論 文 の 内 容 の 要 旨

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論文題目

Comparison of the Neuronal Regeneration Potentials and Neural Growth Factors Expression between Bone Marrow Mesenchymal Stem Cells and Adipose-derived Mesenchymal Stem Cells in Dogs

（犬における骨髄間葉系幹細胞と脂肪由来間葉系幹細胞の神経分化能と神経成長因子発現の比較）

For severe spinal cord injury (SCI) cases, poor prognosis usually is given due to the characteristics of obstacles after SCI. Stem cell transplantation is one of the most promising yet enigmatic treatments for SCI. In contrast to other types of stem cells, mesenchymal stem cells (MSCs), which can be easily harvested with less ethical and tumorgenic concerns, are recognized as a ready-to-use material for clinical trials at present. By using MSCs in treating SCI, many attributions are demonstrated: anti-inflammation, replacing host neural cells, secreting neurotrophic factors, and modulation the glial scar. Nevertheless, several recent studies showed that MSCs did not possess the ability to differentiate directly into neuronal cells following transplantation and pre-differentiate MSCs into neurospheres, a heterogeneous mixture of cellular aggregates, including neural stem and progenitor cells, prove to be more beneficial for treating SCI. Although some cell sources of MSCs including the bone marrow and adipose tissue have

been studied, the suitable one for treating canine SCI is still not clarified. Therefore, the endogenous neuronal cells differentiation potential from canine bone marrow MSCs (cBMMSCs) with that of the adipose tissue-derived MSCs (cADMSCs), both of which are major sources of MSCs, and the levels of neurotrophic factors released from MSCs were compared here. Moreover, the potential of using generated neurospheres as a transplantation material was studied by the animal model.

In chaper 2, cBMMSCs and cADMSCs were isolated and expanded from canine bone marrow and subcutaneous adipose tissue. The proliferation assay was performed by counting the doubling time from first-passage to fourth-passage MSCs. Gene expressions of ectodermal marker, Nestin, βIII-tubulin, GFAP, NCAM, and stem cell maker, NANOG, OCT4, SOX2, were evaluated for cBMMSCs and cADMSCs by RT-PCR. Mesodermal differentiation assay of adipogenic, osteogenic, and chondrogenic lineages were evaluated for cBMMSCs and cADMSCs.

Results indicated that cADMSCs with a stable and shorter doubling interval proliferated faster than cBMMSCs. Both sources of MSCs were differentiated into mesodermal lineages of adipogenic, osteogenic, and chondrogenic successfully, and ectodermal makers of Nestin, βIII-tubulin, NCAM, and stem cells marker of OCT4 and SOX2, were detected by RT-PCR. According to the results, both cBMMSCs and cADMSCs expressed the properties of stem cells in proliferation and multipotent differentiation lineage, and cADMSCs could be harvested in a shorter time for transplantation. Besides, the expression of stem cells markers demonstrated the potential of multipotent differentiation and ectodermal makers expressed prior to any differentiation procedures indicated both MSCs have the potential in differentiating toward neuronal.

In chapter 3, Nestin-positive neurospheres were generated from cBMMSCs and cADMSCs and neuronal cells were differentiated from the generated neurospheres. The harvest rate of neurospheres was counted by cells harvested from generated neurospheres divided by pre-seeded cell numbers. Gene expression of neurospheres was evaluated by RT-PCR and markers were used as chapter 1. The levels of Nestin, OCT4, and SOX2 were compared for MSCs and generated neurospheres by real-time PCR analysis. Neural markers of βIII-tubulin, GFAP, NF200, S100, MAP2, MBP, and Nestin were evaluated by immunofluorescence analysis and the

percentage of fluorescence-positive cells were counted and compared. Electrophysiological property of neuronal differentiated cell was evaluated by patch-clamp analysis.

The mRNA expressions of NANOG, Nestin, OCT4, and SOX2 were upregulated in neurospheres derived from both generated Nestin-positive neurospheres. Moreover, about 2 times of cells could be harvested from neuropsheres generated from cADMSCs than cBMMSCs. After neuronal differerntiation, neuron-like morphology was noted and notably, cBMMSC-derived neuronal cells expressed higher levels of βIII-tubulin. Immunofluorescence analysis detected the expression of neural markers of βIII-tubulin, GFAP, S100, NF200, and MAP2, in differentiated neuron-like cells. However, the electrophysiological properties of neuronal differentiated cell were not noted. According to the results, Although electrophysiological property was not verified for neuronal differentiated cells, the upregulation of the markers of neural stem cells and neural makers expressed in immunofluorescence analysis still indicated the generated neurospheres have the potential in differentiating toward functional neurons.

In chapter 4, NGF and BDNF were selected to evaluate their expression in cBMMSCs and cADMSCs at passage 1 and their generated neurospheres by RT-PCR. The levels of gene expression were compared between cBMMSCs and cADMSCs by semiquantative PCR. ELISA analysis was also performed to analysis the levels of NGF and BDNF released from cBMMSCs and cADMSCs at passage 1-3. Moreover, neural-progenitor like cell line, PC12, co-culture with cBMMSCs and cADMSCs was used to evaluate their effects on neurogenesis.

The results of RT-PCR demonstrated the expression of NGF in both MSCs and downregulated in generated neurospheres. The expression of BDNF was only noted in cADMSCs and also downregulated in generated neurospheres. cADMSCs have significant higher gene expression levels of NGF and BDNF than cBMMSCs. By using ELISA assay, the secretion of NGF was demonstrated in cBMMSCs and cADMSCs, and higher levels of NGF were secreted from cADMSCs at passage 1-2. In contrast, the levels of BDNF were not detected in both. After co-culture with cBMMSCs and cADMSCs for 8 days, compare to control group, more PC12 cells with neurite extension and longer neurite extension were noted, but there were no significant differences between the groups which co-culture with cBMMSCs and cADMSCs. According to the results, cADMSCs could secret higher levels of NGF. For the benefits of neurotrophic factors

releasing, passage 1 and passage 2 cells of cADMSCs could be used for transplantation. However, both types of MSCs could encourage the neuronal differentiation of PC12 indicated that there are still other factors released from MSCs beneficial to the neuronal differentiation.

In chapter 5, the cells from generated neuropsheres were transplanted into canine's spinal cord and the differentiation fate of transplanted cells was studied. 3 dogs were used and divided into 3 groups: cells from cBMMSCs generated neurospheres (B-NS); cells from cADMSCs generated neurospheres (A-NS); and PBS injection (control). The spinal cord was exposed by laminectomy on L2 area and 1×10^6 cells pre-labeled with Hoechst 33342 for 1 hour were directly injected into spinal cord by using a Hamilton® syringe connected to a 30-gauge needle. After 14 days, the dogs were euthanatized and the transplanted site of spinal cord was excised for HE stain and immunofluorescence analysis. Mature neuron marker of MAP2, astrocyte maker of GFAP, and oligodendrocyte maker of MBP were used for evaluated the differentiation fate of transplanted cells.

Hoechst 33342 labeled cells were noted in immunofluorescence analysis, and MAP2 expressed in part of transplanted cells (B-NS: 15%, A-NS: 8%); GFAP was only slightly expressed in B-NS group (3%); and MBP was not expressed in both groups. The transplanted cells integrated well in spinal cord tissues and migrated for at least 4 mm. The results indicated the transplanted cells could survive for at least 14 days *in vivo* after transplantation and high migration activity was noted. Besides, the transplanted cells tend to differentiate into neuron lineage than other types of glial cells and the different results of differentiation between *in vitro* and *in vivo* demonstrated the fate of cell differentiation depend on surround environment. Consequently, the cells harvested from generated neurospheres could be used for transplantation for the purpose in replacing host neuron cells in SCI.

This study highlight that both cBMMSCs and cADMSCs could be differentiated into neurospheres and neuron-like cells *in vitro* and might replace host neuron cells by using the cells derived from neurospheres after transplantation, and therefore, these cells are suitable candidates for cell transplantation. Further, cADMSCs form a more suitable cell source as faster proliferation rate; larger number of cells harvested from cADMSC-derived neurospheres; releasing higher levels of neurotrophic factors.