NOTE *Internal Medicine*

Characterization of CD34$^+$ Cells from Canine Umbilical Cord Blood, Bone Marrow Leukocytes, and Peripheral Blood by Flow Cytometric Analysis

Shigehisa TSUMAGARI[1)]*, Isao OTANI[2)], Koichi TANEMURA[1)], Shinichi NAMBA[1)], Tadatoshi OHTAKI[1)], Hiroshi KAMATA[3)] and Kiichi KANAYAMA[2)]

[1)]*Laboratories of Therigenology,* [2)]*Veterinary Physiology and* [3)]*Veterinary Microbiology, Department of Veterinary Medicine, Nihon University,1866 Kameino, Fujisawa, Kanagawa 252–8510, Japan*

(Received 1 May 2007/Accepted 1 August 2007)

ABSTRACT. Characterization of CD34$^+$ cells in canine bone marrow, umbilical cord blood, and peripheral blood was performed by flow cytometric analysis. The ratio of CD34$^+$CD45^{hi} cells, which are absent in human blood, was high in the CD34$^+$ cell fraction, but 98% of these was suggested B-cells. The remaining CD34$^+$CD45^{lo} cells may comprise canine hematopoietic progenitor cells, and these cells accounted for 0.23 ± 0.07% of the fraction in cord blood, 0.30 ± 0.07% in bone marrow, and 0.02 ± 0.01% in peripheral blood.
KEY WORDS: canine, CD34$^+$, flow cytometry.

J. Vet. Med. Sci. 69(11): 1207–1209, 2007

CD34$^+$ cells have a self-regenerative ability and pluripotentiality as hematopoietic stem cells in humans [3,10], monkeys [1,2], mice [12], and dogs [4, 8,11,14]. The ratio of CD34$^+$ cells in humans is reported to be 1.0 ± 0.3% in cord blood, 0.8 ± 0.4% in bone marrow [9], and 0.03–0.09% in peripheral blood [16]. Although there is relatively little information concerning canine hematopoietic progenitor cells, the ratio of CD34$^+$ cells in bone marrow is reported to be about 1–3% in adult dogs [11] and 18% in immature dogs [15]. However, there has been no report on the cells in peripheral blood in adult dogs and umbilical cord blood. Thus, we characterized CD34$^+$ cells in the canine umbilical cord blood, bone marrow and peripheral blood by flow cytometry.

Peripheral blood (2–3 m*l*) was aseptically collected from the jugular vein into a 5-m*l* heparinized test tube from 8 adult female beagles aged 4.5 ± 2.5 years maintained at our laboratory. Bone marrow samples were collected under general anesthesia. A 15-G bone marrow puncture needle (Allegiance Co., Tokyo, Japan) was inserted via the bone caput of the upper foreleg, shaved and disinfected by the standard method, and bone marrow fluid (1–2 m*l*) was aspirated using a 20-m*l* syringe containing 10 IU/m*l* heparin. Umbilical cord blood was collected from 5 pregnant beagles. Blood (0.2–1.5 m*l*) was collected from the 2 umbilical veins into a heparinized test tube using a 23-G needle and 1-m*l* syringe after the umbilical cord was ligated and fetuses were excised from the placentas on caesarian section at 57 days (1 dog), 58 days (2), 59 days (1), and 62 days (1) of gestation (3, 2, 1, and 1 fetus, respectively).

Fifty μl of the blood sample was mixed with 10 μl of the following antibodies: phycoerythrin-labeled anti-canine CD34 antibody (1H6, Becton Dickinson, Franklin, U.S.A.), FITC-labeled anti-canine CD45 and CD3 antibodies (CA12.10C12 and CA17.2A12, Serotec, Oxford, UK), phycoerythrin-labeled anti-human CD21 antibody (B-ly4, Becton Dickinson), FITC-labeled anti-canine CD4 antibody (YKIX302.9, Serotec), phycoerythrin-labeled anti-canine CD8 antibody (YCATE55.9, Serotec), and biotin-labeled anti-canine CD34 antibody (2E9, Becton Dickinson). CD34, CD45, CD3, CD21, CD4, and CD8 are cell surface antigen markers of stem cells, all leukocytes, T-cells, B-cells, helper T-cells, and cytotoxic T-cells, respectively [13].

The mixture was reacted for 15 min at room temperature, and mixed with 1.4 m*l* of erythrocyte lysing solution (0.83% ammonium chloride, 0.1% potassium hydrocarbonate, and 0.004% ethylene diamine tetraacetic acid (EDTA) disodium salt in distilled water). The mixture was then kept at 37°C for 15 min in the dark, followed by centrifugation at 2,500 × g for 2 min at 4°C. After removing the supernatant using an aspirator, the precipitate was combined with 1 m*l* of 1% bovine serum albumin (BSA)-phosphate buffer saline (PBS) and mixed using a pipette. The mixture was centrifuged again at 2,500 × g for 2 min at 4°C, and the supernatant was removed. Samples containing biotin-labeled antibody were combined with 50 μl of PC5-labeled avidin and mixed using a pipette. After keeping them at 4°C for 10 min, 1 m*l* of wash buffer (PBS containing 1% BSA, 2 mM EDTA disodium salt, and 0.03% sodium azide) was added to all samples and mixed. The samples were similarly centrifuged at 2,500 × g for 2 min at 4°C, and the supernatant was removed. The precipitate was washed with 500 μl of wash buffer, mixed well with 150 μl of 1% PFA-PBS(–), sealed, and stored at 4°C. The samples were transferred to filter tubes immediately before flow cytometry (FACSCalibur, Becton Dickinson,).

The ratios of CD34$^+$ cells in umbilical cord blood, bone marrow, and peripheral blood were 6.1%, 4.7%, and 2.8%,

* CORRESPONDENCE TO: TSUMAGARI, S., Laboratory of Theriogenology, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252–8510, Japan.
e-mail: tsumagar@brs.nihon-n.ac.jp

respectively. The reported ratio was 0.8 ± 0.4% in human bone marrow [9], 0.03–0.09% in human peripheral blood [16], and 1–3% in adult dog bone marrow [4, 5, 7, 11]. Our findings differed from the reports on human bone marrow and peripheral blood, and the ratio was slightly higher than in adult dog bone marrow.

To re-investigate the properties of canine $CD34^+$ cells, we counterstained $CD34^+$ cells for a leukocyte marker, CD45 [6]. $CD34^+$ cells in umbilical cord blood, bone marrow and peripheral blood could be divided into 2 types based on the fluorescent intensity (strong and weak) of CD45: $CD34^+$ $CD45^{hi}$ and $CD34^+$ $CD45^{lo}$ cells, respectively. The ratios of $CD34^+CD45^{hi}$ cells in umbilical cord blood, bone marrow, and peripheral blood (as a representative sample shown on the figure) were 3.21% (Fig. 1, a-R3), 1.40% (Fig. 1, b-R3), and 2.31% (Fig.1, c-R3), respectively. The ratios of $CD34^+CD45^{lo}$ cells in umbilical cord blood, bone marrow, and peripheral blood were 2.91% (Fig. 1, a-R2), 3.27% (Fig. 1, b-R2) and 0.03% (Fig. 1, c-R2), respectively.

The $CD34^+CD45^{lo}$ cell fraction comprises hematopoietic stem cells in humans [6], but many $CD34^+CD45^{hi}$ cells, which are absent in humans, were present in dogs.

To further characterize $CD34^+CD45^{hi}$ cells in peripheral blood, they were counter-stained for a T-cell surface antigen marker, CD3, B-cell marker, CD21, helper T-cell marker, CD4, and cytotoxicity T-cell marker, CD8, respectively. The ratio of $CD3^+$ cells in $CD34^+$ cells was low (0.49%), whereas the ratio of $CD21^+$ cells was high (98.54%) (Fig. 2, left). There were no CD4-positive $CD34^+$ cells (0%), and the ratio of $CD8^+$ cells was also low (1.28%) (Fig. 2, right), suggesting that $CD34^+$ $CD45^{hi}$ cells in the dog might be a part of B-cells.

In contrast, the fluorescent character of the $CD34^+$ $CD45^{lo}$ cells was similar to that of human hematopoietic stem cells [6], suggesting that this cell population comprises canine hematopoietic progenitor cells.

Several samples of umbilical cord blood, bone marrow, and peripheral blood were analyzed based on this finding. As a whole, the ratios of $CD34^+CD45^{lo}$ cells in umbilical cord blood, bone marrow, and peripheral blood were 0.23 ± 0.07% (n=7), 0.30 ± 0.07% (n=5), and 0.02 ± 0.01% (n=5) (Mean ± Standard error), respectively (Fig. 3), showing that the ratio of hematopoietic progenitor cells was one-order lower than the reported ratio of $CD34^+$ cells (1–3%) in adult dogs [4, 5, 7, 11].

Since only 0.2–1.5 m*l* of umbilical cord blood could be collected from a single placenta during caesarian section in beagle bitches, suggesting umbilical blood collection from

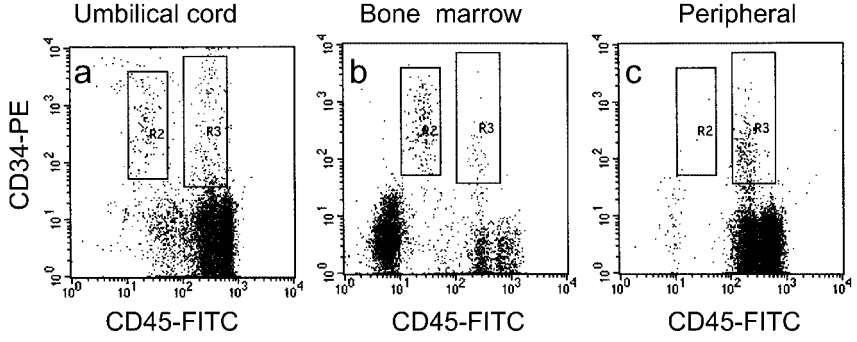

Fig. 1. Counterstaining of canine umbilical cord blood (a), bone marrow (b), and peripheral blood (c) cells for CD34 and CD45; gated for mononuclear cells. R2: $CD34^+CD45^{lo}$, R3:$CD34^+CD45^{hi}$.

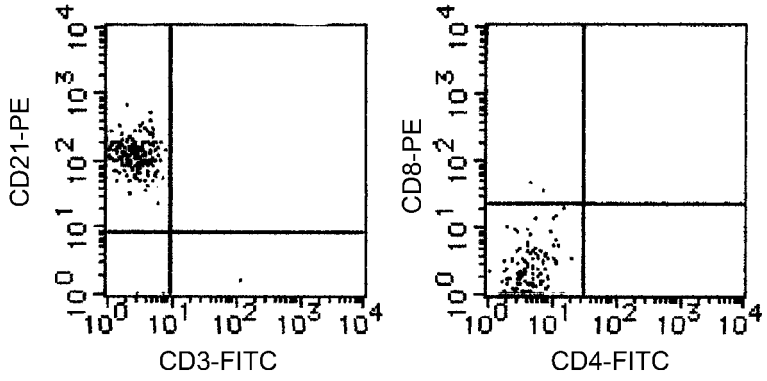

Fig. 2. Counterstaining of canine peripheral blood cells for CD21 and CD3 (left), CD8 and CD4 (right); gated for $CD34^+$ cells.

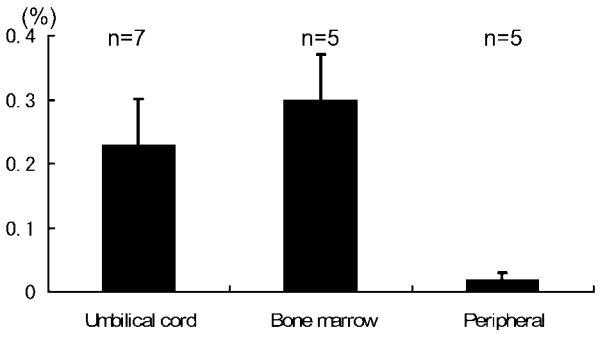

Fig. 3. Population of $CD34^{+}CD45^{lo}$ in canine umbilical cord, bone marrow, and peripheral blood cells. Data is expresses as mean ± SE.

large sized dogs might be more useful on practical cases.

Hereafter $CD34^{+}CD45^{lo}$ fraction from canine samples should be investigated by colony-forming unit-culture and the autologus transplantation of the cells in dogs that received radiation.

REFERENCES

1. Andrews, R.G., Bryant, E.M., Bartelmez, S.H., Muirhead, D.Y., Kitter, G.H., Bensinger, W., Strong, D.M. and Bernstein, I.D. 1992. *Blood* **80**: 1693–1701.
2. Berenson, R.J., Andrews, R.G., Bensinger, W.I., Kalamasz, D., Knitter, G., Buckner, C.D. and Bernstein, I.D. 1988. *J. Clin. Invest.* **81**: 951–955.
3. Besinger, W.I., Buckner, C.D., Rowley, S., Demirer, T., Storb, R. and Appelbaum, F.A. 1996. *Bone Marrow Transpl.* **17** (*Suppl.* 2): 38–39.
4. Bruno, B., Nash, R.A., Wallace, P.M., Gass, M.J., Thompson, J., Storb, R. and McSweeney, P.A. 1999. *Transplantation* **68**: 338–344.
5. Faldyna, M., Sinkora, J., Knotigova, P., Rehakova, Z., Moravkova, A. and Toman, M. 2003. *Vet. Immunol. Immunopathol.* **95**: 165–176.
6. Grantama, J.W., Sutberland, D.R. and Keeney, M. 2001. *Seminars Hematol.* **38**: 139–147.
7. Hagglund, H.G., McSweeney, P.A., Mathioudakis, G., Bruno, B., Georges, G.E., Gass, M.J., Moore, P., Sale, G.E., Storb, R. and Nash, R.A. 2000. *Transplantation* **70**: 1437–1442.
8. Hartnett, B.J., Yao, D., Suter, S.E., Ellinwood, N.M., Henthorn, P.S., Moore, P.E., McSweeney, P.A., Nash, R.A., Brown, J.D., Weinberg, K.I. and Felsburg, P.J., 2002. *Biol. Blood Marrow Transpl.* **8**: 188–197.
9. Kinniburgh, D. and Rusell, N.H. 1993. *Bone Marrow Transplant.* **12**: 489–494.
10. Larochelle, A., Vormoor, J., Hanenberg, H., Wang, J.C., Bhatia, M., Lapidot, T., Moritz, T., Mudoch, B., Xiao, X.L., Kato, I., Williams, D.A. and Dick, J.E. 1996. *Nat. Med.* **2**: 1329–1337.
11. McSweeney, P.A., Rouleau, K.A., Wallace, P.M., Buruno, B., Andrews, R.G., Krizanac-Bengez, L., Sandmaier, B.M., Storb, R., Wayner, E. and Nash, R.A. 1998. *Blood* **91**: 1977–1986.
12. Morel, F., Szilvassy, S.J., Travis, M., Chen, B. and Galy, A. 1996. *Blood* **88**: 3774–3784.
13. Moore, P.F., Rossito, P.V., Danilenko, D.M., Wielenga, J.J., Raff, R.F. and Severns, E. 1992. *Tissue. Antigens* **40**: 75–85.
14. Niemeyer, G.P., Hudson, J., Bridgman, R., Spano, J., Nash, R.A. and Lothrop, C.D. 2001. *Exp. Hematol.* **29**: 686–693.
15. Suter, S.E., Gouthro, T.A., Mcsweeney, P.A., Nash, R.A., Haskins, M.E., Flesburg, P.J. and Henthorn, P.S. 2004. *Vet. Immunol. Immunopathol.* **101**: 31–47.
16. Sutherland, D.R., Keating, A., Nayar, R., Anania, S. and Stewart, A.K. 1994. *Exp. Hematol.* **22**: 1003–1010.