論文の内容の要旨

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論文題目

Exploration of a novel mesenchymal stem cell in canine bone marrow and its therapeutic effects for acute spinal cord injury
（犬の新規骨髄間葉系幹細胞の探索と急性期脊髄損傷に対する治療効果の検討）

Spinal cord injury (SCI) is a relatively common neurological disease, which causes serious problems in many canine cases. The most common causes of SCI in dogs are intervertebral disc disease and trauma caused by motor vehicle accident. The currently available treatments; pharmacological intervention, surgical management and rehabilitation, are generally useful for patients with mild to moderate SCI. However, these conventional treatments are ineffective in many patients with severe SCI. Cell transplantation has recently been recognized as one of the promising therapeutic approach for severe SCI. Although pluripotent cells such as ES cells or iPS cells are widely used in research field. Almost all clinical studies have used less undifferentiated cells such as macrophages, olfactory ensheathing cells and adult stem cells or mesenchymal stem cells (MSCs) harvested from umbilical cord blood, bone marrow, and adipose tissue. Although MSCs have less multipotency compared with ES cells or iPS cells, MSCs are attractive candidates for cell transplantation therapy in veterinary medicine because of easiness to isolate, low cost of expansion, low immunogenicity, low tumorigenic risk, and ability to migrate to the site of injury or inflammation. MSCs are considered to have two major mechanisms for tissue repairing. One is a multipotent ability to differentiate into various cells such as osteocytes, chondrocytes and myocytes. Another is a trophic effect, which supplies curative humoral factors via secretion of growth factors or cytokines to injured tissue. However, several studies have recently documented that transplanted MSCs are most likely to provide therapeutic effects throughout the ability to secrete trophic factors and not via their own integration or differentiation within the host tissue. Hepatocyte growth factor (HGF) is one of the representative trophic factors for tissue repairing and exogenous administration of HGF has been reported to promote functional recovery in rodent and primate models of SCI. However, the effect of recombinant HGF is limited due to its short duration of effect, difficulty of continuous supply of HGF to the target area, and excessive cost. On the other hand, after the injury, pro-inflammatory cytokines such as TNF-α and IL-1β are upregulated at the lesion site during acute to subacute phase and these factors are known to induce HGF secretion by MSCs. Therefore, MSCs transplantation is expected to exhibit therapeutic effects through continuous secretion of HGF, when transplanted cells are successfully delivered and integrated into lesion site

under inflammation.

In dogs, bone marrow mesenchymal stem cells (BMMSCs) and adipose-derived mesenchymal stem cells (ADMSCs) have been well characterized and have already been applied clinically in dogs. However, the use of these cells has certain limitations. BMMSCs are isolated from mononuclear cells in bone marrow but the proportion of MSCs is very low in dogs. The limited numbers of BMMSCs necessitates longer time to obtain sufficient numbers of BMMSCs for clinical application. It may cause the delay of cell transplantation for the proper period of treatment. On the other hand, ADMSCs isolated from stromal cell fragments of fat tissue can be easily expanded. However, ADMSCs have similar problems for clinical use as BMMSCs. They consist of a heterogeneous cell population contaminated by endothelial cells, smooth muscle cells, pericytes, and blood cells such as monocytes and lymphocytes, and purification should be needed to obtain genuine stem cells with high quality. Recently, dedifferentiated fat (DFAT) cells, which are obtained by ceiling culture of adipocytes from subcutaneous or omental adipose tissues, have been newly established and has been reported to exhibit high proliferation capacity and multipotency. DFAT cells show with higher homogeneity and colony-forming efficiency than ADMSCs and are considered to be promising candidates for cell-based therapies for various tissues. Another study showed that adipocytes in calf bone marrow could dedifferentiate into fibroblast-like cells after ceiling culture and exhibited proliferation ability and spontaneous re-differentiation into an adipogenic lineage after confluence. Although the multipotent property of these cells is unknown, adipocytes in bone marrow may generate multipotent cells like DFAT cells and provide enough number of MSCs in a shorter period.

Therefore, the objective of this study was to explore a novel MSC from canine bone marrow using ceiling culture method and compare its MSC potential with that of conventional BMMSCs (Chapter 1). Accordingly, novel canine MSCs were isolated from bone marrow and were named as “bone marrow peri-adipocyte cells (BM-PACs)” Then, the ability to secrete HGF from BM-PACs was evaluated to investigate whether these cells would be a promising cell source for cell transplantation therapy for SCI (Chapter 2). Finally, BM-PACs were transplanted to a mouse severe SCI model to estimate its clinical efficacy and safety of treatment of SCI in dogs (Chapter 3).

In chapter 1, after density gradient isolation of canine bone marrow, floating adipose layer was subjected to ceiling culture and mononuclear cells were cultured to isolate conventional BMMSCs, respectively. Cell proliferation process on ceiling surface was observed by time-lapse microscopy and MSC properties including clonogenicity, proliferation ability, multi-differentiation ability and expression of cell surface antigens were compared between proliferated cells and conventional BMMSCs.

The results of time-lapse microscopy revealed small cells adhering to adipocytes proliferated rapidly with a fibroblastic morphology, and any dedifferentiation of adipocytes as seen in culture of DFAT cells was not observed. The proliferated cells were regarded as novel MSCs and showed significantly greater clonogenicity and proliferation ability than BMMSCs. An in vitro trilineage differentiation assay revealed that the novel

canine MSCs possess adipogenic, osteogenic, and chondrogenic capacities superior to those of BMMSCs. Flow cytometric analysis revealed that the expression of CD73, which plays an important role in cell growth and differentiation, was significantly higher in novel MSCs than in BMMSCs. These results indicate that cells regarded as novel canine MSCs have stem cell characteristics that are superior to those of BMMSCs, but not identical with DFAT cells. Therefore, I named these newly discovered cells bone marrow peri-adipocyte cells (BM-PACs) to distinguish them from DFAT cells. BM-PACs should be considered as a distinct cell population from conventionally cultured BMMSCs and are expected to be more feasible for cell transplantation therapy than BMMSCs in dogs.

In chapter 2, intracellular HGF expressions in BM-PACs and BMMSCs were examined by quantitative RT-PCR and western blotting. Changes in the expression of HGF mRNAs in BM-PACs and BMMSCs after TNF-α and IL-1β stimulation were also evaluated by qRT-PCR. In addition, the amount of HGF secreted by BM-PACs before and after TNF-α and IL-1β stimulation was measured using ELISA. As MSCs are known to secrete several trophic factors besides HGF, Madin-Darby canine kidney (MDCK) cells, which are known to be induced a scattering effect after treated with HGF were used to estimate the bioactivity of HGF secreted by BM-PACs.

Significant up-regulation of HGF mRNA and significant higher intracellular HGF protein in BM-PACs was observed after cytokine induction. The quantity of HGF proteins secreted by BM-PACs increased by over 50 times after TNF-α and IL-1β stimulation. Culture supernatant of BM-PACs stimulated by TNF-α or IL-1β facilitated phosphorylation of tyrosine kinase receptor of HGF, c-MET and its downstream mediators ERK1/2 in MDCK cells. The culture supernatant of BM-PACs also induced scattering effect on MDCK cells, which resulted in loss of cell-cell contact and significantly fewer cell numbers in each colony. Therefore, even though BM-PACs should be considered to release various trophic factors in response to inflammatory cytokines, secreted HGF from BM-PACs dominantly or substantially had bioactive effects on MDCK cells. These results indicated that transplantation of BM-PACs at injured tissue with inflammation is expected to secrete HGF and provide therapeutic effect.

In chapter 3, in order to estimate clinical efficiency and safety of BM-PACs transplantation into severe SCI in dogs, BM-PACs were transplanted intralesionally or intravenously to contusive SCI model in mice. In chapter 3, pro-inflammatory cytokines remarkably enhanced HGF secretion of BM-PACs. Therefore, here, BM-PACs were transplanted in acute phase when the highest expression of these cytokines at lesion site is expected. Severe contusive SCI at T9 was induced in 19 female Balb/c nu/nu mice, and the animals were randomly assigned to intralesional (IL; n = 4), intravenous (IV; n = 7), and control group for each transplanted group (n = 4 each), respectively. Cells were labeled by fluorescent dye, VivoTrack 680, in advance to reveal the localization of transplanted cells and transplanted intralesionally or intravenously within 6 hours post injury. Control groups for IL and IV group were given the same volume of culture media from the each route. Cell distribution and fluorescent intensity of VivoTrack 680-labeled BM-PACs transplanted into living mice was

tracked by in vivo imaging device (IVIS) immediately after transplantation and every week of observation period for 8 weeks after injury. Three mice in IV group were sacrificed after 1 week of transplantation to confirm histologically whether the transplanted cells could be delivered the injured site. The functional recovery of the other mice was also evaluated every week using Basso Mouse Scale (BMS) for locomotion. After 8 weeks, the mice were euthanatized and injured site was collected for histopathological examination.

The fluorescence of VivoTrack 680 was detected only in lung immediately after IV injection, whereas signal intensity was noted in liver, spleen and even in lesion site after 1-2 week of transplantation. Direct fluorescence signals were observed in the extracted lung, liver, spleen and lesion area after 1 week and only in lung after 8 weeks, respectively. After one week of IV transplantation, the fluorescence of VivoTrack 680 was histologically detected within the cytoplasm of macrophages. These results indicated cells were engulfed by macrophages even though they were redistributed and delivered into lesion site within 7 days. However, weak fluorescence signals were still histologically detected in the lesion area of IV group after 8 weeks of transplantation and more cells with signal intensity was observed in IL group. At 8 weeks post transplantation, the mean BMS scores in the IL, IV, and each control group were 5.5 ± 1.3, 5.3 ± 1.0, 2.5 ± 1.0, and 3.5 ± 1.3, respectively. The mean BMS scores of IL were significantly higher than that of control group (p = 0.0037). Histological analysis of contusive spinal cord showed no tumorigenic changes observed in both transplanted group. The sagittal sections stained with LFB at 8 weeks after injury showed a reduction in the area of demyelination in the IL group compared with the control group. IV injection has advantage in the applicability and is minimally invasive to injured site compared to IL injection, but a large number of cells were trapped inside the lungs following IV injection. It was suggested that superior results of IL transplantation related to the number of delivering cells to target lesion areas.

This study demonstrated that isolation of novel MSCs, BM-PACs from canine bone marrow and its therapeutic effects for acute spinal cord injury using a severe SCI model in mice. BM-PACs can be easily harvested by ceiling culture of adipose layer in canine bone marrow and showed superior MSC properties such as proliferation and multilineage differentiation ability to BMMSCs. Significant differences between the two types of cells were detected in the expression of CD73 and it is suggested that CD73 is an important cell surface marker to identify canine mesenchymal stem cells in bone marrow. Further, BM-PACs showed significant higher expression of HGF mRNA than BMMSCs and secretion of HGF from BM-PACs extremely increased in response to pro-inflammatory cytokines such as TNF-α and IL-1β released into injured tissue. Therefore, it was considered BM-PACs contribute to tissue repairing through the prominent HGF secretion ability when transplanted in the acute phase of SCI. Actually, when BM-PACs were transplanted intralesionally into a severe SCI model in mice, tissue sparing was observed and better functional recovery was noted. Although the underlying mechanism of therapeutic effect of BM-PACs for SCI remained unknown in this study, intralesional transplantation of BM-PACs would be applicable in clinical settings. Further studies should be needed to optimize the transplantation strategy of BM-PACs in clinical settings.