Studies on therapeutic mechanisms of bone marrow-derived mononuclear cell and involvement of hepatocyte growth factor in acute spinal cord injury

Summary of Doctoral Thesis

Kiyotaka Arai

Graduate School of Veterinary Medicine and Life Science

Nippon Veterinary and Life Science University

Spinal cord injury is a critical condition that is often encountered in the clinical site. Mild cases are responsive to treatment and allow for functional recovery, but in severe cases, locomotor, sensory and physiological functions do not recover, causing a deterioration of the QOL. In addition, since many spinal cord injury patients live with a deteriorated QOL during their full life span and no effective treatment has yet been established, the development of new treatment is urgently needed. The basis of the treatment of acute spinal cord injury consists of preventing secondary injuries that spread to surrounding normal tissue. On the other hand, in the chronic phase, the purpose of the treatment is to promote axonal re-extension, and to reconstruct neural circuits that have been destroyed. The chronic phase is extremely difficult to treat. However, suppression of secondary damage through treatment in the acute phase allows for a higher possibility of functional recovery during the chronic phase. Cell transplantation therapy has recently been found effective in treating spinal cord injury, and various types of cell for transplantation have been reported. Bone marrow-derived mesenchymal stem cell (BMSC) is a source of transplantation cells commonly used recently. However the problems are that isolation and culture of BMSC are time-consuming, and that they cannot be used in the treatment of the acute phase. Bone marrow-derived mononuclear cell (BM-MNC) is a multicellular population composed of bone marrow cells excluding megakaryocyte and mature erythrocyte, and can easily be conditioned and adjusted by only centrifugation. Therefore, they can be transplanted on the same day as the onset of disease, and have been applied for the acute treatment of various diseases including spinal cord injury. The therapeutic effect of BM-MNC in the treatment of spinal cord injury was first reported in 2001; and since then, therapeutic effects such as their anti-apoptotic effect and angiogenic effect have also been reported.

The therapeutic effects of BM-MNC are believed to be brought about by the paracrine effects of growth factors, but thus far, its detailed mechanisms have elusively uncleared. The therapeutic effect of cell transplantation therapy tends to attract attention, and determination of

details regarding the underlying mechanism could potentially lead to an elucidation of the targets, and a proper timing for transplantation. In addition, elucidation of the main therapeutic mechanism may lead to the development of effective therapeutic methods. The purpose of this study was to determine their therapeutic mechanism, and explore the less invasive and effective therapeutic methods.

Chapter 2. Differentiation of BM-MNC into vascular component in the injured spinal cord

When tissue damage occurs, bone marrow cells are known to migrate to the lesion, after which they get involved in angiogenesis by differentiating into vascular component cells such as vascular endothelial cells, pericytes, or perivascular macrophages. Further, the kinetics of their differentiation into various cells can be different depending on the disease, suggesting that the fate of bone marrow cells is determined by microenvironments specific to the injured tissue. On the basis of such a background, the differentiation kinetics exhibited by BM-MNC at the transplantation site were analyzed in myocardial infarction and hind-limb ischemia models, and the findings revealed that most BM-MNC differentiated into vascular endothelial cells at the transplantation site. Meanwhile, no previous study has analyzed BM-MNC kinetics after transplantation for spinal cord injury. Prostacyclin derived from newly formed blood vessels has recently been found to promote axonal regeneration, and the importance of angiogenesis in the central nervous system has been re-acknowledged. In Chapter 2, in order to elucidate the underlying mechanism behind the reported angiogenesis-promoting effect of BM-MNC in rat models of spinal cord injury, BM-MNC was tracked with green fluorescent protein (GFP), and their capability to differentiate into spinal cord microvascular component cells was clarified. The findings showed that BM-MNC-derived macrophages were transiently localized around blood vessels, and that some of them exhibited immunoreactivity to perivascular macrophage markers. Some BM-MNCs also differentiated into cells such as vascular endothelial cells and

pericytes, but such cells were extremely few in number. Macrophages have been reported to promote angiogenesis through mutual interaction with the vascular endothelial cells, although the detailed mechanism remains unknown. In the treatment of spinal cord injury through BM-MNC transplantation therapy as well, findings have also suggested that BM-MNC-derived macrophages may promote angiogenesis by the interaction with endothelial cells.

Chapter 3. Ability of BM-MNC to produce growth factors in injured spinal cords

Bone marrow cells have been found to be capable of constantly producing high levels of growth factors. A previous report has shown that bone marrow cells migrated to the injured tissue, and produced various growth factors using mice model of myocardial infarction that express GFP only in their bone marrow cells. Similar phenomena have been reported in other disease models e.g. hindlimb ischemia, skin injury and stroke, suggesting that bone marrow cells may be involved in the physiological tissue repair process through the paracrine effects of growth factors. In the same way, BM-MNC that composed of bone marrow cells may also exert therapeutic effects through the paracrine effects of growth factors. In Chapter 3, BM-MNC tracked with GFP was transplanted into a rat model of spinal cord injury to evaluate survivability of BM-MNC at the transplantation site, and to determine what kinds of growth factors they produced. As a result, BM-MNC survived in the injured spinal cord at 7 days after transplantation. However, the number of BM-MNC decreased from 3 to 7 days after transplantation and a few of survived BM-MNCs were immunoreacted with the activated caspase-3 in two time points. The survived BM-MNC was also immunoreacted with hepatocyte growth factor (HGF), vascular endothelial growth factor and monocyte chemotactic protein-1. In particular, the expression rate of HGF was found to be the highest. In addition, the therapeutic effect of BM-MNC was evaluated. As a result, the number of caspase-3-activated cells and demyelinated area were significantly decreased in BM-MNC transplanted group compared with

control. In this chapter, the findings revealed that transplanted BM-MNC survives more than one week and produces various growth factors including HGF in the injured spinal cord. In the injured spinal cord of acute phase, expression levels of c-Met, HGF receptor are known to start increasing rapidly immediately after the injury, regard less of the late increase of HGF production. BM-MNC may bring about therapeutic effect such as anti-apoptotic effect by paracrine HGF that is a deficiency state in acute spinal cord injury.

Chapter 4. Neuroprotective effects of BM-MNC mediated by the paracrine of HGF

HGF is known as a growth factor with various physiological activities including angiogenesis and cytoprotection. Thus far, the therapeutic effect of HGF has been widely confirmed in various models such as cirrhosis and renal failure, amyotrophic lateral sclerosis and multiple sclerosis; and its clinical applications are promising. Particularly, HGF has been reported to strongly inhibit cell death through induction of Bcl-2 expression and inhibition of reactive oxygen species (ROS) production mediated by inactivation of Rac-1. On the basis of the results in Chapter 3, we have speculated that the anti-apoptotic effect found in the treatment of acute phase spinal cord injury was due to the paracrine effects of HGF. Therefore, in Chapter 4, the underlying mechanisms behind the neuroprotective effect of BM-MNC were analyzed with a focus on ROS production and c-Met phosphorylation using rat adrenal pheochromocytoma cell line (PC12), a neuronal cell model. PC12 cell was induced cell death with CoCl2, and at the same time, treated with BM-MNC conditioned media. As a result, BM-MNC phosphorylated c-Met expressed in PC12 cells through the paracrine effects of HGF and significantly reduced the production of endogenous ROS and cell death. These effects were suppressed by SU11274, a c-Met inhibitors, and the latter caused a significant decrease of the cell protective effects of BM-MNC, suggesting that BM-MNC inhibited ROS-induced cell

death by c-Met phosphorylation. In this chapter, the findings revealed that BM-MNC, at least partly, suppresses neuronal cell death induced by intracellular ROS production by activating HGF/Met signaling. The concentrations of the c-Met inhibitors used in this study were low enough not to affect the viability of PC 12 cells itself. Then, the influence of autocrine of HGF by PC12 cells was considered small. In spinal cord injury, production of ROS is induced immediately after injury, and returns to normal within 2 or 3 days. Therefore, BM-MNC transplantation therapy is expected to be most effective when transplantation is performed within two days after injury.

Chapter 5. Comparison of the therapeutic effects of the BM-MNC transplantation and HGF single-dose administration into the injured spinal cord parenchyma during the acute phase

The results in Chapter 4 revealed that the cytoprotective effect of BM-MNC was at least partly mediated by the paracrine effects of HGF. This suggested that administration of HGF instead of BM-MNC during the acute phase of a spinal cord injury may provide a comparable or highly effective. HGF is already reported therapeutic effects including anti-apoptotic effect in spinal cord injury, and its continuous administration into the subarachnoid space by using a catheter as well as its administration through gene transfer, have been devised as therapeutic methods. In this study aimed at delivering HGF more efficiently at the site of the injury, a single-dose HGF administration into the spinal cord parenchyma was performed, and its therapeutic efficacy in the treatment of acute spinal cord injury was compared with that of BM-MNC transplantation therapy. As a result, fractional anisotropy value of diffusion tensor imaging in HGF group showed significantly higher than that of control group at 14 and 28 days after administration. Besides, positive area of neuron, axon, and astrocyte markers in HGF group were significantly preserved compared with control at 28 days after administration, but did not have enough effects compared with BM-MNC transplantation. Our result demonstrated

that single-dose administration of HGF suppressed tissue degeneration, but did not have enough effects compared with BM-MNC transplantation. Further studies are needed to clarify the causes of inferior effect of single-dose HGF administration.

In conclusion, the findings of our study suggest that at the site of injury, BM-MNC produces various kinds of growth factors, mainly HGF, and that HGF secreted by BM-MNC suppresses neuronal cell death by causing a decrease in the production of ROS through phosphorylation of c-Met. Besides, single-dose administration of HGF showed efficacy of a new therapy, although it is not enough effective compared with BM-MNC transplantation in vivo. In addition, our study revealed that BM-MNC exhibited a characteristic behavior that they adhered to blood vessels in an injured spinal cord, suggesting that they were associated with an angiogenesis promoting effect. In the future, more detailed analyses may potentially lead to the finding of new healing mechanisms and to the development of more effective therapeutic methods.