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-

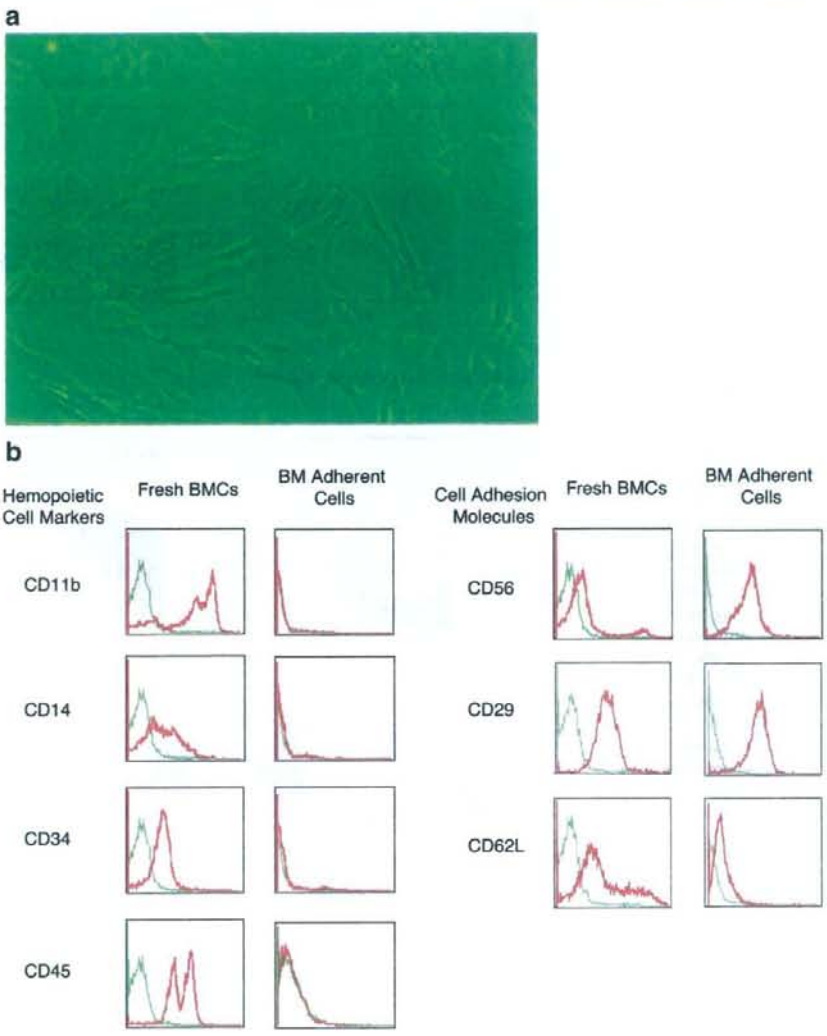

Fig. 1 Phenotypic analysis of monkey BM adherent cells. **a** Morphology of BM adherent cells. Monkey BMCs obtained by the perfusion method were cultured in flasks containing 10% FBS–IMDM, and spindle-shaped adherent cells were obtained 3 weeks later (×100). **b** Comparison of surface phenotypes between 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–

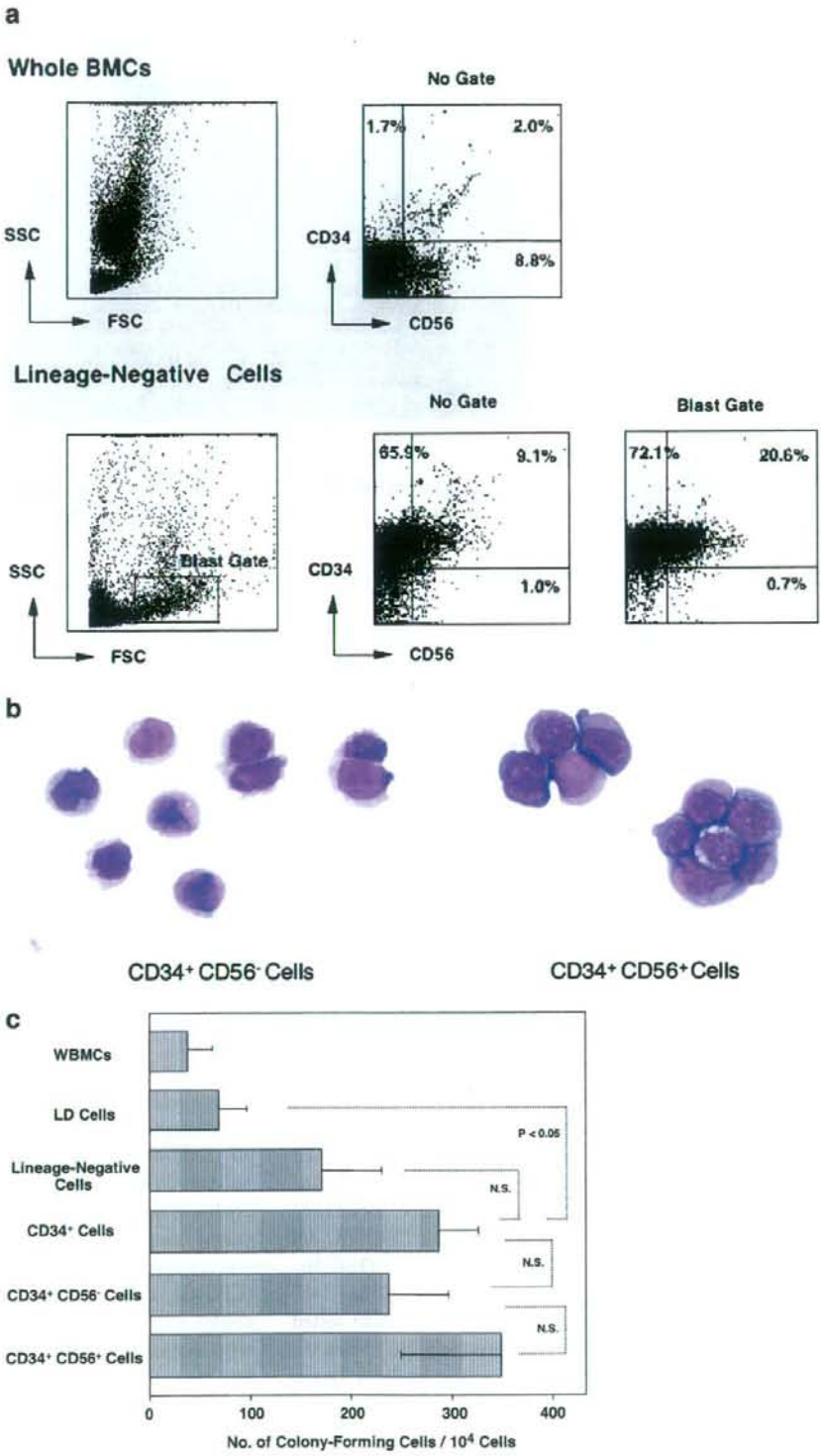

Fig. 2 Expression of CD56 molecule in monkey HSCs. **a** Increase of $CD56^+$ cells in HSC-enriched population. When whole BMCs were double-stained with anti-CD34 and CD56 mAbs, only a few $CD34^+/56^+$cells were detected. However, the percentages of the double-positive cells increased to 9.1% in the 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.

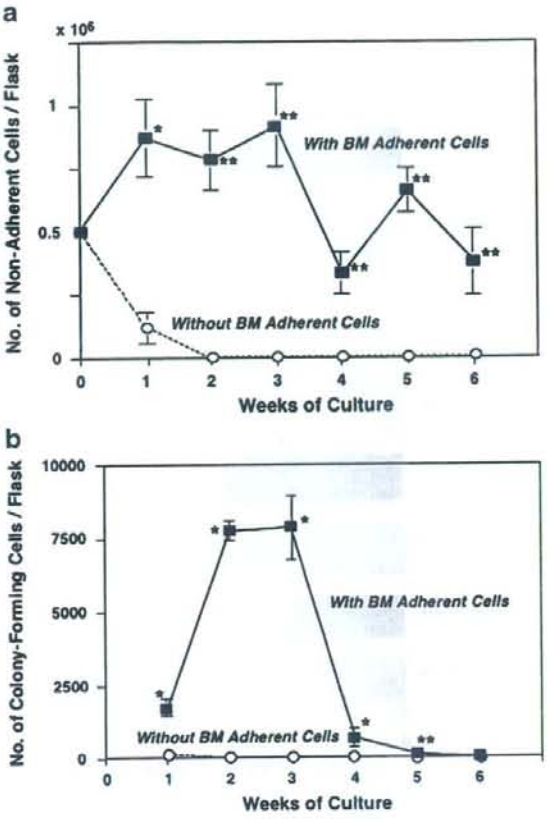

Fig. 3 Long-term hemopoiesis-supporting ability of monkey BM adherent cells. **a** Number of nonadherent cells obtained from long-term culture. Partially purified HSCs were cultured in the presence or absence of BM adherent cells (triplicate), and the number of nonadherent cells was counted every week. 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

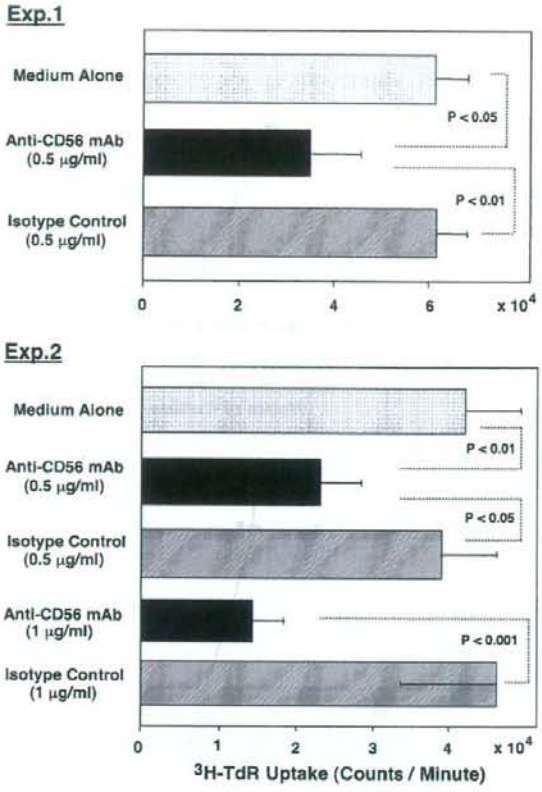

Fig. 4 Inhibitory effect of anti-CD56 mAb on monkey HSC proliferation. Partially purified HSCs were cultured on the BM adherent layer in the presence or absence of anti-CD56 mAb (five wells per sample). As a control, the same concentration of isotype-matched mouse normal IgG was added to the culture.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

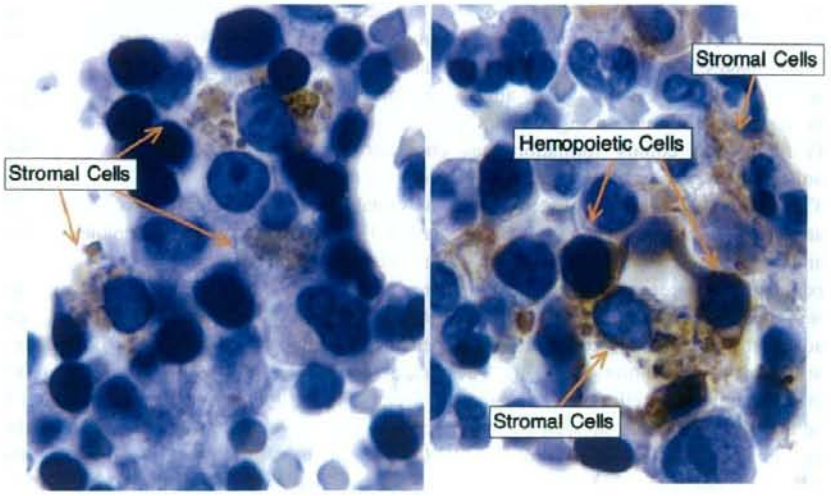

Fig. 5 Detection of CD56-positive stromal and hemopoietic cells in monkey BM tissues. BM sections were stained with anti-CD56 mAb and CD56-positive stromal cells were detected in many places. Immature hemopoietic cells, but not mature ones, were also positively stained with the mAb. The adhesion of the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

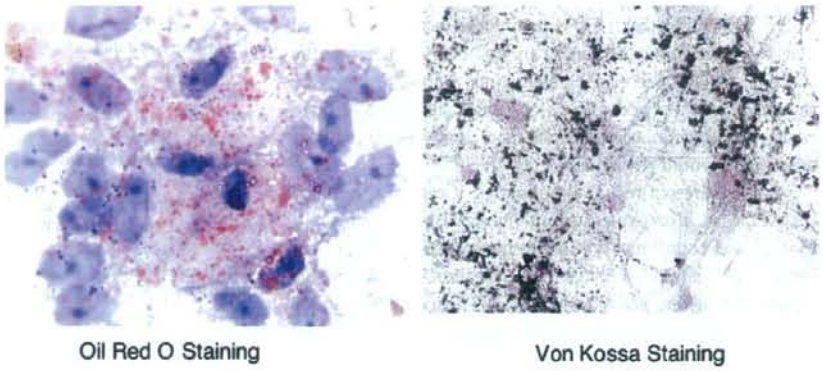

Fig. 6 Differentiation capacity of monkey BM adherent cells. The BM adherent cells were induced to differentiate into adipocytes and osteoblasts. Oil red O staining and von Kossa staining confirmed their differentiations (×1,000). Representative data of three independent experiments

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.

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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

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Prevention of graft-*versus*-host disease by intrabone marrow injection of donor T cells: involvement of bone marrow stromal cells

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Summary

We have developed a new and effective method for bone marrow transplantation (BMT): bone marrow cells (BMCs) are injected directly into the bone marrow (BM) cavity of recipient mice. The intrabone marrow injection of BMCs (IBM-BMT) greatly facilitates the engraftment of donor-derived cells, and IBM-BMT can attenuate graft-*versus*-host reaction (GVHR), in contrast to conventional intravenous BMT (i.v.-BMT). Here, we examine the mechanisms underlying the inhibitory effects of IBM-BMT on GVHR using animal models where GVHR is elicited. Recipient mice (C57BL/6) were irradiated and splenic T cells (as donor lymphocyte infusion: DLI) from major histocompatibility complex-disparate donors (BALB/c) were injected directly into the BM cavity (IBM-DLI) or injected intravenously (i.v.-DLI) along with IBM-BMT. The BM stromal cells (BMSCs) from these recipients were collected and related cytokines were examined. The recipient mice that had been treated with IBM-BMT + i.v.-DLI showed severe graft-*versus*-host disease (GVHD), in contrast to those treated with IBM-BMT + IBM-DLI. The suppressive activity of BMSCs in this GVHD model was determined. The cultured BMSCs from the recipients treated with IBM-BMT + IBM-DLI suppressed the proliferation of responder T cells remarkably when compared with those from the recipients of IBM-BMT + i.v.-DLI in mixed leucocyte reaction. Furthermore, the level of transforming growth factor-β and hepatocyte growth factor in cultured BMSCs from IBM-BMT + IBM-DLI increased significantly when compared with those from the recipients of IBM-BMT + i.v.-DLI. Thus, the prevention of GVHD observed in the recipients of IBM-BMT + IBM-DLI was attributable to the increased production of immunosuppressive cytokines from BMSCs after interaction with host reactive T cells (in DLI).

Keywords: bone marrow stromal cells, donor lymphocyte infusion, graft-*versus*-host disease, intrabone marrow-bone marrow transplantation

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Introduction

Allogeneic bone marrow transplantation (BMT) has been used as a potentially curative therapy for patients with a wide variety of diseases, including haematological disorders, congenital immunodeficiencies, metabolic disorders, autoimmune diseases and solid tumours [1–6]. However, there are several problems to be resolved in allogeneic BMT. One of the important issues is how to control graft-*versus*-host disease (GVHD), which remains a major cause of post-transplantation morbidity and mortality.

We have recently developed intrabone marrow (IBM)-BMT, in which bone marrow cells (BMCs) are injected directly into the bone marrow (BM) cavity [7]. We have found that IBM-BMT allows us not only to use low-dose irradiation as a preconditioning regimen [7,8] but also to suppress GVHD [9], as IBM-BMT can efficiently recruit donor-derived stromal cells [including mesenchymal stem cells (MSCs)] that can support donor-derived haemopoietic stem cells [1,9–12].

It is noted that IBM-BMT can be used to prevent GVHD, even when intensive donor lymphocyte infusion (DLI) is

carried out [9]. We attempted to inject allogeneic T cells as DLI into the BM cavity (IBM-DLI) or intravenously (i.v.-DLI) with IBM-BMT. The prolongation of survival rate and reduction of GVHD were observed clearly in the recipients treated with IBM-BMT + IBM-DLI, but not in those with IBM-BMT + i.v.-DLI [13]. These findings prompted us to examine the regulatory function of BM stromal cells (BMSCs) after interaction with T cells that had been injected into the BM cavity. Evidence has been accumulated that BMSCs play a critical role in the regulation of haemopoiesis by promoting cell-to-cell interactions and constitutively secreting immunoregulatory soluble factors [14–23]. In fact, BMSCs suppress the proliferation of allogeneic T cells in a major histocompatibility complex (MHC)-independent manner [24–31].

In the present study, we examine the suppressive activity of BMSCs that had been in contact with T cells *in vivo*, and evaluate the effect of T cell polarization and several factors produced by BMSCs.

Materials and methods

Mice

C57BL/6 (B6, H-2^b), BALB/c (H-2^d) mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). C57BL/6 mice at the age of 7–9 weeks were used as recipients, and BALB/c mice at the age of 7–9 weeks were used as donors. All mice were kept in our animal facilities under specific pathogen-free conditions. All animal procedures were performed in accordance with protocols approved by the Animal Experimentation Committee, Kansai Medical University.

Irradiation

C57BL/6 mice were irradiated at 8·5 Gy (1·0 Gy/min) from a ^{137}Cs source (Gammacell 40 Exactor, Nordion, International Inc., Ottawa, Ontario, Canada) 1 day before the BMT.

Bone marrow transplantation and donor lymphocyte infusion

Bone marrow cells were flushed from the femoral and tibial bones of the BALB/c mice, and then suspended in RPMI-1640. The BMCs were then filtered through a 70-μm nylon mesh (Becton Dickinson Labware, Franklin Lakes, NJ, USA) to remove debris, washed and adjusted to $1{\cdot}5 \times 10^9$ cells/ml in RPMI-1640. The BMCs, thus prepared, were injected directly into the BM cavity as described previously [7]. Briefly, the region from the inguen to the knee joint was shaved and a 5-mm incision was made on the thigh. The knee was flexed to 90 degrees and the proximal side of the tibia was drawn to the anterior. A 26-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and then inserted into the BM cavity. Using a microsyringe (10 μl; Hamilton Co., Reno, NV, USA) containing the donor BMCs ($1{\cdot}5 \times 10^9$ cells/ml), the donor BMCs were injected from the said bone holes into the BM cavity of the left tibia (10^7 cells/7 μl/tibia) (IBM-BMT). In some groups, BMCs were injected intravenously.

T cells were purified from the spleens by positive selection by a MACS® system using CD4 and CD8α microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) after depletion of red blood cells, or by an EPICS ALTRA flow cytometer (Coulter, Hialeah, FL, USA) after staining with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-CD4/CD8 monoclonal antibodies (mAbs) (BD Pharmingen, San Diego, CA, USA).

Splenic T cells were injected into the BM cavity of the right tibia (10^7 cells/7 μl/tibia: intrabone marrow T cell injection as DLI; IBM-DLI) or injected intravenously (i.v.-DLI; 10^7 cells/0·5 ml) into the recipient mice along with the IBM-BMT. Recipients treated with IBM-BMT alone (without DLI) served as negative controls (termed NO-DLI) [13].

Preparation of freshly isolated BMSCs

Three days after the DLI, BMCs were flushed from the right tibial bones of the recipient mice, and non-haemopoietic MSC-enriched cells (defined as $CD45^-/CD106^+$ cells) were sorted immediately by an EPICS ALTRA flow cytometer (Coulter, Hialeah, FL, USA) after staining with FITC- or PE-conjugated anti-CD45/CD106 mAbs (BD Pharmingen, San Diego, CA, USA). Freshly isolated non-haemopoietic BMSCs-enriched populations, sorted as $CD45^-/CD106^+$ cells, were prepared from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI, or IBM-BMT alone (NO-DLI). Haemopoietic BMC-enriched populations, sorted as $CD45^+/CD106^-$ cells, were also prepared from the recipients and used as controls.

Preparation of cultured BMSCs

Bone marrow cells from the right tibia, into which T cells had been injected as DLI, were collected from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) 3 days after treatment, and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). Two days later, non-adherent cells were removed. Adherent cells were detached using trypsin-ethylenediamine tetraacetic acid, and passaged when 80% confluence was reached and then replated. After 2 weeks (short-term culture) or 3 months (long-term culture) the cultures were discontinued, and BMSCs were harvested and used for further experiments, including mixed leucocyte reaction (MLR) and real-time reverse transcription–polymerase chain reaction (RT–PCR) assay. The culture-expanded BMSCs from the recipients of IBM-BMT +

IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) were stained with FITC-anti-CD45 and PE-anti-CD106 mAbs and analysed by a fluorescence activated cell sorter (FACScan) (BD Pharmingen).

Mixed leucocyte reaction

Various numbers of freshly prepared (defined as $CD45^-/CD106^+$ BM cells) or cultured BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.V-DLI or IBM-BMT alone (without DLI) were added to the culture of one-way MLR (4-day culture) where 2×10^5 responder $CD4^+$ T cells from BALB/c mice were stimulated with 12 Gy irradiated stimulator spleen cells (2×10^5 cells) from B6 mice in a 96-well flat-bottomed plate in a total volume of 0·2 ml. $CD45^+/CD106^-$ haemopoietic cells or whole BMCs served as controls for BMSCs added to the culture. The cultures were pulsed with 0·5 μCi of [^{3}H]-TdR for the last 16 h of the culture period.

Activation of T cells with concanavalin A

Splenic T cells (2×10^6 cells) from BALB/c mice were cultured with 2·5 μg/ml of concanavalin A (ConA) for 4 days. Activated T cells, thus prepared, were used as a positive control in real-time RT–PCR assay and enzyme-linked immunosorbent assay (ELISA) to detect cytokines.

Flow cytometric analyses of intracellular cytokines

CD4-enriched T cells from BALB/c mice were cultured with irradiated stimulator spleen cells from B6 mice with cultured BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) in round-bottomed plates (RPMI-1640) with 10% FCS. Cells were harvested 6 days later and stained with biotin-conjugated anti-H-$2K^d$ (visualized by streptavidin–peridinin chlorophyll-Cy5·5) and FITC-anti-CD4 mAb (BD Pharmingen) to detect responder (donor) CD4 T cells. The cells were next fixed and permeabilized with Cutofix/Cytoperm solution™ (BD Pharmingen). Intracellular cytokines were detected after the staining of cells with PE-anti-interleukin (IL)-2, -interferon (IFN)-γ or -IL-4 using an Intracellular Cytokine Staining Kit® (BD Pharmingen). Cells stained with isotype control cocktail (BD Pharmingen) served as a control. The stained cells were analysed by a FACScan® (Becton Dickinson Co., Mountain View, CA, USA).

Real-time RT–PCR assay

Cytokine messages of BMSCs were determined by real-time RT–PCR. We prepared some primers for transforming growth factor (TGF)-β (forward: TTTCGATTCAGCGCT CACTGCTCTTGTGAC, reverse: ATGTTGGACAACTGCT CCACCTTGGGCTTGC), hepatocyte growth factor (HGF) (forward: AAGAGTGGCATCAAGTGCCAG, reverse: CTG GATTGCTTGTGAAACACC), IL-2 (forward: TGGAGCA GCTGTTGATGGAC, reverse: CAATTCTGTGGCCTGCTT GG), IL-4 (forward: ACAGGAGAAGGGACGCCAT, reverse: GAAGCCCTACAGACGAGCTCA), IL-10 (forward: GGTT GCCAAGCCTTATCGGA, reverse: ACCTGCTCCACTGC CTTGCT) and IL-15 (forward: CATCCATCTCGTGCTAC TTGTGTT, reverse: CATCTATCCAGTTGGCCTCTGTTT) (Nisshinbo, Chiba, Japan).

Real-time RT–PCR was conducted on a DNA engine Opticon2 System (MJ Japan Ltd, Tokyo, Japan) by using SYBR Green I as a double-stranded DNA-specific binding dye and continuous fluorescence monitoring. The cycling conditions consisted of a denaturation step for 10 min at 95°C, 40 cycles of denaturation (94°C for 15 s), annealing (60°C for 30 s) and extension (72°C for 30 s). After amplification, melting curve analysis was performed with denaturation at 95°C, then continuous fluorescence measurement from 65°C to 95°C at 0·1°C/s. All reactions were run at least in duplicate, and included control wells without cDNA.

Detection of cytokines in MSC culture supernatant

Mesenchymal stem cell culture supernatants were collected 2 weeks later, and the amounts of IL-2, IL-4, IFN-γ and TGF-β were determined by ELISA kits.

Statistical analyses

Non-parametric analyses (Mann–Whitney *U*-test and log-rank test) were performed using StatView software (Abacus Concepts, Berkley, CA, USA). Values of $P < 0.05$ were considered statistically significant.

Results

In vitro immunosuppressive effects of BMSCs on T cell proliferation

Three days after DLI, BMCs were collected from the recipients, and non-haemopoietic BMCs (defined as $CD45^-/CD106^+$ cells) were isolated immediately as shown in Fig. 1a. The average number of these sorted cells per mouse were as follows. $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + IBM-DLI: 31 033 ± 2450 cells (four mice), $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + i.v.-DLI: 29 850 ± 2728 cells (four mice), $CD45^-/CD106^+$ cells from the recipients of IBM-BMT alone (without DLI): 36 630 ± 5244 cells (four mice). There were no statistical differences among these groups regarding the yields of $CD45^-/CD106^+$ cells. The sorted $CD45^-/CD106^+$ cells from these recipients were added to the culture of one-way MLR. As shown in Fig. 2, all the $CD45^-/CD106^+$ cells isolated from

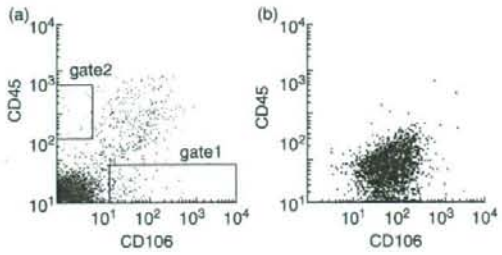

Fig. 1. Flow cytometric profiles of freshly isolated and cultured bone marrow stromal cells (BMSCs). (a) Non-haemopoietic mesenchymal stem cell-enriched cells, defined as $CD45^-/CD106^+$ cells, were sorted immediately (gate 1) from the recipient of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI) after the staining of cells with fluorescein isothiocyanate (FITC)-anti-CD45 and phycoerythrin (PE)-anti-106 monoclonal antibodies (mAbs). The dot-plot profile of $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) was similar to (a). Haemopoietic bone marrow cell-enriched populations, sorted as $CD45^+/CD106^-$ cells (gate 2), were also prepared from the recipients, and used as controls. (b) Cultured BMSCs (for 2 weeks) obtained originally from the right tibia of the recipients of IBM-BMT + IBM-DLI were stained with FITC-anti-CD45 and PE-anti-106 mAbs, and analysed by a fluoresence activated cell sorter scan. The dot-plot profile of cultured BMSCs from the recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) was similar to (b).

the BM of IBM-DLI, i.v.-DLI and IBM-BMT alone (without DLI) suppressed MLR only slightly, but not significantly (not statistically significant among three groups). This is the case when haemopoietic $CD45^+/CD106^-$ cells or whole BMCs were added to the culture. Thus, non-haemopoietic BMCs freshly isolated from the site of IBM-DLI could not significantly suppress T cell proliferation in MLR. This might be due to the heterogeneity of non-haemopoietic BMCs. Therefore, we next examined the inhibitory effect of cultured BMSCs after IBM-DLI.

Three days after DLI, BMCs were collected from the recipients, and cultured in DMEM with 10% FCS for 2 weeks, as shown in *Materials and methods*. The phenotypes of BMSCs, thus prepared, were negative for CD45 and CD34, but positive for CD90 and CD106 (Fig. 1b). These BMSCs were added to the culture of MLR to examine their suppressive effects.

As shown in Fig. 3a and b, the BMSCs prepared from the recipients treated with IBM-BMT + IBM-DLI significantly suppressed MLR in a dose-dependent fashion when compared with those from the recipients treated with IBM-BMT + i.v.-DLI. It is surprising that the BMSCs from the recipients of IBM-BMT + IBM-DLI still showed a suppressive effect on T cell proliferation even after long-term culture (3 months) when compared with those prepared from the recipients of IBM-BMT + i.v.-DLI (Fig. 3c), suggesting that the suppressive effects of BMSCs on the BM (IBM-DLI) are long-lasting.

The frequency of IFN-γ- and IL-4-producing T cells after coculture with BMSCs

To examine the effects of BMSCs on T cell polarization, CD4-enriched T cells from donor BALB/c mice were cultured with irradiated stimulator spleen cells from B6 mice and BMSCs cultured from the recipients of IBM-BMT + IBM-

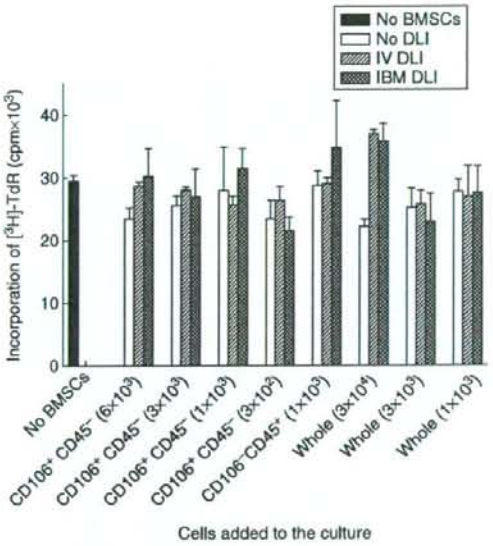

Fig. 2. Effect of freshly isolated bone marrow stromal cells (BMSCs) on T cell proliferation. Non-haemopoietic mesenchymal stem cell-enriched cells, defined as $CD45^-/CD106^+$ cells, were sorted immediately (gate 1) from the recipient of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) after the staining of cells with fluorescein isothiocyanate-anti-CD45 and phycoerythrin-anti-10^4 monoclonal antibodies (mAbs). Haemopoietic cells in the bone marrow (BM), defined as $CD45^+/CD106^-$ cells, were also obtained by a cell sort (gate 2). Graded numbers of $CD45^-/CD106^+$ BMSCs (3×10^2–6×10^3), $CD45^+/CD106^-$ haemopoietic cells (1×10^3) or whole BM cells (1×10^3–3×10^4) were added to the culture of one-way mixed leucocyte reaction where 2×10^5 responder $CD4^+$ T cells from BALB/c mice were stimulated with 12 Gy irradiated stimulator spleen cells (2×10^5 cells) from B6 mice in a 96-well flat-bottomed plate in a total volume of 0·2 ml and cultured for 96 h. The cultures were pulsed with 0·5 μCi of [^{3}H]-TdR for the last 16 h of the culture period. This figure shows the representative result of three experiments. The data are expressed as mean counts per minute ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient).

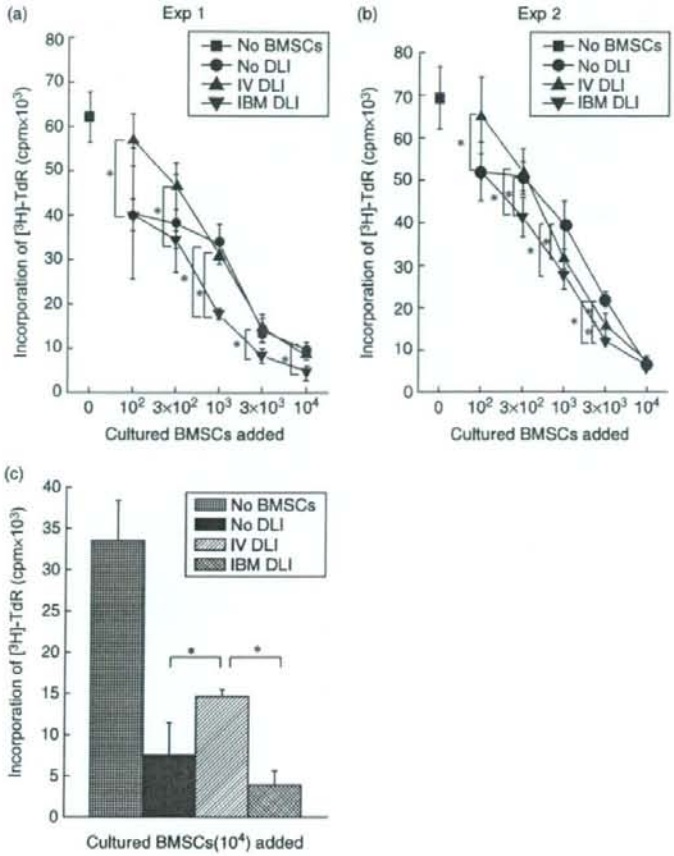

Fig. 3. Inhibitory effect of cultured bone marrow stromal cells (BMSCs) on T cell proliferation. Bone marrow cells from the right tibia were collected from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) and cultured for 2 weeks. Graded numbers of cultured BMSCs (10^2–10^4 cells) were added to the culture of one-way mixed leucocyte reaction (MLR) (a, b). BMSCs were obtained after the long-term culture (cultured for 3 months), and were added to the culture of one-way MLR (c). The data in figures are expressed as mean counts per minute ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient). Symbols in the boxes represent origins of cultured BMSCs. *Statistically significant when compared with MLRs performed in the groups ($P < 0.05$).

DLI, IBM-BMT + i.v.-DLI, or IBM-BMT alone (without DLI). The development of T helper 1 (Th1) or Th2 cells was defined by intracellular staining of IFN-γ or IL-4. The frequency of IL-4-producing cells was slightly but significantly higher in the culture with BMSCs from IBM-BMT + IBM-DLI than in that with BMSCs from IBM-BMT + i.v.-DLI (Fig. 4a and b *versus* 4c and summarized in 4g). Conversely, the percentage of IFN-γ-producing cells was lower in the culture with BMSCs from IBM-BMT + IBM-DLI than in that with BMSCs from IBM-BMT + i.v.-DLI (Fig. 4d and e *versus* 4f, and summarized in 4g). Furthermore, this is the case when intracellular IL-2 was examined (data not shown). Thus, the polarization of Th2 cells is facilitated strongly after co-culture with the BMSCs from the recipients of IBM-BMT + IBM-DLI, while Th1 cells are induced dominantly by co-culture with the BMSCs from the recipients of IBM-BMT + i.v.-DLI. These findings suggest strongly that T cells injected into the BM cavity can modulate the function of BMSCs after their interaction.

Bone marrow stromal cells produce immunoregulatory cytokines: TGF-β and HGF

Previous reports have shown that BMSCs can modify T cell functions by soluble factors [18,19]. Therefore, we attempted to identify molecules involved in the immune modulation by BMSCs. First, we determined the levels for IL-2, IL-10, IFN-γ and TGF-β in the culture supernatant of BMSCs using an ELISA. The culture supernatants of enriched T cells stimulated with ConA served as a control. As shown in Table 1, IL-2, IL-10 or IFN-γ were not detected in the culture supernatants of BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI), while a significant amount of TGF-β was detected in the culture supernatants of BMSCs from the recipients of IBM-BMT + IBM-DLI, but not in those from the recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). These results indicate that TGF-β secreted from the BMSCs obtained from the recipients of IBM-BMT + IBM-DLI

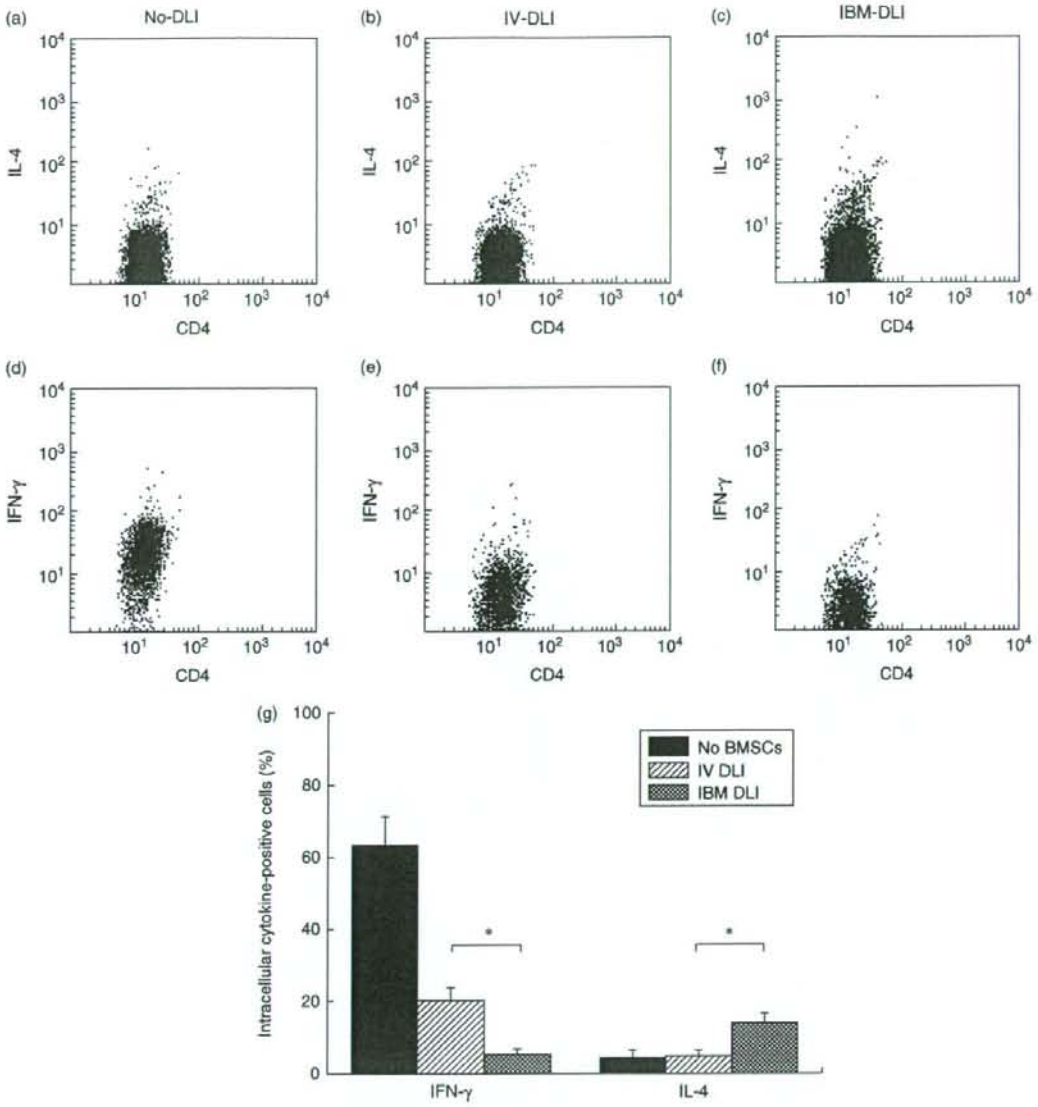

Fig. 4. Interaction of bone marrow stromal cells (BMSCs) with T cells and induction of T helper 2 cells. $CD4^+$ cell-enriched T cells from BALB/c mice were cultured with irradiated stimulator spleen cells from B6 and BMSCs cultured from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) in a round-bottomed plate (RPMI-1640) with 10% fetal calf serum. Cells were harvested 6 days later and stained with biotin-conjugated anti-$H\text{-}2K^d$ (visualized by streptavidin–peridinin chlorophyll-Cy5·5) and fluorescein isothiocyanate-anti-CD4 monoclonal antibody (mAb) to detect responder (donor) CD4 T cells. The cells were next fixed and permeabilized and intracellular cytokines were detected after the staining of cells with phycoerythrin-anti-interleukin (IL)-4 and -interferon (IFN)-γ mAbs. Representative dot-plot profiles of $CD4^+/IL\text{-}4^+$ cells (a, b, c) or $CD4^+/IFN\text{-}\gamma^+$ cells (d, e, f) are shown, co-cultured with BMSCs from the recipients of IBM-BMT alone (without DLI) (a, d), IBM-BMT + i.v.-DLI (b, e), or IBM-BMT + IBM-DLI (c, f). Cells in dot-plot profiles were gated positively as $H\text{-}2K^{d+}$ responder cells. Cells stained with isotype control cocktail served as a control. (g) Representative result of three experiments. Columns represent mean percentage of IFN-γ or IL-4 bearing cells ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient). Symbols in the boxes represent origins of cultured BMSCs. *Statistically significant when compared with intracellular cytokines performed in the groups ($P < 0.05$).

Table 1. Measurement of cytokines.

	No DLI[†]	i.v.-DLI	IBM-DLI	T cells with ConA[‡]
IL-2 (pg/ml)	0	0	0	87·3 ± 15·5
IFN-γ (pg/ml)	0	0	0	1418·2 ± 369·4
IL-10 (pg/ml)	0	0	0	1114·6 ± 103·1
TGF-β (ng/ml)	0	0·27 ± 0·4	14·2 ± 2·4	0·6 ± 0·5

[†]Bone marrow stromal cell (BMSC) culture supernatants from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI, or IBM-BMT alone (without DLI) were collected 2 weeks later. The cell supernatants were analysed for the amount of interleukin (IL)-2, IL-4, interferon (IFN)-γ and trandforming growth factor (TGF)-β by enzyme-linked immunosorbent assay. [‡]Splenic T cells from BALB/c mice were activated with concanavalin A (ConA) and used as a positive control.

might be one of the candidates for attenuation of GVHD in our model system.

It has been reported that HGF also inhibits T cell proliferation or activation [18,19]. Therefore, we next determined in the culture supernatants of BMSCs whether the levels of HGF in BMSCs increased after IBM-BMT + IBM-DLI. We measured HGF (and also TGF-β) in the message level by a quantitative real-time RT–PCR because no ELISA kit is available to detect murine HGF. As shown in Fig. 5a (HGF) and 5b (TGF-β), the relative levels of both HGF and TGF-β were significantly higher in the BMSCs from the recipients of IBM-BMT + IBM-DLI than in those from the recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). Furthermore, as summarized in Table 2, we did not detect substantial levels of IL-2, IL-4 or IL-15 mRNA in BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). However, it is noted that a slight but significant level of IL-10 message was detected only in the BMSCs from recipients of IBM-BMT + IBM-DLI, but not in those from recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). Therefore, T cells injected directly into the BM cavity can induce the production of suppressive cytokines from BMSCs, and BMSCs might exert their inhibitory effect on T cell activation or proliferation via HGF and/or TGF-β.

Discussion

Transplantation biology has been one of the major advances in medicine during the last few decades. BMT, in particular, can cure a variety of malignancies by exploiting graft-*versus*-tumour effects exerted by the lymphocytes. In this proce-

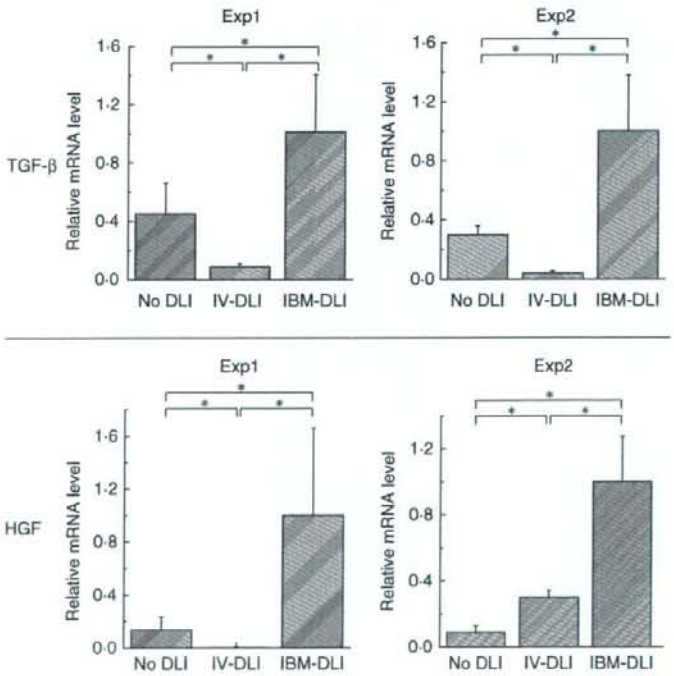

Fig. 5. Production of transforming growth factor (TGF)-β and hepatocyte growth factor (HGF) in bone marrow stromal cells (BMSCs). Culture expanded BMSCs from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) were used for analysis of cytokine messages by real-time PCR. After DNase I treatment, cDNA was synthesized, amplified using HGF or TGF-β primer, and visualized with SYBR Green by real-time reverse transcription–polymerase chain reaction. Relative intensity of HGF or TGF-β mRNA was calculated on the basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) intensity. Columns represent relative cytokine message levels of TGF-β and HGF. Each column shows mean ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient), and we performed two separate experiments. Symbols in the boxes represent origins of cultured BMSCs. *Statistically significant when compared with cytokine message performed in the groups ($P < 0·05$).

Table 2. Analyses of cytokine messages by real-time reverse transcription–polymerase chain reaction (RT–PCR).

Cytokines examined	No DLI†	i.v.-DLI	IBM-DLI	T cells with ConA‡
IL-2	0·47 ± 0·3§	0·31 ± 0·2	0·47 ± 0·3	8·51 ± 6·1
IL-4	0	0	0·025 ± 0·03	1277·2 ± 357·4
IL-10	0	0	2·7 ± 2·3	95 000 ± 16 000
IL-15	0	0	0	n.d.

†Culture expanded bone marrow stromal cells (BMSCs) from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI, or IBM-BMT alone (without DLI) were used for analysis of cytokine messages by real-time RT–PCR. After DNase I treatment, cDNA was synthesized, amplified using interleukin (IL)-2, IL-4, IL-10 or IL-15 primer, and visualized with SYBR Green by real-time RT–PCR. ‡Splenic T cells from BALB/c mice were activated with concanavalin A (ConA) and used as a positive control.

§Relative intensities of soluble factors were calculated on the basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Numbers in the table represent mean intensities of cytokines ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient). We performed two separate experiments. n.d., not done.

dure, one of the major problems to be solved is GVHD. We have developed recently a new protocol for BMT: IBM-BMT can induce persistent allogeneic donor-specific tolerance without the use of immunosuppressants after the treatment, even when the radiation doses are reduced to sublethal levels. Therefore, we have aimed to develop a new strategy for the successful engraftment of donor-derived haematolymphoid cells without developing GVHD even in the presence of T cells in the donor inoculum. We have found that GVHD could be alleviated when BMCs containing T cells were inoculated into the BM cavity [9]. We compared the severity of GVHD induced by the intravenous injection of T cells (i.v.-DLI) with that induced by the IBM injection of T cells (IBM-DLI). Acute GVHD was observed in recipients treated with IBM-BMT + i.v.-DLI, while reduced GVHD was seen in those treated with IBM-BMT + IBM-DLI. However, the mechanisms underlying this inhibition still remain unresolved and therefore we focused on the function of BMSCs, because T cells can interact with BMSCs in the BM cavity after the IBM-DLI. The ability of MSCs to interact with immune cells and to modulate their response has important implications in the transplantation biology. We have carried out experiments in which the sorted $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) were added to the culture of one-way MLR. The inhibitory ability of non-haemopoietic BMCs to activated T cells was insufficient (Fig. 2). However, cultured BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI and IBM-BMT alone (without DLI) showed an immunosuppressive effect in MLR in a dose-dependent fashion (Fig. 3). Furthermore, of interest and of importance is that the cultured BMSCs from the recipients of IBM-BMT + IBM-DLI suppressed MLR strongly even in small numbers (10^2–3×10^3) when compared with BMSCs from the recipients of IBM-BMT + i.v.-DLI.

Furthermore, the conversion of Th1 cells (defined by intracellular staining of IFN-γ) was clearly inhibited while the polarization of Th2 cells (defined by intracellular staining of IL-4) was facilitated by BMSCs from the recipients treated with IBM-DLI. In contrast to this, BMSCs from the recipients of i.v.-DLI prompted the polarization of Th1 cells (Fig. 4). These data suggest that BMSCs from the recipients of IBM-BMT + IBM-DLI interact with naive T cells to convert Th2 cells, which might be beneficial for GVHD management.

Several recent reports have described how BMSCs produce soluble factors, including TGF-β and HGF, which regulate T cell proliferation [18,21,22,32]. In our present study, BMSCs from the recipients of IBM-BMT + IBM-DLI produced significantly higher amounts of HGF and TGF-β than those from the recipients of IBM-BMT + i.v.-DLI and IBM-BMT alone (without DLI) (Fig. 5 and Table 1).

Collectively, our findings indicate clearly that BMSCs can interact with T cells that have been injected into the BM cavity as IBM-DLI, and that the function(s) of BMSCs might somehow be modulated by this interaction to produce inhibitory cytokines and to possess the ability to convert Th0 cells to Th2 cells, but not to Th1 cells. It should be noted that the modulated features of BMSCs were maintained for at least 6 weeks, thus leading to the reduction of GvH responses. We have shown, in our GVHD model, that IBM-DLI (*in vivo* injection of donor T cells into the BM cavity) (but not i.v.-DLI) can attenuate GVHD. Therefore, our present study provides the basic information that IBM-BMT is an excellent strategy to engraft donor cells efficiently along with attenuation of GVHD, even when some quantities of T cells are contaminated in BMC preparations. Thus, IBM-BMT can control GVHD easily.

T cells can recognize MHC determinants on BMSCs *in vivo*, and the BMSC recognized by T cells can modulate their functions. Therefore, we are now investigating subcellular processes after the T–BMSC interaction and identifying molecules, other than MHC, to be essential for this interaction.

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References

1 Ikehara S. Bone marrow transplantation: a new strategy for intractable disease. Drugs Today 2002; **38**:103–11.

2 Ikehara S, Ohtsuki H, Good RA *et al.* Prevention of type I diabetes in non-obese diabetic mice by allogeneic bone marrow transplantation. Proc Natl Acad Sci USA 1985; **22**:7743–7.

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 USA 1987; **84**:6555–7.

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**:1233–8.

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-lpr/lpr mice by transplantation of bone marrow plus bones (stromal cells) from the same donor. J Immunol 1994; **152**:3119–27.

6 Nakagawa T, Nagata N, Hosaka N, Ogawa R, Nakamura K, Ikehara S. 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**:263–8.

7 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**:3292–9.

8 Taira M, Inaba M, Takata K *et al.* Treatment of streptozotocin-induced diabetes mellitus in rats by transplantation of islet cells from two major histocompatibility complex disparate rats in combination with intra bone marrow injection of allogeneic bone marrow cells. Transplantation 2005; **79**:680–7.

9 Nakamura K, Inaba M, Sugiura K *et al.* Enhancement of allogeneic hematopoietic stem cell engraftment and prevention of graft-versus-host diseases (GvHD) by intra-bone marrow-bone marrow transplantation plus donor lymphocyte infusion. Stem Cells 2004; **22**:125–34.

10 Hashimoto F, Sugiura K, Inoue K, Ikehara S. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells *in vivo*. Blood 1997; **89**:49–54.

11 Sugiura K, Hisha H, Ishikawa J *et al.* Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells *in vitro*. Stem Cells 2001; **19**:46–58.

12 Ikehara S. A novel strategy for allogeneic stem cell transplantation: perfusion method plus intra-bone marrow injection of stem cells. Exp Hematol 2003; **31**:1142–6.

13 Fukui J, Inaba M, Ueda Y *et al.* Prevention of graft-versus-host disease by intra-bone marrow injection of donor T cells. Stem Cells 2007; **25**:1595–601.

14 Golde DW, Gasson JC. Hormones that stimulate the growth of blood cells. Sci Am 1988; **259**:62–71.

15 Gordon MY. Extracellular matrix of the marrow microenvironment. Br J Haematol 1988; **70**:1–4.

16 Dexter TM. Regulation of hemopoietic cell growth and development: experimental and clinical studies. Leukemia 1989; **3**:469–74.

17 Majumdar MK, Thiede MA, Haynesworth SE, Bruder SP, Gerson SL. Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. J Hematother Stem Cell Res 2000; **9**:841–8.

18 Muriel S, Francoise N, Aurelie T *et al.* Bone marrow mesenchymal stem cells suppress lymphocyte proliferation *in vitro* but fail to prevent graft-versus-host disease in mice. J Immunol 2006; **176**:7761–7.

19 Di Nicola M, 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**:3838–43.

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**:3722–9.

21 Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into non-human primates. Blood 2003; **101**:2999–3001.

22 Lazarus HM, Haynesworth SE, Gerson SL, Rosenthal NS, Caplan AI. *Ex vivo* expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. Bone Marrow Transplant 1995; **16**:557–64.

23 Sudeepta A, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005; **105**:1815–22.

24 Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation 2003; **75**:389–97.

25 Le Blanc K. Immunomodulatory effects of fetal and adult mesenchymal stem cells. Cytotherapy 2003; **5**:485–9.

26 Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003; **57**:11–20.

27 Potian JA, Aviv H, Ponzio NM, Harrison JS, Rameshwar P. Veto-like activity of mesenchymal stem cells: functional discrimination between cellular responses to alloantigens and recall antigens. J Immunol 2003; **171**:3426–34.

28 Djouad F, Plence P, Bony C *et al.* Immunosuppressive effect of

mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 2003; **102**:3837–44.

29 Beyth S, Borovsky Z, Mevorach D *et al.* Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood 2005; **105**:2214–9.

30 Meisel R, Zibert A, Laryea M, Göbel U, Däubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 2004; **103**:4619–21.

31 Augello A, Tasso R, Negrini SM *et al.* Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. Eur J Immunol 2005; **35**:1482–90.

32 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**:42–8.

Analysis of Tolerance Induction Using Triple Chimeric Mice: Major Histocompatibility Complex-Disparate Thymus, Hemopoietic Cells, and Microenvironment

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Background. Although bone marrow transplantation (BMT) has become a valuable strategy for the treatment of various intractable diseases in recent years, success rates remain low in elderly patients because of low thymic function. We have previously shown that fetal thymus transplantation (TT) with BMT is effective for elderly recipients in mice.
Methods. We performed fully major histocompatibility complex (MHC)-mismatched fetal TT from B6 (H-2^b) mice plus allogeneic BMT from C3H/HeN (H-2^k) mice by intra-bone marrow-BMT (IBM-BMT) using congenitally athymic nude (nu/nu) BALB/c (H-2^d), or BALB/c adult-thymectomized recipients to obtain triple chimeras. We next carried out the IBM-BMT+TT using senescence-accelerated mouse P1 strain (SAMP1) to examine whether this method would be applicable to aging mice.
Results. Triple chimeric mice survived for a long period with sufficient T-cell functions comparable to the mice treated with BMT plus MHC-matched TT, whereas those without TT survived for a short period with insufficient T-cell reconstitution. Almost all the hematolymphoid cells were derived from donor bone marrow cells. Interestingly, they showed tolerance to all three types of MHC determinants with donor-derived thymic dendritic cells in TT. Triple chimeric SAMP1 also survived for long periods with T-cell functions restored in contrast to non-TT SAMP1 recipients.
Conclusion. These findings suggest that third party combined TT with allogeneic IBM-BMT may be more advantageous for elderly recipients with low thymic function, than IBM-BMT alone (without TT).

Keywords: Thymus transplantation, MHC, IBM-BMT.

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In recent years, allogeneic bone marrow transplantation (BMT) has proven to be effective in the treatment of hematologic disorders (including leukemia, lymphoma, aplastic anemia) and congenital immunodeficiencies (*1*). Using various animal models, we have found that allogeneic BMT can be used to treat autoimmune diseases such as insulin-dependent diabetes mellitus, a certain type of non–insulin-dependent diabetes mellitus, systemic lupus erythematosus, rheumatoid arthritis, chronic pancreatitis, and chronic glomerulonephritis, and also be applicable to solid cancers and organ transplantation (*2–9*). These results suggest that BMT is likely to become a powerful tool in the treatment of a wide range of diseases.

However, BMT has some problems. The success rate of allogeneic BMT is very low in elderly patients (*10–12*), who run the high risk of complications, including interstitial pneumonitis, graft-versus-host disease (GVHD), systemic infections, and relapses of primary disease. One reason is markedly reduced thymic functions because of involution, leading to insufficient or erratic T-cell development (*13–15*). We have previously demonstrated that fetal thymus transplantation (TT) with BMT from the same donor is effective in survival, reconstitution, and treatment of autoimmune diseases in aged mice (*16*). However, the thymus cannot always be obtained from the same young donor in BMT, and the induction of tolerance has not yet been studied in detail for TT.

We have recently developed intra-bone marrow (IBM)-BMT, in which bone marrow cells (BMCs) are directly injected into the BM cavity (*17*). This method allows us not only to use low-dose irradiation as a preconditioning regimen but also to effectively suppress GVHD (*18*); IBM-BMT promotes efficient proliferation of BMCs in the microenviron-

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