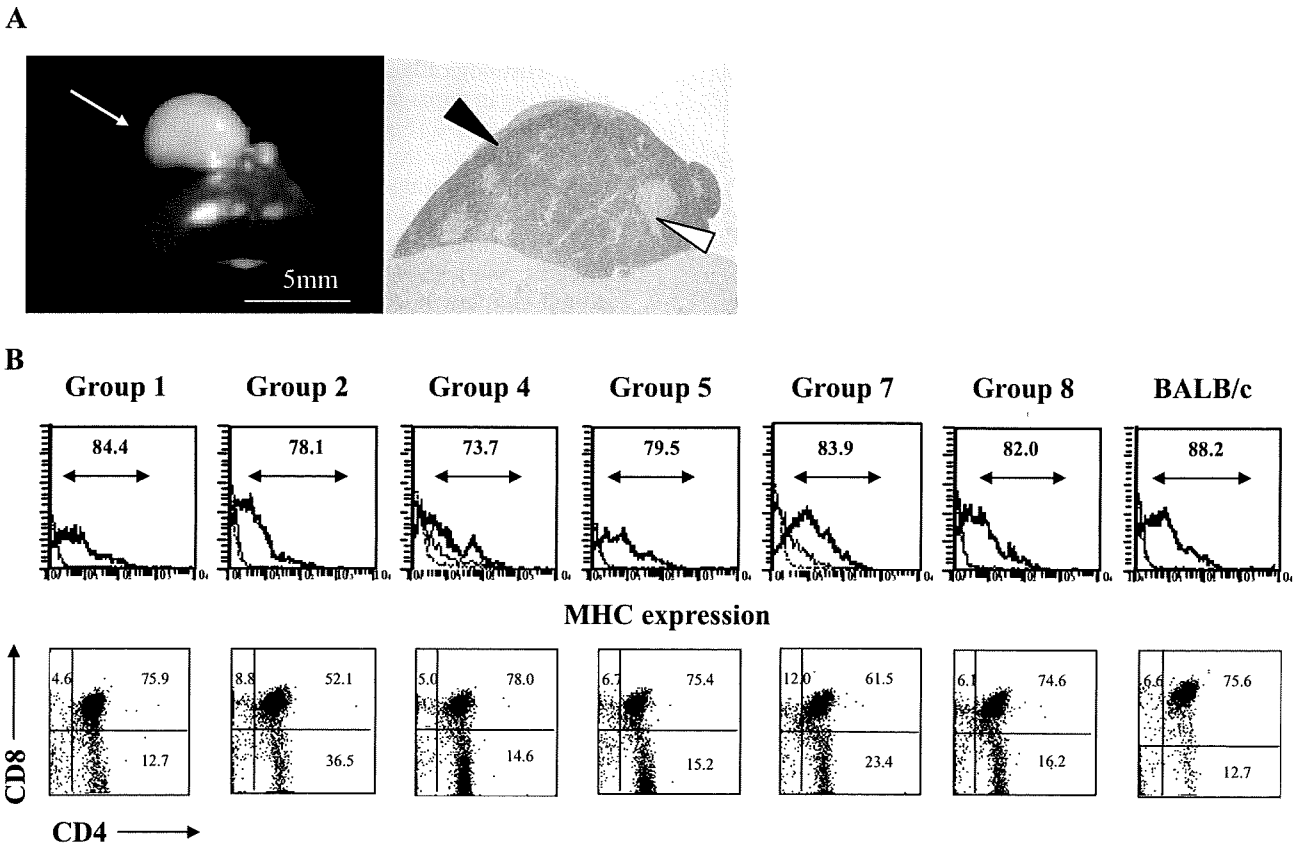

FIGURE 2. Analysis of histology, chimerism, and thymocyte subsets in transplanted thymus from experimental groups. (A) Macroscopic (*left*) and microscopic findings (*right*) in transplanted thymus from group 1 at 2 months after transplantation are shown. (B) Thymus tissue was engrafted (*arrow*), and cortical (*closed arrowhead*) and medullary (*open arrowhead*) areas displayed fine construction. FACS profile for chimerism (*upper*) and CD4- and CD8-thymocyte subsets (*lower*) are shown in transplanted thymus from groups 1, 4, and 7 for triple chimeras, from groups 2, 5, and 8 for MHC-matched TT (described in Table 1), and from untreated BALB/c as controls. Thick line, BMC type; thin line, thymus type; dotted line, recipient type in histogram by H-2K^b, H-2K^d or H-2K^k staining, as described in Table 1. CD4 and CD8 double-staining in thymocytes from transplanted thymi. Representative profiles are shown for three or four mice in each group.

(without TT; Table 1). Donor-derived CD4$^+$ and CD8$^+$ T cells as well as B220$^+$ B cells were generated well in the triple chimeric SAMP1, although very few lymphocytes were seen in the mice without TT (Fig. 5A). In functional analyses, spleen cells from the triple chimeric mice showed significant responses to both Con A and LPS, the levels being comparable to untreated BALB/c mice (Fig. 5B). In contrast, untreated SAMP1 and the mice treated by IBM-BMT alone (without TT) showed low responsiveness to Con A, but responded to LPS. In MLR assays, the triple chimeric mice also showed tolerance to all three types of MHC determinants, but responsiveness to the fourth party (DBA/1; Fig. 5C).

DISCUSSION

In the present study we have established a triple chimeric mouse model consisting of donor 1 giving MHC-disparate-thymus, donor 2 giving MHC-disparate-BMCs, and 3, the recipient MHC-disparate microenvironment (disparate to donors 1 and 2). It should be noted that we use the fresh and nontreated fetal thymus as TT. The immature and high proliferative potential may help support and reconstitute BMC-derived T cells, and the resident immature T cells may suppress alloreactivity. This beneficial effect might be adapted well for aged hosts. Although there are ethical issues involved in obtaining the thymus graft for clinical use, taking such immature thymus tissues was previously approved by patients with congenital heart diseases for treatment of DiGeorge Syndrome and human immunodeficiency virus in-fection (*28*, *29*). In addition, a method of regenerating the thymus has been developed (*30*), and the thymus graft could be obtained from aborted fetuses. Thymus transplantation might be clinically applicable in the near future.

The triple chimeras showed significantly long survival with BMC-derived chimerism comparable to the mice with MHC-matched TT and BMC donors (Table 1). Both the triple chimeras and the mice with MHC-matched donor TT and BMC survived more than 6 months (data not shown). In addition, the triple chimeric mice also showed the normal T-cell reconstitution and functions, which were comparable to the mice with MHC-matched TT (Figs. 1B and 3). In contrast, the nude and ATx chimeric mice that had been treated with IBM-BMT alone showed a significantly short survival with low

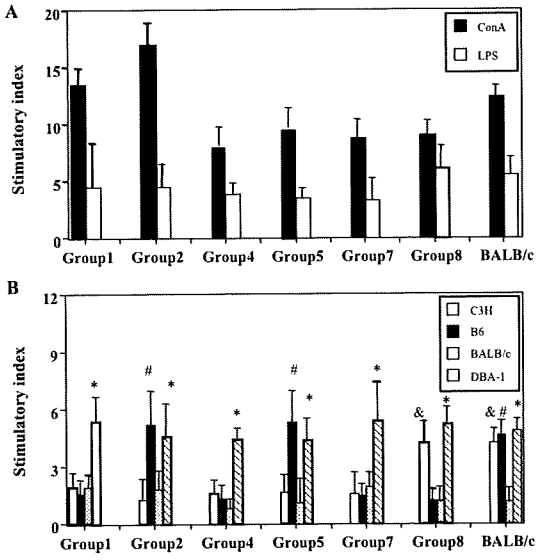

FIGURE 3. Mitogen responses and MLR in experimental groups. Mitogen responses to Con A and LPS (A) and MLR (B) in splenocytes are shown from groups 1, 4, and 7 for triple chimeras, from groups 2, 5, and 8 for MHC-matched TT, and from group 3 for absence of TT (as described in Table 1), and from untreated BALB/c mice as controls. Representative results are shown for three experiments in each group. SI was calculated as the average ^{3}H-TdR incorporation of triplicate samples of responding cells with either mitogen or stimulating cells/^{3}H-TdR incorporation of responding cells in medium alone. There were no significant statistical difference for Con A and LPS responses in triple chimeras, their MHC-matched TT (groups 1 and 2; groups 4 and 5; groups 7 and 8) and BALB/c mice (A). *$P<0.05$ compared with C3H, B6 and BALB/c in group 1, compared with C3H and BALB/c in group 2, compared with C3H, B6 and BALB/c in group 4, compared with C3H and BALB/c in group 5, compared with C3H, B6, and BALB/c in group 7, compared with B6 and BALB/c in group 8, and compared with BALB/c in BALB/c mice; #$P<0.05$ compared with C3H and BALB/c in group 2, compared with C3H and BALB/c in group 5, and compared with BALB/c in BALB/c mice; &$P<0.05$ compared with B6 and BALB/c in group 8 and compared with BALB/c in BALB/c mice. Data were shown as means±SD.

T-cell reconstitution, as expected. These results strongly suggest that, even with fully MHC-mismatched TT, hematopoietic stem cells can develop and reconstitute well in the MHC-mismatched microenvironment with long survival.

The exact mechanisms underlying the supply of sufficient T cells even with fully MHC-mismatched thymus are unknown. One possibility is that MHC type is not related to host survival with sufficient hemopoiesis and thymopoiesis, although the MHC-matched combination is better than the MHC-mismatched combination (*31*, *32*). The second possibility is that some donor BMC-derived BM and thymic stromal cells support the hemopoiesis and/or thymopoiesis as MHC-matched stromal cells. Actually, we have previously found that donor-derived bone marrow stromal cells (including MSCs) migrate into the thymus where they are engaged in positive selection and also negative selection (*33*). Further study is needed to find the origins of the stromal cells in the transplanted thymus and BM in the triple chimeric mice.

Histologically, triple chimeric mice did not show apparent organ damage, although a few lymphocytes had infiltrated the organs (Fig. 1A). In addition, triple chimeric mice showed tolerance to all three types of MHC determinants (Fig. 3B), indicating the acceptance of three types of skin grafts (data not shown). Because the skin grafts were accepted for a long time (>5 months), long-term tolerance could be induced, although we did not carry out the MLR assays using double chimeric mice at that time. These results suggest that there were at least no lethal GVHR or HVGR in the triple chimeric mice.

Regarding the mechanisms underlying tolerance induction, it is likely that thymic DCs, which can delete autoreactive T cells by negative selection (*27*), play a crucial role in our present study as well as previous reports (*34–36*). We have found both BMC-derived and thymus-derived (resident) DCs in the transplanted thymus of triple chimeric mice, although hardly any recipient-type DCs were found in the thymus (Fig. 4). It has been reported that marrow stromal cells (including MSCs) can induce tolerance in the prethymic process (*20*, *21*, *37*, *38*). In this regard, IBM-BMT would facilitate contact between donor-derived HSCs and MSCs, which results in preventing GVHD and HVGR (*17–19*). Regarding postthymic tolerance induction, a recent report shows that stromal cells of the lymph node induce tolerance in naive T cells by expressing tissue-specific antigen (*39*). They might also induce tolerance of host MHC in the microenvironment (including the lymph node). The specific mechanism for tolerance induction needs further analyses.

Finally, we have found that triple chimerism is also effective in SAMP1, which offers an animal model for senescence in humans; triple chimeric SAMP1 survived longer than SAMP1 with IBM-BMT alone (without TT; Fig. 4). The former also improved T-cell functions and showed tolerances to all three MHC determinants (Fig. 5). However, SAMP1 treated by IBM-BMT alone (without TT) showed a few donor-derived cells, indicating chimeric resistance in the absence of functional T cells. In fact, it has been reported that aged recipients show increased sensitivity to irradiation (*40*). However, in this respect, we have recently found that TT overcomes chimeric resistance in MRL/lpr mice with Fas gene defect (*41*). In addition, the triple chimeric mice in SAMP1 did not show the loss of activity, alopecia, and increased lordokyphosis, which are characteristic symptoms in aged SAMP1 (*22*, *23*).

It has also been shown that allogeneic BMT with TT elevates T-cell functions in the recipients with thymic deficiency in mice (*16*, *42*, *43*) and humans (*44*). The decline in T-cell functions with age leads to increased incidences of development of ailments such as autoimmune disease, malignancy, or infection (*11*, *45*). BMT plus TT may thus be effective not only in curing primary disease but also in preventing other diseases by improving T-cell functions.

Based on the findings, simultaneous multiple organs, tissues, or cell transplantation such as the heart, liver, kidney, islets, or dopamine-producing cells from the different donors might be applicable. In addition, the three models (nude, ATx, and SAMP1), which are primarily used for elderly patients with thymic deficiency, might also represent models for

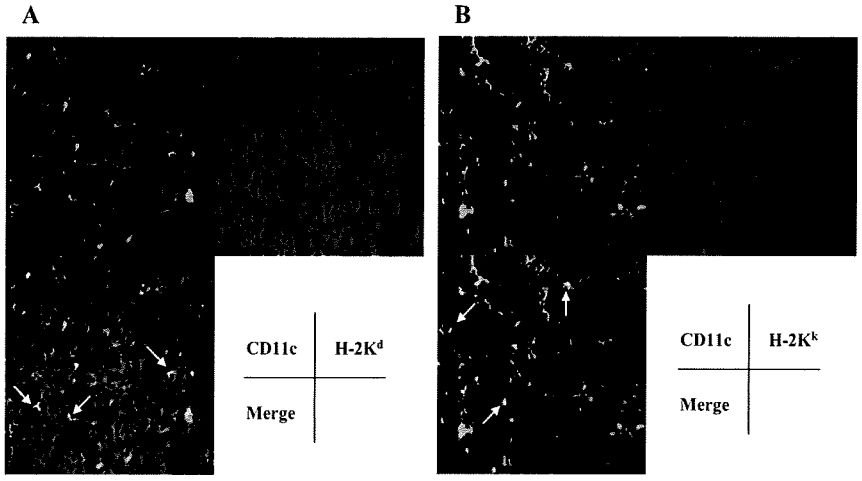

FIGURE 4. BMC- and thymus-derived dendritic cells (DCs) in transplanted thymus. The transplanted thymic lobe from group 7 at 2 months after transplantation was stained using FITC-conjugated CD11c mAb and PE-avidin/biotin-conjugated H-2K^d:BMC type (A), or H-2K^k:thymus type (B) mAb. Expressions were evaluated under confocal microscopy. Both BMC-derived (H-2K^{d+} CD11c$^+$) and thymus-derived DCs (H-2K^{k+} CD11c$^+$) were found in the transplanted thymus (*arrows*). Representative profiles are shown for three experiments.

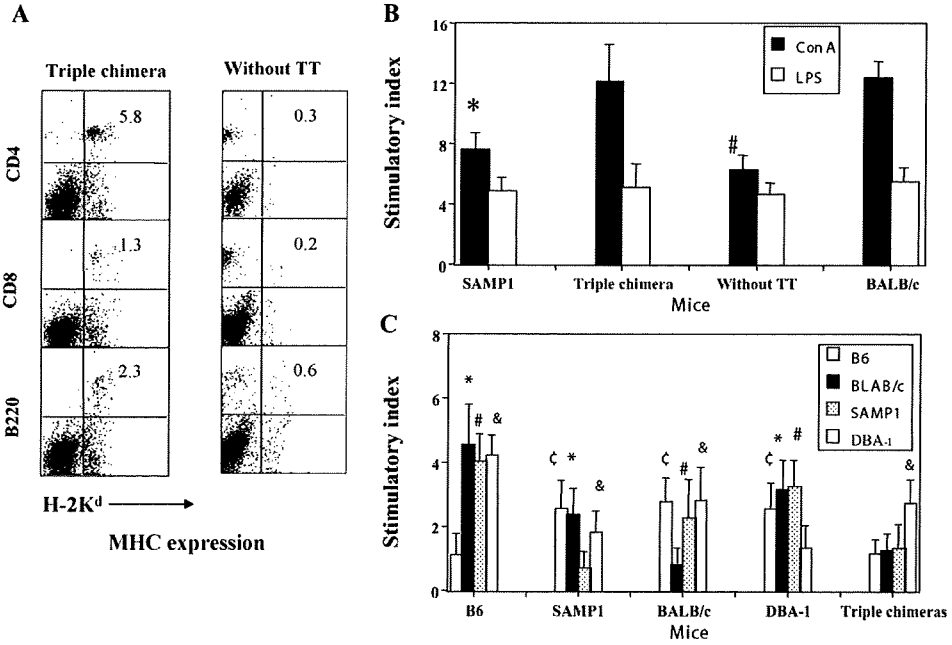

FIGURE 5. BMC-derived lymphocyte subsets, mitogen responses, and MLR in spleen from SAMP1 mice treated by triple chimeric transplantation or absence of TT. BMC-derived CD4$^+$ and CD8$^+$ T cells and B220$^+$ B cells in spleen were analyzed from triple chimeric mice or mice with absence of TT by flow cytometry. (A) Donor BMCs were from BALB/c mice (H-2^d). (B) Mitogen responses in splenocytes to Con A and LPS are shown from untreated SAMP1 mice, mice treated with triple chimeric transplantation, chimeras without TT 2 months after transplantation, and untreated BALB/c mice as controls. (C) MLR in splenocytes from untreated B6, SAMP1, BALB/c, DBA-1, and SAMP1 mice treated for triple chimeric transplantation 2 months after transplantation. Representative results are shown for three experiments. SI was calculated as the average ^{3}H-TdR incorporation of triplicate samples of responding cells with either mitogen or stimulating cells/^{3}H-TdR incorporation of responding cells in medium alone. (B) $^{*\#}P<0.05$ compared with Con A in triple chimeric mice. (C) $^*P<0.05$ compared with B6 in B6 mice, compared with SAMP1 in SAMP1 mice, and compared with DBA1 in DBA 1 mice; $^\#P<0.05$ compared with B6 in B6 mice, and compared with DBA1 in DBA1 mice; $^\&P<0.05$ compared with B6 in B6 mice, compared with SAMP1 in SAMP1 mice, compared with BALB/c in BALB/c mice, and compared with B6, BALB/c, or DBA1 in triple chimeric mice; $^\phi P<0.05$ compared with SAMP1 in SAMP1 mice, compared with BALB/c and BALB/c mice, and compared with DBA1 in DBA1 mice. Data were shown as means±SD.

DiGeorge Syndrome or patients with thymectomy after surgery for congenital heart disease. Although the recent nonmyeloablative protocol for BMT induced less complication than the conventional method in older people (*46*), the addition of TT method in the present study might facilitate improved results with some rejuvenating. If some ethical issues are resolved, fetal thymus plus IBM-BMT may be a valuable strategy for the treatment of various diseases.

ACKNOWLEDGMENTS

The authors thank Ms. Y. Tokuyama, Ms. R. Hayashi, and Ms. A. Kitajima for their technical assistance and Mr. Hil-

ary Eastwick-Field, Mr. Brian O'Flaherty, and Ms. K. Ando for their help in the preparation of the manuscript.

REFERENCES

1. Ikehara S. Bone marrow transplantation: A new strategy for intractable disease. *Drugs Today* 2002; 38: 103.
2. Ikehara S, Ohtsuki H, Good RA, et al. Prevention of type I diabetes in nonobese diabetic mice by allogenic bone marrow transplantation. *Proc Natl Acad Sci U S A* 1985; 22: 7743.
3. Yasumizu R, Sugiura K, Iwai H, et al. Treatment of type 1 diabetes mellitus in non-obese diabetic mice by transplantation of allogeneic bone marrow and pancreatic tissue. *Proc Natl Acad Sci U S A* 1987; 84: 6555.
4. Than S, Ishida H, Inaba M, et al. Bone marrow transplantation as a strategy for treatment of non-insulin-dependent diabetes mellitus in KK-Ay mice. *J Exp Med* 1992; 176: 1233.
5. Ishida T, Inaba M, Hisha H, et al. Requirement of donor-derived stromal cells in the bone marrow for successful allogeneic bone marrow transplantation. Complete prevention of recurrence of autoimmune diseases in MRL/MP-Ipr/Ipr mice by transplantation of bone marrow plus bones (stromal cells) from the same donor. *J Immunol* 1994; 152: 3119.
6. Nakagawa T, Nagata N, Hosaka N, et al. Prevention of autoimmune inflammatory polyarthritis in male New Zealand black/KN mice by transplantation of bone marrow cells plus bone (stromal cells). *Arthritis Rheum* 1993; 36: 263.
7. Nishimura M, Toki J, Sugiura K, et al. Focal segmental glomerular sclerosis, a type of intractable chronic glomerulonephritis, is a stem cell disorder. *J Exp Med* 1994; 179: 1053.
8. Kaneda H, Adachi Y, Saito Y, et al. Long-term observation after simultaneous lung and intra-bone marrow-bone marrow transplantation. *J Heart Lung Transplant* 2005; 24: 1415.
9. Koike Y, Adachi Y, Suzuki Y, et al. Allogeneic intrabone marrow-bone marrow transplantation plus donor lymphocyte infusion suppresses growth of colon cancer cells implanted in skin and liver of rats. *Stem Cell* 2007; 25: 385.
10. Klingemann HG, Storb R, Fefer A, et al. Bone marrow transplantation in patients aged 45 years and older. *Blood* 1986; 67: 770.
11. Weiner RS, Bortin MM, Gale RP, et al. Interstitial pneumonitis after bone marrow transplantation. Assessment of risk factors. *Ann Intern Med* 1986; 104: 168.
12. Ringden O, Paulin T, Lonngvist B, et al. An analysis of factors predisposing to chronic graft-versus-host disease. *Exp Hematol* 1985; 13: 1062.
13. Hirokawa K, Utsuyama M, Kasai M, et al. Understanding the mechanism of the age-change of thymic function to promote T cell differentiation. *Immunol Lett* 1994; 40: 269.
14. Mackall CL, Gress RE. Pathways of T-cell regeneration in mice and humans: Implications for bone marrow transplantation and immunotherapy. *Immunol Rev* 1997; 157: 61.
15. Aspinall R. Age-associated thymic atrophy in the mouse is due to a deficiency affecting rearrangement of the TCR during intrathymic T cell development. *J Immunol* 1997; 158: 3037.
16. Hosaka N, Nose M, Kyogoku M, et al. Thymus transplantation, a critical factor for correction of autoimmune disease in aging MRL/+mice. *Proc Natl Acad Sci U S A* 1996; 93: 8558.
17. Kushida T, Inaba M, Hisha H, et al. Intra-bone marrow injection of allogeneic bone marrow cells: A powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. *Blood* 2001; 97: 3292.
18. Nakamura K, Inaba M, Sugiura K, et al. Enhancement of allogeneic hematopoietic stem cell engraftment and prevention of GVHD by intra-bone marrow bone marrow transplantation plus donor lymphocyte infusion. *Stem Cells* 2004; 22: 125.
19. Ikehara S. A novel strategy for allogeneic stem cell transplantation: Perfusion method plus intra-bone marrow injection of stem cells. *Exp Hematol* 2003; 31: 1142.
20. Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 2003; 101: 3722.
21. Blanc KL, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004; 363: 1439.
22. Takeda T, Hosokawa M, Takeshita S, et al. A new murine model of accelerated senescence. *Mech Ageing Dev* 1981; 17: 183.
23. Odagiri Y, Uchida H, Hosokawa M, et al. Accelerated accumulation of somatic mutations in the senescence-accelerated mouse. *Nat Genet* 1998; 19: 116.
24. Hosono M, Hanada K, Toichi E, et al. Immune abnormality in relation to nonimmune diseases in SAM mice. *Exp Gerontol* 1997; 32: 181.
25. Miller JF. Studies on mouse leukaemia. Leukaemogenesis by cell-free filtrates inoculated in newborn and adult mice. *Br J Cancer* 1960; 14: 93.
26. Nagahama T, Sugiura K, Lee S, et al. A new method for tolerance induction: Busulfan administration followed by intravenous injection of neuraminidase-treated donor bone marrow. *Stem Cell* 2001; 19: 425.
27. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol* 2003; 21: 685.
28. Markert ML, Boeck A, Hale LP, et al. Transplantation of thymus tissue in complete DiGeorge syndrome. *N Engl J Med* 1999; 341: 1180.
29. Markert ML, Hicks CB, Bartlett JA, et al. Effect of highly active antiretroviral therapy and thymic transplantation on immunoreconstitution in HIV infection. *AIDS Res Hum Retroviruses* 2000; 16: 403.
30. Zhang L, Sun L, Zhao Y. Thymic epithelial progenitor cells and thymus regeneration: An update. *Cell Res* 2007; 17: 50.
31. Chen BJ, Cui X, Sempowski GD, et al. Hematopoietic stem cell dose correlates with the speed of immune reconstitution after stem cell transplantation. *Blood* 2004; 103: 4344.
32. Hashimoto F, Sugiura K, Inoue K, et al. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vivo. *Blood* 1997; 89: 49.
33. Li Y, Hisha H, Inaba M, et al. Evidence for migration of donor bone marrow stromal cells into recipient thymus after bone marrow transplantation plus bone grafts: A role of stromal cells in positive selection. *Exp Hematol* 2000; 28: 950.
34. Kamano C, Vagefi PA, Kumagai N, et al. Vascularized thymic lobe transplantation in miniature swine: Thymopoiesis and tolerance induction across fully MHC-mismatched barriers. *Proc Natl Acad Sci U S A* 2004; 101: 3827.
35. Nobori S, Shimizu A, Okumi M, et al. Thymic rejuvenation and the induction of tolerance by adult thymic grafts. *Proc Natl Acad Sci U S A* 2006; 103: 19081.
36. Duncan SR, Capetanakis NG, Lawson BR, et al. Thymic dendritic cells traffic to thymi of allogeneic recipients and prolong graft survival. *J Immunol* 2002; 109: 755.
37. Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002; 30: 42.
38. Nicola MD, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002; 99: 3838.
39. Lee JW, Epardaud M, Sun J, et al. Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. *Nat Immunol* 2007; 8: 181.
40. Rugh R. The relation of sex, age, and weight of mice to microwave radiation sensitivity. *J Microw Power* 1976; 11: 127.
41. Hosaka N, Ryu T, Miyake T, et al. Treatment of autoimmune diseases in MRL/lpr mice by allogenic bone marrow transplantation plus adult thymus transplantation. *Clin Exp Immunol* 2007; 147: 555.
42. Hirokawa K, Utsuyama M. Combined grafting of bone marrow and thymus, and sequential multiple thymus graftings in various strains of mice. The effect on immune functions and life span. *Mech Ageing Dev* 1989; 49: 49.
43. Van den Brink MR, Alpdogan O, Boyd RL. Strategies to enhance T-cell reconstitution in immunocompromised patients. *Nat Rev Immunol* 2004; 4: 856.
44. Fulop T, Larbi A, Wikby A, et al. Dysregulation of T-cell function in the elderly: Scientific basis and clinical implications. *Drugs Aging* 2005; 22: 589.
45. Kudlacek S, Willvonseder R, Stohlawetz P, et al. Immunology and aging. *Aging Male* 2000; 3: 137.
46. Falda M, Busca A, Baldi I, et al. Nonmyeloablative allogeneic stem cell transplantation in elderly patients with hematological malignancies: Results from the GITMO (Gruppo Italiano Trapianto Midollo Osseo) multicenter prospective clinical trial. *Am J Hematol* 2007; 82: 863.

Ann Hematol (2008) 87:797–807
DOI 10.1007/s00277-008-0513-9

ORIGINAL ARTICLE

Contribution of neural cell adhesion molecule (NCAM) to hemopoietic system in monkeys

Junko Kato · Hiroko Hisha · Xiao-li Wang · Tomomi Mizokami · Satoshi Okazaki · Qing Li · Chang-ye Song · Masahiko Maki · Naoki Hosaka · Yasushi Adachi · Muneo Inaba · Susumu Ikehara

Received: 4 September 2007 / Accepted: 21 May 2008 / Published online: 25 June 2008

Abstract Neural cell adhesion molecules (CD56) are important adhesion molecules that are mainly expressed on neural cells and natural killer cells. Although freshly isolated cynomolgus monkey bone marrow cells (BMCs) contained only a few CD56-positive cells, almost all the BM adherent cells (obtained after a 2- to 3-week culture of the BMCs) were stained positively with anti-CD56 monoclonal antibody (mAb). The BM adherent cells showed uniformly fibroblastic morphology and were negative for hematolymphoid markers (CD4, CD8, CD11b, CD14, CD34, and CD45). Adipogenesis and osteogenesis were observed under inductive culture conditions. The BM adherent cells had the ability to support hemopoiesis of hemopoietic stem cells (HSCs) in vitro, and the proliferation of HSCs was significantly inhibited by the addition of anti-CD56 mAb to the coculture system. CD56 molecules were also expressed on HSCs because about 20% of an HSC-enriched population (lineage-negative and blast-gated cells) was positive for CD56. In addition, the immunostaining of monkey BM sections revealed that many stromal cells were CD56-positive, and some CD56-positive stromal cells came into direct contact with CD56-positive hemopoietic cells. These results indicate that the CD56 molecule is expressed on both HSCs and BM stromal cells (containing MSCs) in monkeys, and therefore it can be speculated that CD56 also contributes to the human hematopoietic system.

Keywords NCAM · MSCs · Bone marrow stromal cells · Cynomolgus monkey

Equal contributors: J. Kato and H. Hisha

J. Kato · H. Hisha · X.-l. Wang · T. Mizokami · S. Okazaki · Q. Li · C.-y. Song · M. Maki · N. Hosaka · Y. Adachi · M. Inaba · S. Ikehara
1st Department of Pathology, Kansai Medical University, Moriguchi, Osaka, Japan

H. Hisha · S. Ikehara
Department of Transplantation for Regeneration Therapy (Sponsored by Otsuka Pharmaceutical Co. Ltd.), Kansai Medical University, Moriguchi, Osaka, Japan

H. Hisha · M. Maki · N. Hosaka · Y. Adachi · M. Inaba · S. Ikehara
Regeneration Research Center for Intractable Diseases, Kansai Medical University, Moriguchi, Osaka, Japan

J. Kato
Japan Cell Net, Kyoto, Japan

S. Ikehara (✉)
Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan
e-mail: ikehara@takii.kmu.ac.jp

Introduction

The bone marrow (BM) long-term culture system, established by Dexter et al. [1] and Whitlock and Witte [2], has demonstrated the importance of direct interactions between hemopoietic cells and BM stromal cells in order to induce active proliferation and differentiation of hemopoietic progenitor–stem cells and to maintain long-term hemopoiesis. The stromal cells provide niches for hemopoietic stem cells (HSCs) by expressing adhesion molecules and secreting cell matrix molecules and growth factors. Several important adhesion molecules, such as VLA-4, VLA-5, VCAM-1, ICAM-1, and CD44, have been found and their functions have been well elucidated [3–5]. To find other

important interaction molecules for hemopoiesis, we previously established a monoclonal antibody (mAb; anti-PA6) against a mouse BM stromal cell line (PA6) [6]. The mAb inhibits pseudoemperipolesis and suppresses the proliferation of HSCs, suggesting that it reacts with molecules responsible for the interaction between HSCs and stromal cells. Affinity chromatography and mass peptide fingerprinting revealed that the PA6 protein is a neural cell adhesion molecule (NCAM, CD56) [7]. We also established a stromal cell line (FMS/PA6-P) from fetal mouse bone marrow cells (BMCs) using the anti-PA6 mAb. The cell line is highly positive for CD56 and has a higher ability to support hemopoiesis than other stromal cell lines. Moreover, the cell line has characteristics of mesenchymal stem cells (MSCs) because it can differentiate into adipocytes, osteoblasts, and vascular endothelial cells [8]. These results indicate that CD56 is expressed not only on stromal cells but also on MSCs in the murine system.

The CD56 molecule has been identified in retinal tissues of chick embryos by Thiery et al. [9] in 1977 and was shown to be an important adhesion molecule involved in the morphogenesis of neural cells in embryonic development [9, 10]. The CD56 molecule belongs to the immunoglobulin superfamily, and three isoforms (NCAM-120, NCAM-140, and NCAM-180), differing in molecular size of the intracellular domain, are known. NCAM-180 is expressed mainly on neural cells, whereas NCAM-120 and NCAM-140 are expressed on cardiac muscle, skeletal muscle, some T cell populations, and natural killer (NK) cells. However, the expression of the CD56 molecule on hematolymphoid cells, except for NK cells, has not thus far been well elucidated, although there are a few reports showing that CD56 is highly expressed on some malignant cells: plasma cells in multiple myeloma [11] and leukemic cells in acute myeloid leukemia [12]. As for BM stromal cells, it has been reported that human BM endosteal cells express CD56 [13] and that a human osteoblastic cell line U2-OS, derived from osteoblastoma, has the capacity to support hemopoiesis and is highly positive for CD56 [14].

Recently, we have established a new method for harvesting monkey BMCs (the perfusion method) [15–17]. By this method, the contamination of the collected BMCs with peripheral blood was reduced to a minimum, compared with the conventional aspiration method. To examine whether the contribution of CD56 to hemopoiesis is a feature common to all species, we attempted to investigate the expression of CD56 on monkey BM adherent cells that were obtained by the culture of BMCs collected from cynomolgus monkeys using the perfusion method. It is known that CD56 molecules regulate cell migration, homing, proliferation, and maturation by homophilic (CD56 binding to CD56) as well as heterophilic (CD56 binding to a number of proteins and extracellular matrix molecules) interactions. Therefore, we examined whether CD56 molecules were also expressed on HSC-enriched populations and whether the interaction between HSCs and stromal cells through CD56 molecules is important for the proliferation and differentiation of HSCs. Recent research has demonstrated that human and murine BMCs contain MSCs and the MSCs can be obtained easily by the culture of BMCs. In the present study, we also investigate whether the monkey BM adherent cells, expressing CD56 molecules, have characteristics of MSCs.

Materials and methods

Monkeys

Normal cynomolgus monkeys (3 to 6 years old; 3- to 6-kg body weight) were obtained from Keari (Osaka, Japan). The monkeys were free of intestinal parasites and were seronegative for tuberculosis, herpes B, hepatitis A, and hepatitis B viruses. All surgical procedures and postoperative care of animals were carried out in accordance with the guidelines of the National Institutes of Health for care and use of primates. Experiments using these monkeys were conducted in accordance with protocols approved by the university's committee for animal research.

Harvesting of monkey BMCs

Monkeys were anesthetized using Ketalar (5 mg; Sankyo Co. Ltd.; Tokyo Japan) and an analgesic agent, Pentagin (Sankyo Co. Ltd.), and BMCs were then collected from the long bones (humerus and femur) and the ilium by the perfusion methods established in our laboratory [15–17]. In brief, one BM puncture needle was inserted into the proximal side of the long bone and the other was inserted into the distal side. In the case of the ilium, one needle was inserted into the end of the iliac crest and the other into the edge of the iliac crest. A syringe containing 0.5 ml of heparin was connected to the needle and a 30-ml syringe containing 30 ml of phosphate-buffered saline (PBS) was connected to the other needle. The PBS was gently pushed from the syringe into the BM cavity, and the PBS containing BMCs was collected in the syringe containing heparin.

Adherent cell culture of monkey BMCs

Monkey whole BMCs (1×10^7) were cultured in flasks (25 cm^2, #35-3014; BD Falcon, Franklin Lakes, NJ, USA) containing IMDM (Gibco, Grand Island, NY, USA)

supplemented with 10% fetal bovine serum (FBS; lot No. 5-1064; BioSolutions International, Melbourne, Australia) at 37 °C under 5% CO_2. Every 4 or 5 days, the culture medium containing nonadherent cells was removed and fresh medium was added to the flasks. During the culture, BM adherent cells began to proliferate and reached confluence 2–3 weeks later.

Flow cytometry

Monkey BM adherent cells were washed twice with 0.02% ethylene diamine tetraacetic acid (EDTA)–PBS and the adherent cells were detached from the flasks using trypsin–EDTA treatment. The thus-prepared cells were stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) against nonhuman primate CD4, CD8, CD11b, CD14, CD29, CD31, CD34, CD45, CD56, and CD62L (BD Biosciences, San Jose, CA, USA). Fresh BMCs were also stained with these mAbs. The stained cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA, USA).

Purification of monkey HSCs

Low-density (LD) BMCs ($\rho < 1.077$) were purified from fresh or frozen monkey BMCs by discontinuous density gradient centrifugation using Ficoll-Paque™ PLUS (#17-1440-02; GE Healthcare Bio-Science, Uppsala, Sweden; http://www.pnu.com). The LD cells were incubated with mAb (mouse immunoglobulin G (IgG) class) cocktails against nonhuman primate lineage markers (CD3, CD9, CD11b, CD14, CD16, and CD20) and then incubated twice with sheep antimouse IgG-conjugated immunobeads (#110.31; Dynal Inc., Oslo, Norway; http://www.dynal.no) with gentle agitation at a 3:1 bead-to-cell ratio. The immunobead-rosetted cells were removed using a magnetic particle concentrator. The remaining nonrosetted cells (lineage-negative cells) were considered as partially purified HSCs and used for culture on the BM adherent layer.

In some experiments, the partially purified HSCs were double-stained with PE-labeled anti-nonhuman primate CD34 mAb (#550619; BD Biosciences) and FITC-labeled antihuman CD56 mAb (cross-reactive with homologous monkey epitope, #0562; Exalpha, Watertown, MA, USA) and then analyzed using a FACScan. $CD34^+/56^+$ and $CD34^+/56^-$ populations included in a "blast" gate were sorted from the double-stained cells using EPICS ALTRA (Beckman Coulter, Inc., Fullerton, CA, USA). The sorted cells were morphologically analyzed by staining with May–Giemsa reagents and also functionally analyzed by clonal cell culture using methylcellulose assay.

Long-term culture of monkey HSCs

Monkey BMCs were passed through a cell strainer (70 μm mesh size, #REF352350; BD Falcon, Bedford, MA, USA) and divided into passed cells and nonpassed cells. The nonpassed cells were composed of small tissue fragments of BM and small bone pieces and therefore contained a higher percentage of adherent cells than the passed cells. These nonpassed cells were cultured in order to acquire the BM adherent cells, whereas the passed cells were frozen and stored in liquid nitrogen until BM adherent cell layers were formed.

The nonpassed cells were cultured in flasks (125 cm^2, #35-3136; BD Falcon) containing IMDM Gibco supplemented with 10% FBS (lot No. 5-1064, BioSolutions International), and a confluent adherent cell layer was formed 3 weeks later. The adherent cells were trypsinized, collected, and then subcultured in flasks (25 cm^2; 10^6 cells per flask). When the BM adherent cells had become subconfluent 2–3 days later, partially purified HSCs that were obtained from the frozen cell strainer-passed cells of the same monkey were inoculated on the BM adherent layer (5×10^5 cells per flask; triplicate). The cells were cultured in IMDM supplemented with 10% FBS (lot No. 5-1064) and a low concentration of human cytokines (IL-3, TPO, and FLT-3 ligand: 2 ng/ml, SCF: 9 ng/ml). TPO and SCF were kindly donated by the Kirin Brewery Co. Ltd. (Tokyo, Japan). Every week, half of the culture medium in the flasks (containing nonadherent cells) was removed and fresh medium was added to the flasks. The numbers of nonadherent cells per flask were counted and the nonadherent cells were then used for methylcellulose assays.

Methylcellulose assays

The colony-forming ability of the nonadherent cells obtained from the long-term culture was assessed using methylcellulose assays. Appropriate numbers of these cells were plated in 12-well plates (#3815-012; Iwaki, Chiba, Japan) in a volume of 1 ml of MethoCult GF H4434 (StemCell Technologies Inc., Vancouver, British Columbia, Canada), consisting of optimal concentrations of human cytokines (SCF, EPO, IL-3, GM-CSF, and G-CSF), 30% FBS, 1% bovine serum albumin, 2 mM L-glutamine, 100 μM 2-mercaptoethanol, and 0.9% methylcellulose (triplicate). The plates were incubated for 14 days at 37 °C under 5% CO_2, and the numbers of colonies were counted under an inverted microscope.

Whole BMCs, LD cells, and lineage-negative cells were also examined using methylcellulose assays to determine their ability to generate colonies. In addition, we investigated the colony formation of the sorted $CD34^+/56^+$ and $CD34^+/56^-$ cells.

Addition of anti-CD56 antibody in HSC proliferation assay system

To obtain BM adherent cells, cell strainer-nonpassed BMCs were cultured in flasks as described above. The BM adherent cells were trypsinized, collected, and cultured at a concentration of 3,000 cells per well in 96-well plates (#3595; Corning, Inc., NY, USA). When the BM adherent cells had become subconfluent, the partially purified HSCs prepared from the frozen BMCs of the same monkey were inoculated on the BM adherent layer (10^4 cells per well). The cells were cultured in IMDM supplemented with 10% FBS (lot No. 5-1064) and human cytokines (IL-3, TPO, and FLT-3 ligand: 2 ng/ml, SCF 9 ng/ml). In some wells, antihuman CD56 mAb (mouse IgG class, cross-reactive with homologous monkey epitope, #0561, Exalpha) was added at a concentration of 0.5 or 1 μg/ml. As a control, isotype-matched normal mouse IgG (#349040; BD Biosciences) was added at the same concentrations. Five wells were prepared for each condition. These cells were cultured for 12 days, after which ^{3}H-thymidine (^{3}H-TdR) was introduced into each well. After 24 h, the cells were harvested and the incorporation of ^{3}H-TdR was measured.

Induction of monkey BM adherent cells to adipocytes and osteoblasts

Osteogenic differentiation was induced in the subconfluent BM adherent cells by incubating them in an osteogenic medium: IMDM containing 10% FBS (lot No. A01120-494, PAA Laboratories GmbH, Linz, Austria), 0.1 μM dexamethasone, 0.2 mM L-ascorbic acid, and 10 mM β-glycerophosphate. The medium was changed every 2 or 3 days. Osteogenic differentiation was assessed by von Kossa staining 4 weeks after the initial osteogenic induction.

To induce adipogenic differentiation, the subconfluent BM adherent cells were incubated with an adipogenic medium: IMDM containing 10% FBS (lot No. A01120-494), 1 μM dexamethasone, 5 μg/ml human insulin, and 4.5 mg/ml D-glucose. The medium was changed every 3 or 4 days. Adipogenic differentiation was assessed by oil red O staining 5 weeks after the initial adipogenic induction.

Histological examinations

Monkey BM tissues were fixed with 10% neutral formalin. The sections (4 μm) were prepared and stained with anti-CD56 mAb. Briefly, the sections were deparaffinized with xylene and methanol and incubated with 3% hydrogen peroxide in methanol for 10 min to block the reactivity of endogenous peroxidase. They were washed in PBS and incubated with protein blocking agent (484360, Thermo Electron Corporation, Pittsburgh, PA, USA) for 5 min and then incubated with mouse antihuman CD56 mAb (cross-reactive with homologous monkey epitope, clone: 1B6, #413331, Nichirei Bioscience Inc., Tokyo, Japan) for 1 h. After being washed with PBS, they were incubated with peroxidase-labeled goat antimouse immunoglobulin polyclonal Ab (Dako Cytomation EnVision kit/HRP, Dako Cytomation, Glostrup, Denmark) at room temperature for 30 min. They were visualized using metal-enhanced 3,3'-diaminobenzidine.

Statistics

Statistical differences were analyzed by Student two-tailed *t* test. Each experiment was carried out three or more times. Reproducible results were obtained, and therefore representative data are shown in the figures.

Results

Immunophenotypic analyses of monkey BM adherent cells

Monkey BMCs were collected from cynomolgus monkeys by the perfusion method. After a 2- to 3-week culture, BM adherent cells showing uniform fibroblastic morphology were obtained (Fig. 1a). Surface markers of freshly isolated BMCs and the culture-expanded BM adherent cells were assessed by flow cytometry. Although the fresh BMCs contained only a few CD56-positive cells, almost all the BM adherent cells were stained positively with anti-CD56 mAb (Fig. 1b). The BM adherent cells were negative for lymphoid markers (CD4 and CD8, data not shown), an endothelial cell maker (CD31, data not shown), and hemopoietic cell markers (CD11b, CD14, CD34, and CD45; Fig. 1c) but highly positive for CD29 and weakly positive for CD62L (Fig. 1b); CD29 and CD62L are the representative cell adhesion molecules expressed on hemopoietic cells, BM stromal cells, and MSCs. The negative staining for CD11b, CD14, and CD31 indicated that the BM adherent cells did not contain macrophages, dendritic cells, or endothelial cells. It is generally accepted that CD73 and CD105 are representative markers of human MSCs. However, the mAbs reacting with monkey CD73 and CD105 homologs are not commercially available and we could not therefore confirm the expression of CD73 and CD105 molecules on the monkey BM adherent cells in the present study.

Expression of CD56 on monkey HSC-enriched population

Next, we attempted to analyze whether the CD56 molecule is also expressed on the HSC-enriched population. When whole BMCs were double-stained with anti-CD34 and anti-

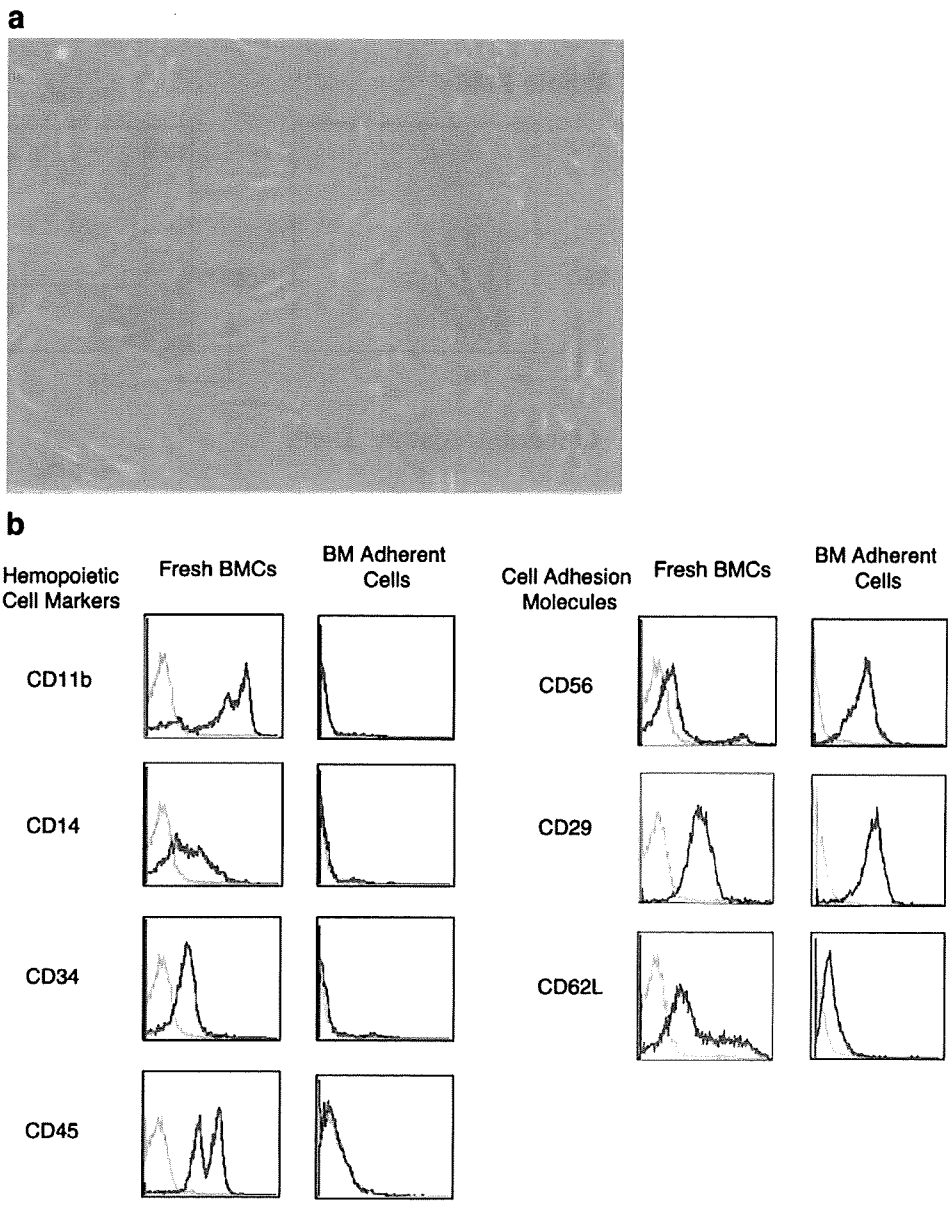

Fig. 1 Phenotypic analysis of monkey BM adherent cells. **a** Morphology of BM adherent cells. Monkey BMCs obtained by the perfusion method were cultured in flasks containing 10% FBS–IMDM, and spindle-shaped adherent cells were obtained 3 weeks later (×100). **b** Comparison of surface phenotypes between fresh BMCs and BM adherent cells. Freshly isolated BMCs and culture-expanded BM adherent cells were stained with a panel of mAbs reacting with hematolymphoid markers and cell adhesion molecules, and their phenotypes were analyzed using a FACScan. The *green lines* indicate the cells stained with isotype-matched control Abs. Representative staining patterns of five independent experiments

CD56 mAbs, only 2% were double-positive cells (Fig. 2a). We took the $CD34^-/56^+$ cells (8.8%) to be NK cells, a variety of T cell populations, and some stromal cells. After the enrichment of the HSCs, the percentage of $CD34^+/56^+$ cells increased to 9.1% in the lineage-negative population. When the cells in the "blast" window on the SSC/FSC dot plot profile were analyzed, the percentage of $CD34^+/56^+$ cells further increased and reached 20.6%. The $CD34^+/56^+$ cells and $CD34^+/56^-$ cells in the "blast" gate were sorted and stained with May–Giemsa reagents (Fig. 2b). When the $CD34^+/56^+$ cells were spun onto the cytospin slides for the May–Giemsa staining, marked cell aggregations were observed, but this was not the case with the $CD34^+/56^-$ cells. Both the $CD34^+/56^+$ and $CD34^+/56^-$ cells showed large nuclei with narrow cytoplasms, indicating that these cells have the characteristics of HSCs, although the $CD34^+/56^+$ cells are slightly larger in size. As expected, the $CD34^+/56^+$ and $CD34^+/56^-$ cells had the ability to differentiate into erythroid and myeloid lineage cells in the clonal hemopoietic colony assay using MethoCult GF H4434; burst-forming unit erythroid (BFU-E), colony-forming unit granulocyte (CFU-G), colony-forming unit-macrophage (CFU-M), and colony-forming unit granulocyte–macrophage (CFU-GM) were detected. There was no significant difference between the two populations in the cellularity of the clonal hemopoietic colonies and in the number of the colonies (Fig. 2c). None of the most primitive colony-forming cells, colony-forming unit granulocyte–erythroid–macrophage–

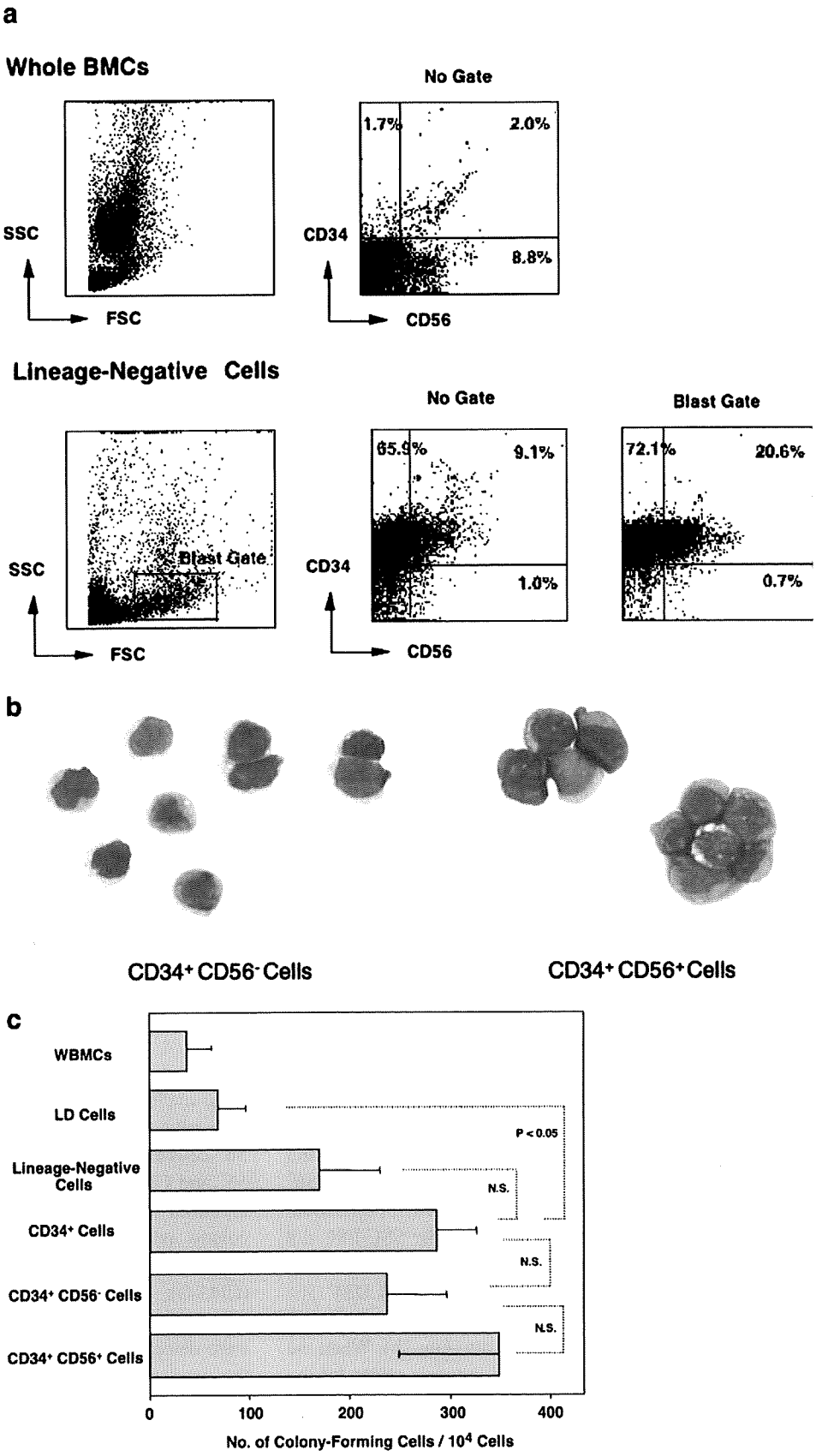

Fig. 2 Expression of CD56 molecule in monkey HSCs. **a** Increase of $CD56^+$ cells in HSC-enriched population. When whole BMCs were double-stained with anti-CD34 and CD56 mAbs, only a few $CD34^+/56^+$cells were detected. However, the percentages of the double-positive cells increased to 9.1% in the HSC-enriched population (lineage-negative cells) and a further increase in the percentages was observed in the "blast" gated population of the SSC/FSC profile. Representative staining patterns of five independent experiments. **b** Morphology of $CD34^+/56^-$ cells and $CD34^+/56^+$cells. The $CD34^+/56^-$ cells and the $CD34^+/56^+$ cells included in the "blast" window were sorted and then stained with May–Giemsa reagents. Both populations express a phenotype of HSCs (×1,000). **c** Number of hemopoietic colony-forming cells in various cell populations. Whole BMCs, LD cells, lineage-negative cells, $CD34^+$ cells, the $CD34^+/56^-$ cells, and the $CD34^+/56^+$ cells were incubated in methylcellulose containing optimal concentrations of human cytokines (MethoCult GF H4434) to obtain the number of hemopoietic colony-forming cells (triplicate). Mean±SD of five independent experiments

megakaryocytes were detected in either the $CD34^+/56^+$ or $CD34^+/56^-$ populations, since the MethoCult formulation, which we used in the present study, was designed for the induction of optimal colony formations for human BMCs.

Long-term hemopoiesis-supporting ability of monkey BM adherent cells

We have previously shown that major histocompatibility complex (MHC) restriction exists between HSCs and BM stromal cells in the murine hemopoietic system; purified HSCs can proliferate and differentiate to a higher extent in MHC-compatible stromal cells than in MHC-incompatible environments [18–20]. Therefore, in the present study, monkey partially purified HSCs were cultured on a BM stromal cell layer derived from the same monkey, and the hemopoiesis-supporting ability of the BM adherent cells was examined. Monkey BMCs were divided into cell strainer-passed cells and nonpassed cells. The nonpassed cells were cultured in order to obtain a BM adherent cell layer, whereas the passed cells were stored in liquid nitrogen until a BM adherent cell layer was formed. Partially purified HSCs were prepared from the frozen BMCs and cocultured with the BM adherent layer. As a control, the HSCs were cultured in the absence of the BM adherent layer.

When the HSCs were cocultured with the BM adherent cells, marked proliferation of the HSCs was observed from the first week and the hemopoiesis was maintained at higher levels for up to 3 weeks of culture (Fig. 3a). The nonadherent cells recovered from the culture flasks at 1 to 3 weeks of culture contained immature and mature hemopoietic cells of all lineages: myelocytes, erythroblasts, granulocytes, macrophages, and megakaryocytes (data not shown). On the other hand, the nonadherent cells recovered at and after 4 weeks of culture contained mainly granulocytes and macrophages (data not shown). The nonadherent cells were then assessed for their ability to form clonal hemopoietic colonies using MethoCult. As shown in Fig. 3b, high colony formation was observed in the nonadherent cells recovered at 2 to 3 weeks of culture. These colonies were mainly composed of granulocytes and/or macrophages (CFU-G, CFU-M, and CFU-GM), and a few erythroblasts (BFU-E) were detected. In contrast, no increase in the number of nonadherent cells was observed in the control culture without the BM adherent cells, and the cell number reached the baseline at 2 weeks of culture (Fig. 3a). These data show that the BM adherent cells have the capacity to support the hemopoiesis of HSCs at least for 6 weeks, whereas the proliferation and differentiation of HSCs cannot be induced without the BM adherent layer.

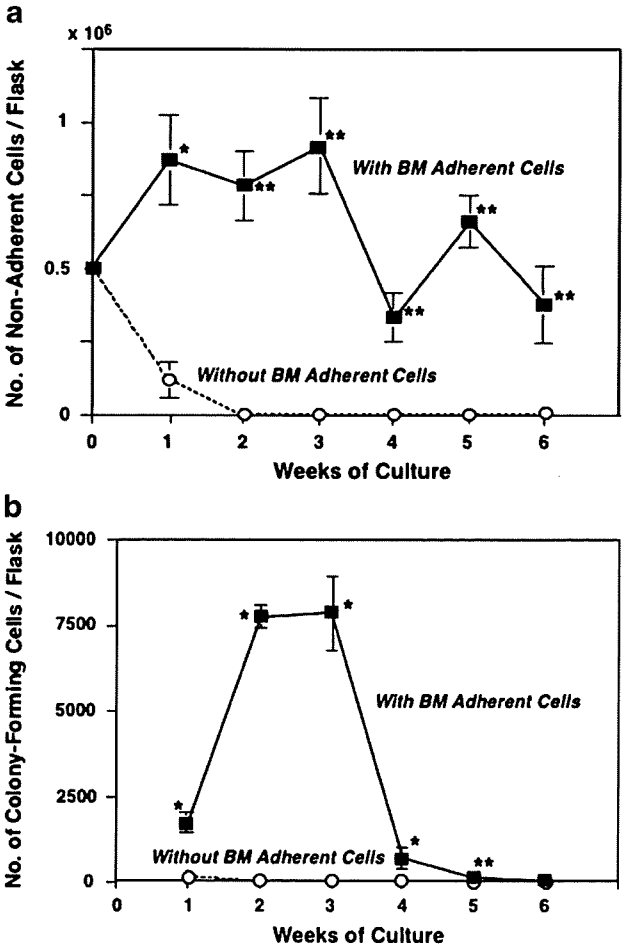

Fig. 3 Long-term hemopoiesis-supporting ability of monkey BM adherent cells. **a** Number of nonadherent cells obtained from long-term culture. Partially purified HSCs were cultured in the presence or absence of BM adherent cells (triplicate), and the number of nonadherent cells was counted every week. Mean±SD of three flasks. $*P<0.005$, $**P<0.0001$ significantly different from the control (without BM adherent cells). Representative data of five independent experiments. **b** Number of hemopoietic colony-forming cells in nonadherent cells recovered from culture flask. The nonadherent cells recovered from the long-term culture were evaluated for their colony-forming ability using methylcellulose assay (MethoCult GF H4434; triplicate). Mean±SD of three wells. $*P<0.0001$, $**P<0.005$ significantly different from the control (without BM adherent cells). Representative data of three independent experiments

Inhibitory effects of anti-CD56 antibody against proliferation of monkey HSCs

CD56 molecules were expressed on both HSCs and BM adherent–stromal cells (Figs. 1b and 2a) and the monkey BM adherent cells showed an ability to support hemopoiesis (Fig. 3). These findings suggest that the CD56 molecules play an important role in hemopoiesis in monkeys. Therefore, we next examined whether anti-CD56 mAb inhibits the proliferation of HSCs on the BM adherent cells when added to the coculture system of the partially purified HSCs and the

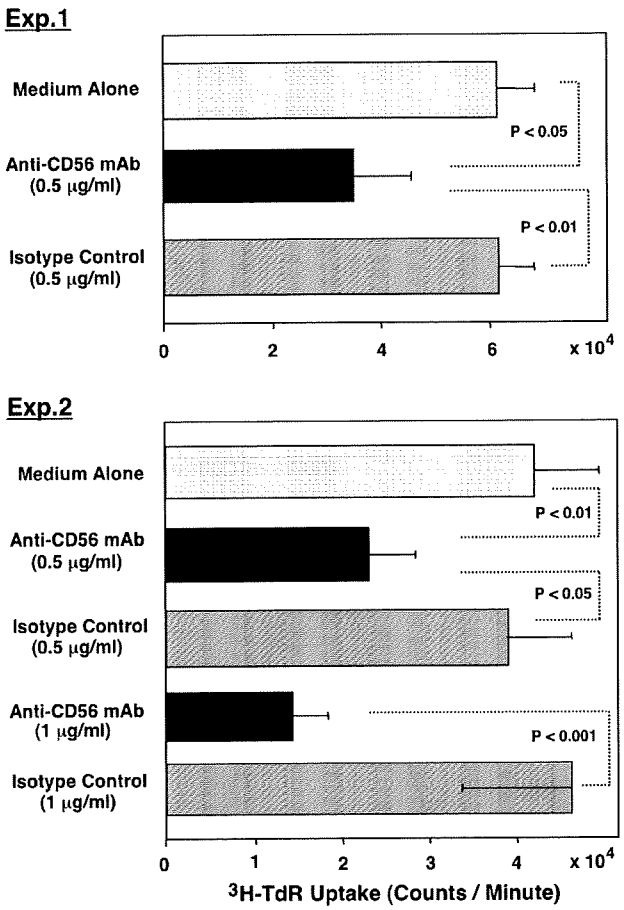

Fig. 4 Inhibitory effect of anti-CD56 mAb on monkey HSC proliferation. Partially purified HSCs were cultured on the BM adherent layer in the presence or absence of anti-CD56 mAb (five wells per sample). As a control, the same concentration of isotype-matched mouse normal IgG was added to the culture. Mean±SD of five wells. Representative data of three independent experiments

BM adherent cells. With the addition of anti-CD56 mAb, the number of cells adhering to the BM adherent cell layer decreased significantly (data not shown). As shown in Fig. 4, a marked decrease in the uptake of ^{3}H-TdR was seen in the wells with the anti-CD56 mAb, whereas no decrease was observed in the wells with isotype-matched control Ab. The proliferation of the HSCs was inhibited in a dose-dependent manner, as shown in experiment 2 in Fig. 4.

Detection of CD56-positive cells in monkey BM sections

We examined whether BMCs expressing CD56 molecules indeed exist in monkey BM tissues. The BM sections were immunostained with anti-CD56 mAb, and the cells showing stromal-cell-like features (characteristic nuclei with clear nucleolus and abundant cytoplasms) were stained positively with the anti-CD56 mAb. Some immature hematopoietic cells were also stained with the mAb. In addition, tight adhesions between the CD56-positive hemopoietic cells and the CD56-positive stromal cells were observed in some places (Fig. 5).

Differentiation ability of monkey BM adherent cells into osteoblasts and adipocytes

BM adherent cells were examined to see whether they have any characteristics of MSCs; we investigated their ability to differentiate into osteoblasts and adipocytes. BM adherent cells were cultured in the inductive medium into osteoblasts and adipocytes for 4 and 5 weeks and then stained with von Kossa and oil red O reagents, respectively. As shown in Fig. 6, calcium deposits were detected by the von Kossa staining, and intracellular lipid droplets were also detected

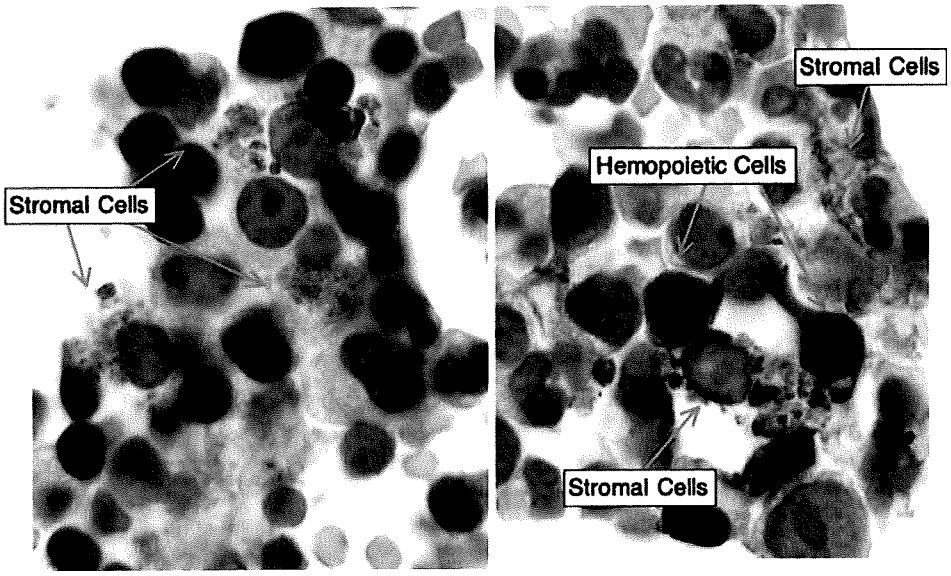

Fig. 5 Detection of CD56-positive stromal and hemopoietic cells in monkey BM tissues. BM sections were stained with anti-CD56 mAb and CD56-positive stromal cells were detected in many places. Immature hemopoietic cells, but not mature ones, were also positively stained with the mAb. The adhesion of the CD56-positive stromal cells and the CD56-positive immature hemopoietic cells was observed (×1,000). Representative data of six independent experiments

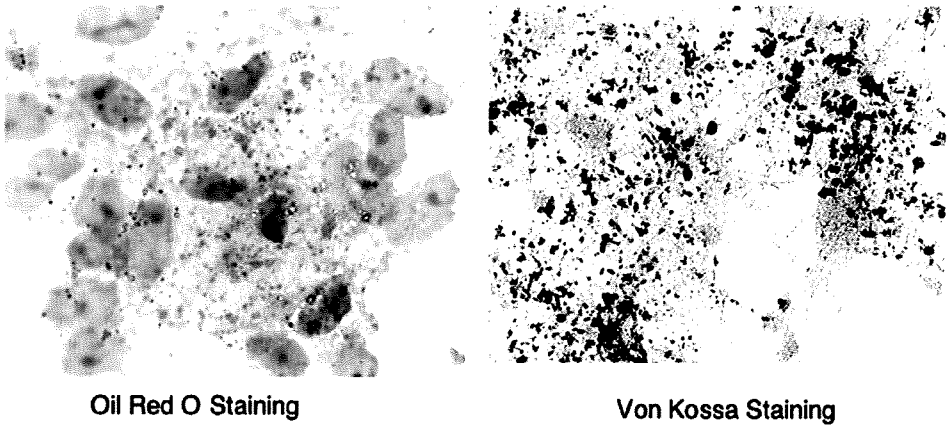

Fig. 6 Differentiation capacity of monkey BM adherent cells. The BM adherent cells were induced to differentiate into adipocytes and osteoblasts. Oil red O staining and von Kossa staining confirmed their differentiations (×1,000). Representative data of three independent experiments

by the oil red O staining, indicating that the cells do have the potential to differentiate into osteoblasts and adipocytes.

Discussion

Studies of the hemopoietic system of nonhuman primates have provided important information for understanding the mechanisms of human hemopoiesis [21, 22]. Recently, Lee et al. [23] demonstrated that BM adherent cells, prepared from the BMCs of fetal rhesus monkeys, had a high proliferative potential and had an ability to differentiate towards adipogenic, chondrogenic, and osteogenic lineages, indicating that the BM adherent cells have characteristics of MSCs. They also cultured adult BMCs collected by the conventional aspiration method from the iliac crest and obtained BM adherent cells, although the differentiation capacity of the adult BM adherent cells was not evaluated. In addition, Devine et al. have isolated BM adherent cells by culturing baboon BMCs obtained by the aspiration method, and the cells were shown to be MSCs; the cells were capable of differentiating along adipogenic and osteogenic lineages. When lethally irradiated baboons were administered the autologous (gene-marked) MSCs in conjunction with autologous HSCs by the intravenous route, the engraftment of the HSCs was facilitated, and the transgene was detected in the posttransplant BM biopsies [24]. These reports clearly demonstrate that cell populations that can be considered as MSCs exist in BMCs of nonhuman primates, as in humans and mice. We have previously shown that BMCs collected by the perfusion method contain a significantly higher number of hemopoietic colony-forming cells than those collected by the aspiration method [16, 17] since peripheral blood contamination was reduced to the minimum level in the perfusion method. There was a tendency for the number of colony-forming unit fibroblast (CFU-F) to be higher in the BMCs collected by the aspiration method than in those by the perfusion method, although the differences were not significant [17]. Thus, the perfusion method, which requires only two holes for the insertion of syringes in the bones and can be used to collect a sufficient number of BMCs with just one or two perfusions, provides a safe and convenient tool for harvesting BMCs that contain a high number of HSCs and CFU-F.

The present study clearly shows that CD56 is expressed on both BM adherent cells and HSCs (Figs. 1b and 2a) and that their interactions through the CD56 molecules are important for hemopoiesis because the proliferation of HSCs was suppressed markedly by the addition of anti-CD56 mAb (Fig. 4). As shown in Fig. 2c, the $CD34^{+}/56^{+}$ cells generated a comparable number of hemopoietic colonies to the $CD34^{+}/56^{-}$ cells in the methylcellulose assay and also showed the morphology of HSCs (Fig. 2b), indicating that the $CD34^{+}/56^{+}$ cells represent a population of HSCs but not artifacts produced by the HSC purification process. Our previous reports [7, 8] have shown that CD56 is expressed on mouse BM adherent cells as well as a stromal cell line (FMS/PA6-P) established from fetal mouse BMCs and contributes greatly to the supporting capacity of these stromal cells. However, we could not detect the expression of CD56 on highly purified mouse HSC populations, such as $Lin^{-}Sca\text{-}1^{+}$, $Lin^{-}CD34^{-/+}$, $Lin^{-}CD38^{-/+}$, or $Lin^{-}c\text{-}kit^{low/+}$ cells [7]. Therefore, it is conceivable that the CD56 molecules on mouse BM stromal cells might interact with HSCs through heterophilic bindings. Indeed, there are many reports showing that CD56 binds to heparan sulfate proteoglycan (extracellular matrix molecule) [25] and fibroblast growth factor receptors [26] in murine and chicken neural systems, although the biological significances of the heterophilic bindings have not yet been fully elucidated. The homophilic bindings of CD56 molecules are known to induce cell-to-cell tight adhesions because zipper-like CD56 complexes are generated by multiple cis- and trans-homophilic bindings of CD56. In this study, we provide evidence that CD56 molecules are also expressed on HSC-enriched populations in the monkey hemopoietic

system, indicating that HSCs can interact with BM stromal cells by homophilic bindings, in addition to heterophilic bindings. Such homophilic interactions would induce the tight adhesions between HSCs and stromal cells and, as a result, stable cellular adhesions and long-lasting cellular interactions would be achieved.

From the present results in monkeys, it can be speculated that CD56 is also expressed on human BM stromal cells (MSCs) and contributes to human hemopoiesis. Indeed, our preliminary study showed that 67% of commercially available human MSCs were positive for CD56. Thus, CD56 might be used as a new marker for MSCs in mice, monkeys, and humans. Moreover, BM stromal cells expressing CD56 molecules were detected in human BM sections by immunohistochemical staining. We also found that $CD34^{+}/56^{+}$ cells were contained in an HSC-enriched population of human cord blood cells at the percentage of 3–6% (manuscript in preparation). There is a possibility that human BMCs contain a higher amount of $CD34^{+}/56^{+}$ cells than human cord blood cells, although we have not assessed this yet.

Several adhesion molecules are known to regulate the interactions between hemopoietic stem–progenitor cells and BM stromal cells; for example, β-catenin–N-cadherin, integrin–VCAM, and $\beta_1$integrin–osteopontin interactions [27]. The present results indicate the important role of CD56 in the hemopoietic system, but they do not rule out the contribution of other adhesion molecules. We have very recently found that HSCs from MRL/lpr mice (autoimmune-prone mice) exhibit enhanced adhesion to the FMS/PA6-P stromal cell line, established in our laboratory [8], in vitro after the onset of autoimmune diseases: this is attributed to the increased expression of CD56 on the HSCs, but no increase was found in other adhesion molecules such as CD44, CD62L, VLA-4, and VCAM-1. The proliferation of HSCs on the FMS/PA6-P cells was significantly suppressed by the addition of anti-CD56 mAb, suggesting that the interaction between HSCs and stromal cells through CD56 molecules plays a major role in the abnormal proliferation (no MHC restriction) of HSCs in MRL/lpr mice [28].

More work is required before one can say that CD56 molecules contribute to hemopoiesis to a higher extent than other adhesion molecules. However, our previous [7, 8, 28] and present studies provide new understandings of the regulation mechanisms of hemopoiesis through CD56 molecules. To fully understand the physiological significance of CD56, we are now investigating the functional role of CD56 molecules in the monkey BM adherent cells and human MSCs.

Acknowledgments This work was supported by a grant from Haiteku Research Center of the Ministry of Education, a grant from the Millennium program of the Ministry of Education, Culture, Sports, Science, and Technology, a grant from the Science Frontier program of the Ministry of Education, Culture, Sports, Science, and Technology, a grant-in-aid for scientific research (B) 11470062, grants-in aid for scientific research on priority areas (A) 10181225 and (A) 11162221, and Health and Labor Sciences research grants (Research on Human Genome, Tissue Engineering, Food Biotechnology) and also a grant from the Department of Transplantation for Regeneration Therapy (Sponsored by Otsuka Pharmaceutical Company, Ltd.), a grant from Molecular Medical Science Institute, Otsuka Pharmaceutical Co., Ltd., and a grant from Japan Immunoresearch Laboratories Co., Ltd. (JIMRO).

We are grateful to Ms. Y. Tokuyama, K. Hayashi, and A. Kitajima for their excellent technical assistance. We also thank Mr. Hilary Eastwick-Field and Ms. K. Ando for their help in the preparation of the manuscript.

References

1. Dexter TM, Allen TD, Lajtha LG (1977) Conditions controlling the proliferation of hematopoietic stem cells in vitro. J Cell Physiol 91:335–344
2. Whitlock CA, Witte ON (1982) Long-term culture of B-lymphocytes and their precursors from murine bone marrow. Proc Nat Acad Sci U SA 79:3608–3612
3. Dorshkind K (1990) Regulation of hemopoiesis by bone marrow stromal cells and their products. Ann Rev Immunol 8:111–114
4. Verfaillie CM (1998) Adhesion receptors as regulators of the hematopoietic process. Blood 92:2609–2612
5. Charbord P (2001) The hematopoietic stem cell and the stromal microenvironment. Therapie 56:383–384
6. Izumi-Hisha H, Soe T, Ogata H et al (1991) Monoclonal antibodies against a preadipose cell line (MC3T3-G2/PA6) which can support hemopoiesis. Hybridoma 10:103–112
7. Wang X, Hisha H, Taketani S et al (2005) Neural cell adhesion molecule contributes to hemopoiesis-supporting capacity of stromal cell lines. Stem Cells 23:1389–1399
8. Wang X, Hisha H, Taketani S et al (2006) Characterization of mesenchymal stem cells isolated from mouse fetal bone marrow. Stem Cells 24:482–493
9. Thiery JP, Brackenbury R, Rutishauser U et al (1977) Adhesion among neural cells of the chick embryo. J Bio Chem 252:6841–6845
10. D'Eustachio P, Owens GC, Edelman GM et al (1985) Chromosomal location of the gene encoding the neural cell adhesion molecule (N-CAM) in the mouse. Proc Nat Acad Sci U S A 82:7631–7635
11. Van Camp B, Durie BGM, Spier C et al (1990) Plasma cells in multiple myeloma express a natural killer cell-associated antigen: CD56 (NKH-1; Leu-19). Blood 76:377–382
12. Cruse JM, Lewis RE, Pierce S et al (2005) Aberrant expression of CD7, CD56, and CD79a antigens in acute myeloid leukemias. Exp Mol Patho 79:39–41
13. Sillaber C, Walchshofer S, Mosberger I et al (1999) Immunophenotypic characterization of human bone marrow endosteal cells. Tissue Antigens 53:559–568
14. Nelissen JMDT, Torensma R, Pluyter M et al (2000) Molecular analysis of the hemopoiesis supporting osteoblastic cell line U2-OS. Exp Hematol 28:422–432
15. Kushida T, Inaba M, Ikebukuro K et al (2000) A new method for bone marrow cell harvesting. Stem Cells 18:453–456
16. Kushida T, Inaba M, Ikebukuro K et al (2002) Comparison of bone marrow cells harvested from various bones of cynomolgus monkeys at various ages by perfusion or aspiration methods: a preclinical study for human BMT. Stem Cells 20:155–162

17. Inaba M, Adachi Y, Hisha H et al (2007) Extensive studies on perfusion method plus intra-bone marrow-bone marrow transplantation using cynomolgus monkeys. Stem Cells 25:2098–2103
18. Hisha H, Nishino T, Kawamura M et al (1995) Successful bone marrow transplantation by bone grafts in chimeric-resistant combination. Exp Hematol 23:347–352
19. Hashimoto F, Sugiura K, Inoue K et al (1997) Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vivo. Blood 89:49–54
20. Sugiura K, Hisha H, Ishikawa J et al (2001) Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vitro. Stem Cells 19:46–58
21. Lee CC, Fletcher MD, Tarantal AF (2005) Effect of age on the frequency, cell cycle, and lineage maturation of rhesus monkey (*Macaca mulatta*) $CD34^+$ and hemopoietic progenitor cells. Pediatr Res 58:315–322
22. Shepherd BE, Kiem H-P, Lansdorp PM et al (2007) Hematopoietic stem cell behavior in non-human primates; HSC behavior in non-human primates. Blood 110:1806–1803 (prepublished online)
23. Lee CCI, Ye F, Tarantal AF (2006) Comparison of growth and differentiation of fetal and adult rhesus monkey mesenchymal stem cells. Stem Cells and Develop 15:209–220
24. Devine SM, Bartholomew AM, Mahmud N et al (2001) Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. Exp Hematol 29:244–255
25. Storms SD, Rustishauser U (1998) A role for polysialic acid in neural cell adhesion molecule heterophilic binding to proteoglycans. J Biol Chemi 273:27124–27129
26. Kiryushko D, Korshunova I, Berezin V et al (2006) Neural cell adhesion molecule induces intracellular signaling via multiple mechanisms of Ca^+ homeostasis. Mol Biol Cell 17:2278–2286
27. Yin T, Li L (2006) The stem cell niches in bone. J Clin Invest 116:1196–1201
28. Wang X, Hisha H, Cui W et al (2007) The characteristics of hematopoietic stem cells from autoimmune-prone mice and the role of neural cell adhesion molecules in abnormal proliferation of these cells in MRL/lpr mice. Haematologica 92:300–307

STEM CELLS®

TRANSLATIONAL AND CLINICAL RESEARCH

Intra-Bone Marrow Injection of Donor Bone Marrow Cells Suspended in Collagen Gel Retains Injected Cells in Bone Marrow, Resulting in Rapid Hemopoietic Recovery in Mice

MING SHI,[a] YASUSHI ADACHI,[a,b,c] AKIO SHIGEMATSU,[a] NAOKO KOIKE-KIRIYAMA,[a] WEI FENG,[a] SEJI YANAI,[a] CUI YUNZE,[d] ZHE-XIONG LIAN,[e] JUAN LI,[f] SUSUMU IKEHARA[a,b,c]

[a]First Department of Pathology, [b]Transplantation Center, and [c]Regeneration Research Center for Intractable Diseases, Kansai Medical University, Osaka, Japan; [d]Japan Immunoresearch Laboratories, Gunma, Japan; [e]Division of Rheumatology/Allergy and Clinical Immunology, University of California at Davis, Davis, California, USA; [f]School of Public Health, Jilin University, Changchun City, China

Key Words. Intra-bone marrow bone marrow transplantation • Collagen gel • Colony-forming units of spleen • Reconstitution • Enhanced green fluorescent protein

ABSTRACT

We have recently developed an innovative bone marrow transplantation (BMT) method, intra-bone marrow (IBM)-BMT, in which donor bone marrow cells (BMCs) are injected directly into the recipient bone marrow (BM), resulting in the rapid recovery of donor hemopoiesis and permitting a reduction in radiation doses as a pretreatment for BMT. However, even with this IBM injection, some of the injected BMCs were found to enter into circulation. Therefore, we attempted to modify the method to allow the efficient retention of injected BMCs in the donor BM. The BMCs of enhanced green fluorescent protein transgenic mice (C57BL/6 background) were suspended in collagen gel (CG) or phosphate-buffered saline (PBS), and these cells were then injected into the BM of irradiated C57BL/6 mice. The numbers of retained donor cells in the injected BM, the day 12 colony-forming units of spleen (CFU-S) counts, and the reconstitution of donor cells after IBM-BMT were compared between the CG and PBS groups. The number of transplanted cells detected in the injected BM in the CG group was significantly higher than that in the PBS group. We next carried out CFU-S assays. The spleens of mice in the CG group showed heavier spleen weight and considerably higher CFU-S counts than in the PBS group. Excellent reconstitution of donor hemopoietic cells in the CG group was observed in the long term (>100 days). These results suggest that the IBM injection of BMCs suspended in CG is superior to the injection of BMCs suspended in PBS. STEM CELLS *2008;26:2211–2216*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Bone marrow transplantation (BMT) was originally developed to treat congenital immunodeficiencies and hematologic disorders [1, 2]. Recently, BMT-related methods have been improved, because of the discoveries of more effective immunosuppressants, powerful antibiotics, antithymocyte globulin, and fractionated irradiation, all of which add up to a better prognosis [3, 4]. BMT has, at this stage, been carried out for the treatment not only of immunodeficiencies and hemopoietic diseases but also of autoimmune diseases and solid malignant tumors [5–10]. However, BMT is still a difficult procedure because of the risk of lethal side effects, such as infection, graft-versus-host disease (GVHD), graft failure, and so on [11–13]. Recently, we developed a new and powerful BMT method: intra-bone marrow (IBM)-BMT [14]. In this method, donor bone marrow cells (BMCs) are injected directly into the recipient bone marrow (BM), and a much greater number of donor hemopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) can therefore be inoculated into the recipient BM than by conventional i.v. BMT, resulting in the rapid reconstitution of donor hemopoietic cells and permitting a reduction in radiation doses as a pretreatment for BMT [14, 15]. In addition, we have shown that the IBM-BMT can be used for organ transplantation, with the engrafted organs surviving long-term without the use of immunosuppressants [16, 17]. Collagen gel (CG) was originally used for the three-dimensional cell culture systems for isolated cells [18]. Cellmatrix (Nitta Gelatin, Inc., Yao, Japan, http://www.nitta-gelatin.com) is an acidic soluble type I collagen that is liquid on ice but forms a gel when warm. In this paper, we show that the CG helps retain more injected donor BMCs in the

Author contributions: M.S.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; Y.A.: conception and design, provision of study material or patients, collection and/or assembly of data, manuscript writing, final approval of manuscript; A.S., N.K.-K., W.F., and C.Y.: collection and/or assembly of data, final approval of manuscript; S.Y., Z.-X.L., and J.L.: data analysis and interpretation, final approval of manuscript; S.I.: financial support, administrative support, manuscript writing, final approval of manuscript.

Correspondence: Susumu Ikehara, M.D., Ph.D., First Department of Pathology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi City, Japan 570-8506. Telephone: 81-6-6992-1001, ext. 2470; Fax: 81-6-6994-8283; e-mail: ikehara@takii.kmu.ac.jp Received January 12, 2008; accepted for publication May 24, 2008; first published online in STEM CELLS *EXPRESS* July 3, 2008. 1066-5099/2008/$30.00/0 doi: 10.1634/stemcells.2008-0035

recipient BM upon IBM-BMT than phosphate-buffered saline (PBS), resulting in excellent reconstitution of donor cells and permitting a reduction in radiation doses as a pretreatment for BMT.

MATERIALS AND METHODS

Mice

C57BL/6 mice (B6) were purchased from SLC (Shizuoka, Japan, http://www.jslc.co.jp) for use as recipients, and enhanced green fluorescent protein (eGFP) transgenic (tg) mice, for use as donors, were kindly donated by Dr. Okabe (Osaka University, Osaka, Japan) [19]. These mice were used at 8–10 weeks of age. The university's committee for animal research approved all experiments.

Reagents

The antibodies (Abs) used in this study were as follows: phycoerythrin (PE)-labeled anti-mouse CD3 Ab, anti-mouse B220 Ab, anti-mouse Gr-1 Ab, peridinin chlorophyll protein (PerCP)-Cy5.5-labeled anti-mouse CD45 Ab, biotin-labeled anti-CD3 Ab, biotin-labeled anti-B220 Ab, biotin-labeled anti-CD11c Ab, biotin-labeled anti-Mac-1 Ab, biotin-labeled anti-NK1.1 Ab, biotin-labeled TER119 Ab, and biotin-labeled anti-Gr1 Ab (BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml). Cellmatrix, a purified collagen solution for cell culture, was purchased from Nitta Gelatin, Inc.

Whole-Body Irradiation of Recipient Mice

Gamma radiation was delivered by a Gammacell 40 Exactor (MDS Nordion, Kanata, ON, Canada, http://www.mds.nordion.com) with two ^{137}Cs sources. Recipient mice were irradiated with 7, 8, 9 or 10 Gy, the day before BMT.

Preparation of the Collagen Gel Matrix

The collagen gel matrix, Cellmatrix, was prepared following the manufacturer's instructions. That is, solutions A, B, and C were mixed at a ratio of 8:1:1 and were kept on ice to prevent gel formation until use. This is because the mixture is liquid on ice but becomes a gel when it is warmed. Hereafter, this mixture is referred to as collagen gel.

Treatment of Donor BMCs

BMCs were flushed from the medullary cavities of the femurs and tibias of donor mice with PBS. After gentle dissociation, the BMC suspension was filtered through a 70-μm nylon mesh (Becton Falcon, Franklin Lakes, NJ, http://www.bd.com). The BMCs were counted.

The BMCs were then divided into two groups: the CG group and the PBS group. The BMC suspension was centrifuged, and the supernatant was aspirated. The BMCs were suspended in 4°C PBS for the PBS group and ice-cold CG for the CG group and adjusted to 10^7, 10^8, or 10^9 cells per milliliter.

Bone Marrow Transplantation

One day after irradiation, the BMCs of eGFP tg mice were transplanted into recipient mice directly into the bone cavity via the intra-bone marrow route (IBM-BMT), as previously described [14]. Briefly, the mice were anesthetized, and the area from the inguinal region to the knee joint was shaved. The tibia was gently drilled with a 26-gauge needle through the patellar tendon into the BM cavity. BMCs suspended in PBS or CG were aspirated into a microsyringe (50 μl; Ito, Fuji, Shizuoka, Japan, http://www.ito-ex.co.jp) and then kept at room temperature. The BMCs (10^5, 10^6, or 10^7 in 10 μl) were then injected into the BM cavity using the microsyringe.

Analysis of Donor Cells in Recipient BM, Peripheral Blood, or Spleen

To detect injected donor BMCs in the recipient BM, the BMCs of eGFP tg mice (10^7 in 10 μl) or cultured MSCs (5×10^5 in 10 μl) were transplanted into the BM of B6 mice the day after 10-Gy irradiation. One hour after IBM-BMT, the B6 mice were sacrificed and BMC-injected bones were flushed to obtain the BMCs. The BMCs were then stained with biotin-labeled lineage Abs (anti-Gr1, anti-Mac-1, anti-TER119, anti-B220, anti-NK1.1, anti-CD3, and anti-CD11c Abs), PE-labeled anti-Sca-1 Ab, and PerCP-Cy5.5 labeled anti-CD45 Ab, followed by incubation with allophycocyanin (APC)-coupled streptavidin. The cells were analyzed using a FACSCalibur instrument (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com).

To detect donor-lineage$^-$ Sca-1$^+$ c-kit$^+$ cells (KSL cells) in the recipient mice after BMT, the BMCs of eGFP tg mice (1×10^6) were injected into 9-Gy-irradiated recipient B6 mice intravenously or into the left tibia (l-tibia). Spleen cells and BMCs from the BMC-injected l-tibia and noninjected right tibia (r-tibia) were obtained from the recipient mice, independently, at 5, 7, or 9 days after BMT. We calculated the numbers of nuclear cells using an SF-3000 autoanalyzer (Sysmex, Kobe, Japan, http://www.sysmex.co.jp/en) and stained the cells with APC-labeled anti-c-kit Ab (BD Pharmingen), PE-labeled anti-Sca-1 Ab (BD Pharmingen), and biotin-labeled lineage Abs followed by staining with PerCP-Cy5.5-coupled streptavidin (BD Pharmingen). The percentages of KSL cells in the spleen and the BM were analyzed using the FACSCalibur. The total numbers of donor KSL cells, which are eGFP$^+$, in the spleen and the BM were calculated by using the total cell numbers and percentage of donor KSL cells in the cells.

To detect donor-derived peripheral blood nuclear cells, the peripheral blood (PB) of the recipient mice was examined at 2 weeks, 1 month, 2 months, and 100 days after BMT. The PB of each mouse was divided into three lots, and samples from each mouse were stained with PE-conjugated CD3 and PerCP-Cy5.5-conjugated CD45, PE-conjugated B220 and PerCP-Cy5.5-conjugated CD45, and PE-conjugated Gr1 and PerCP-Cy5.5-conjugated CD45. To examine the cells retained in the injected bone, the recipient mice were sacrificed 1 hour after IBM-BMT. The BMCs were flushed from the tibia that had been previously injected and were then suspended in ice-cold PBS. This step was carried out on ice. The number of BMCs was counted, and 10^6 BMCs were stained with PerCP-Cy5.5-conjugated CD45. The stained cells were analyzed using a FACSCalibur instrument equipped with CellQuest software.

Percentages of Donor Cells by FACS Analyses

Leukocytes were first gated by CD45$^+$ cells, which were estimated as nuclear cells. The percentage of donor T lymphocytes was estimated as eGFP$^+$/CD3$^+$cells. The percentages of donor B lymphocytes and granulocytes were estimated as eGFP$^+$/B220$^+$ cells and eGFP$^+$/Gr-1$^+$ cells, respectively.

Statistical Analysis

The results are represented as means $\pm$ SD. The Student t test was used to determine a statistical significance. A p value $<.05$ was considered a significant difference.

RESULTS

CG Retains Injected Donor BMCs in Recipient BM

First, we examined whether CG can retain more injected cells in the injected BM than PBS. We obtained BMCs from eGFP tg mice and suspended the BMCs in ice-cold CG (Cellmatrix) or PBS. These were then warmed to room temperature (>20°C) in a syringe, since Cellmatrix is liquid at 4°C but changes into the gel state when warm. The BMCs suspended in CG or PBS were injected directly into the BM, as previously described [14]. The mice were sacrificed 1 hour after

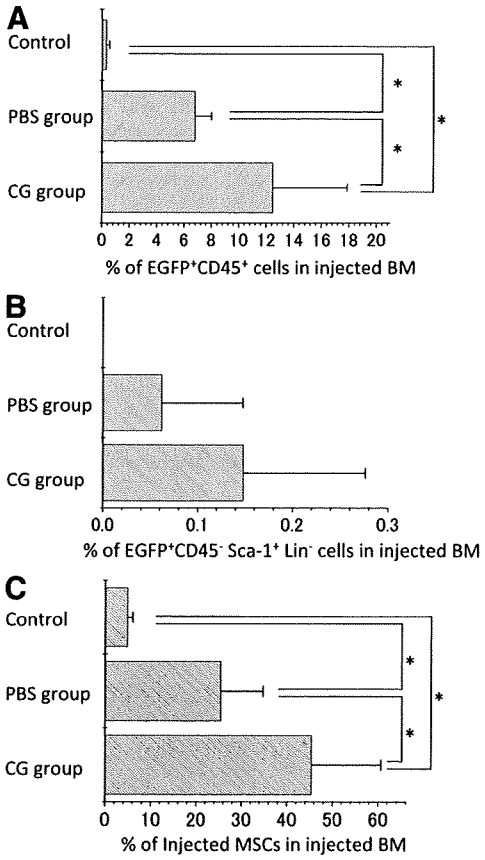

Figure 1. CG efficiently helps retain donor cells in recipient BM. Recipient (B6) mice were irradiated at 10 Gy 1 day before intra-bone marrow (IBM) bone marrow transplantation (BMT). BMCs were obtained from eGFP transgenic (tg) mice (donor mice). The BMCs (1×10^7/10 μl) suspended in PBS (PBS group) or CG (CG group) were injected into the BM of the B6 mice. Control mice were injected with only PBS into the BM. One hour after IBM-BMT, the mice were sacrificed, and percentage of $CD45^+$ donor cells (**A**) and percentage of $CD45^-$lineage$^-$Sca-1^+ donor cells (**B**) in the BM injected with donor BMCs were analyzed by FACScan. $n = 3$ (control; mice injected with only PBS), 6 (PBS group), and 6 (CG group). The cells from control mice show autofluorescence. *, $p < .05$. (**C**): B6 mice were irradiated at 10 Gy 1 day before IBM-BMT. Cultured MSCs (5×10^5 cells per 10 μl) from eGFP tg mice suspended in PBS (PBS group) or CG (CG group) were injected into the BM of the B6 mice. Control mice were injected with only PBS into the BM. One hour after IBM-BMT, the mice were sacrificed, and percentages of donor cells were analyzed by FACScan. $n = 3$ (control; mice injected with only PBS), 5 (PBS group), and 6 (CG group). The cells from control mice show autofluorescence. *, $p < .05$. Abbreviations: BM, bone marrow; CG, collagen gel; EGFP, enhanced green fluorescent protein; MSC, mesenchymal stem cell; PBS, phosphate-buffered saline.

IBM-BMT, and the percentages of donor cells in the injected BM were examined. As shown in Figure 1A, many more donor BMCs remained in the injected BM cavity in the CG group than in the PBS group. Next, we examined whether IBM-BMT using CG helps more mesenchymal stem cells to be retained in the injected recipient BM. As shown in Figure 1B, CG helped more MSCs to be retained in the recipient BM, but there was no significant difference between the groups, possibly because of the large SDs. Therefore, we transplanted cultured MSCs of eGFP tg mice into the BM of B6 mice and analyzed the percentages of donor MSCs in the recipient BM. As shown in Figure 1C, a significantly larger number of donor MSCs existed in the recipient BM in the CG group than in the PBS group ($p < .05$). These results suggest that CG can help more injected cells (both HSCs and MSCs) be retained in the injected BM than PBS.

CG Retains KSL Cells in the Injected Site, Followed by Helping Proliferation and Migration of KSL Cells into the Spleen and Noninjected Sites

To examine the kinetics of the KSL cells in the recipients, we carried out IBM-BMT or i.v. BMT using BMCs suspended in PBS or CG from eGFP tg mice into B6 mice. As shown in Figure 2, on day 5, KSL cells were found only in the BM of the BMC-injected l-tibia in both PBS and CG groups; the number of KSL cells in the BMC-injected l-tibia was significantly higher in the CG group than in the PBS group. However, we could not detect KSL cells in the BM in the case of the i.v. group. In contrast, a greater number of KSL cells were detected in the spleen in the case of i.v. BMT. On day 7, we could still detect a high number of KSL cells in the BMC-injected l-tibia in the CG group. The KSL cells also appeared even in the noninjected r-tibia in the CG group, although no KSL cells were detected in the noninjected r-tibia in the PBS group in the case of IBM-BMT. On day 9, we still detected many KSL cells in the BMC-injected l-tibia in the CG group. There were no significant differences between the other groups due to the increases in the numbers of KSL cells. These findings suggest that immature hemopoietic progenitor cells (KSL cells) proliferate inside the BMC-injected bone cavity, followed by an acceleration of the migration of the KSL cells into the noninjected bone and the spleen in the CG group.

CG Group Shows Significantly Higher Day 12 Colony-Forming Units of Spleen Counts After IBM-BMT Than PBS Group

Next, we carried out day 12 colony-forming units of spleen (CFU-S) assays to examine the hemopoietic stem cell/immature hemopoietic progenitor activity after IBM-BMT using CG or PBS (Fig. 3). In the CG group, 16.1 ± 4.4 colonies per spleen (106.8 ± 35.9 mg of spleen weight) were observed on day 12, whereas 6.6 ± 3.4 colonies per spleen (68.2 ± 15.5 mg of spleen weight) were observed in the PBS group ($p < .05$). These results suggest that hemopoietic stem cell/progenitor cells can be more effectively retained in the CG group than in the PBS group.

Reduced Radiation Doses As Pretreatment for BMT

As described above, we have shown that CG can effectively help donor BMCs be retained in the recipient BM after IBM-BMT, resulting in the formation of significantly higher CFU-S counts. Next, we examined whether radiation doses could be reduced by the use of IBM-BMT with CG (Fig. 4). First, we irradiated recipient B6 mice at 8 Gy and reconstituted them with eGFP mouse BMCs by IBM-BMT. In this experiment, both the PBS and CG groups showed good reconstitution of donor hemopoietic cells 1 month after BMT. There was no significant difference between the two groups: the mean and SDs of the percentage of donor cells in the PBS group and the CG group were 89.3% ± 2.0% and 85.6% ± 5.0%, respectively, at 1 month after IBM-BMT. We therefore reduced the radiation dose (8 to 7 Gy). With 7 Gy of radiation, 1 month after BMT, significantly higher percentages of reconstitution with donor cells were observed in the CG group than in the PBS group. These results suggest that the IBM injection of BMCs suspended in CG is effective in reducing irradiation doses as a pretreatment.

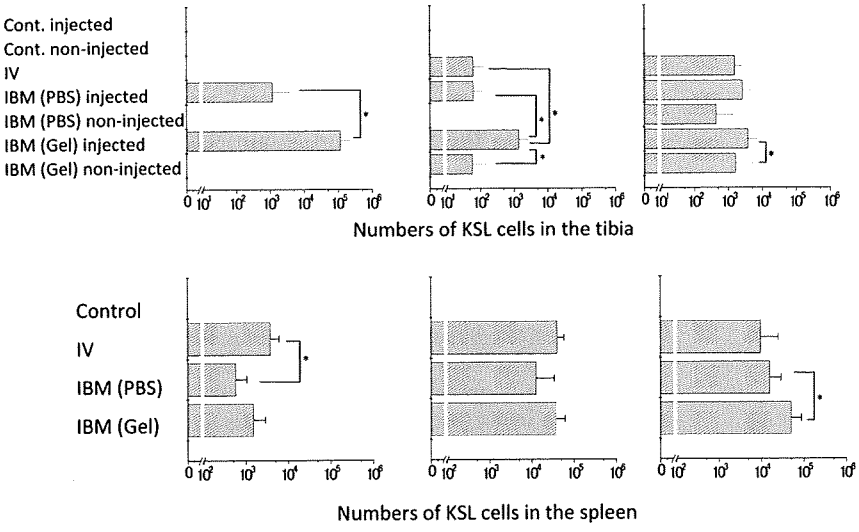

Figure 2. Collagen gel (CG) augments increment of KSL cells in injected bone marrow (BM) followed by increment of KSL cells in noninjected BM and spleen. B6 mice were irradiated at 9 Gy 1 day before bone marrow transplantation (BMT). BMCs from enhanced green fluorescent protein (eGFP) transgenic (tg) mice (1×10^6 cells per 10 μl) suspended in CG were injected into the left tibia. In the IV group, 1×10^6 BMCs from eGFP tg mice suspended in PBS were injected intravenously. As a control, the mice were injected with only PBS into the left tibia. The mice were sacrificed 5, 7, or 9 days after BMT. Total cell numbers of the spleen, the left tibia (injected tibia), and right tibia (noninjected tibia) were calculated with Sysmex, and percentages of donor-derived KSL cells were examined with FACSCalibur. Next, we calculated the total number of eGFP-positive KSL cells in the spleen, the BMC-injected tibia, and the noninjected tibia. $n = 5$–11. *, $p < .05$. Abbreviations: IBM, intra-bone marrow; IV, intravenous; KSL, donor-lineage$^-$ Sca-1$^+$ c-kit$^+$; PBS, phosphate-buffered saline.

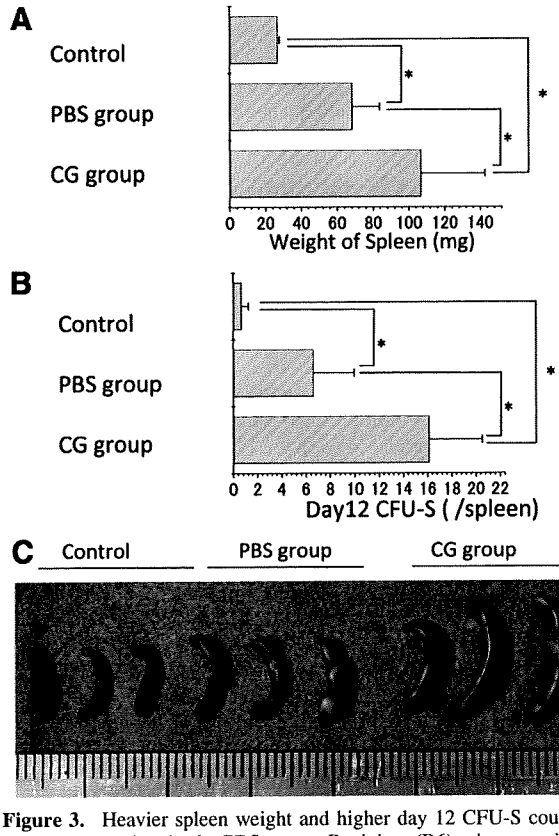

Figure 3. Heavier spleen weight and higher day 12 CFU-S counts in the CG group than in the PBS group. Recipient (B6) mice were irradiated at 9 Gy, 1 day before intra-bone marrow (IBM) bone marrow transplantation (BMT). BMCs (1×10^5 cells per 10 μl) from enhanced green fluorescent protein transgenic mice were injected into the bone marrow of the B6 mice. Twelve days after IBM-BMT, the spleens of the B6 mice were weighed, and numbers of CFU-S were counted. **(A):** Means and SDs of the weight of spleens. **(B):** Means and SDs of CFU-S. **(C):** Representative photograph of spleens. There were three mice in the control group (mice injected with only PBS), five in the PBS group, and eight in the CG group. *, $p < .05$. Abbreviations: CFU-S, colony-forming units of spleen; CG, collagen gel; PBS, phosphate-buffered saline.

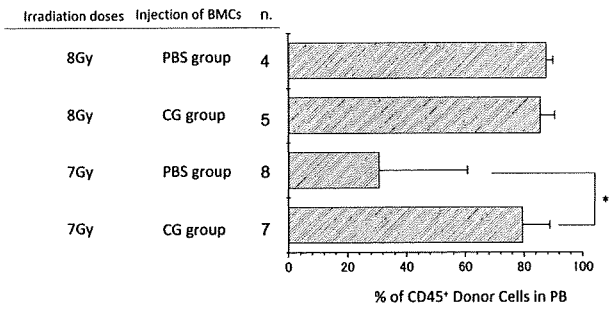

Figure 4. Better reconstitution of donor hemopoietic cells in the CG group than the PBS group. Recipient (B6) mice were irradiated at 7 or 8 Gy, 1 day before intra-bone marrow (IBM) bone marrow transplantation (BMT). BMCs (1×10^6 cells per 10 μl) from enhanced green fluorescent protein mice were injected into the bone marrow of the B6 mice. One month after IBM-BMT, nuclear cells in the PB were obtained from the mice, and percentages of donor cells were analyzed by FACScan. *, $p < .05$. Abbreviations: BMC, bone marrow cell; CG, collagen gel; PB, peripheral blood; PBS, phosphate-buffered saline.

Next, we examined whether cells of various lineages can differentiate in the CG group. As shown in Table 1, donor BMCs had differentiated into multilineage hemopoietic cells in both the CG and PBS groups. The percentages of donor cells (all lineage cells) in the CG group were higher than those in the PBS group even on day 100 after IBM-BMT (Table). We also calculated the percentage of donor cells in various lineages at 6 months after IBM-BMT and obtained results similar to those at 100 days after IBM-BMT (data not shown). These results suggest that CG is effective in reconstituting recipients with donor cells by IBM-BMT not only in short-term observation but also in long-term observation after IBM-BMT.

DISCUSSION

In the present study, we have shown that IBM-BMT using CG helps transplanted BMCs to be retained in the recipient BM upon IBM-BMT and permits a reduction in radiation doses as a pretreatment for BMT. In 2001, we reported that IBM-BMT promotes the rapid recovery of donor hemopoiesis in BMT [14] and that IBM-BMT can be used to treat intractable autoimmune diseases in MRL/Mp-lpr/lpr mice, which are resistant to conventional BMT

Table 1. Short-term (1 month) and long-term (100 days) reconstitution of donor-derived hemopoietic cells between the PBS group and the CG group

		% Of donor cells			
	n	CD45	CD3	B220	Gr-1
1 Month after IBM-BMT					
PBS group	8	30.8 ± 30	22.6 ± 16.2	51.0 ± 35.1	34.1 ± 35.0
CG group	7	79.5 ± 9.3[a]	52.5 ± 17.5[a]	91.4 ± 4.2[a]	85.8 ± 7.0[a]
100 Days after IBM-BMT					
PBS group	8	31.3 ± 40.3	16.8 ± 23.3	35.9 ± 43.3	26.6 ± 38.0
CG group	7	87.3 ± 8.7[a]	57.1 ± 17.4[a]	94.7 ± 3.3[a]	80.1 ± 20.8[a]

[a] $p < .05$ versus PBS group.
Abbreviations: CG, collagen gel; IBM-BMT, intra-bone marrow bone marrow transplantation; PBS, phosphate-buffered saline.

therapy [14]. Since then, we have shown the advantages of IBM-BMT not only in the treatment of hemopoietic diseases but also in organ transplantations and solid malignant tumors [20–24]. In these papers, we have shown that IBM-BMT induces rapid recovery of donor hemopoietic cells, easily induces tolerance in the recipients, reduces the severity of GVHD, and permits a reduction in radiation doses as a pretreatment for BMT. The mechanisms underlying the effects of IBM-BMT are (a) the efficacy of injection of donor BMCs (the direct injection of HSCs into the recipient BM) and (b) the injection of donor MSCs into the recipient BM. However, as there is an abundance of blood vessels in the BM, we have found that some of the injected BMCs get into the blood vessels, and thereby into peripheral circulation, even with IBM-BMT. Therefore, there was a need to modify the method of IBM-BMT to promote a greater retention of the injected BMCs at the site of injection in the BM. In this paper, we used CG (Cellmatrix) to help retain the donor BMCs in the recipient BM. Cellmatrix is liquid on ice but becomes a gel when warmer [18]. Therefore, we expected that the warmed CG containing BMCs would prevent the BMCs entering into circulation. As shown in Figure 1, we found a significantly higher number of donor BMCs in the injected bone of the recipients when donor BMCs were suspended in CG. As we expected, higher CFU-S counts were found in the CG group than in the PBS group, and the peripheral blood cells showed better reconstitution with donor hemopoietic cells. These results suggest that the more BMCs are injected and are retained in the recipient BM upon IBM-BMT, the better the reconstitution of the recipient mice. It has been reported that the interaction of stromal cells (particularly MSCs) and HSCs is crucial for the differentiation of hemopoietic cells and the maintenance of HSCs [16, 25, 26]. Therefore, it is necessary that the injected HSCs migrate to the BM and interact with stromal cells, including MSCs, for the production of mature hematopoietic cells and the maintenance of the HSCs. Recently, we have found that unique HSCs exist in the human cord blood [27]. These HSCs cannot migrate to the BM when they are injected into the vein. However, they can differentiate into mature hematopoietic cells and produce HSCs, resulting in long-term hematopoiesis. Therefore, we assume that some HSCs cannot participate in hematopoiesis if they are injected into the vein, possibly because of a lack of some important receptor(s) for migration to the bone marrow niche. These results and concepts suggest that BMT via the vein results in the loss of some multipotent HSCs and that IBM-BMT is effective in retaining the injected HSCs in the BM. However, in the present study, some of the HSCs were found to have entered into peripheral circulation even after IBM-BMT. IBM-BMT using CG could help retain more HSCs in the BM than conventional IBM-BMT. As shown in Figure 2, it is likely that KSL cells (immature hemopoietic progenitor cells) of the CG group proliferate more rapidly in the injected bone than those of the PBS and IV groups even 5 days after BMT, and that they migrate to the other bones and the spleen more effectively, resulting in the expansion of hemopoiesis followed by high CFU-S counts. Thus, CG is a candidate for improving conventional IBM-BMT, since more donor BMCs can be retained in the recipient BM upon IBM-BMT and since CG itself does not induce severe inflammation or foreign body granuloma when it is injected into mice (data not shown).

CONCLUSION

Collectively, our results show that CG helps retain more injected donor BMCs in the recipient BM upon IBM-BMT than PBS, resulting in excellent reconstitution of donor cells and permitting a reduction dose as a pretreatment for BMT in mice.

ACKNOWLEDGMENTS

We thank Y. Tokuyama, K. Hayashi, and A. Kitajima for expert technical assistance, and we also thank Hilary Eastwick-Field and K. Ando for the preparation of the manuscript. This work was supported by a grant from the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science, and Technology; the Department of Transplantation for Regeneration Therapy (sponsored by Otsuka Pharmaceutical Company, Ltd.); the Molecular Medical Science Institute; Otsuka Pharmaceutical Co., Ltd.; Japan Immunoresearch Laboratories Co., Ltd.; and Scientific Research 18590388. M.S. and Y.A. contributed equally to this work.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

1 Gatti RA, Meuwissen HJ, Allen HD et al. Immunological reconstitution of sex-linked lymphopenic immunological deficiency. Lancet 1968;2:1366–1369.

2 Bach FH, Albertini RJ, Joo P et al. Bone marrow transplantation in a patient with the Wiskott-Aldrich syndrome. Lancet 1968;2:1364–1366.

3 Gocheva L. Total body irradiation prior to bone marrow transplantation; some aspects of fifty year experience. J BUON 2004;9:147–160.

4 Carella AM. Treatment of hematological malignancies with allogeneic nonmyeloablative stem cell transplantation: Conditioning regimens with fludarabine. Hematol J 2004;5(suppl 1):S68–S75.