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Bone marrow cells contribute to regeneration of damaged glomerular endothelial cells

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Bone marrow cells contribute to regeneration of damaged glomerular endothelial cells.

Background. There is accumulating evidence that adult bone marrow (BM) cells show unexpected plasticity, and can differentiate into a wide range of specialized cells. In the case of intrinsic renal glomerular cells, BM-derived cells have been reported to differentiate into both mesangial cells and podocytes. However, there is controversy on recruitment of glomerular endothelial cells, although endothelial cells in other tissues are known to be recruited from the BM.

Methods. Sprague-Dawley (SD) rats and SD rats made chimeric by transplantation of bone marrow cells from enhanced green fluorescent protein (EGFP) transgenic littermate rats, were injected with anti-Thy-1.1 antibody, followed by unilateral nephrectomy (1-kidney model). Chimeric rats used in 1-kidney model were sacrificed for histologic examination at weeks 2, 4, 8, and 11. We examined isolated glomeruli and frozen sections of kidneys from rats of each group at weeks 2 and 11 by confocal laser scan microsopy (CLSM), both immunohistologically and three dimensionally.

Results. In the 1-kidney group, using chimeric rats transplanted with EGFP(+) bone marrow cells, most rats died, presumably of uremia, after 8 to 11 weeks. A CLSM study using isolated glomeruli and frozen sections of kidneys revealed that bone marrow-derived PECAM-1(+), RECA-1(+) cells, and OX-7(+) cells contributed to the structural support for the glomerular capillaries during the chronic course. Global glomerular sclerotic lesions and diffuse tubular atrophic changes, with interstitial cell infiltration, were remarkable at weeks 8 and 11.

Conclusion. Bone marrow–derived endothelial progenitor cells participated in glomerular endothelial cell turnover after severe damage. Treatment that could target bone marrow–derived endothelial progenitor cells and promote angiogenesis in regions of progressive glomerular lesions may be a promising therapeutic approach for preventing end-stage renal disease.

Key words: bone marrow–derived endothelial progenitor cells, reconstitution of glomerular endothelial cells, EGFP-transgenic rats, progressive glomerulosclerosis.

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The circulatory system is indispensable for maintaining homeostasis of the interior milieu by supplying the body tissues with blood in doses proportionate to their requirements for oxygen and nutrients, and for removal of waste products, including carbon dioxide, for excretion by the kidneys and lungs. Microvascular endothelial cells are known to play active roles in preserving the fundamental structure of tissues and controlling blood flow. Therefore, endothelial cell turnover and recruitment have been the focus of research in many physiopathologic conditions. There is growing evidence that adult bone marrow cells show unexpected plasticity, and can differentiate into a wide range of specialized cells, such as hepatocytes [1], skeletal muscle cells [2], cardiomyocytes [3], and neuronal cells [4]. In the case of renal glomerular cell components, bone marrow (BM)-derived cells have been reported to differentiate into both mesangial cells (MCs) and podocytes [5]. However, there is controversy concerning recruitment of glomerular endothelial cells (GECs) [6–8], although it is well known that endothelial cells in other tissues are recruited from BM [9].

In a recent study [10], we presented a useful experimental model for analyzing the pathogenesis of progressive glomerulosclerosis caused by unilateral nephrectomy following MC injury (1-kidney model). In comparison with reversible 2-kidney model (conventional anti-Thy-1 model), regeneration of glomerular endothelial cells was remarkably suppressed, leading to irreversible glmerulosclerosis with renal insufficiency. An important observation was the positive association between impairment of vascular regeneration and development of progressive glomerulosclerosis, suggesting that angiogenesis and vascular remodeling in the 1-kidney model may be different from those in the 2-kidney reversible model.

Here, we report that BM-derived endothelial progenitor cells (EPCs) play a pivotal role in the recruitment of GECs in progressive 1-kidney model of anti-Thy1 antibody nephritis induced in chimeric rats transplanted with green fluorescent protein (GFP)-positive BM cells.

METHODS

Animals

Transgenic Sprague-Dawley (SD) rats carrying the enhanced green fluorescent protein (EGFP) transgene (EGFP rat) were purchased from Japan SLC, Inc. (Hamamatsu, Japan; by permission of Dr. M. Okabe, Osaka University), and were maintained and cross-mated to obtain littermates of GFP-positive and -negative (wild-type) rats. Rats were kept at the animal facility of Niigata University School of Medicine; they were allowed free access to standard laboratory diet and tap water. The EGFP rats were originally established by use of the same construct and technique described for production of EGFP transgenic mice [11]. The expression of EGFP was under the control of the cytomegalovirus enhancer and the chicken beta-actin promoter that were derived from an expression vector, pCAGGS. All experimental protocols were approved by the animal committee of Niigata University.

Bone marrow transplantation (BMT)

When rats were 5 weeks old, BMTs were performed by use of SD rats (wild-type, EGFP−) as recipients, and their siblings (EGFP+) as donors. EGFP-positive rat BM was collected by flushing bone shafts of femurs, tibiae, and humeri of EGFP rats with phosphate-buffered saline (PBS). After sieving through 75-μm and 50-μm meshes, the cells were resuspended at a concentration of 1 to 2×10^8 cells/mL and kept on ice until use. Littermate rats of wild-type, EGFP(−) were lethally irradiated (10 Gy per animal with an x-ray generator (PS-3000 SB Cs-137; Pony Industry Co., Ltd., Osaka, Japan), and within 4 hours after irradiation, BM cells were administered via the tail vein. The chimeric rats (wild-type rats that had received EGFP+ BMT) were not treated with any drugs after BMT.

The nephritogenic antibody (Ab): 1–22-3 Ab

1–22-3 Ab, the nephritogenic anti-Thy 1 monoclonal antibody, was prepared as described previously [12]. The nephritogenicity of the 1–22-3 Ab appeared to be higher than that of the commercially available anti-Thy 1.1 Ab, OX-7 [13].

Experimental protocol

First, we checked the nephritogenicity of 1–22-3 Ab and rat strain differences in susceptibility to nephritis induced in the 2-kidney model (sham operation group) and 1-kidney model (uninephrectomized group), using SD rats of wild type. These rats were injected with 1–22-3 Ab, and sacrificed at week 12 after injection. Second, male rats of wild type were given BMT from their EGFP-positive brothers at 5 weeks of age. These chimeric rats were divided into the 2 groups. In the 1-kidney model, chimeric rats were injected intravenously with 0.5 mg of 1–22-3 Ab and uninephrecomized 30 minutes thereafter. Four chimeric rats were sacrificed at weeks 2, 4, and 8, and 3 chimeric rats were sacrificed at 11 week after the injection of 1–22-3 Ab. As a control, 3 chimeric rats, without injection of 1–22-3 Ab, were sacrificed at week 17 after BMT.

Biochemical analysis of serum

At sacrifice, blood was collected from all chimeric rats used, and total serum protein (TP), serum albumin (Alb), creatinine (Cre), and blood urea nitrogen (BUN) were measured.

Urinary analysis

Chimeric rats with and without anti-Thy1 nephritis were individually housed in metabolic cages, with free access to water, for collection of 24-hour urine specimens on day 3, and weeks 1, 2, 4, 8, and 11 after the injection of 1–22-3 Ab. The amount of urinary protein excreted was determined by the biuret method with bovine serum albumin (BSA) as a standard.

Light microscopy

Tissue for light microscopy was fixed in 10% neutral-buffered formalin. Paraffin sections were cut at 4-μm and stained with hematoxylin-eosin (HE). In addition, kidney sections were stained with periodic acid–Schiff (PAS) and periodic acid methenamine silver (PAM), sections from liver and lung were stained with Azan.

Examination of cell markers on frozen sections and in isolated glomeruli by immunofluorescence

Indirect immunofluorescence was performed on 4-μm frozen sections followed by fixation with cold acetone. Isolated glomeruli from rat kidneys were prepared by a mechanical sieving method, as described previously [14]. Isolated glomeruli were fixed with 2% paraformaldehyde for 2 hours, and washed with PBS and treated with 1 mg/mL collagenase-dispase for 15 minutes at room temperature, followed by 0.04% Triton X 100 for 5 minutes. Glomeruli were then stained with the primary antibody at 4° overnight, and washed with PBS. Incubation with the second antibody was for 1 hour at room temperature and stained with the primary antibodies, OX-7, anti-rat Thy1 mouse Ab (a hybridoma producing OX-7 IgG1 was purchased from European Collection of Animal Cells Porton Down, Salisbury, UK) for identifying glomerular MC, and antirat PECAM-1, and antirat RECA-1 mouse Abs (Serotec, Ltd., Kindlington, UK) for identifying glomerular endothelial cells (GECs). Rabbit anti-von Willebrand's factor (vWF) antibody (Dako, Glostrup, Denmark) was used

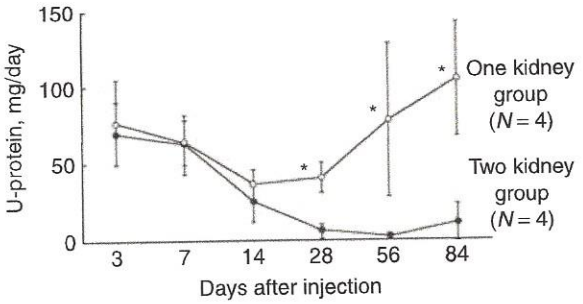

Fig. 1. Urinary protein excretion in the 1- and 2-kidney models of wild SD rats. Urinary protein excretion was determined in 24-hour urine specimens at the indicated times after injection of 1–22-3 Ab. Data are expressed as mean values ± SD. *$P < 0.05$ vs. 2-kidney group.

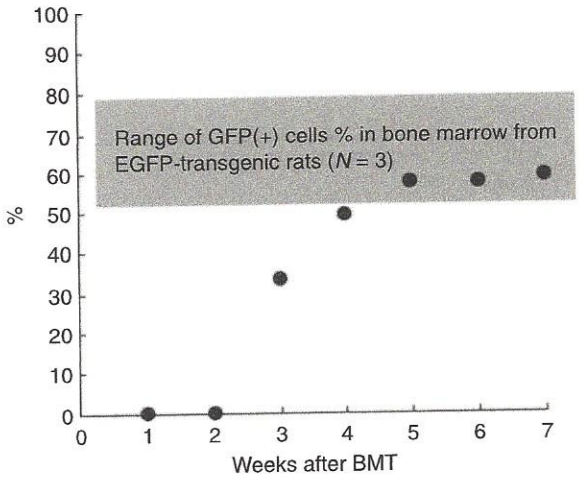

Fig. 2. Bone marrow reconstitution by bone marrow transplantation from EGFP-positive donors. Smear specimens of bone marrow cells from EGFP(−) recipients after bone marrow transplantation from EGFP(+) donors were examined with fluorescent microscopy. The percentage of donor EGFP-positive cells in the recipient's bone marrow after bone marrow transplantation were calculated at the indicated times. The shaded zone shows the range (average ± SD) of GFP(+) bone marrow cells from 3 EGFP-transgenic rats. The closed circles showed the averages of EGFP(+) cells in the recipients' chimeric bone marrow after bone marrow transplantation.

as a functional marker of vascular endothelial cells [12]. Rabbit antirat laminin antibody (Sigmal Chemical Co., St. Louis, MO, USA) was used as a staining marker of basement membrane. The secondary Ab was tetramethyl rhodamine B isothiocyanate (TRITC)-conjugated anti-mouse and -rabbit immunoglobulins (Igs) Ab (Dako). Of note, 3-time washings with PBS after antibody incubation were limited to 5 minutes duration, due to the high water-solubility of EGFP. For conventional immunofluorescece of the sections and 3-dimensional analysis of isolated glomeruli, we used confocal laser scanning immunofluorescent microscopy (CLSM) with the MRC-1024 confocal imaging system (Bio-Rad Laboratories, Hemel Hempstead, UK) [15].

Statistics

Data are expressed as mean ± SD. Statistical significance was evaluated by the Student t test or nonparametric Mann-Whitney U test. A value of $P < 0.05$ was taken to denote statistical significance.

RESULTS

The reversible 2-kidney and irreversible 1-kidney models in SD rats

To confirm the nephritogenicity of monoclonal Ab 1–22-3 and rat strain difference in susceptibility to nephritis, as described previously [10], in the 2-kidney model (sham operation group) and 1-kidney model (uninephrectomized group) using SD rats of wild type, urinary protein excretion was determined for 12 weeks (Fig. 1). There was a significant difference in the results of urinary analysis from 4 weeks onwards, after disease induction, between 1- and 2-kidney models. In the 1-kidney group, urinary protein excretion was 40.2 ± 10.0 mg/24h at 4 weeks, and increased with time to 104.5 ± 37.2 mg/24h at 12 weeks, while urinary protein excretion decreased to normal levels in the 2-kidney model with time. Serum creatinine values were significantly higher in the 1-kidney group than in the 2-kidney group at week 12 after injection of 1–22-3 Ab, 0.49 ± 0.03 and 0.35 ± 0.04 mg/dL, respectively [($P < 0.05$) (0.38 ± 0.05 mg/dL in age-matched, control rats ($N = 5$)].

General features of 1-kidney model in chimeric rats transplanted with EGFP-positive BM cells

We confirmed the nephritogenicity of monoclonal Ab 1–22-3 in SD rats made transgenic to carry the EGFP (EGFP rat). Then, BMT studies using SD rat were done to examine whether bone marrow–derived progenitor cells are involved in recruitment of cellular components to glomeruli after severe damage, resulting in progressive glomerulosclerosis in 1-kidney model. To avoid as far as possible interfering phenomenon, such as a graft-versus-host reaction, in chimeric rats, BM cells obtained from sibling EGFP-positive rats were transplanted to their littermate rats.

To examine BM reconstitution by BMT from EGFP-positive donors, and to determine the time-point for induction of glomerular injury, the recipient's BM was examined by fluorescent microscopy 1 to 7 weeks after BMT. The ratio of donor EGFP positive to negative BM cells in the recipient's BM increased with time, and, at 5 weeks, reached the normal ratio of EGFP-positive to -negative cells seen in EGFP transgenic rats without treatment (Fig. 2). Therefore, a time-point of 5 weeks after

Table 1. Laboratory data after disease induction (1-kidney model) in chimeric rats transplanted with EGFP(+) bone marrow cells

	Urinary protein excretion *mg/day*	Serum TP *g/dL*	BUN *mg/dL*	Creatinine *mg/dL*
2 week ($N = 4$)	59.8 ± 30.5^a	6.2 ± 0.4	55.3 ± 4.7^a	0.52 ± 0.15
8 week ($N = 4$)	42.9 ± 9.9^a	4.7 ± 0.4^a	257.5 ± 57.0^a	2.59 ± 0.85^a
11 week ($N = 3$)	230.0 ± 50.1^a	4.5 ± 0.5^a	320.4 ± 70.5^a	3.81 ± 0.91^a
Control ($N = 3$)	8.5 ± 5.0	5.7 ± 0.4	34.2 ± 7.8	0.44 ± 0.06

Each value is expressed as mean ± SD. Five of 8 chimeric rats in the 1-kidney model died presumably of renal insufficiency during the period 8 to 11 weeks after disease induction. The surviving 3 rats were sacrificed for examination at 11 weeks after disease induction. Control: bone marrow chimeric rats, 17 weeks after bone marrow transplantation, without anti-Thy-1 antibody injection.
[a] $P < 0.01$ vs. control.

BMT was selected for induction of glomerular injury. Three rats of wild type that were irradiated without transplantation of EGFP-positive BM cells died on days 4, 8, and 9, respectively, after total body irradiation (TBI). On the contrary, rats of wild type that received BMT from EGFP-positive littermates following TBI were still alive 5 months thereafter, without any apparent histopathologic findings at sacrifice (data not shown).

Urinary protein excretion and serum biochemical data after 1–22-3 Ab injection in the control, and 1-kidney chimeric groups is shown in Table 1. Proteinuria and serum levels of creatinine in control chimeric rats appeared to be normal. In the 1-kidney group, serum BUN and creatinine values were significantly higher than those in the 2-kidney group at 8 weeks, reaching levels indicating chronic renal insufficiency (Table 1). Rats in the 1-kidney group were severely ill and malnourished, as reflected by reduced levels of total serum proteins, restricting levels of proteinuria. Impressively, 5 of 8 rats in the 1-kidney group died between 8 and 10 weeks, showing generalized emaciation, presumably due to uremia.

Histologically, no pathologic findings were observed in the control chimeric rats at 17 weeks after BMT (Fig. 3A). At week 2 after injection of 1–22-3 Ab, there were mesangioproliferative, and persistent mesangiolytic lesions with mild sclerosis in the 1-kidney group (Fig. 3B). At week 4, severe mesangial matrix expansion with glomerular sclerotic lesions was often observed in the 1-kidney group (data not shown). As shown in Figure 3C, in the 1-kidney group at week 8, glomerular sclerotic lesions and diffuse tubular atrophic changes with interstitial cell infiltration were much more dominant. At week 11 when examined finally, globally sclerotic glomeruli and remarkable interstitial cell infiltration and fibrosis were found diffusely (Fig. 3D).

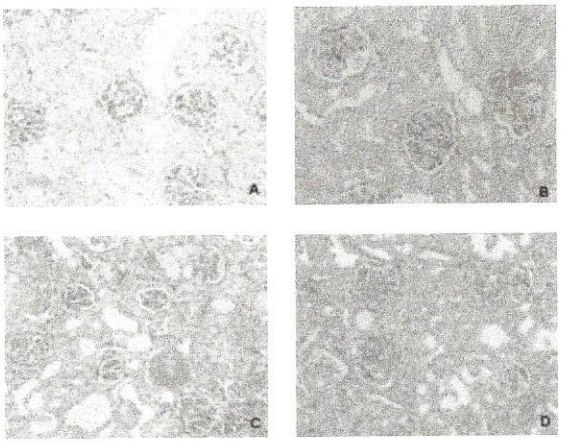

Fig. 3. Light microscopic findings in the kidney in the 1-kidney model using chimeric SD rats transplanted with EGFP(+) bone marrow cells. There were no pathologic findings in the control chimeric rat at 17 weeks after bone marrow trasplantation (*A*). At week 2 after injection of 1–22-3 Ab, diffuse mesangial cell proliferation and mesangial matrix expansion was found. Persistent mesangiolytic lesions were also found (*B*). At week 8 after Ab injection, severe mesangial matrix expansion with severe sclerotic lesions was observed in the majority of the glomeruli. Exudation into the Bowmann's space and urinary casts were also found (*C*). Global glomerular sclerosis and diffuse tubular atrophic changes with interstitial cell infiltration and fibrosis became much more prominent at week 11 (PAS stain).

Identification of EGFP-positive cells recruited within glomeruli

In the present work, we have focused on the role of BM-derived, nonhematopoietic progenitor cells in the regeneration of resident glomerular cells after severe glomerular damage, leading to progressive glomerulosclerosis.

Two weeks after administration of 1–22-3 Ab in chimeric rats, we examined the recruitment of bone marrow–derived cells into the glomeruli in the 1-kidney group by immunofluorescence with cell-specific markers. Figure 4 shows intraglomerular cells expressing EGFP and either OX-7 as a mesangial cell marker, or PECAM-1 and RECA-1 as endothelilal cell markers. Distorted staining patterns of mesangial cells and loss of capillary densities indicated that mesangial cell injury, followed by destructive changes of glomerular capillaries, persisted. By computer-aided superimposition of the 2 fluorescence images, colocalization of EGFP and OX-7 or EGFP and PECAM-1 or RECA-1 generated a yellow fluorescence. There were many EGFP(+) cells, some of which were costained with OX-7 (Fig.4C) and PECAM-1 (Fig. 4F) in the 1-kidney group. Other numerous EGFP(+) cells and particle-like cell components without costaining for OX-7 or PECAM-1 seemed to be bone marrow–derived inflammatory cells, such as polymorphonuclear leukocytes and monocytes, and platelets, as the acute phase of inflammation still persisted 2 weeks after disease induction.

Light microscopic findings in the present study showed that progressive glomerulosclerotic lesions, associated with renal insufficiency, occurred in the 1-kidney model, as described previously [10]. To investigate microvascular regeneration and formation of glomerulosclerotic

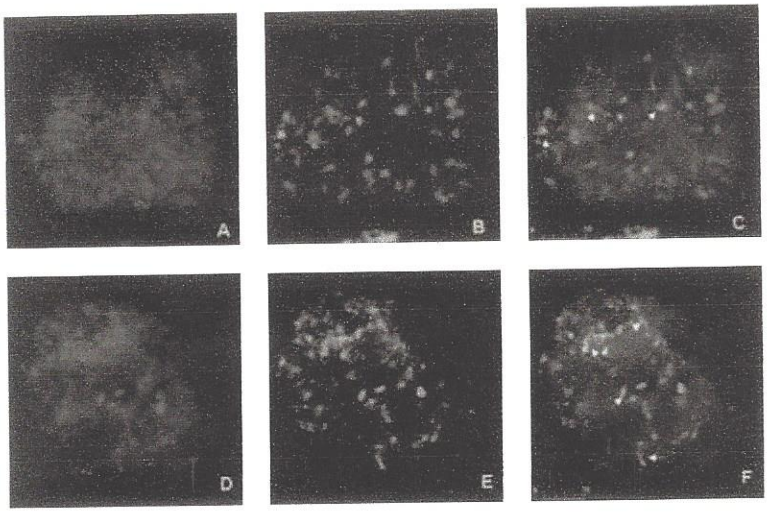

Fig. 4. Recruitment of EGFP (+) bone marrow cells into the glomeruli at 2 weeks after the injection of 1–22-3 Ab, in the 1-kidney model. Isolated glomeruli were incubated with monoclonal antibody to OX-7 as a mesangial cell maker (*A*) or monoclonal antibody to PECAM-1 (*D*), as an endothelial cell marker, followed by staining with TRITC-conjugated anti-mouse Igs Ab (red color). By computer-aided superimposition of the two fluorescence images, EGFP(+) cellular colocalization of OX-7 (*C*) or PECAM-1 (*F*), generated a yellow fluorescence (arrows). There are many EGFP(+) cells that were costained with OX-7 or PECAM-1 in the 1-kidney group (C, D). Of note, other numerous EGFP(+) cells and particle-like cell components without mesangial or endothelial cell-markers were found. The scales were indicated by μm.

lesions, glomeruli isolated from a chimeric rat in the 1-kidney group that survived, despite renal insufficiency, were examined at week 11 after injection of 1–22-3 antibody. The density of glomerular capillaries evaluated by anti-PECAM-1 or anti-RECA-1 staining decreased remarkably in the 1-kidney group compared to that of normal control groups (Fig. 5). Furthermore, cellular colocalization of EGFP(+), PECAM-1 (Fig. 5C), or RECA-1 (Fig. 5D) suggested strongly that bone marrow–derived EPCs were incorporated into the damaged capillary beds. CLSM, in combination with immunostaining and optical sectioning of isolated glomeruli, allowed 3-dimensional analysis and characterization of EGFP-positive BM-derived cells within glomeruli. Sequential images of glomerular capillary tufts (2 μm in thickness) showed EGFP(+) cells in a mosaic pattern mingled with endothelial cells indicated by PECAM-1 staining, as shown in Figure 6.

To examine more precisely the recruitment of bone marrow–derived EPCs into the severely damaged glomerular vasculatures, frozen kidney sections fixed with acetone were used for immunostaining, although detectablity for EGFP-positive cells was relatively low, probably due to the high water solubility of EGFP. Some glomerular endothelial cells immunostained with anti-PECAM-1 or RECA-1 antibody were colocalized with EGFP, showing the involvement of bone marrow–derived EPCs in the regeneration of glomerular microvasculature (Fig. 7). At the same time, there were very few EGFP(+) cells costained with the mesangial cell marker OX-7 (Fig. 8). In terms of endothelial function after recruitment of bone marrow–derived endothelial precursor cells, we examined the production of von Willebrand's factor (vWF) in glomerular endothelial cells using immunofluorescence. Figure 9 showed colocalization and proximal localization of vWF with EGFP-positive cells within glomeruli, suggesting that glomerular endothelial cells recruited from bone marrow exhibited endothelial specific functions.

During the time course examined, we never found abnormal depositions of autologous immunoglobulins and C3 in the glomeruli in any experimental group (data not shown).

DISCUSSION

Regeneration of the microvasculature after injury is indispensable for maintaining tissue- and organ-specific structures and functions. Recent studies have revealed that glomerular capillary repair is the key to determining the progression of glomerular pathology. In models of glomerulonephritis exhibiting recovery from injury, such as anti-Thy-1 glomerulonephritis and Habu snake venom–induced glomerular injury, vascular remodeling is an essential step leading to full recovery of glomerular architecture [16, 17]. In addition, there is increasing evidence that impaired angiogenesis may occur in the diseased kidney, resulting in irreversible renal sclerosis [10, 18, 19].

It is now established that BM-derived EPCs can differentiate into endothelial cells that contribute to angiogenesis or vasculogenesis in postnatal neovascularization [9, 20–22]. There have been no published data showing that loss of glomerular endothelial cells (GECs) due to injury is followed by recruitment of BM-derived EPCs, although the BM has been reported to serve as a reservoir for MCs [6, 7]. Very recently, Rookmaaker et al, using a rat allogenic BM transplant model, reported that bone marrow–derived endothelilal cells could be recruited and integrated into the glomerular structure in anti-Thy1-glomerulonephritis [8]. These latter authors showed that donor-derived EPCS could be detected with a specific

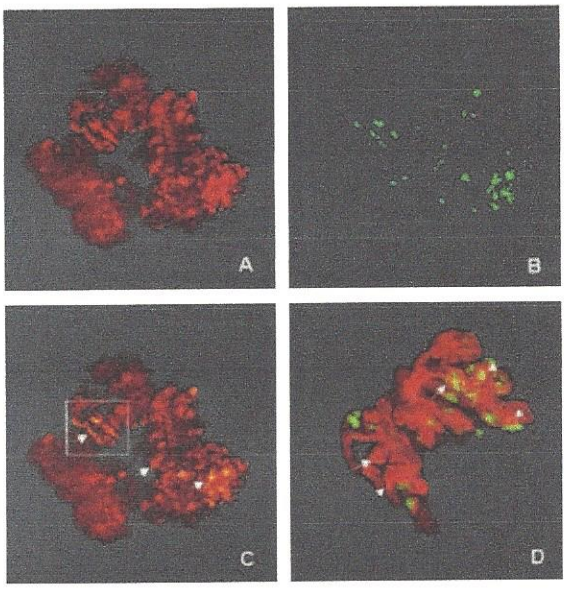

Fig. 5. Recruitment of EGFP(+) bone marrow cells into the glomeruli 11 weeks after the injection of 1–22-3 Ab in the 1-kidney model. Isolated glomeruli were incubated with monoclonal antibodies to PECAM-1 (*A*) or RECA-1 (*D*), followed by staining with TRITC-conjugated antimouse Igs Ab. Microvascular structures composed of cellular colocalization of EGFP(+) and PECAM-1 were found in contact with nonvascular lesions, presumably sclerotic lesions (*C*, arrows). The same colocalization of RECA-1 used for the other endothelial marker was also observed (D).

monoclonal antibody that is able to recognize the difference in histocompatiblity antigens between donor and recipient MHCI. There is discrepancy between published results, using a similar model of reversible anti-Thy-1 glomerulonephritis, between Rookmaaker's and another paper of Ito et al [7], in which bone marrow–derived cells replaced a part of mesangial cells, but not endothelial cells in the glomerulus.

Here, we showed that BM-derived EPCs can differentiate into GECs as well as MCs during the chronic phase of irreversible, anti-Thy 1 Ab-mediated glomerulonephritis. Before going into a discussion of the significance of recruitment of BM-derived EPCs after damage, we wish to consider reasons for the differences between our present data and between previous papers concerning involvement of bone marrow–derived EPCs. First, in our present study, we analyzed the contribution of EPC in the model of progressive glomerulosclerosis induced by a 1-shot injection of anti-Thy 1 antibody followed by unilateral nephrectomy (1-kidney model) [10] using SD rats with transplanted, GFP-positive BM cells. In comparison with reversible anti-Thy-1 model (2-kidney model), glomerular microvasculatures suffered from more severe injury, and showed disturbed regeneration after disease induction, as described previously [10]. There is increasing evidence that bone marrow stem cell "plasticity" is not a normal function of EPCs, but that the EPCs are likely to be incorporated into the lesions of severe injury under selective pressure, due to their atypical function [23, 24]. In Rookmaaker's work [8], as described above, allogeneic bone marrow transplantation from WAG/RijHsd to Brown Norway/RijHsd may affect the function of EPCs or the degree of injury induced in the glomerular microvasculature. Second, isolated glomeruli fixed immediately with 2% paraformaldehyde or frozen sections fixed with acetone followed by short-time washings were used for detection and analysis of EGFP-positive cells, in contrast to the study by Ito et al, who performed immunohistologic studies using thin frozen sections from the kidneys previously perfused, and fixed with 10% buffered formalin via the abdominal aorta [7]. The isolation procedure for glomeruli, in combination with analysis using CLSM, causes little mechanical injury to intraglomerular cells, as shown previously [15]. Tissue sectioning, followed by extensive washing during immunostaining, may result in loss of GFP from fragile endothelial cells, even after fixation, because GFP is very water soluble and easily diffuses through damaged cell membranes. Third, there are differences in the nephritogenicities of the anti-Thy-1 antibodies used for induction of glomerulonephritis. Ito et al used OX-7, a commercially available MAb of IgG1 subclass, and Rookmaaker et.al used ER4, Mab of IgG2a [25], while we used another monoclonal Ab, called 1–22-3 (IgG3 subclass), which we produced, and which appeared to be more nephritogenic than OX-7 [13].

Recently, it has been highlighted that bone marrow cells have the capacity to fuse with embryonic stem cells and differentiate in vitro [26]. In addition, Wang et al have shown that regenerated hepatocytes, in a mouse model of tyrosinemia, could arise from the stable fusion of bone marrow–derived cells with host hepatocytes [27]. Analyzing these data, cell fusion appears to be a very low-frequency event, occurring at the level of once in 10^4 to 10^5 cells [24]. On the other hand, Bailey et al have excluded the possibility of cell fusion as the principal mechanism responsible for the generation of functional endothelial cells from stem cells in the mouse portal vein endothelium [28]. Very recently, using the sophisticated method of the Cre/lox recombinase system, in combination with β-galactosidase and EGFP expression, it was demonstrated that epithelial cells in the lung, liver, and skin developed from bone marrow–derived stem cells without cell fusion [29]. Although we cannot exclude the possibility that cell fusion of bone marrow–derived cells with intrinsic microvascular endothelial cells of recipient origin may occur, it is reasonable to consider that bone marrow–derived EPCs can repair the damaged glomerular capillaries without cell fusion in the 1-kidney model of anti-Thy-1 glomerulonephritis. This is because

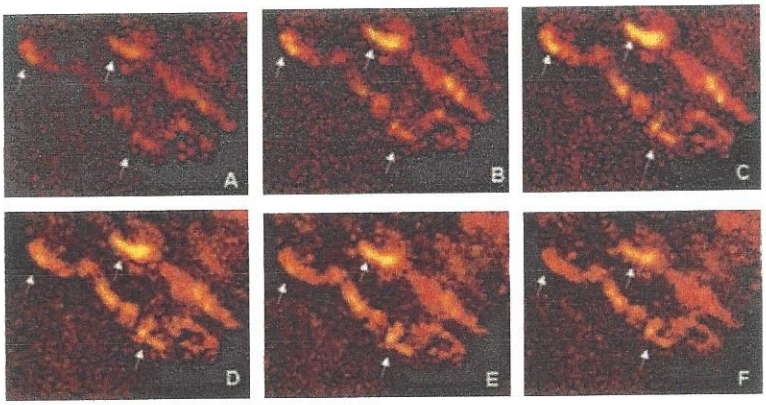

Fig. 6. Sequential images of an isolated glomerulus (2 μm in thickness), obtained by optical sectioning of the same area shown in Figure 5 **(indicated by the white-line square), revealed that some glomerular capillary tufts were composed of EGFP(+) cells in a mosaic pattern, as indicated by PECAM-1 staining (arrows).**

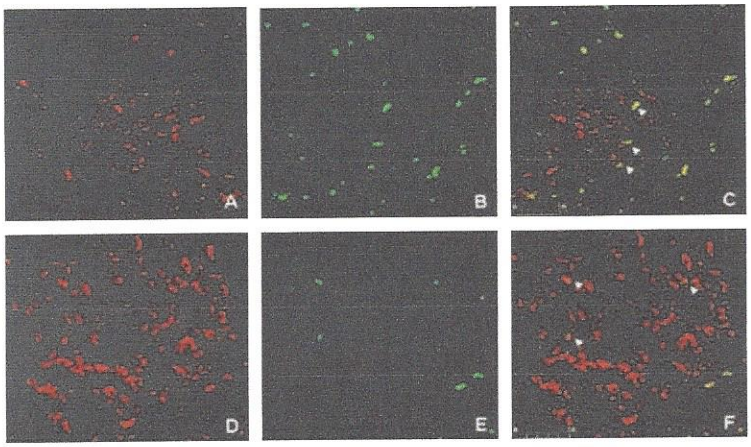

Fig. 7. EGFP(+) cellular colocalization of endothelial cell markers in frozen kidney sections 11 weeks after the injection of 1–22-3 Ab in the 1-kidney model. Frozen specimens fixed with acetone were incubated with monoclonal antibodies to RECA-1 (*A*) or PECAM-1 (*D*), followed by staining with TRITC-conjugated antimouse Igs Ab. EGFP(+) cellular colocalization of RECA-1 (*C*) or PECAM-1 (*F*) was found within a glomerulus (arrows). The scales were indicated by μm.

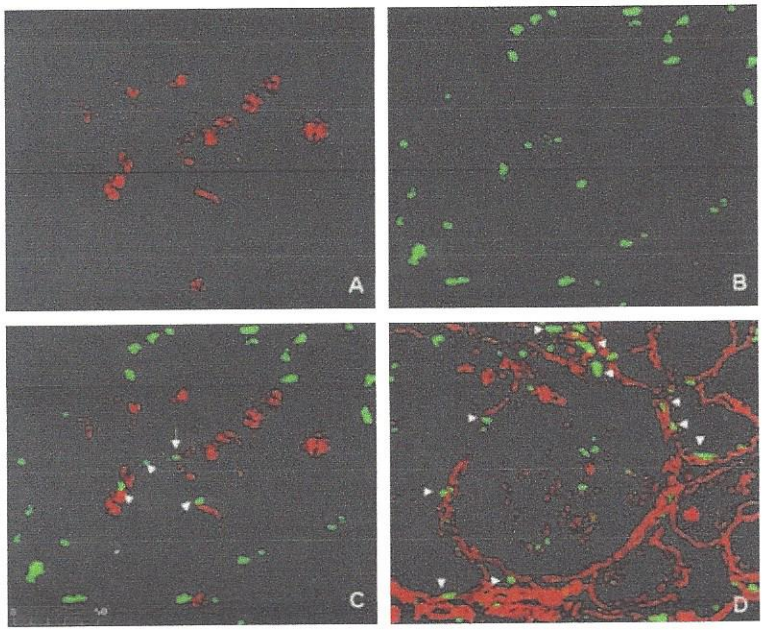

Fig. 8. EGFP(+) cells localized in the interstitium and EGFP(+) cellular colocalization of mesangial cell marker, OX-7. Frozen specimens fixed with acetone were incubated with monoclonal antibody OX-7, followed by staining with TRITC-conjugated antimouse Igs (*A*). EGFP(+) cellular colocalization of OX-7 was rarely found within glomeruli (arrows). With the aid of basement membrane staining by rabbit antilaminin antibody, followed by staining with TRITC-conjugated antirabbit Igs, many EGFP(+) cells were found in the interstitium (arrow heads), as described by Ito et al [7]. The scale was indicated by μm.

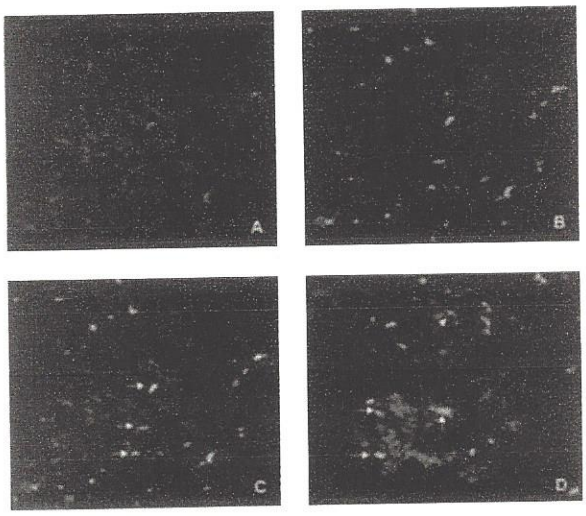

Fig. 9. EGFP(+) cellular colocalization of a specific, functional marker of endothelial cell. Frozen specimens fixed with acetone were incubated with monoclonal antibodies, RECA-1 (*A*) or rabbit anti-vWF (*D*) antibody, followed by staining with TRITC-conjugated antimouse Igs or antirabbit Igs, respectively. GFP(+) cellular colocalization of RECA-1 (*C*) was found. In addition, colocalization (arrow) and proximate localization (arrow heads) of vWF with GFP(+) cells were observed (D). The scale was indicated by μm.

of the higher frequency of recruitment of GFP(+) bone marrow–derived cells with specific endothelial markers and exhibiting endothelial specific functions, such as expression of PECAM-1, cell adhesion molecule, and production of vWF. It is well known that cell fusion of normal somatic cells yields multinuclear cells of specific fusion, resulting in loss of cell function, such as proliferation [24]. Alvarez-Dolado et al reported that the hepatocytes and cardiomyocytes that have undergone fusion down-regulate the expression of GFP, and thus, could possibly inactivate the donor genes overtime [30]. Further study is needed in our experimental model to provide the direct evidences that GFP(+) vasculature is not due to the cell fusion.

It is worthwhile discussing the pathomechanisms involved in inducing revascularization in glomerular injury from the angle of chronic ischemia. Our comparative study in the 1- and 2-kidney models showed that there was a positive association between impairment of vascular regeneration and the development of glomerulosclerosis [10]. Loss of capillary density and blood flow may result in poor delivery of oxygen and nutrients to the damaged area, producing chronic ischemia [19]. There was a dramatic decrease of capillary density by day 3 after disease induction in the acute phase of the ATS model [10]. Semiquantitative analysis by reverse transcription-polymerase chain reaction (RT-PCR) indicated that mRNA expression of PECAM-1 and VEGF was significantly suppressed by day 14 in the 1-kidney group compared to the 2-kidney group. Asahara et al reported extensive studies on the contribution of VEGF to postnatal neovascularization in ischemia and/or reperfusion injury [31–33]. They showed evidence that VEGF may modulate both differentiation of EPC in vitro, as well as the kinetics of mobilization from BM in vivo, suggesting that VEGF has multipotential roles in endothelial cell growth, cell differentiation, and chemoattractive mobilization of BM mononuclear cells, including EPC. The intrinsic cellular source of VEGF is the glomerular visceral epithelial cells [34]. In a physiologic setting, hydrostatic pressure across glomerular capillary walls prevents fluid, containing biologically active substances such as VEGF and hepatocyte growth factor from flowing back from the Bowman's space to the endocapillary region. Interestingly, real-time observation of glomerular blood flow in the ATS model revealed that there was hemostasis within microaneurysms at day 3. The blood flow within glomeruli was retarded with less injury; the level of blood flow was about one tenth of that seen in normal control subjects [35]. In such a pathologic setting, the occurrence of hemostasis as seen at day 3 in the ATS model, VEGF produced by glomerular visceral epithelial cells may exhibit biological activity on the damaged endocapillary lesions, resulting in neovascularization by both vascular sprouting from the remaining vessels and/or by recruitment of EPCs from BM.

CONCLUSION

In remodeling of glomerular structures after damage, the revascularization process is a key factor in determining prognosis of glomerular injury, as described above. From a therapeutic viewpoint, future studies on delivering bone marrow–derived endothelial EPCs, or passive administration of bone marrow–derived EPCs and subsequent activation of angiogenesis and/or vasculogenesis by transfer of angiogenetic factors such as VEGF, HGF, and the relevant genes will be needed to halt, or promote recovery from irreversible progression to glomerulosclerosis.

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