

Depletion of endothelial progenitor cells in the peripheral blood of patients with ulcerative colitis

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Received September 5, 2006; Accepted October 24, 2006

Abstract. There is strong evidence to suggest that endothelial progenitor cells (EPCs) play a significant role in reendothelialization and subsequent tissue repair. This study examined the role of EPCs in inflammatory bowel disease, a disease in which impairment of mucosal healing has been implicated. Peripheral blood mononuclear cells obtained from 50 patients with ulcerative colitis (UC), 29 patients with Crohn's disease (CD), 14 patients with infectious colitis, and 35 normal control subjects were cultured in EPC medium, harvested after 7 days, and characterized by immunocytochemistry and flow cytometry. Colony assay for hematopoietic progenitor cells was also performed. Patients with active UC had a significantly decreased number of circulating EPCs as compared with healthy controls ($p=0.0013$), patients with inactive UC ($p=0.0099$), patients with active CD ($p=0.0235$) and patients with infectious colitis ($p=0.0002$). On the other hand, patients with infectious colitis had a significantly increased number of circulating EPCs as compared with healthy controls ($p=0.0406$), patients with active UC ($p=0.0002$), and patients with active CD ($p=0.0316$). In patients with UC, the number of circulating EPCs was correlated with the serum hemoglobin levels ($r=0.485$, $p=0.007$) and inversely with the platelet count

($r=-0.372$, $p=0.0382$). The number of hematopoietic progenitor cell colonies was comparable among patients with UC, patients with CD, patients with infectious colitis, and healthy controls. Our observations indicate that the number of circulating EPCs in patients with UC is significantly reduced. Further studies are needed to define the mechanisms that underlie the reduction in the number of circulating EPCs and to better understand the pathophysiological consequences of this event in patients with UC.

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are both characterized by refractory inflammatory damage in the gastrointestinal tract, dominance of the processes of injury over those of repair (1,2). In general, intestinal mucosal repair consists of two major processes, epithelial regeneration and mesenchymal reconstruction. The former involves epithelial restitution, cell proliferation, maturation, and differentiation and the latter includes angiogenesis, fibrosis, and smooth muscle regeneration. Angiogenesis, a complex multistep process in the formation of new blood vessels from pre-existing vessels, is central to tissue repair, as the ingrowth of newly formed vessels is necessary to ensure the supply of oxygen and nutrients to the regenerating tissue (3,4). While available data to date suggest that angiogenesis and inflammation frequently occur together, evidence of the pathophysiologic relevance of angiogenesis in inflammatory bowel disease (IBD) is extremely limited (5-8).

There is now increasing evidence to suggest that neo-vascularization may not solely be the result of angiogenesis, but may also involve bone marrow-derived endothelial progenitor cells (EPCs). EPCs have been isolated from circulating mononuclear cells in the peripheral blood and have also been shown to be incorporated into foci of

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Key words: angiogenesis, endothelial progenitor cells, hematopoietic progenitor cells, Crohn's disease, ulcerative colitis

Table I. Clinical and laboratory characteristics in healthy control subjects, patients with infectious colitis, patients with ulcerative colitis and patients with Crohn's disease.

	Healthy control	Infectious colitis	Ulcerative colitis	Crohn's disease
No. of patients	35	14	50	29
Age (years) ^a	29.6±1.2	29.4±3.0	37.1±1.9	31.3±2.5
Sex (female/male)	13/22	6/8	19/31	8/21
Disease duration (years) ^a			7.1±1.0	9.6±1.4
Disease extent			Entire/left-sided/rectal =23/13/11	Ileal/colonic/ileocolic =3/2/24
Treatment (None/5-aminosalicylate/steroids)			6/35/25	3/22/10
Disease activity (active/inactive)			27/23	19/10
Leukocyte (/μl) ^a (active/inactive)		6679±653	8206±906/6233±437	7563±787/7129±320
Granulocyte (%) ^a (active/inactive)		66.6±3.1	73.4±3.2/61.7±2.2	74.2±3.2/77.8±3.4
Lymphocyte (%) ^a (active/inactive)		27.1±3.45	21.3±3.1/32.6±2.0	20.3±3.4/16.8±3.3
Monocyte (%) ^a (active/inactive)		5.9±0.8	5.3±0.5/5.5±0.4	5.4±0.6/5.4±0.7
Platelet (10 ⁴ /μl) ^a (active/inactive)		21.9±1.9	32.6±2.5/25.5±1.8	26.2±2.6/22.7±0.9
Erythrocyte (10 ⁴ /μl) ^a (active/inactive)		465±14	421±16/484±15	461±16/485±21
CRP (mg/dl) ^a (active/inactive)		0.9±0.3	1.4±0.7/0.1±0.0	2.5±0.7/0.1±0.1

^amean ± SEM.

neovascularization in adults, consistent with the notion of postnatal vasculogenesis (9-13). These EPCs are mobilized endogenously in response to tissue damage (14) and/or exogenously in response to stimulation by humoral factors, such as vascular endothelial growth factor (15), granulocyte colony-stimulating factor (G-CSF) (16), erythropoietin (17), and stromal derived factor 1 (18). The involvement of EPCs in some disease conditions, including acute myocardial infarction (19,20), acute lung injury (21,22), chronic renal disease (23,24), rheumatoid arthritis (25,26), diabetes mellitus (27,28) and cancers (29), is now well documented. However, to date, there are no reports on the role of EPCs in patients with IBD.

On the basis of all the above observations, we hypothesized that there might be an alteration in the number of circulating number of EPCs in the peripheral blood in cases of IBD. Our results provide further insight into the pathophysiology of IBD, and also provide a useful framework of reference for developing therapeutic strategies for patients with IBD.

Materials and methods

Patients and samples. Blood samples were obtained from 50 patients with UC and 29 with CD (Table I). The diagnoses were based on characteristic clinical, endoscopic, radiological and histological features. In the UC group, clinical disease activity was analyzed according to the Lichtiger index (30). In

the CD group, clinical disease activity was assessed by scoring from the International Organization for the Study of Inflammatory Bowel Disease (IOIBD) (31). Healthy, age- and sex-matched subjects served as normal controls for the assay (n=35). Patients with infectious colitis served as acute inflammatory controls (n=14). IBD patients were treated with 5-aminosalicylate, steroids or both. Subjects were excluded from this study if they had diabetes mellitus, cardiovascular disease or any condition that may influence EPC kinetics (19-29). All experiments were approved by the Kurume university medical ethics committee. Informed consent was obtained from every participant.

In vitro cultures of EPCs. EPCs were cultured according to previously described techniques (9,11). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation. After purification and three washings, 2x10⁶ PBMCs were plated on gelatin-coated 6-well plates. Cells were cultured in Medium 199 supplemented with 20% fetal bovine serum (FBS), bovine pituitary extract to stimulate cell growth, heparin (100 μg/ml), and antibiotic-antimycotic (Gibco, Tulsa, OK, USA). Five microscopic fields from one well were examined for each sample on Day 7 of culture, and the number of EPCs was expressed as number of cells/mm². Intraassay (n=20) and interassay (n=10) coefficients of variation were <5%.

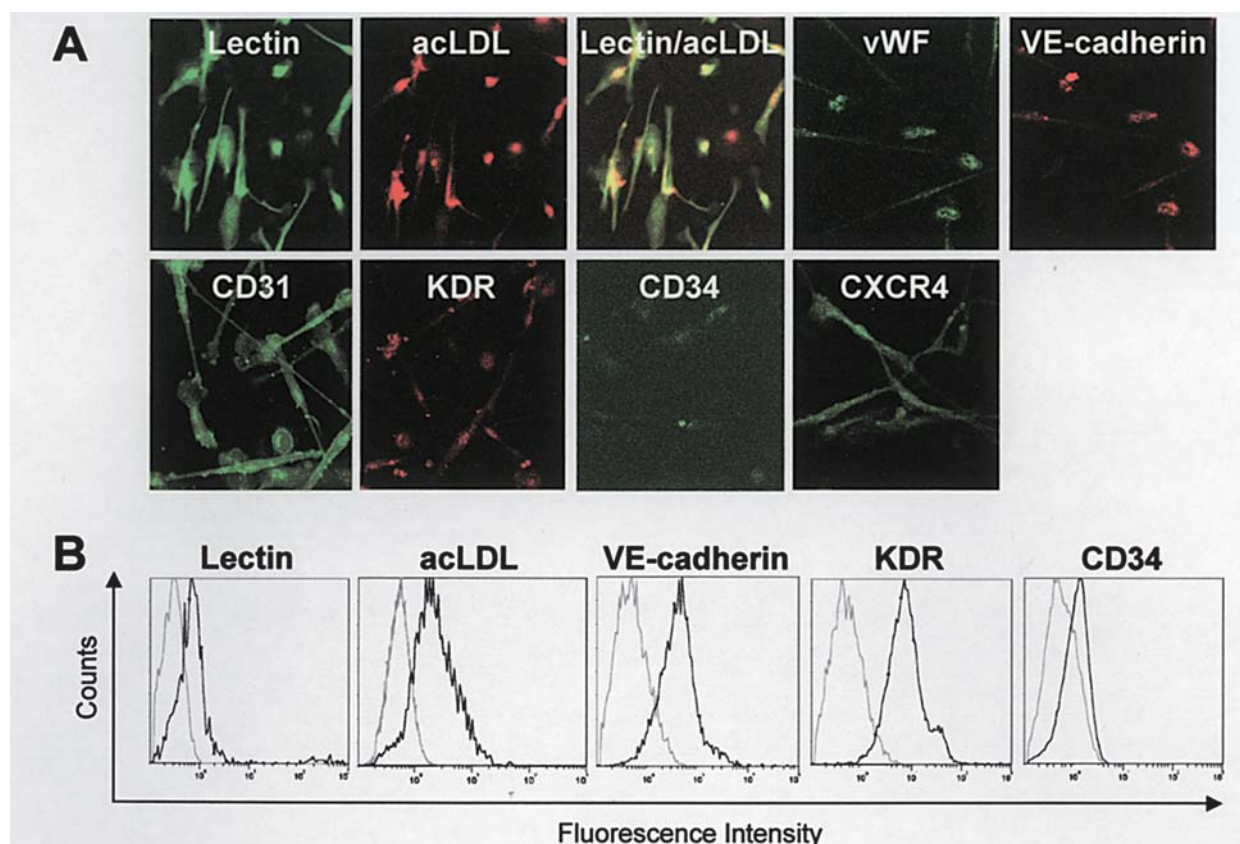


Figure 1. Characteristics of EPCs derived from peripheral mononuclear cells. (A) Immunofluorescence images of lectin (green), acetylated LDL (acLDL; red), both lectin and acLDL (yellow), von Willebrand factor (vWF; green), vascular endothelium (VE)-cadherin (red), CD31 (green), kinase domain-containing receptor (KDR; red), CD34 (green), and CXCR4 (green) expression in EPCs. Original magnification, $\times 200$. (B) Flow cytometric analyses of lectin, acLDL, VE-cadherin, KDR and CD34 expression in EPCs. Plots show isotype controls (gray lines) vs specific antibody staining (black lines). Representative findings of healthy subjects were shown.

Fluorescence microscopy. EPCs on Day 7 of culture were fixed in equal volumes of acetone and ethanol at -20°C for 10 min. To block nonspecific binding of the primary antibodies, dishes were preincubated with Protein Block Serum-Free (Dako, Kyoto, Japan) at room temperature for 20 min. As primary antibodies, antibodies recognizing lectin (Sigma, St. Louis, Mo, USA), acetylated LDL (acLDL; Biomedical Technologies, Stoughton, MA, USA), von Willebrand factor (vWF; Dako), vascular endothelium (VE)-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD31 (Dako), kinase domain-containing receptor (KDR; Santa Cruz Biotechnology), CD34 (BD, Franklin Lakes, NJ, USA) and CXCR4 (BD) were used. Dishes were incubated at room temperature for 60 min with primary antibodies in PBS and for 60 min with immunofluorescence-labeled secondary antibody. Finally, to protect fluorescence staining, the specimens were embedded in Vectorshield (Vector Laboratories, Burlingame, CA, USA).

Flow cytometric analysis. For flow cytometry, EPCs on Day 7 of culture were detached with PBS with 1 mM ethylenediaminetetraacetic acid (EDTA) followed by repeated gentle flushing through a pipette tip. The cells in suspension were labeled with immunofluorescence-tagged antibodies recognizing lectin, acLDL, VE-cadherin (Santa Cruz Biotechnology), KDR (Sigma) and CD34. Isotype-identical antibodies served as negative controls. Cells were fixed with 1% formaldehyde

and analyzed by quantitative flow cytometry with the use of an EPICSXL (Beckman Coulter, Fullerton, CA, USA).

In vitro cultures of HPCs. For analyses of hematopoietic progenitor cells (HPCs), including burst forming unit-erythroid (BFU-E); colony forming unit (CFU)-granulocyte macrophage (GM); CFU-granulocyte (G); and CFU-granulocyte, erythroid, macrophage, and megakaryocyte (GEMM), cells were suspended at a concentration of 1×10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM) containing 2% FBS. The cell suspensions were mixed with 10 volumes of methylcellulose-based semisolid culture medium, MethoCultTM GF H4434 (StemCell Technologies, Vancouver, Canada), containing 50 ng/ml of recombinant human stem cell factor, 10 ng/ml of granulocyte macrophage-CSF, 10 ng/ml of interleukin (IL)-3, and 3 U/ml of erythropoietin. Aliquots (1.1 ml) were plated in duplicate in 35-mm dishes (Falcon, Easy gripTM, Franklin lakes, NJ, USA) and incubated for 14 days in a humidified atmosphere containing 5% CO_2 at 37°C (20,32). To quantify the mesenchymal progenitor cells *in vitro*, the CFU-fibroblast (F) assay (MesenCultTM basal medium for human mesenchymal stem cells, StemCell Technologies Inc.) was performed.

Statistics. Results were expressed as medians with interquartile ranges (IQRs), unless stated otherwise. Data were evaluated

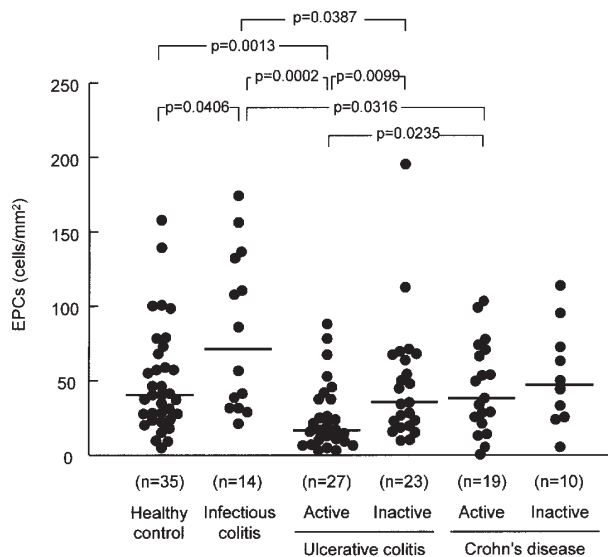


Figure 2. Quantitation of circulating EPCs in patients with ulcerative colitis, patients with Crohn's disease, patients with infectious colitis and in healthy control subjects. Solid lines represent the median values.

by Mann-Whitney's U test. Correlations were performed by determining the Spearman correlation coefficients. A p-value of <0.05 was considered to be significant. Statistical evaluations were performed using SPSS for Windows, version 12.0 (SPSS Inc, Chicago, IL, USA).

Results

Characterization of EPCs. As the levels of circulating EPCs in the peripheral blood are too low to be directly measured, an indirect method has been widely used to determine the number of EPCs in the peripheral blood (9,11). When PBMCs

were allowed to grow in culture plates coated with fibronectin, a subset of the PBMCs differentiated into EPCs and $>80\%$ of the attached spindle-shaped cells were found to express the endothelial lineage marker. According to this established protocol, we isolated PBMCs and analyzed the number of EPCs. The EPCs were characterized as adherent cells doubly positive for lectin and acLDL, and their endothelial and hematopoietic phenotype was additionally confirmed by documenting the expression of well-established markers for EPCs, including vWF, VE-cadherin, CD31, KDR, CD34 and CXCR4 by immunostaining. Representative photomicrographs of the immunostaining are shown in Fig. 1A. Flow cytometric analysis further confirmed the endothelial and hematopoietic phenotype of the EPCs used in the present study (Fig. 1B).

Quantitation of circulating EPCs. Patients with active UC (n=27) had significantly reduced numbers of circulating EPCs as compared with healthy controls (n=35, $p=0.0013$), patients with inactive UC (n=23, $p=0.0099$), patients with active CD (n=19, $p=0.0235$) and patients with infectious colitis (n=14, $p=0.0002$). On the other hand, patients with infectious colitis (n=14) had significantly increased numbers of circulating EPCs as compared with healthy controls (n=35, $p=0.0406$), patients with active UC (n=27, $p=0.0002$), and patients with active CD (n=19, $p=0.0316$) (Fig. 2). The result was quite similar in the analysis of patients not receiving steroid treatment. The circulating number of EPCs was significantly lower in patients with active UC (n=12) than in healthy controls (n=35, $p=0.0115$), patients with inactive UC (n=14, $p=0.1166$), patients with active CD (n=13, $p=0.2108$) and patients with infectious colitis (n=14, $p=0.0014$). The number of EPCs was significantly higher in patients with infectious colitis (n=14) than in healthy controls (n=35, $p=0.0406$), patients with active UC (n=12, $p=0.0014$), and patients with active CD (n=13, $p=0.0347$). Table II shows the relationship between circulating

Table II. Relationship between circulating EPCs and laboratory and clinical variables in patients with ulcerative colitis, patients with Crohn's disease and patients with infectious colitis.

	Ulcerative colitis		Crohn's disease		Infectious colitis	
	r	n	r	n	r	n
Leukocyte	0.177	32	0.358	19	-0.125	14
Erythrocyte	0.313	32	0.071	18	0.047	14
Hemoglobin	0.485 ^b	32	0.015	18	0.123	14
Platelet	-0.372 ^c	32	0.174	18	-0.310	14
CRP	-0.056	41	-0.200	21	-0.410	14
Clinical disease activity ^a	-0.224	47	-0.120	28		
Neutrophil	0.187	32	0.263	14	-0.212	14
Eosinophil	0.105	32	-0.280	14	0.496	14
Basophil	0.226	32	0.030	14	-0.093	14
Lymphocyte	0.128	32	-0.234	14	-0.096	14
Monocyte	0.337	32	0.447	14	-0.078	14

^aClinical disease activity was quantified by the Lichtiger index in cases of UC and by the IOIBD score in cases of CD. ^b $p=0.007$, ^c $p=0.0382$.

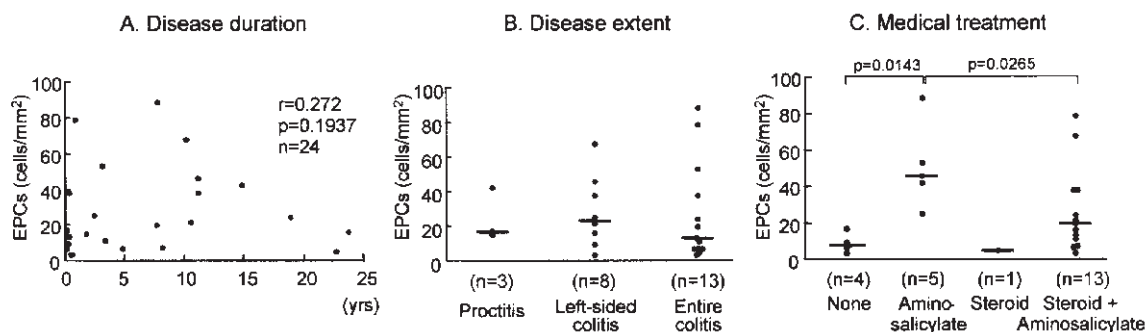


Figure 3. Number of circulating EPCs in patients with active ulcerative colitis according to the disease duration (A), the disease extent (B) and the treatment strategy employed (C). Solid lines represent the median values.

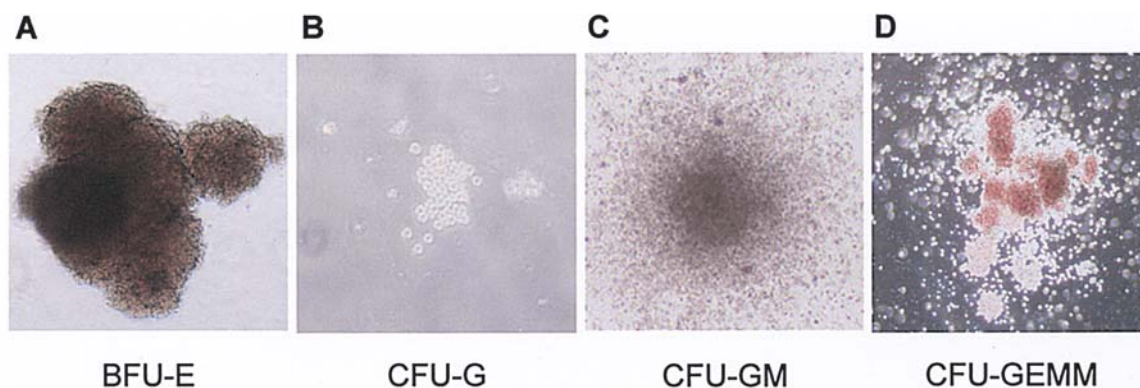


Figure 4. Photomicrograph of a typical colony-forming units representing BFU-E (A), CFU-G (B), CFU-GM (C) and CFU-GEMM (D). Representative images from one healthy subject are shown. Original magnification, x200. CFU-E produces one to 2 clusters containing a total of 8-200 erythroblasts; BFU-E produces a colony containing >200 erythroblasts; CFU-G produces ≥ 40 granulocytes and CFU-M produces ≥ 40 macrophages; CFU-GM produces a colony containing ≥ 40 granulocyte and macrophage cells; CFU-GEMM contains erythroid cells as well as ≥ 20 granulocytes, macrophages and megakaryocytes.

EPCs and laboratory and clinical variables. In patients with UC, the number of circulating EPCs was correlated with the serum hemoglobin level ($n=32$, $r=0.485$, $p=0.007$) and inversely related with the platelet count ($n=32$, $r=-0.372$, $p=0.0382$). No significant correlation of the circulating number of EPCs was observed with the degree of clinical disease activity ($n=47$, $r=-0.224$, $p=0.1235$). Subanalyses performed for active UC patients (Fig. 3) showed that while the number of EPCs was decreased in cases with active disease, no correlation of the number was observed with the disease duration or disease extent. Patients treated with 5-aminosalicylate alone, but not 5-aminosalicylate plus steroids, had significantly higher number of EPCs in the peripheral blood than those not receiving medical treatment ($p=0.0143$). Also, patients treated with 5-aminosalicylate alone had higher numbers of circulating EPCs than those treated with 5-aminosalicylate plus steroids ($p=0.0265$). However, the numbers of EPCs in all groups were too small to allow any reasonable conclusions to be drawn.

Quantitation of circulating HPCs. HPC is a putative precursor of EPCs (9-13). We assessed the overall trend in the different types of hematopoietic colonies generated from PBMCs using a CFU assay for HPCs (Fig. 4). Colony formation of HPCs was found in every subject. Nevertheless, in contrast to the results of the EPC culture assay, the colony-forming ability of each HPC, including BFU-E, CFU-G+GM and CFU-GEMM, was

comparable among patients with UC, patients with CD, patients with infectious colitis, and healthy controls (Table III). In addition, we attempted to quantify the number of CFU-F colonies derived from the PBMCs using a CFU assay for mesenchymal cells; however, no colony formation was observed in any of the subjects with UC, CD, infectious colitis, or healthy controls.

Discussion

One theory of the pathogenesis of IBD suggests that there is a basic defect of mucosal healing in this condition, resulting in chronic intestinal inflammation (1,2,33). This study examined the role of EPCs in the pathophysiology of IBD.

Our data first demonstrated a reduction in the number of circulating EPCs in patients with UC. Previous studies have reported a significant reduction in the circulating number of EPCs in other chronic diseases, such as rheumatoid arthritis (25) and diabetes mellitus (27,28), as compared with the numbers in healthy individuals. Interestingly, no such reduction in the circulating number of EPCs was observed in CD, another chronic inflammatory condition. Although the precise pathogenesis of UC and CD is not fully understood, available evidence indicates that the pathogenetic mechanisms underlying the two conditions are different. Interestingly, cases with the infectious colitis, an acute inflammatory condition, were

Table III. Evaluation of the total number of colonies derived from hematopoietic cells obtained by PBMCs cultured *in vitro* from healthy control subjects, patients with infectious colitis, patients with ulcerative colitis, and patients with Crohn's disease.

	Healthy control (n=45)	Infectious colitis (n=11)	Ulcerative colitis		Crohn's disease	
			Active (n=25)	Inactive (n=16)	Active (n=15)	Inactive (n=11)
BFU-E	18.0±25.3	11.0±18.9	9.5±15.4	15.8±20.8	14.5±9.3	23.0±28.6
CFU-G+GM	4.0±4.6	3.0±4.4	2.5±5.6	2.3±5.3	5.0±3.4	3.5±5.1
CFU-GEMM	0.0±0.5	0.0±0.4	0.0±0.6	0.0±0.5	0.0±0.5	0.0±0.9
Total	24.5±32.6	13.5±21.1	16.5±17.9	19.3±25.8	22.5±18.9	34.0±36.5

BFU-E, burst-forming unit-erythroid; CFU-G, colony-forming unit-granulocyte; CFU-GM, colony-forming unit-granulocyte, macrophage; CFU-GEMM, colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte. As CFU-G and CFU-GM were sometimes indistinguishable, we calculated the sum of CFU-G and CFU-GM and represented this as CFU-G+GM. The results, expressed as number of CFU-hem/1x10⁵ PBMCs, are shown as median ± IQR.

found to show higher numbers of circulating EPCs, suggesting that prolonged response to chronic inflammation is necessary for this pathophysiologic change. Although no direct correlation between the circulating number of EPCs and the degree of clinical disease activity has been observed in UC, it should be noted that the number of EPCs in the peripheral blood was correlated with some laboratory markers, including the serum hemoglobin level and platelet count (34).

There are several possible explanations for the reduction in the number of circulating EPCs in patients with UC. The reduction could be a byproduct of a number of mechanisms. The existence of multi-lineage- or single lineage-committed HPCs was demonstrated in the peripheral blood in the aforementioned patient groups with inflammatory conditions, with no quantitative differences in the HPC levels among the groups. Taking into consideration the contention that HPC is one putative precursor of EPCs, (9-13) the ability of HPCs to differentiate into EPCs may be impaired in patients with UC. If this were true, the factor responsible for this reduced ability of the HPCs to differentiate this event may play a crucial role in the pathophysiology of UC. Further studies are required to define the possible factors involved, such as intracellular signaling molecules, cytokines and growth factors.

An alternative explanation for the reduction in the number of EPCs in the peripheral blood might be consumption of circulating EPCs at the site of disease. It is conceivable that EPCs may be trapped in the inflamed intestinal vessels, resulting in a reduction of their numbers in the peripheral blood. In fact, this hypothesis is supported by recent observations of EPC enrichment in the inflamed rheumatoid arthritis joint (26). This hypothesis may be difficult to prove due to the lack of exclusive markers for EPCs (12,13). However, it seems unlikely, at least in intestinal inflammatory conditions, since the reduction of the cell number in the peripheral blood was observed only in UC, but not in other diseases, such as CD or infectious colitis. Moreover, no correlation of the EPC number was observed with the UC disease extent. In addition, we must rule out the possibility of the effect of medications used in IBD patients, such as steroids or 5-aminosalicylate, on the peripheral blood count of EPCs. However, this hypothesis also seems unlikely since the reduction in the

number of circulating EPCs was consistently observed in UC regardless of the steroid treatment, and the alteration of the circulating number of EPCs by the treatment with steroids and/or 5-aminosalicylate was observed in UC but not in CD (data not shown). To date, there are no reports on the effects of these drugs on hematopoiesis or EPC mobilization. Additional studies are needed to define the mechanisms that underlie the reduction in the circulating number of EPCs and to better understand the patho-physiological consequences of this event in cases of UC.

Recently, several reports have indicated bone marrow cells as a novel source of cells to regenerate the damaged intestine. Okamoto *et al* demonstrated that bone marrow-derived epithelial cells are distributed in the intestinal epithelial layer, particularly during epithelial regeneration after mucosal damage (35). Brittan *et al*, on the other hand, found that a portion of intestinal myofibroblasts, which play an important role in wound healing, are derived from the bone marrow (36). There is some circumstantial evidence to suggest that bone marrow-derived cells may be recruited into the damaged intestine, where they may repopulate and promote tissue repair. Oyama *et al* reported that autologous hematopoietic stem cell transplantation attenuated disease activity in patients with refractory CD (37). Dieckgraefe *et al* showed that the administration of GM-CSF, a potent stimulant for hematopoiesis, was effective for treating patients with active CD (38). These results in humans were supported by the results of animal studies demonstrating that bone marrow cells (39,40) or their inducers (41,42) could ameliorate experimental colitis.

The role of bone marrow-derived EPCs in the regeneration of the endothelium in mature vessels has not yet been clarified. Several experimental studies indicated a significant contribution of EPCs in reendothelialization during the recovery process from disease. A previous study showed that bone marrow-derived EPCs participated in neovascularization in patients with fatal acute myocardial infarction with a history of allogeneic bone marrow transplantation (43). Another report showed that EPCs were mobilized from bone marrow and accumulated within the ischemic border zone after acute myocardial infarction in animals (11). Together with these beneficial effects of EPCs, the reduction in the number of

circulating EPCs observed in UC may cause insufficient regeneration of the endothelium, which may lead to chronicity of the disease. Defining the mechanisms that underlie EPC reduction in UC may yield important insight into the pathogenesis of the disease. Furthermore, breaking this vicious cycle with EPCs themselves or their inducers might be of benefit in the treatment of UC.

One of the limitations of the present study was the lack of exclusive markers that distinguish EPCs from native endothelial cells (12,13). Indeed, mature endothelial cells may also circulate in the peripheral blood. However, the number of circulating mature endothelial cells was low, even in patients with acute myocardial infarction (10 to 100 cells/ml blood) (44), whereas the circulating number of EPCs ranged from 0.3 to 1×10^4 cells/ml (45,46). Moreover, mature endothelial cells have a low proliferative activity and do not participate in neovascularization (43,47,48).

In conclusion, the results of this study indicate that the circulating number of EPCs is significantly reduced in cases of UC, and that this reduction might constitute an important pathophysiological event in cases with the disease. Further studies designed to specifically investigate the role of EPCs in the chronically inflamed bowel in cases of IBD are needed.

Acknowledgements

We thank Ms. Kazuyo Handa and Drs Atsushi Toyonaga, Osamu Tsuruta, Asuka Suzuki, Nobuo Tomiyasu, Toru Nakamura, Eitaro Taniguchi and Kazunori Harada (Division of Gastroenterology, Kurume University), Ms. Ritsuko Seki (Division of Hematology, Kurume University), Drs Yoshiaki Takeshita, Toshifumi Shimada and Yosuke Katsuda (Division of Cardiovascular Medicine, Kurume University), and Dr Haruchika Masuda (Department of Physiology, Tokai University) for their invaluable help.

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