In recent years, BMSCs have become an attractive source of stem cells for the regeneration of damaged tissues in clinical applications. This is because they are able to self-renew with high proliferative capacity and have potential for mesodermal differentiation. Studies of transplantation of BMSCs have been performed in rodents. Clinical applications of canine BMSCs in dogs with fractures and spinal cord injury have been reported. Only a limited number of BMSCs can be obtained from bone marrow, and culture and cell passage is necessary to prepare a greater number of BMSCs.

Evaluation of methods for cell harvesting and the biological properties at successive passages of canine bone marrow stromal cells

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Objective—To compare methods for harvesting canine bone marrow stromal cells (BMSCs) and determine the biological properties of canine BMSCs at successive passages in vitro.

Sample—BMSCs collected from the femurs of 9 Beagles.

Procedures—a fibroblast assay was performed to compare 2 methods for harvesting BMSCs: the aspiration and perfusion method. Flow cytometric analysis was performed to evaluate the cell surface markers. Changes in proliferative activity were analyzed by examining radioactivity of hydrogen 3-thymidine. Cell senescence was studied via senescence-associated β-galactosidase staining, and differentiation properties (osteogenesis and adipogenesis) were estimated in association with passage.

Results—The aspiration method yielded significantly more fibroblasts than the perfusion method. The cells harvested by both methods gave positive results for CD44 and CD90 and negative results for CD34 and CD45. After induction, the cells had osteogenic and adipogenic phenotypes. The biological properties of BMSCs harvested by the aspiration method were estimated in association with passage. With increasing number of passages, the proliferative activity was reduced and the proportion of cells with senescence-associated β-galactosidase staining was increased. The capacity of differentiation was reduced at passage 3.

Conclusions and Clinical Relevance—The aspiration method was superior for collection of BMSCs. In early passages, canine BMSCs had the proliferative activity and potential of osteogenic and adipogenic differentiation, but this decreased with increased number of passages. Consideration of passage will be important to the success of any strategy that seeks to regenerate tissue though the use of BMSCs. (Am J Vet Res 2012;73:1832–1840)

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ABBREVIATIONS
BMSC Bone marrow stromal cell
CFU-F CFU fibroblast
DMEM Dulbecco modified Eagle medium
FBS Fetal bovine serum
MSC Mesenchymal stem cell
SAβ-gal Senescence-associated β-galactosidase
by bone marrow aspiration according to the method of Thomas and Storb.\(^5\) Peripheral-blood lymphocytes reportedly cause immune responses, and it is difficult to obtain fresh whole bone marrow cells without peripheral blood lymphocyte contamination. Kushida et al.\(^10,11\) have reported a new method for bone marrow cell harvesting, known as the perfusion method; bone marrow fluid from long bones or the ilium is flushed with saline (0.9% NaCl) solution. The resulting bone marrow cells contain BMSCs; fibroblastic adherent cells grow in vitro after culturing of the bone marrow cells. The perfusion method minimizes the contamination of T cells by peripheral blood and gives a significantly higher number of hematopoietic progenitor cells.\(^10,11\)

In rodents, BMSCs have usually been collected by cutting off both ends of the tibia and flushing out the marrow.\(^1\) This method is similar to the perfusion method. However, to our knowledge, no study has yet compared methods for harvesting canine BMSCs.

Bone marrow stromal cells have to be cultured over a few weeks from fresh whole bone marrow cells to accumulate sufficient purified cultured BMSCs for transplantation. The important variable, particularly because of decreased proliferation and the propensity toward senescence, is the effect of cell passage on the differentiation capacity of BMSCs. The potential of osteogenic and adipogenic differentiation is reportedly reduced with increasing number of passages in human and rodent BMSCs.\(^13,14\) Changes in biological properties in successive passages differ with cell source and animal species.\(^15\) Many promising applications of tissue engineering require cell expansion following harvest, so the principles of ex vivo handling must be understood to develop clinical techniques and therapeutics on the basis of canine BMSCs. A few studies have been made of the biological properties and potential of canine BMSCs. Previous studies\(^16\) of canine BMSCs have focused on their osteogenic, adipogenic, and chondrogenic differentiation potential. Kamishina et al.\(^17\) reported that growth kinetic patterns depend on the initial cell seeding densities, with the greatest increase at lower density. However, it is not clear whether the proliferating canine BMSCs maintained their characteristics permanently. The aim of the study reported here was to compare the aspiration and perfusion methods for harvesting canine BMSCs and to estimate their biological properties with passage in culture, for future therapeutic application.

**Materials and Methods**

**Bone marrow collection**—Samples of bone marrow were collected from 9 Beagles 4 to 8 years of age (median, 7.0 years), under sterile conditions. All surgical procedures and the postoperative care of animals were performed in accordance with the guidelines of Osaka Prefecture University.

All dogs were anesthetized with propofol (5.0 mg/kg, IV), and anesthesia was maintained with isoflurane. Bone marrow fluid was collected as follows: in the aspiration method, bone marrow fluid (4 mL) was aspirated by use of 13-gauge Jamshidi needles from the proximal end of the diaphysis of the femur into sterilized 10-mL syringes containing 2,000 U of heparin; in the perfusion method, the bone marrow fluid was aspirated as in a previous study.\(^10,11\) Briefly, 2 sterilized 13-gauge Jamshidi needles were inserted into the proximal and distal ends of the diaphysis of the femur. One needle was connected via an extension tube to a 50-mL syringe to collect the bone marrow fluid. The other needle was connected to a syringe containing 50 mL of saline (0.9% NaCl) solution with heparin. The saline solution was infused gently through the needle into the bone marrow cavity, to flush out the bone marrow cells via the other needle. The extracted bone marrow cells were collected in a syringe, and the process was repeated twice on the same limb.

**Isolation and culture of BMSCs**—The bone marrow perfusate was centrifuged, and the precipitates were suspended in 15 mL of Dulbecco PBS solution. The mononuclear cells were isolated by density centrifugation with a lymphocyte separation solution\(^1\) at 400 × g for 30 minutes at room temperature (approx 20°C). The Buffy coat at the interface was collected, mixed with 20 mL of Dulbecco PBS solution, and centrifuged at 300 × g for 5 minutes. The precipitated cells were washed twice with Dulbecco PBS solution. The number of cells was determined with a hemacytometer. Enriched mononuclear cells were plated in 25-cm\(^2\) tissue culture flasks\(^2\) for a growth kinetics assay at a density of 1.5 × 10\(^3\) cells/cm\(^2\) in DMEM\(^6\) containing 10% FBS\(^5\) and 1% antibiotic-antimycotic solution,\(^4\) with incubation at 37°C in a humidified 5% carbon dioxide environment. Nonadherent cells were removed by replacing the medium at 48 hours after plating. The culture medium was changed 3 times/wk. After 14 days, the adherent BMSCs were transferred to another culture at a concentration of 8.0 × 10\(^3\) cells/cm\(^2\). These cells were termed passage 1 (P1). Cells were released with 0.05% trypsin and 0.53mM EDTA.\(^3\) Cells were passaged upon reaching approximately 70% to 80% confluence. The released cells were collected by centrifuging at 300 × g for 5 minutes, washed with PBS solution, and replated at 8.0 × 10\(^3\) cells/cm\(^2\) for subsequent passages prior to P5.

**CFU-F assay**—To estimate the proportion of BMSCs in the canine bone marrow, a CFU-F assay was performed according to the method described by Kamishina et al,\(^17\) with modifications. Enriched mononuclear cells were plated on 60-mm cultured dishes for CFU-Fs at a density of 3.0 × 10\(^4\) cells/cm\(^2\), cultured for 12 days in DMEM containing 10% FBS and 1% antibiotic-antimycotic solution, and incubated at 37°C in a humidified 5% carbon dioxide environment. Colonies were stained with Giemsa stain. The number of cells in each colony was determined by counting; colonies containing > 50 cells were scored. All assays were performed in duplicate.

**Osteogenesis**—The cells were seeded at a density of 4.0 × 10\(^3\) cells/cm\(^2\) in a 12-well plate\(^1\) and cultured. Osteogenic differentiation was induced by canine osteoblast differentiation medium.\(^4\) The medium was changed every 3 days. Negative control cells were maintained in cell culture medium containing 10% FBS. After 14 days of induction, differentiation of BMSCs into the osteoblastic phenotype was evaluated by von Kossa staining for mineralized matrix deposition.
was regarded as positive when there was mineral deposition in the newly formed cell matrix in the well, as described by Bonab et al.\textsuperscript{10} Osteogenic differentiation was performed with P1 to P3 in duplicate.

**Adipogenesis**—The cells were seeded at a density of $1.0 \times 10^4$ cells/cm$^2$ in a 12-well plate and cultured until confluency was attained. Adipogenic induction was set up as described by Pittenger et al.\textsuperscript{1} Briefly, the cells were treated for 72 hours with an adipogenic induction medium consisting of DMEM, 10% FBS, 1mM dexamethasone,\textsuperscript{1,11} 0.2mM indomethacin,\textsuperscript{11} 0.1 mg of insulin/mL, and 1mM 3-isobutyl-1-methylxanthine.\textsuperscript{11} Then, cells were treated for 24 hours with adipogenic maintenance medium consisting of DMEM, 10% FBS, and 0.1 mg of insulin/mL. This cycle was repeated 4 times. For the negative control, cells were cultured in cell culture medium containing 10% FBS. After 16 days of induction, the cells were fixed in 4% paraformaldehyde and stained with oil Red O solution to visualize the formation of fat droplets. Results were considered positive when there was a cytoplasmic droplet of staining with oil Red O, as described by Bonab et al.\textsuperscript{10} Adipogenic induction was performed with P1 to P3 in duplicate.

**Figure 1**—Photograph of 60-mm plates used to determine canine progenitor cell activities via in vitro CFU-F assay of bone marrow cells harvested by aspiration or perfusion methods. Giemsa stain.

**Figure 2**—Results of flow cytometric analysis of canine BMSCs harvested by the aspiration method (green). Isotype control samples are also included in each panel (red line). Notice that canine BMSCs had positive results for CD44 and CD90 and negative results for CD34 and CD45.
Flow cytometric analysis—To evaluate cell surface markers, flow cytometric analysis was performed. Cells were detached from the flasks with 0.05% trypsin and 0.53 mM EDTA and collected by centrifugation. Aliquots containing $1.5 \times 10^5$ cells were washed in fluorescence-activated cell sorting buffer (Dulbecco PBS solution containing 0.5% FBS and 0.1% sodium azide) and incubated in 100 µL of fluorescence-activated cell sorting buffer with fluorescent isothiocyanate and phycoerythrin-labeled antibodies for CD34 (1/25), CD44 (1/50), CD45 (1/10), and CD90 (1/50) for 30 minutes on ice. Isotype control was used in a negative control sample. Data were analyzed by recording 10,000 events on a flow cytometer. The flow cytometric analysis was performed with P1, P3, and P5 samples harvested by the aspiration method and P1 sample harvested by the perfusion method.

Proliferative activity of BMSCs—Cells harvested by the aspiration method ($2.0 \times 10^4$) were incubated in 200 µL of DMEM containing 10% FBS and 1% antibiotic-antimycotic solution at 37°C for 24 hours, in a 96-well plate. Proliferation of the cells was evaluated by measurement of radioactivity of hydrogen 3-thymidine ($92.5 \text{kBq/mL}$) incorporated into DNA during a further 16 hours of incubation. The proliferative activity was performed with P1 to P5 in triplicate.

SAβ-gal staining—The cells harvested by the aspiration method were seeded as in a 12-well plate and were cultured to 70% to 80% confluence. The cells were stained with a senescence detection kit. In preliminary experiments, all cells stained for lysosomal β-galactosidase at a pH of 4.0, which therefore acts as a positive control for this staining protocol, and did not stain at a pH of 7.5, whereas only senescent cells stained for SAβ-gal at a pH of 6.0. The cells were incubated overnight (maximum, 16 hours) at 37°C with X-gal substrate at a pH of 6.0 according to the manufacturer’s protocol. After staining, the cells were observed at 200X magnification and counted under a microscope. More than 100 cells were counted, and the percentage of blue-stained cells was calculated from the means from the 2 wells with P1 to P5.

Expansion characteristics—Structural changes, the number of cells, and the cumulative population doublings were estimated with the cells harvested by the aspiration method. Structural changes were observed via microscope for each passage. The number of BMSCs was calculated with the following formula:

$$\text{BMSCs} = \frac{(N_h/N_1)}{N_{h-1}}$$

where $N_h$ is the cell harvest number, $N_1$ is the inoculum cell number, and $N_{h-1}$ is the previous cell harvest number. The cumulative population doublings were calculated with the following formula:
where \( x \) is the cumulative population doublings. To determine the cumulated doubling level, the population doubling was calculated for each passage and then added to the population doubling levels of the previous passages with P1 to P5.

**Statistical analysis**—Data are presented as mean ± SEM values. Values were compared by use of the unpaired \( t \) test between 2 groups and by use of 1-factor ANOVA among > 3 groups. The differentiation rate of osteogenesis and adipogenesis was analyzed via a Fisher exact test. Values of \( P < 0.05 \) were considered significant.

**Results**

Bone marrow cells were collected from the femur via the aspiration and perfusion methods. Means of the total numbers of bone marrow mononuclear cells from aspiration and perfusion before culture were \( 1.74 \times 10^8 \) cells ± \( 0.45 \times 10^8 \) cells (n = 9 samples) and \( 1.67 \times 10^8 \) cells ± \( 0.19 \times 10^8 \) cells (4), respectively. Progenitor cell activities determined by in vitro CFU-F assays (Figure 1) revealed that bone marrow cells collected by the aspiration method generated a significantly (\( P = 0.01 \)) higher number of CFU-Fs than those harvested by the perfusion method, when assayed 12 days later (\( 76.0 \pm 9.0 \) vs \( 3.3 \pm 2.2; n = 4 \) samples). The frequency of canine BMSCs harvested by the aspiration method was estimated to be \( 0.0090 \pm 0.0011\% \), greater than those harvested by the perfusion method (\( 0.0004 \pm 0.0003\% \)). The cell surface markers of these cells (P1) were comparable to those reported in human and rodent BMSCs. Flow cytometric analysis revealed that the cells harvested by the aspiration method gave positive results for CD44 and CD90 and negative results for CD34 and CD45 (Figure 2). The cells harvested by the perfusion method also gave the same results as the aspiration method. Therefore, these cells conformed to the cell surface markers of human and rodent BMSCs.

To determine their osteogenic and adipogenic differentiation potential, the canine BMSCs were cultured in each medium containing inducers. The cells (P1) yielded negative staining results with von Kossa and oil Red O staining prior to differentiation (Figure 3). After 14 days of osteogenic induction, all 9 samples of P1 harvested by the aspiration method and all 4 samples of P1 harvested by the perfusion method yielded positive results with von Kossa staining for mineral deposition in the newly formed cell matrix. Control cultures did
not form cell aggregates and yielded negative staining results for mineral deposition. After 16 days of adipogenic induction, 8 of the 9 samples of P1 harvested by the aspiration method and 3 of the 4 samples of P1 harvested by the perfusion method contained cytoplasmic droplets. These cytoplasmic droplets were stained with oil Red O. These changes were not observed in cells grown in control medium. The cells harvested by the perfusion method also gave the same results as the aspiration method.

The proliferative activity, structural changes, SAβ-gal staining, and differentiation for osteogenesis and adipogenesis with passage in culture were performed with BMSCs harvested by the aspiration method. The total number of BMSCs cultured at P5 was $7.7 \times 10^8$ to $2.4 \times 10^{10}$. There were large variations among dogs for the total number of BMSCs. To calculate the cumulated doubling level, the population doubling for each passage was calculated and then added to the population doubling levels of previous passages. The cumulative population doublings decreased with increasing number of passages (Figure 4; $n = 9$; P1 vs P2, P3, P4, and P5 [$P = 0.01$]; P2 vs P3 [$P < 0.05$]; P2 vs P4 and P5 [$P = 0.01$]; P3 vs P5 [$P < 0.05$]), and the radioactivity of the $^3$H-thymidine decreased with increasing number of passages (Figure 5; $n = 5$; P1 vs P2 [$P < 0.05$]; P1 vs P3, P4, and P5 [$P = 0.01$]; P2 vs P3, P4, and P5 [$P < 0.05$]). The proliferation of BMSCs was scarcely changed in P4 and P5 cells. The spindle-shaped cells typically became predominant in P1. When passaged, these long spindle-shaped cells became flattened and assumed large fibroblastic morphology in late passage (Figure 6). These changes were consistent across all bone marrow samples harvested by the aspiration method from the various donors. The SAβ-gal staining was then performed (Figure 7). The percentage of SAβ-gal cells in late passage was greater than for cells in early passage (Figure 8; $n = 4$; P1, P2, and P3 vs P4 and P5 [$P = 0.01$]; P1 vs P3 [$P < 0.05$]). To study the differentiation capacity for osteogenesis and adipogenesis, samples from each dog’s passage were analyzed. After osteogenic induction, 9 of 9 of P1, 7 of 9 of P2, and 0 of 9 of P3 had an osteogenic phenotype. After adipogenic induction, 8 of 9 of P1, 6 of 9 of P2, and 2 of 9 of P3 had an adipogenic phenotype. The capacity of differentiation for osteogenesis and adipogenesis was different in each dog under identical culture conditions. The differentiative potential for osteogenesis and adipogenesis in P1 samples was greater than for cells in P3 samples ($P < 0.05$). Flow cytometric analysis revealed that P3 and P5 samples...
Results of the present study clearly revealed that canine BMSCs harvested by the aspiration method were more abundant than those harvested by the perfusion method. The numbers of canine BMSCs harvested by the perfusion method were higher than that reported for murine BMSCs\(^{21,22}\) and canine BMSCs\(^{17,23}\). The CFU-F number might be influenced by the culture conditions (eg, FBS concentration or type of basal medium),\(^{24}\) so further studies should be performed to determine the optimal culture conditions for canine BMSCs.

Canine bone marrow cells have usually been collected by bone marrow aspiration.\(^{16,17}\) Two studies\(^{10,11}\) compared methods for harvesting hematopoietic progenitor cells in vitro. The perfusion method was superior to the aspiration method for preventing contamination of bone marrow cells by peripheral RBCs and lymphocytes, which may cause an immune response.\(^{11}\) The number of hematopoietic progenitor cells harvested via the perfusion method was greater than for those harvested via the aspiration method.\(^{10,11}\) Although the perfusion method should have been superior to the aspiration method for collection of canine BMSCs from the femur, the present study found otherwise. It has been reported that BMSCs are adhered in the arterial perivascular space near the inner surface of the cortical bone.\(^{2}\) Therefore, the continuous negative pressure in the bone marrow cavity caused by the aspiration method may enhance the collection of BMSCs. In the present study, we did not compare the aspiration and perfusion method with regard to the cell surface markers, proliferative activity, and senescence. Further study would be needed to determine clearly how useful are cells harvested by the aspiration and perfusion methods.

Flow cytometric analysis revealed that these cells (at P1) were positive for CD44 and CD90 and negative for CD34 and CD45. Expression of CD44 and CD90 was in agreement with other reports for human,\(^{15}\) mouse,\(^{26}\) cat,\(^{27}\) and canine BMSCs.\(^{16,18}\) On the basis of the morphological characteristics and results of flow cytometric analysis, these cells were considered to be BMSCs. In vivo and in vitro studies\(^{1,20}\) indicate that BMSCs include a small number of MSCs. There are no MSC-specific cellular markers, so their identification depends on their ability to adhere to culture-associated plastic. There is a clear need for specific markers and methods of detection, enumeration, and isolation of MSCs from bone marrow.

In the present study, we wished to determine the biological properties of BMSCs harvested by the aspiration method because this method yielded significantly more CFU-Fs than did the perfusion method. The potential of differentiation for osteogenesis and adipogenesis was different in each dog under identical culture conditions. In P3, no samples had an osteogenic phenotype and only 2 of 9 samples had an adipogenic phenotype after induction. It has been reported that BMSCs from aged rats have less bone formation capacity than BMSCs from young donors.\(^{8}\) It has been also reported that aging is associated with a decrease in BMSC number.\(^{31}\) In the present study, the bone marrow cells were collected from dogs 4 to 8 years of age. The variation in biological samples may be associated with the quantity of MSCs in the extracted bone marrow cells. Further study would be needed to clarify the relevance of aging and the quantity of MSCs in dogs. It is reported that bone morphogenetic protein-2 is a further inducer of osteogenesis in short-term cultures of canine BMSCs.\(^{32}\) Addition of bone morphogenetic protein-2 might have facilitated further osteogenic induction. The CD106-positive BMSCs were less osteogenic and more adipogenic than were CD106-negative BMSCs.\(^{33}\) The selected cells might be useful for transplantation in diseases such as delayed union of fractures. Human BMSCs largely lose their in vitro differentiation capacity with greater numbers of passages.\(^{18}\) Murine BMSCs have osteogenic and adipogenic differentiation in cultures of P6, but this potential decreases with age and passage.\(^{11}\) The proliferative activity and differentiation capacity of BMSCs might be influenced by culture conditions (eg, growth factor and cell seeding densities), but it is possible that canine BMSCs lost the potential for osteogenic and adipogenic differentiation earlier than in other animals. It is reported that BMSCs have the ability to differentiate into chondrocytes,\(^{19}\) skeletal muscle fibers,\(^{34}\) cardiomyocytes,\(^{35}\) and hepatocytes\(^{36}\) as well as osteoblasts and adipocytes in vitro. Further studies would be needed to estimate the multipotential of canine BMSCs with passage in culture.

Proliferative activity decreased with increasing number of passages, and the potential for osteogenic and adipogenic differentiation was reduced at P3. Results of the present study suggest that passage should be stopped at P2 for therapeutic applications.

Nonmultipotent stromal cells inhibit the proliferation and differentiation of MSCs in vitro.\(^{3}\) The BMSCs might preserve their multilineage potential if they are isolated by use of MSC-specific markers. Culture under low oxygen conditions favors CFU-F expansion while maintaining the MSC characteristic immunophenotype and differentiative potential.\(^{3}\) Identification of factors that preserve the proliferative and differentiative potential of the cells will be important for future therapeutic applications.
Cell shape is known to be correlated with cell growth. In general, when cells from freshly isolated tissue are used for the culture, they proliferate well. When the cells are repeatedly passaged, their shape flattens and the cells undergo only slow proliferation. This phenomenon is known as cellular senescence. Cellular senescence is related to the tumor suppressive system, and the expression of senescence-associated genes such as p16 and p21 is enhanced in senescent cells. It is reported that p16 and p21 are associated with SAβ-gal positivity. Staining of SAβ-gal is easy and is widely used by researchers of senescence.

In the present study, canine BMSCs were flattened and assumed large fibroblast morphology in late passage; these flat BMSCs with low proliferative activity were positive for SAβ-gal. These results suggest that cell shape and growth activity of BMSCs are closely related to each other and that this is due to cellular senescence. It has been reported that senescent human BMSCs lose their stem cell characteristics. Bone marrow stromal cells should therefore be considered for therapeutic applications only in the early stages of in vitro culture.

Results of the present study indicated that the aspiration method was superior to the perfusion method for collection of canine BMSCs. The results suggested that canine BMSCs entered senescence and began to lose their capacity with increased number of passages. Consideration of passage will be important to the success of any strategy that seeks to regenerate tissue though the use of BMSCs.

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