Proliferation Capacity, Neuronal Differentiation Potency and Microstructures after the Differentiation of Canine Bone Marrow Stromal Cells into Neurons

Kazuya EDAMURA1)*, Koji KURIYAMA1), Kenichi KATO1), Rei NAKANO1), Kenji TESHIMA1), Kazushi ASANO1), Tsuneo SATO2) and Shigeo TANAKA1)

1)Laboratory of Veterinary Surgery, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa, 252–0880, Japan
2)Laboratory of Veterinary Pathology, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa, 252–0880, Japan

(Received 19 August 2011/Accepted 1 February 2012/Published online in J-STAGE 15 February 2012)

ABSTRACT. We examined the proliferation capacity and neuronal differentiation potency of canine bone marrow stromal cells (BMSCs). In addition, the microstructures of neuron-like cells after neuronal differentiation were observed under a scanning electron microscope. Canine BMSCs grew to confluence at 10.0 ± 2.5 days, and 3.8 ± 2.1 × 10^6 BMSCs were collected in one passage. Approximately 65% of canine BMSCs changed to neuron-like morphology after neuronal differentiation, and nearly all neuron-like cells stained positive against neuron-specific enolase. In addition, microstructures such as the cellular organelles, filaments and growth cones of these cells bore a close resemblance to those of the original mature neurons. These results suggested that canine BMSCs might be capable of differentiating into neurons.

KEY WORDS: bone marrow stromal cell, canine, differentiation, microstructure, neuron.


Despite the progress in the treatment of spinal cord injury (SCI) in recent years, it is still difficult to recover from severe paralysis. Recently, spinal cord regenerative therapy using stem cells has been clinically applied as one option for the treatment of severe SCI in human medicine [3, 11, 15]. Among the stem cells, bone marrow stromal cells (BMSCs) and olfactory ensheathing glia (OEG) have been the focus of research and clinical application for spinal cord regenerative therapy in dogs [5, 7, 8, 12, 14, 17].

BMSCs are adherent cells in the culture of bone marrow aspirates, but are not hematopoietic cells [13]. BMSCs are capable of differentiating into bone [2, 6], cartilage [9], fat [2], and muscle [18]. Furthermore, differentiation into neurons expressing mature neuronal markers has been reported in human [19], feline [10], rat [19] and mouse [16] models. BMSCs are easy to isolate in dogs and expand without serious technical problems [6]. The most significant practical advantages of using BMSCs are that autologous transplantation is possible, the costs of culturing are lower, and the risk of teratoma formation is very low [13]. Some studies have shown that canine BMSCs can differentiate into neuron-like cells expressing mature neuronal markers [5, 7, 8]. For these reasons, our laboratory is currently investigating BMSCs for use in spinal cord regenerative therapy in dogs.

Recently, the experimental study and clinical trial of regenerative therapy for SCI using autologous BMSCs also been performed in dogs [5, 12]. Surprisingly, there have been almost no reports regarding basic research on canine BMSCs, although this is very important for the clinical application of canine BMSCs. Therefore, the purpose of the present study was to investigate the number of days until the canine BMSCs reach confluence and the number of cells collected in one passage. In addition, the percentage of total cells that changed to a neuron-like morphology and the percentage of cells shown to stain positive against a mature neuronal marker after neuronal differentiation were examined. We also observed the microstructures of the neuron-like cells differentiated from canine BMSCs under a scanning electron microscope (SEM).

Six male beagle dogs (2.3 ± 1.8 years old) were used in the present study. This study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the College of Bioresource Sciences of Nihon University. All dogs were premedicated intravenously with midazolam hydrochloride (0.2 mg/kg) and butorphanol tartrate (0.2 mg/kg). Anesthesia was induced with an intravenous injection of propofol (4.0 mg/kg) and maintained with 1.5–2.0% isoflurane in 100% oxygen given by an endotracheal tube. Butorphanol tartrate (0.2 mg/kg) was again administered intravenously for pain relief before awakening. Two ml of bone marrow was aspirated from the humerus using strong suction with a heparinized syringe. Mononuclear cells were then separated by density gradient centrifugation using a Histopaque-1077 (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.). Following collection, the mononuclear cells were transferred to a 25 cm^2 culture flask and static-cultured in...
Canine BMSCs were cultured in a QX-capsule (QX-102; QuantomiX, Ltd., Nes-Ziona, Israel) and differentiated into neurons by the same methods as described above. Differentiated canine BMSCs were fixed in 2% glutaraldehyde (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) and stained with 2% phosphotungstic acid (Sigma-Aldrich). The microstructures of these cells were observed by SEM (S-3500N; Hitachi High-Technologies Co., Tokyo, Japan).

The data for these experiments are presented as the means ± standard deviations. Statistical analyses were performed using the SigmaStat® 3.5 (Systat Software Inc., Chicago, IL, U.S.A.) software package and the Student’s t-test. Values of \( p \) less than 0.05 were considered to be significant.

A small number of the cells adhered at 1 day of culture (Fig. 1). Most of the adherent cells exhibited a fibroblast-like spindle shape (Fig. 1). Morphologically, canine BMSCs appeared very similar to their rodent, feline and human counterparts. Canine BMSCs proliferated remarkably and grew to confluency at 10.0 ± 2.5 days (Fig. 1). The mean number of BMSCs was 3.8 ± 2.1 × 10^6 cells at the first confluency.

In the differentiation group, a large number of canine BMSCs changed to the neuron-like morphology with multipolar, rounded cell bodies and long, narrow processes (Fig. 1D, E). Canine BMSCs changed to round-shaped cells after less than 1 hr of exposure to neuron differentiation media.
Several sharp processes extended from these cells within 2 hr (Fig. 1D). The formation of a synapse-like network was observed after 12 hr of neuronal differentiation (Fig. 1E). Approximately 65.7 ± 19.7% of canine BMSCs changed to the neuron-like morphology. The percentage of neuron-like differentiated cells in the differentiation group was significantly higher than that (0.9 ± 1.0%) in the control group.

Nearly all neuron-like cells stained positive against NSE (Fig. 1F). Some of the cells that did not change to the neuron-like morphology also stained positive against NSE. The NSE-positive ratios in the differentiation and control groups were 88.5 ± 8.6% and 0.8 ± 1.2%, respectively, and a significant difference was detected between the 2 groups.

The canine BMSCs successfully proliferated in the QX-capsule and changed to the neuron-like morphology after neuronal differentiation. SEM observation revealed that the cellular organelles were located around the nucleus in the cell body; B: Many filaments ran from the edge of the cell body into the axon-like structure; C, D: At the tip of the axon-like structure, a growth cone and a synapse-like connection were observed.

In the present study, we were able to culture and expand the adherent cells isolated from canine bone marrow. BMSCs are defined as cells that are isolated from bone marrow and have a propensity to adhere to culture plastic [1]. Our cultured cells also grew out of bone marrow cell suspensions as a result of their selective attachment to the plastic surface of the culture flask. In addition, most of the adherent cells exhibited a fibroblast-like spindle shape in this study (Fig. 1). Morphologically, our cultured adherent cells resembled BMSCs of dogs [7] as well as BMSCs of other species [10, 16, 19]. These results suggested that our cultured adherent cells were BMSCs.

To the best of our knowledge, there have been only a few reports on the number of days until canine BMSCs reach confluency or the number of canine BMSCs collected in one passage [7, 12]. In the present study, canine BMSCs grew to confluency at about 10 days, and approximately $3.8 \times 10^6$...
BMSCs were collected per 2 ml of bone marrow in one passage. There have been a few reports on the transplantation of autologous BMSCs for the treatment of dogs with SCI [5, 12]. In those studies, a total of 1.4 × 10^6 to 1.0 × 10^7 BMSCs were transplanted [5, 12]. It is considered that at least the same number of cells may be necessary for transplantation in clinical cases of dogs with SCI. By our method, one to three passages may be necessary to obtain a sufficient number of canine BMSCs for transplantation.

Although various types of neuronal differentiation media have been used to induce the differentiation of BMSCs, Woodbury et al. reported that treatment with 2% DMSO and 200 μM BHA was the most effective [19]. Based their findings, we used media containing 2% DMSO and 200 μM BHA to differentiate canine BMSCs into neurons in the present study. We found that approximately 65% of canine BMSCs changed to the neuron-like morphology, and approximately 90% of the neuron-like cells were NSE-positive in the differentiation group. The present study was able to replicate the experiment reported by Woodbury et al. using canine BMSCs. The time frame for the changing to the neuron-like morphology of canine BMSCs was very similar to that of rat and human BMSCs [19]. They reported that more than 50% of adult rat BMSCs changed to the neuron-like morphology and NSE-positive ratio was 78% after 5 hr of neuronal differentiation [19]. NSE-positive ratio of canine BMSCs in the present study was higher than that of rat BMSCs reported by Woodbury et al. [19]. As for the difference of NSE-positive ratio between dog and rat, the time from induction to measurement of the ratio might have influenced. These results suggested that at least 90% of canine BMSCs might have neuronal differentiation potency.

Using SEM, we were able to observe the microstructures of the neuron-like cells differentiated from canine BMSCs in detail. In addition, the QX-capsule was found to be a superior culture substrate for observing the microstructures of these living cells. To the best of our knowledge, there have been no reports in which cultured or differentiated canine BMSCs have been observed under an electron microscope. In this study, the microstructures of the neuron-like cells were very similar to those of the original mature neurons.

These results suggested that, in all likelihood, the canine BMSCs actually did differentiate into neurons. However, it was not fully clarified whether canine BMSCs truly have neuronal differentiation potency in this experiment. One previous study reported that canine BMSCs might differentiate preferentially toward the astrocyte lineage [7]. Another study reported that the neuron-like cells differentiated from BMSCs did not show typical neuronal properties such as action potentials or voltage-gated Na+ and K+ currents and were not mature neurons [4]. Therefore, additional investigations will be needed to clarify whether canine BMSCs can differentiate into mature neurons.

In conclusion, we succeeded in the culturing and proliferation of canine BMSCs in this study. Canine BMSCs changed to neuron-like morphology after neuronal differentiation, and nearly all neuron-like cells stained positive against NSE. In addition, the microstructures of the neuron-like cells bore a close resemblance to those of the original mature neurons. These results suggested that canine BMSCs might be capable of differentiating into neurons. However, further basic research is needed to evaluate the potential of canine BMSCs for spinal cord regenerative therapy.

ACKNOWLEDGMENTS. The authors would like to thank M&S Instruments Inc. for their technical support with the SEM. This study was supported in part by a Nihon University Research Grant for Assistants and Young Researchers (05–048) and by a Grant-in-Aid for Young Scientists (B; 18780240) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES


