. Such human X chromosome$^+$ cells were identified both along villi and at the bottom of crypt (Fig. 4*F*), which is a candidate location of intestinal stem cells (17–19). The results of xenogeneic transplantation assay demonstrated that regeneration of epithelial cells along with reconstitution of lymphocytes in recipient intestine was feasible with transplantation of human CD34$^+$ progenitor cells.

DISCUSSION

In the present study, we used clinical and experimental transplantation settings in order to examine the capacity of human hematopoietic progenitor cells to generate gastrointestinal epithelial cells in allogeneic and xenogeneic recipients.

In previous studies using murine hematopoietic stem/progenitor cells, Krause et al. (5) reported with the murine syngeneic transplantation that the incidence of murine BM-derived epithelial cells in small intestine and large intestine was 0.54% and 0.12%, respectively. Jiang et al. (20) suggested that multipotential adult progenitor cells existed in murine BM. Wagers et al. (21) concluded that a single hematopoietic stem cell had little capacity to differentiate into nonhematopoietic lineages. Our experimental xenogeneic transplantation assay enables us to examine whether human progenitor cells enriched by the expression of CD34 possess the capacity to generate epithelial cells. Human CB- and BM-derived CD34$^+$ cells highly enrich hematopoietic stem/progenitor cells as judged by the presence of multilineage progenies in BM of the recipient mice. However, without clonal studies, we cannot exclude the possibility that mesenchymal stem cells or tissue-specific stem/progenitor cells in hematopoietic tissues give rise to mature epithelial cells in the gastrointestinal system.

In terms of technical issues, we successfully performed FISH analysis on clinical and experimental specimens combined with immunofluorescence studies to identify donor chromosome$^+$ cytokeratin$^+$ cells as CB- or BM-derived epithelial cells. The simultaneous FISH and immunofluorescence studies on the same specimens, not using serial sections, enabled the identification of donor-derived epithelial cells accurately. Although FISH analysis has been a helpful tool for analyzing specimens derived from sex-mismatched transplantation, previous studies have been criticized on the basis of analytical points. One of the criticisms of previous FISH studies was that a tiny dot indicating the presence of donor chromosome could be localized at a different level of the nucleus due to cell overlay (22). Both in allogeneic and xenogeneic transplantations, X-Z images, reconstructed from serial X-Y images captured by laser-scanning confocal microscopy, clearly demonstrated that the hybridization signal for human chromosome existed inside the nucleus, which supported the specificity of FISH analyses in the present study. Also, in sex-mismatched transplantations, Bianchi et al. (23) suggested that microchimerism of fetal-derived cells in pregnant females could be an alternative reason for the presence of Y chromosome$^+$ epithelial cells. Our analyses of pediatric and juvenile recipients demonstrated that Y chromosome$^+$ epithelial cells of gastrointestinal tract differentiated from transplanted hematopoietic tissue-derived cells, not reminiscent of fetal-derived cells.

Considering the potential of future therapy, we quantified the incidence of CB- and BM-derived epithelial cells. The incidence of donor-derived epithelial cells was higher in allogeneic transplantation than that in xenogeneic transplantation. The lower incidence in the xenogeneic recipients may be due to major histocompatibility complex disparity between different species,

which can disturb the dynamic differentiation from hematopoietic progenitors to epithelial cells. Also, considering the differentiative capacity of $CD34^+$ cells, the incidence of donor-derived epithelial cells was lower in the xenogeneic environment mirroring the lower level of hematopoietic chimerism in the xenogeneic recipients. CB- and BM-derived epithelial cells were identified in various anatomical sites such as at the bottom of crypt and at villi. The irradiation or chemotherapeutic agents used as conditioning for transplantation could cause mild to moderate intestinal injury. Also, several allogeneic recipients exhibited severe GVHD in the intestine. In analyses of specimens with various degrees of injury, however, the clusters of donor-derived epithelial cells were not identified. If CB or BM cells are considered as a potential source of future therapy, the capacity of CB- or BM-derived stem/progenitor cells should be compared with that of other candidate cell sources, including endodermal progenitor cells (24).

Korbling et al. (25) and Okamoto et al. (26) reported donor-derived epithelial cells in sex-mismatched BM and MPB transplantation. Meignin et al. (27) recently argued that few donor-derived epithelial cells existed in recipients showing varying grades of GVHD. To clarify the controversial results from clinical analyses, we established an experimental xenotransplantation assay, in which purified human hematopoietic progenitor cells could be analyzed for their differentiative capacity. The significant correlation between the dose of $CD34^+$ cells and incidence of donor-derived epithelial cells was not identified in clinical transplantation settings, presumably due to a number of variables (disease, cell source, conditioning, GVHD, and the time intervals to analysis). Xenogeneic transplantations minimize other variables to examine the capacity of $CD34^+$ cells. We identified that human CB- and BM-derived $CD34^+$ cells were capable of generating epithelial cells in vivo, which was not apparent in the transplantation of $CD34^-$ cells. Although the capacities of CB and BM did not differ significantly in the present study, CB could be used as a source for allogeneic and autologous progenitor cells as well as BM in future regenerative medicine.

Molecular mechanisms underlying stem cell plasticity have yet to be clarified. Cell fusion between donor-derived stem cells and mature cells of recipient origin may account for seemingly donor stem cell-derived progeny (28, 29). As hepatocytes and myocytes tend to fuse in physiological condition, the mechanism for gastrointestinal epithelial cells needs to be clarified. Sex-chromosome painting in the FISH analysis of clinical specimens cannot determine the possibility of cell fusion, because thin sections do not include the whole chromosomes. Xenogeneic assays enabled us to evaluate the possibility of cell fusion by using both human chromosome and mouse centromere probes on the same specimens. Double FISH analyses demonstrated that the cells positive for human chromosome and cytokeratin were not labeled with anti-mouse centromere probe. Based on these findings, it is concluded that fusion between stem cells and epithelial cells is not the only mechanism at least in intestine and that plasticity may account for the possibility that the hematopoietic cells give rise to epithelial cells in the gastrointestinal system. Altogether, the results of the present study demonstrated the capacity of purified human $CD34^+$ cells to generate gastrointestinal epithelial cells in clinical and experimental transplantation settings across histocompatibility barriers. The xenotransplantation model can be used to gain insights into the mechanism underlying generation of CB- or BM-derived epithelial cells.

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Table 1

Generation of male-derived epithelial cells in female gastrointestinal tracts[a]

Case	Cell	Age (days)	Diagnosis[b]	Conditioning[c]	$CD34^+$ cells ($\times 10^6$/kg)	Day of biopsy (posttransplant)	GVHD (stage; grade)	Tissue[d]	Incidence of Y^+ epithelial cells
1	BM	7	ALL	TBI/CA/CY	n.e.	d202	gut 1; II	S	34/1902 (1.8%)
2	BM	21	CML	Bu/CY	n.e.	d35	gut 3; III	C	11/2045 (0.5%)
2	BM	21	CML	Bu/CY	n.e.	d39	gut 3; III	D	25/1746 (1.4%)
2	BM	21	CML	Bu/CY	n.e.	d39	gut 3; III	S	24/1863 (1.3%)
2	BM	21	CML	Bu/CY	n.e.	d39	gut 3; III	E	7/1568 (0.4%)
3	BM	15	RAEB	TBI/CA/CY	0.6	d33	gut 1; II	S	11/1648 (0.7%)
4	BM	12	CAEBV	Bu/CY	6.1	d35	gut 1; II	S	29/2341 (1.2%)
5	BM	7	AML	Bu/CY	8.3	d51	None	S	41/2174 (1.9%)
6	CB	7	RAEB-T	TBI/CY	0.27	d36	gut 3; III	I	13/1850 (0.7%)
7	CB	11	ALL	TBI/CA/CY	n.e.	d42	gut1; II	S	9/1512(0.6%)
8	PB	6	NK lymphoma	TBI/Bu/PAM	13.8	d26	gut 1; II	S	27/2011 (1.3%)

[a]The presence of epithelial cells was determined by the positivity of human Y chromosome and the expression of cytokeratin. [b]ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; RAEB, refractory anemia with excess of blast; CAEBV, chronic active EB virus infection; AML, acute myelogenous leukemia; RAEB-T, refractory anemia with excess of blast in transformation; NK lymphoma, Natural Killer cell lymphoma. [c]TBI, total body irradiation; CA, cytosine arabinoside; CY, cyclophosphamide; Bu, busulfan; PAM, melphalan. [d]S, stomach; C, colon; D, duodenum; E, esophagus; I, small intestine.

Table 2

Incidence of human epithelial cells in xenogeneic gastrointestinal tissue[a]

Mouse	Cell	Tissue[b]	Time of Analysis	Human CK$^+$	Total cells	Incidence
1	CB	I	9 w	15	5301	0.28%
1	CB	S	9 w	10	4016	0.25%
2	CB	I	11 w	12	4223	0.24%
2	CB	S	11 w	8	3455	0.23%
3	CB	I	12 w	9	3210	0.28%
3	CB	S	12 w	11	2852	0.39%
4	CB	I	11w	21	4168	0.50%
4	CB	S	11w	18	3101	0.58%
5	CB	I	10w	13	3985	0.33%
5	CB	S	10w	9	2948	0.31%
6	CB	I	10w	17	3056	0.56%
6	CB	S	10w	8	2692	0.30%
7	BM	I	12 w	7	3483	0.20%
8	BM	I	12 w	10	3615	0.30%
8	BM	S	12 w	4	2661	0.15%
9	BM	I	11w	9	3168	0.28%
9	BM	S	11w	6	2549	0.24%
10	BM	I	11w	7	2879	0.24%

[a]Human CB- and BM-derived CD34$^+$ cells were transplanted into newborn NOD/SCID/β2M^{null} mice. At 9–12 wk posttransplantation, recipient gastrointestinal tissues were analyzed for the presence of human chromosome$^+$ cytokeratine$^+$ (CK$^+$) epithelial cells. [b]I, small intestine; S, stomach.

Fig. 1

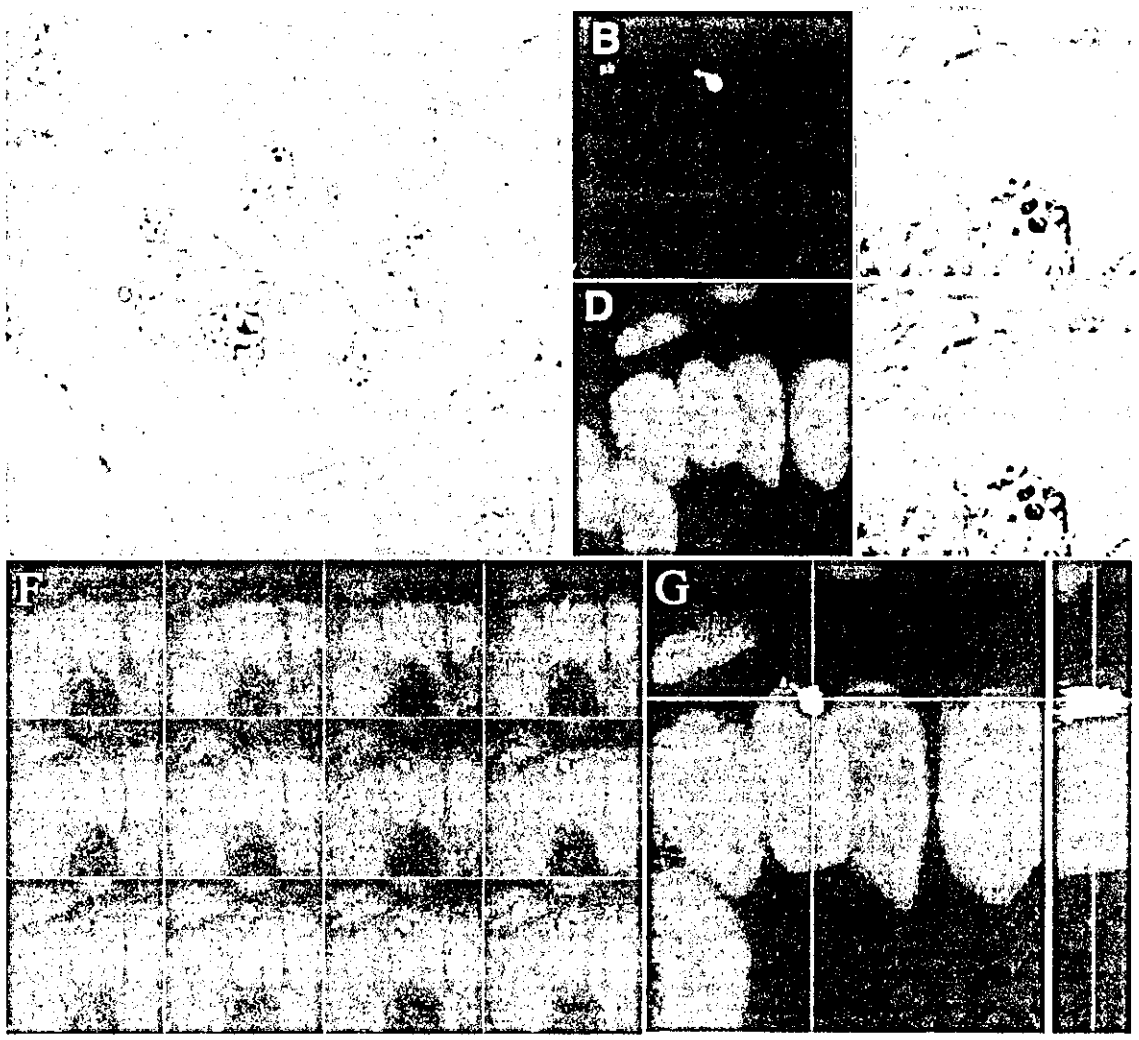

Figure 1. Localization of signal for Y chromosome in the nucleus. In sex-mismatched allogeneic transplantation, donor-derived intestinal epithelial cells were examined with FISH analyses. ***A)*** Low-magnified image showed that a coronal section of crypt contained a donor-derived cell (arrow) identified with the Spectrum Green-conjugated Y chromosome probe. ***B)*** The existence of the male-derived cell was confirmed with Spectrum Green-conjugated Y-chromosome probe in a higher magnified view. ***C)*** Nomarsky imaging revealed the detailed morphology of the cells. ***D)*** Nuclei of the cells were stained with DAPI. ***E)*** Images ***B***, ***C***, and ***D*** merged. ***F)*** The signals for Y chromosome and nuclei staining are shown in 12 serial images obtained from different levels at 0.4 μm interval. ***G)*** The location of signal for human X chromosome was determined at the same level of nucleus by X-Z image.

Fig. 2

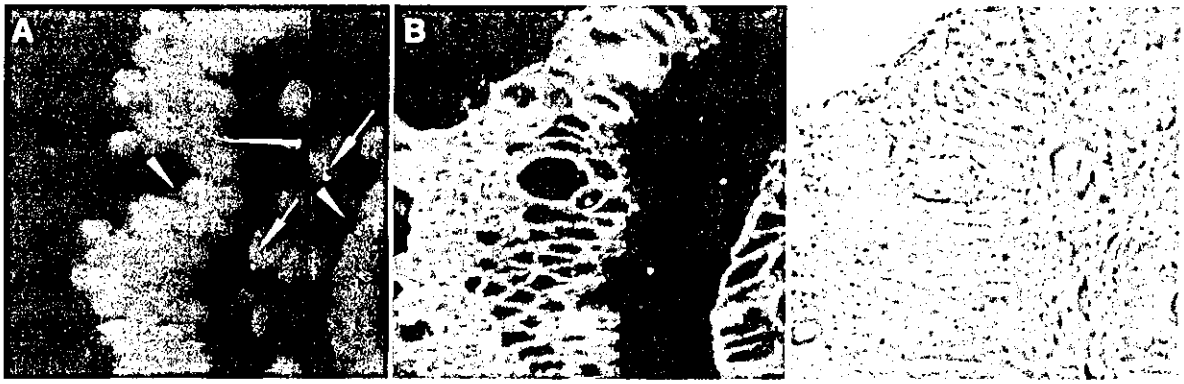

Figure 2. Identification of male-derived epithelial cells in female recipients. FISH analysis and immunostaining were performed on the same specimen for further characterization of male-derived cells. *A)* The specimen was subjected to FISH analysis using Spectrum Green-conjugated Y-chromosome probe. Nuclei of the cells were stained with DAPI. *B)* Two (arrow heads in *A*) of the donor-derived cells were positively stained with anti-cytokeratin antibody, suggesting that these cells were epithelial cells. The other two cells (arrows in *A*) were non-epithelial cells, as judged by negative staining for cytokeratin. *C)* Nomarsky image of the specimen is shown.

Fig. 3

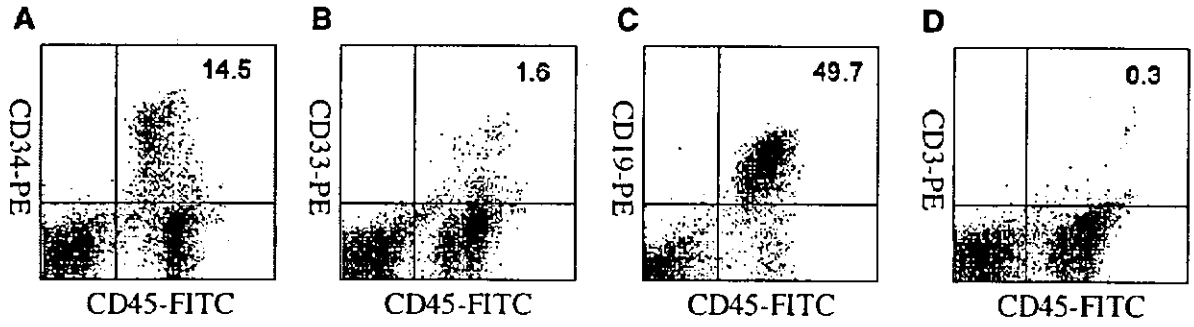

Figure 3. Hematopoietic chimerism in xenogeneic transplantation. Three months after CB-derived $CD34^+$ cells were transplanted into newborn NOD/SCID/$\beta2M^{null}$ mice, BM cells of recipient mice were stained with PE-conjugated anti-human CD34 (*A*), CD33 (*B*), CD19 (*C*), and CD3 (*D*) antibodies along with FITC-conjugated anti-human CD45 antibody. Number indicates the percentage of double-positive cells.

Fig. 4

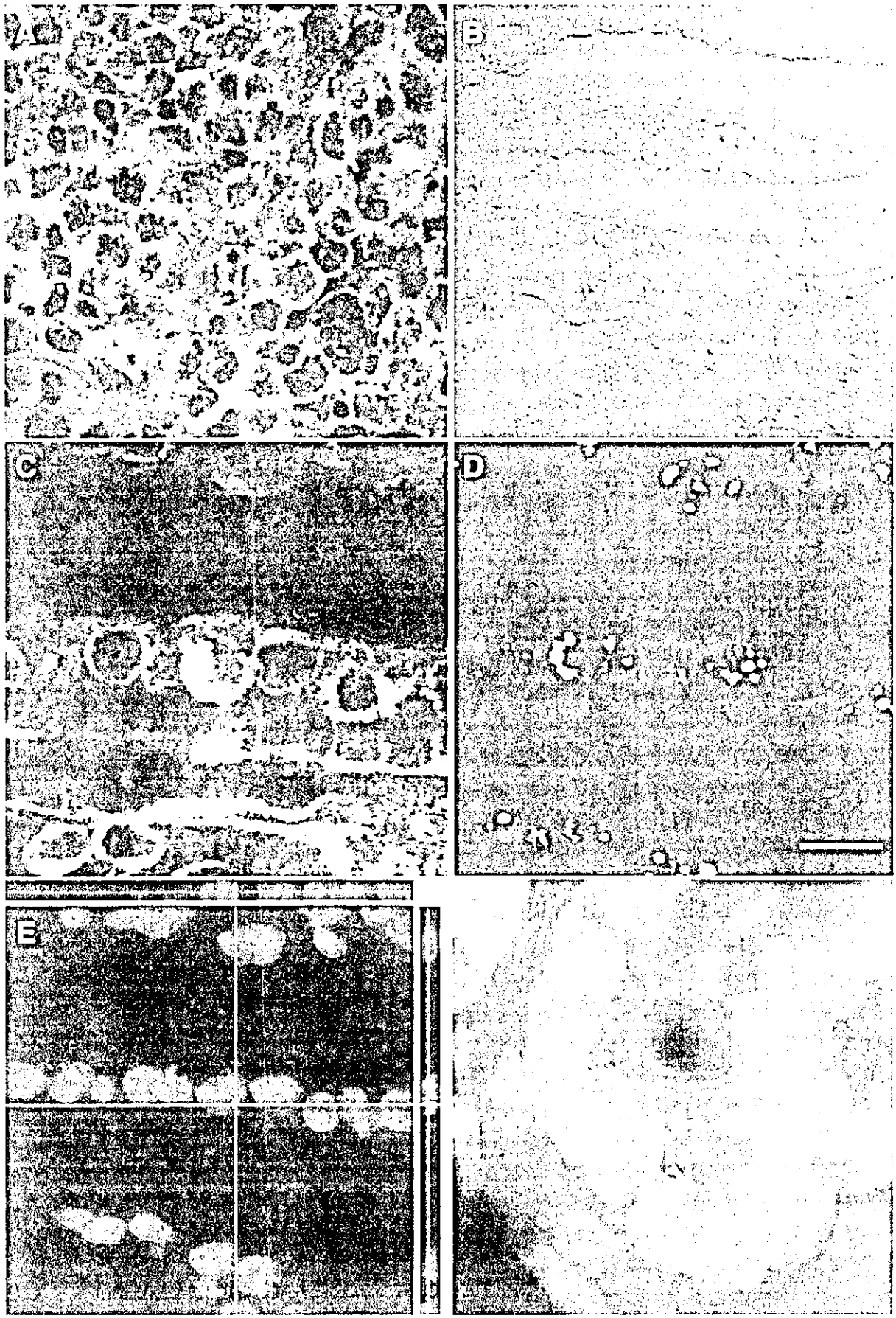

Figure 4. Human X chromosome-positive epithelial cell was not generated by the fusion between human cell and murine epithelial cell. Human CB-derived $CD34^+$ cells were transplanted into newborn NOD/SCID/$\beta 2M^{null}$ recipients. At 3 months posttransplantation, in vivo generation of human intestinal epithelial and lymphoid cells was examined by laser scanning confocal microscopy. ***A)*** The lymphoid tissue derived from a recipient intestine was subjected to immunostaining with anti-human CD45 antibody (green) and FISH analysis with human X chromosome probe (red). The majority of the cells in lymphoid tissue of the recipient mice were human hematopoietic cells as judged by the positivity of human X chromosome and human CD45. ***B–E)*** After FISH analyses and immunofluorescent studies were performed on the same specimen derived from recipient intestine, four-color (FITC, Spectrum Orange, Cy5, DAPI) analyses were executed with laser scanning confocal microscopy along with Nomarsky imaging. ***B)*** Nomarsky image showed that human cell identified with anti-human X chromosome probe (red) was identified in organized sequence of villi. ***C)*** The cell with human X chromosome was confirmed as an epithelial cell by positive staining with Cy5-conjugated anti-cytokeratin antibody. ***D)*** The majority of the cells in the same specimen were murine cells, which were identified with FITC-conjugated mouse pan-centromeric probe. The cell identified with human X chromosome probe did not possess any mouse centromeres in the nucleus. Bar represents 10 μm. ***E)*** The hybridization signal for human X chromosome was located inside nucleus as judged by X-Z image reconstructed from serial X-Y images. ***F)*** Such human cells were also identified at the bottom of crypt.

Characterization and Distribution of Bone Marrow–Derived Cells in Mouse Cornea

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Purpose. Bone marrow (BM)–derived stem cells are thought to possess extensive differentiation capacity. The present study was conducted to investigate the characteristics and distribution of these cells in the normal mouse cornea.

Methods. BM cells and BM-derived hematopoietic stem/progenitor cells (HSCs) from enhanced GFP (eGFP) transgenic mice (lin^-, $Sca\text{-}1^+$) were intravenously transplanted into irradiated wild-type C57BL/6 mice. At 4 to 6 months after transplantation, the mice were killed, and their whole corneas examined by histologic and immunohistochemical methods (CD11c, CD11b, and CD45).

Results. At 2 weeks after BM cell transplantation, GFP^+ cells gradually migrated into the cornea from the limbal area. At 2 to 6 months, they were distributed over the entire cornea. In cross sections of whole cornea, GFP^+ cells comprised 27.3% ± 11.1% (BM) and 24.0% ± 8.01% (HSC) of total cells in the peripheral corneal stroma. In the center of the corneal stroma, GFP^+ cells were 7.58% ± 2.63% (BM) and 8.06% ± 1.76% (HSC) of total cells. Immunohistochemistry showed that GFP^+ $CD11c^+$, $CD11b^+$, $CD11c^-$, and $CD11b^-$ cells occupied the entire corneal stroma.

Conclusions. The present study provides direct evidence of the distribution of BM-derived cells in the mouse cornea. Immunohistochemical study showed that some of these cells are BM-derived antigen-presenting cells such as dendritic cells and macrophages. Some elements of BM-derived cells may continue to exist in the corneal stroma. (*Invest Ophthalmol Vis Sci.* 2005;46:497–503) DOI:10.1167/iovs.04-1154

Adult somatic stem cells have been isolated from several tissue sources including neurons,[1,2] retina,[3] corneal limbal epithelium,[4,5] and bone marrow (BM).[6–8] It had been thought that somatic stem cells preferentially generate differentiated cells of the same lineage as their tissue of origin. However, recent studies suggest that tissue-specific stem cells can differentiate into lineages other than their tissue of origin and that, with respect to the developmental potential of different adult cell types, there is far more plasticity than previously thought. Particular attention has been focused on the plasticity of BM-derived stem cells. They are reported to possess extensive differentiation capacities and can differentiate into several epithelial types such as liver, lung, and skin.[9] Furthermore, BM-derived mesenchymal stem cells can differentiate in vitro not only into mesenchymal cells, but also into cells with visceral mesoderm, neuroectoderm, and endoderm characteristics.[10] These findings suggest that BM-derived stem cells may have the ability to transdifferentiate into a variety of tissues, including those of the eye.

Normal corneal tissue is located in the anterior segment of the eye, and it participates in several major functions. It is the gateway into the eye of visual images and plays a critical role in maintaining corneal transparency and avascularity. It is composed of three layers: the corneal epithelium, stroma, and endothelium. Corneal epithelial stem cells exist in the basal cell layer of the limbal region[4,5] and in the transitional zone between the cornea and conjunctiva. They are supported by the limbal vascular arcade. Little is known about stem cells of the corneal stroma and endothelium, and the origin of these cells is not well understood.

From an immunologic point of view, the normal avascular cornea was thought to be an immune-privileged site without functional antigen-presenting cells (APCs) and largely devoid of BM-derived cells. Therefore, higher success rates would be expected with corneal than other organ transplants. This notion has lost favor since the demonstration of large numbers of resident BM-derived cells of different lineages—for example, macrophages and dendritic cells—in both the epithelium and stroma of the normal cornea.[11–13] Until now, indirect evidence obtained by immunohistochemical studies has shown these cells to be present and important questions, such as the original cell type and the physiological and functional significance of these progenitors, remain unanswered.

We are the first to attempt the characterization and clarification of the distribution of BM-derived cells in the normal mouse cornea. In the current study, we sought to acquire a direct demonstration by transplanting BM cells from enhanced green fluorescence protein (eGFP) transgenic mice using our unique protocol.[14–16] We transplanted GFP-labeled BM cells and hematopoietic stem/progenitor cells (HSCs) into syngeneic C57BL/6 (wild-type) mice and found BM-derived cells distributed in the mouse cornea. We then evaluated the characteristics of these BM-derived cells by immunohistochemical studies.

Materials and Methods

Experimental Animals

The mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental

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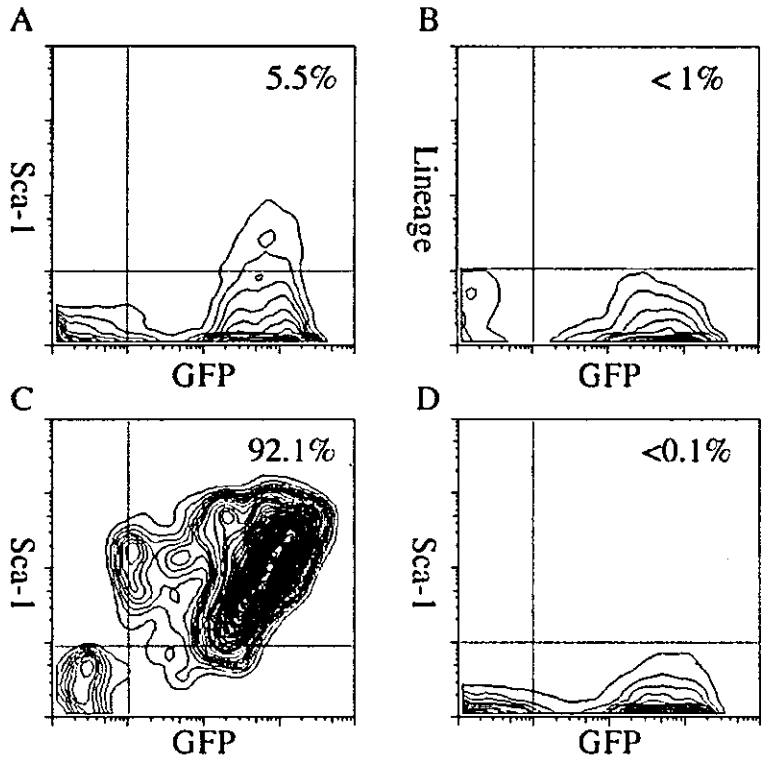

FIGURE 1. Enrichment of hematopoietic stem cells confirmed by flow cytometry. Original BM cells (A). After lineage depletion (B). Positive (C) and negative (D) selection for Sca-1$^+$ cells. After negative and positive selection, the lin$^-$ Sca-1$^+$ cell purity of all GFP$^+$ cells exceeded 95%. Each percentage represents the amount of double-positive cells among all nucleated cells.

procedures were approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine and Kyushu University. Adult (n = 3) and newborn (n = 6) C57BL/6 mice were the recipients of BM cell and HSC transplants, respectively. BM cells were obtained from mice that transgenically express GFP, driven by the chicken β-actin promoter.[17]

Bone Marrow Transplantation

To observe directly the migration of BM-derived cells into the mouse cornea, we used BM cell transplantation.[16] Female eGFP mice (8–10 weeks old) were killed by cervical dislocation while under deep ether anesthesia, and BM cells were obtained by flushing the femurs with sterile phosphate-buffered saline (PBS). The BM cells were washed several times in sterile PBS, filtered twice through a nylon mesh (pore size, 70 μm), counted, and resuspended in PBS at 5×10^7 cells/mL. To generate chimeric mice, all BM cells (6×10^6 to 1×10^7) derived from eGFP transgenic mice were intravenously injected into 8-week-old C57BL/6 recipients that had been lethally x-irradiated with 9 Gy. Their eyes were protected with lead shields to prevent radiation retinopathy. These BM cell transplant recipients were then maintained under special pathogen-free conditions, and successful BM cell transplantation was confirmed by the identification of GFP$^+$ cells in peripheral blood at 2 weeks after transplantation. The corneas of three mice were carefully studied by fluorescence biomicroscopy until 6 months after transplantation. We also used these corneas for histologic and immunohistochemical studies.

Hematopoietic Stem Cell Transplantation

To characterize BM-derived stem/progenitor cells in the mouse cornea, we performed HSC transplantation.[14,15] BM cells were harvested from femurs and tibias of 8- to 12-week-old eGFP mice. Single-cell suspensions of donor cells were prepared by repeated serial passage through a 23-gauge needle. To deplete mature hematopoietic cells, the BM cells were incubated with lineage-specific antibodies (B220, CD3, Gr-1, Mac-1, and TER 119) for 30 minutes 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, NY). Cells not bound to the immunobeads were further purified for Sca-1$^+$ cells. The purity of lineage$^-$ cells was higher than 92% in all experiments. After negative selection of mature hematopoietic and immune cells, positive selection of Sca-1$^+$ cells was performed as just described. After negative and positive selection, the purity of lin$^-$ Sca-1$^+$ cells of all the eGFP$^+$ cells exceeded 95% (Fig. 1).[14,15] To obtain high cell purity, samples were applied twice to columns in each experiment. The resultant 10^4 lin$^-$ Sca-1$^+$ cells were transplanted into C57BL/6 mice within 2 days of their birth. The HSC transplant recipients were maintained under special pathogen-free conditions for 4 weeks. Successful HSC transplantation was confirmed by the identification of GFP$^+$ cells in the peripheral blood at 4 weeks after transplantation. At 4 to 5 months after HSC transplantation, six mice were used for histologic and immunohistochemical studies.

Antibodies

The primary antibodies (all from BD-PharMingen, San Diego, CA) used in this study were purified hamster anti-mouse CD11c (clone HL3), purified rat anti-mouse CD45 (clone 30-F11), and RPE-conjugated rat anti-mouse CD11b (clone M1/70). Secondary antibodies were Cy3-conjugated goat anti-hamster IgG and Cy3-conjugated donkey anti-rat IgG (Vector Laboratories, Inc., Burlingame, CA).

Immunohistochemistry

Immunohistochemical studies of markers for APCs were performed by using a previously reported method[11–13] and a modified version of our method.[18,19] Briefly, freshly excised corneas were fixed for 60 minutes at 4°C in 4% paraformaldehyde, extensively washed with PBS, fast frozen in liquid nitrogen, and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek II; Miles Laboratories, Elkhart, IN).

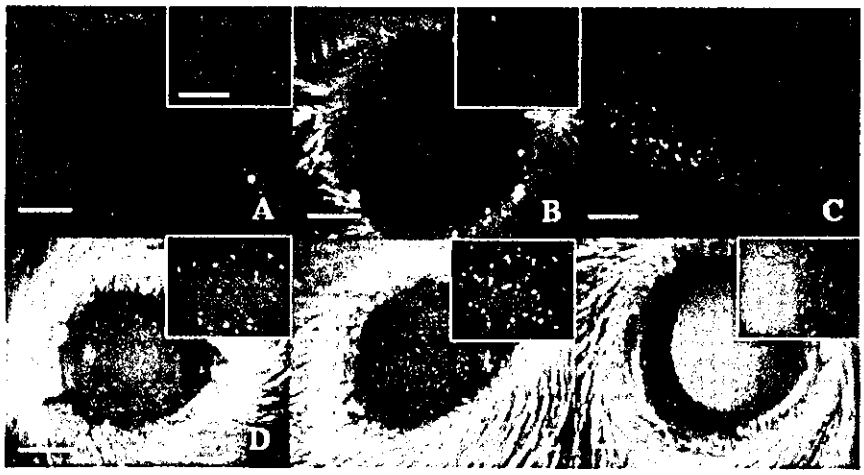

FIGURE 2. Representative time-course slit lamp photographs of murine eyes after BM cell transplantation. (A) One week, (B) 2 weeks, and (C) a high magnification of (B) at the limbal area; (D) 1, (E) 2, and (F) 6 months after transplantation. *Boxes* contain data from the center of the cornea. In the early stages (within the first week) after BM cell transplantation, we observed no GFP^+ cells in the recipient mouse cornea (A). Within 2 weeks, there was intense staining for GFP^+ cells in the periphery of the cornea (B, C). Within 2 months, GFP^+ cells appeared to migrate into the center of the cornea. Their numbers increased in both the periphery and center of the cornea (D, E). Starting at 2 months after BM transplantation, cell density reached a plateau that persisted up to 6 months (F). Scale bars: (A, B, D–F) 1 mm; (C and *insets*) 250 μm.

Cryostat sections (7 μm in thickness) were placed on gelatin-coated slides, air-dried, and rehydrated in PBS at room temperature for 15 minutes. To block nonspecific binding, the tissues were incubated with both anti-Fc receptor mAb (CD16/32; BD PharMingen, San Diego, CA) and 2% bovine serum albumin (BSA) for CD11c and CD11b and with 2% BSA and 10% donkey serum for CD45 at room temperature for 30 minutes. Then the sections were incubated at room temperature for 1 hour with the primary antibody and washed three times in PBS containing 0.15% Triton X-100 (PBST) for 15 minutes. The controls were incubated with the appropriate normal rat and hamster IgG (Dako, Kyoto, Japan) at the same concentration as, but without, the primary antibody. After staining with the primary antibody (CD11c, CD45), the sections were incubated at room temperature for 1 hour with appropriate secondary antibodies, Cy3-conjugated goat anti-hamster IgG, and Cy3-conjugated donkey anti-rat IgG. After several washes with PBS, the sections were coverslipped using antifade mounting medium, with or without propidium iodide (PI; Vectashield; Vector Laboratories) and examined under a confocal microscope (Fluoview; Olympus, Tokyo, Japan).

Quantitative Evaluation

For statistical assessment of corneal cell distribution and characterization, four different fields and six different sections of each cornea were analyzed (24 areas/eye). For analytical purposes, each cornea was divided into central and peripheral areas. The central area was defined as the area within 1 mm of the center and the peripheral area as that within a 1- to 1.5-mm radial distance from the center.

RESULTS

Migration of BM Cells into the Cornea

In the early stages (first week) after BM cell transplantation, there were no GFP^+ cells in the recipient mouse cornea (Fig. 2A). Within 2 weeks of transplantation, some GFP^+ cells appeared in the periphery of the cornea. However, only a small number of GFP^+ cells were present in the center of the cornea (Figs. 2B, 2C). Within 2 months, the number of GFP^+ cells in both the periphery and center of the cornea gradually increased. From 2 months after BM cell transplantation, the cell density reached a relative plateau that persisted up to 6 months (Figs. 2D–F). Our quantitative analysis of GFP^+ cells in the mouse cornea is summarized in Figure 3.

Distribution of BM Cells and HSCs

To determine whether there were BM-derived GFP^+ cells in the recipient cornea, we performed histologic analysis under a dual-channel fluorescence microscope. Cross-sections of recipient corneas showed that most of the GFP^+ cells were distributed in the peripheral corneal stroma and that cell density gradually decreased toward the center (Fig. 4A–D). In the entire corneal epithelium, we noted only a small number of

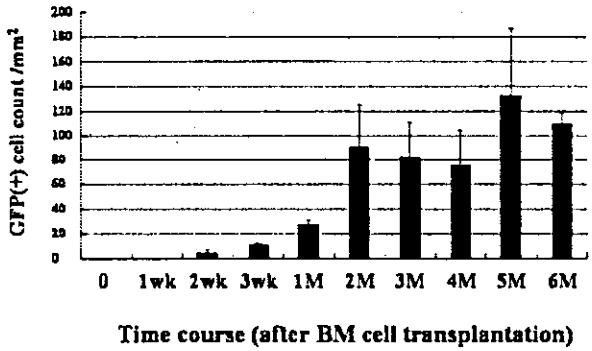

FIGURE 3. Quantitative analysis of GFP^+ cells in the mouse cornea at the indicated times after BM cell transplantation. During the first 2 months, the number of GFP^+ cells gradually increased. Thereafter, cell density reached a relative plateau that persisted up to 6 months after transplantation.

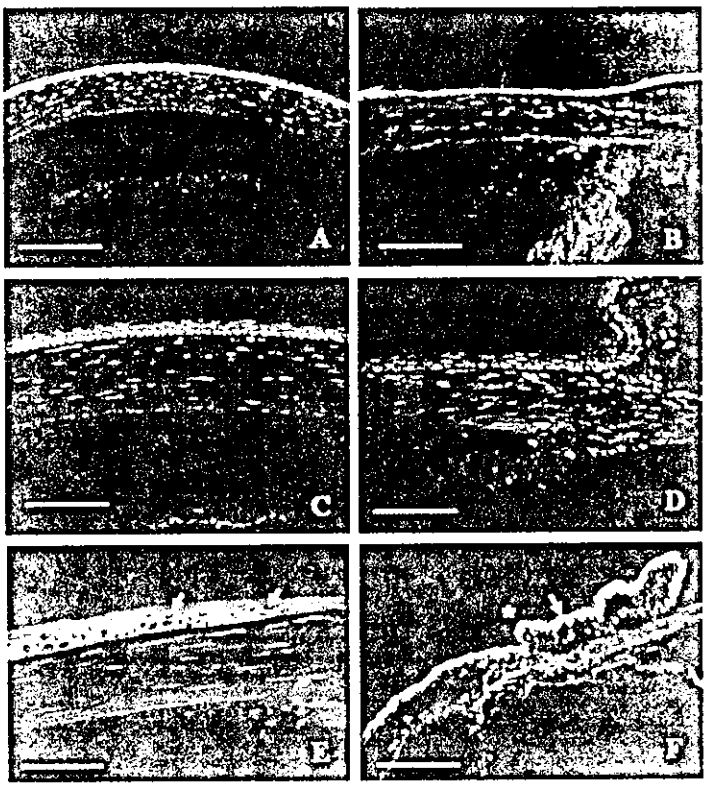

FIGURE 4. Representative cross sections of recipient corneas (A, B, E, BM transplantation; C, D, F, HSC transplantation) show that most of the GFP$^+$ cells were distributed in the peripheral corneal stroma and that the cell density gradually decreases toward the center (A, C, central area; B, D, peripheral area). Only a small number of GFP$^+$ cells were observed throughout the epithelium (E, *arrows*). In contrast, in conjunctival epithelium, several GFP$^+$ cells were noted (F, *arrow*; area to the right of the conjunctiva ✱). Cell nuclei were stained with PI (*red*). Scale bars: (A, B, F) 200 μm; (C, D, E) 100 μm.

these cells (Fig. 4E), whereas in the conjunctival epithelium we observed several GFP$^+$ cells (Fig. 4F). The percentage of GFP$^+$ cells per section was calculated as the number of GFP$^+$ cells divided by the total number of PI$^+$ cells × 100. In the peripheral cornea of mice receiving BM cell transplants, GFP$^+$ cells were 2.03% ± 1.87% (epithelium) and 27.3% ± 11.1% (stroma). At the center of the cornea, they were 0.93% ± 0.65% (epithelium) and 7.58% ± 2.63% (stroma). By contrast, in the peripheral corneas of mice transplanted with HSC, GFP$^+$ cells were 0.78% ± 0.51% (epithelium) and 24.0% ± 8.01% (stroma). At the center of the cornea, they were 0.58% ± 0.4% (epithelium) and 8.06% ± 1.76% (stroma; Fig. 5). The differences between epithelium and stroma in each category were statistically significant (Mann-Whitney test, $P < 0.01$).

Immunohistochemical Analysis

To characterize BM-derived GFP$^+$ cells in corneal tissue, primarily the corneal stroma, we used fluorescence immunohistology with antibodies to the leukocyte markers CD11c, CD11b, and CD45. Negative control sections, incubated with normal rat and hamster IgG but without the primary antibody, exhibited no discernible specific immunoreactivity over the entire region.

CD11c$^+$ or CD11b$^+$ indicate cells coexpressing GFP and CD11c or GFP and CD11b, respectively. The percentage of CD11c$^+$ or CD11b$^+$ cells was calculated by dividing the respective number of cells by the total number of GFP$^+$ cells × 100. In the corneal peripheral stroma of BM cell recipients, we observed 19.4% ± 9.93% CD11c$^+$ cells and 38.7% ± 16.3% CD11b$^+$ cells. In the central stroma, 15.3% ± 8.94% were CD11c$^+$ cells and 48.7% ± 13.1% were CD11b$^+$ cells. In the corneal peripheral stroma of HSC recipients, there were 35.7% ± 14.0% CD11c$^+$ cells and 56.7% ± 22.4% CD11b$^+$ cells. In the central stroma, 41.5% ± 17.8% were CD11c$^+$ cells and 53.7% ± 13.9% were CD11b$^+$ cells (Figs. 6, 7, 8). Most GFP$^+$ cells in the cornea were immunostained with CD45 in both BM- and HSC-recipients (Fig. 9). Asterisks in Figure 8 indicate statistically significant difference between CD11c$^+$ and CD11b$^+$ (Mann-Whitney test; $^*P < 0.01$, $^{**}P < 0.05$).

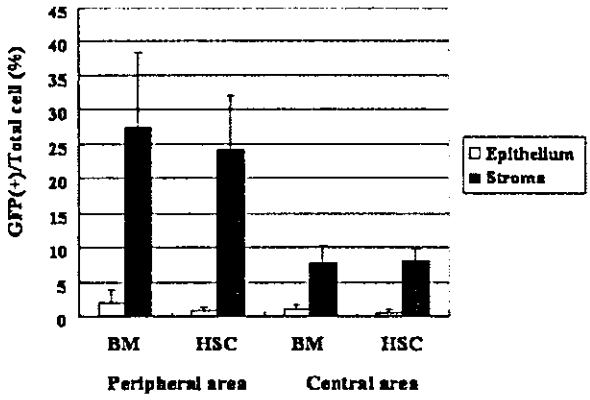

FIGURE 5. Distribution of GFP$^+$ cells in the cornea of mice transplanted with BM cells or HSCs. The percentage of GFP$^+$ cells per section was calculated as the number of GFP$^+$ cells divided by the total number of PI$^+$ cells plus GFP$^+$ cells × 100. Most of the GFP$^+$ cells were distributed in the peripheral corneal stroma. Cell density gradually decreased toward the center. In the entire area covered by epithelium, there were only a few GFP$^+$ cells. The differences between epithelium and stroma in each category were statistically significant (Mann-Whitney test; $P < 0.01$).

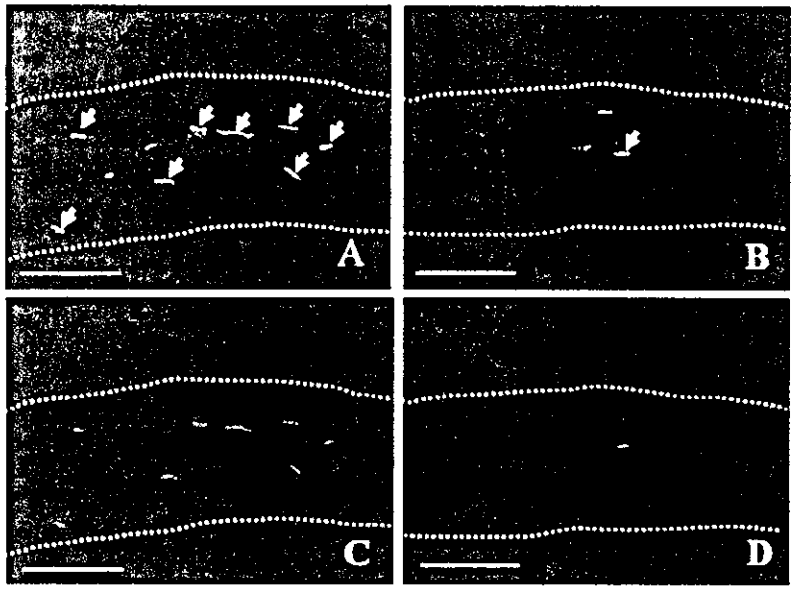

FIGURE 6. Representative immunohistochemical staining for CD11c (*red*) in the peripheral (A, C) and central (B, D) cornea of mice receiving HSC transplants. Some GFP^+ cells (*green*) immunostained with CD11c (*arrows*). Note the presence of GFP^+CD11c^- cells throughout the transplant-recipient cornea. GFP (A, B), CD11c (C, D). *Dotted lines*: perimeter of whole corneal tissue. Scale bars, 100 μm.

DISCUSSION

The cornea is a transparent, avascular tissue, with integrity maintained by various factors derived from the tear film and aqueous humor. Although the normal cornea does not contain vessels, there is indirect immunohistochemical evidence that it is endowed with a significant number of resident BM-derived APCs.[11–13] Hamrah et al.[11] reported that corneal epithelium contains major histocompatibility complex (MHC) class II-negative Langerhans' cells and corneal stroma a large number of resident BM-derived cells of different lineages. These cells were not only macrophages but also $CD11c^+$ dendritic cells. Brissette-Storkus et al.[12] also documented that the normal murine corneal stroma contains a significant number of $CD45^+$ leukocytes and that most of these cells are monocytes or macrophages. However, to date, there has been no direct demonstration of their existence. BM-derived stem cells, such as hematopoietic- and mesenchymal stem cells, have extensive differentiation capacity.[8,9] We considered two possible mechanisms of BM-derived cell differentiation: One is that BM-derived stem cells that have differentiated into APCs such as Langerhans' cells or macrophages migrate into corneal tissue. Alternatively, BM-derived stem cells transdifferentiate into corneal cells such as corneal keratocytes, and function in the cornea. We examined these possibilities using our unique protocol and found that some BM-derived cells were definitely distributed in the cornea. We also determined that these cells are partially of BM-derived APC lineage, a finding that directly confirms the cell origin of

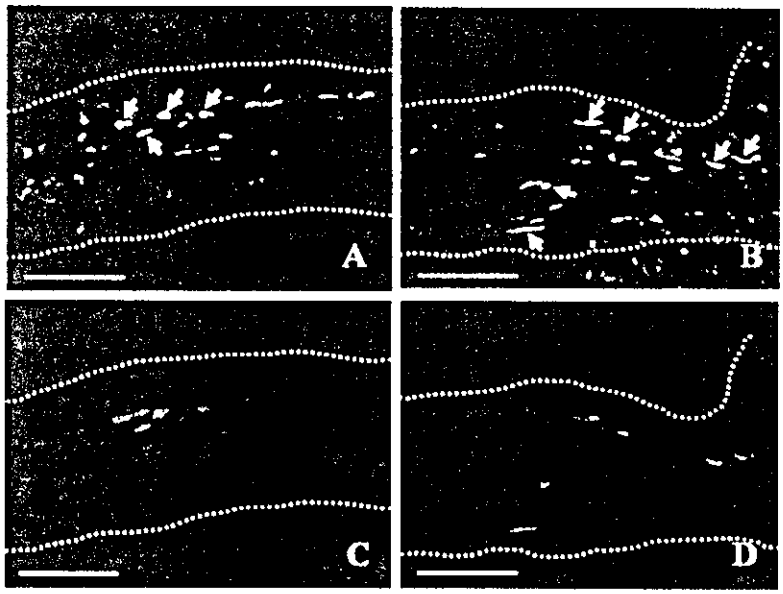

FIGURE 7. Representative immunohistochemical staining for CD11b (*red*) in the cornea of mice receiving BM cell (A, C) or HSC (B, D) transplants. Some GFP^+ cells (*green*) immunostained with CD11b (*arrows*). Note the GFP^+CD11b^- cells dispersed throughout the transplant-recipient cornea. GFP (A, B), CD11b (C, D). *Dotted lines*: whole corneal tissue. Scale bars, 100 μm.