論文の内容の要旨

論文題目　Neurotropism and neurorestorative mechanisms in umbilical cord derived-mesenchymal stromal cells

（臍帯由来間葉系細胞の神経向性と神経修復機序に関する検討）

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Neonatal brain damage during the perinatal period is the cause of cerebral palsy later in life, and is a condition which places a great burden on not only the infant but also the family who raise. Those who present central nervous symptoms such as consciousness disorder, breathing / nursing disorder and spasms etc. in the neonatal period are collectively referred to as having "neonatal encephalopathy", and this is a condition that is by no means rare, and is incident in approximately 2 people per 1000 births in Japan. There are expectations for cell therapies with neuroprotective action accompanied by neurotrophic factor secretions for "neonatal encephalopathy" that do not have effective treatments once they have manifested.

Here I focused on umbilical cord-derived mesenchymal cells (MSC) as a new treatment for suppressing the onset of cerebral palsy. Among the sources of MSC, the human umbilical cord (UC) is a rich source of MSC, and they have advantages as a source; (1) not physically invasive for the donor and have no ethical issues, because the umbilical cord, which is the source of the cells, has been discarded as medical waste; (2) easy to sample, store and transport because they are an abundant source associated with childbirth; (3) have pluripotency to various tissue cells including the nervous system; (4) thought to be useful as a cell therapy for cerebral palsy because they have low immunogenicity while having sufficient immunosuppressive ability. Recently many clinical trials using UC-MSCs have been conducted for the purpose of immunomodulatory, anti-inflammatory and tissue repair. Among them, clinical studies on neurological disorders have already been conducted, and the target diseases include spinocerebellar degeneration, spinal cord injury, traumatic encephalopathy, cerebral palsy in adolescence and early adulthood. Based on the above, by clarifying the usefulness of UC-MSCs and its mechanism of action as a subject for neonatal encephalopathy, it is thought that novel cell therapy using UC-MSCs is effective in preventing the onset of cerebral palsy and lead to the improvement of QOL for children with cerebral palsy, as well as their families.

In addition, because supplemented fetal bovine serum (FBS) in the medium with which UC-MSCs are cultured introduces the possibility of xenogeneic antigens and infections including

bovine spongiform encephalopathy, lower antigenic and safer medium is needed for clinical use. Therefore, in this study, I used the serum-free new medium, RM medium, for the culture of UC-MSCs for clinical use as an alternative to FBS containing α-MEM medium, and I investigated the restorative effect of UC-MSCs cultured with RM medium in a neonatal brain injured model.

Therefore the objectives of this study are to investigate neurotropism of UC-MSCs including potentials of neurogenic differentiation and migration towards injured cells, to evaluate the neurorestorative effect and the mechanisms of UC-MSCs in a neonatal brain injuries *in vivo*, and finally to prove validity of the mechanisms *in vitro.*

At first I differentiated UC-MSCs by culturing in neurogenic differentiation medium. UC-MSCs shifted to a neuronal-like bipolar morphology, with long thin processes and a depressed proliferation rate, in response to culturing in neurogenic differentiation medium for about 72 hours. Then I revealed UC-MSCs can form neurospheres by culturing on spheroid plates supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), and the expression of neural biomarkers was compared amongst cells derived from four groups: (1) undifferentiated UC-MSCs, (2) differentiated UC-MSCs, (3) undifferentiated UC-MSC-neurospheres, and (4) differentiated UC-MSC-neurospheres. I found that there were more neural markers expression in differentiated UC-MSCs and differentiated UC-MSC-neurospheres compared to their undifferentiated counterparts. Next I examined the migration ability of UC-MSCs toward normal or glucose-depleted neurogenic cells using Transwell migration assays because MSCs are known to migrate toward sites of tissue damage. The number of migrating cells co-cultured with glucose-depleted SH-SY5Y cells was significantly higher compared to that with normal SH-SY5Y cells in all groups. These results *in vitro* suggest UC-MSCs have neurotropism including neurogenic differentiation, neurosphere formation and migration towards injured cells.

Then, I investigated the restorative effects of UC-MSCs cultured with serum-free medium in the neonatal IVH model *in vivo*. IVH is a common complication in preterm neonates and is responsible for high mortality and subsequent disabilities such as cerebral palsy, though there are no effective therapies available at present. I first established an animal model of neonatal IVH using newborn postnatal day 5 B6 Albino mice. The mice in the IVH group showed significantly decreased traveled distance and rearing number compared to those in the control group, and both UC-MSCs cultured with αMEM and serum-free RM improved behavioral outcome of IVH mice. Moreover the RM group exhibited significant behavioral improvement compared to the control group. Histopathological analysis revealed that the IVH group exhibited more GFAP-positive glial cells, decreased white matter thickness (hypomyelination), and increased TUNEL-positive apoptotic cells in

the periventricular area than control group. UC-MSCs cultured with RM significantly attenuated these periventricular reactive gliosis, hypomyelination, and periventricular cell death observed after IVH. As for tracing the UC-MSCs in IVH mice, the luciferase gene was transfected into UC-MSCs and performed *in vivo* imaging analysis. In the mice injected with UC-MSCs, prominent luminescent signals were detected first in the lung, spreading to the neck and head, and no luminescent signals were detected 21 days after injection. Furthermore, human Alu genes were detected 1 day after UC-MSC injection mostly in lungs and brains. However, Alu elements were not detected 3 weeks after UC-MSC injection. Moreover, HLA Class 1 and CD105 double positive cells were observed in the perivascular region of the mouse brain in the UC-MSCs injected mice but disappeared 21 days after injection. To assess the key factors in the repair of damaged tissue in the IVH + UC-MSCs group, mouse sera and cerebrospinal fluid (CSF) were collected, and human brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and hepatocyte growth factor (HGF) were measured using the multiplex beads immunoassay. Human BDNF and HGF were detected in IVH + UC-MSCs group. RT-PCR results also revealed BDNF and HGF were elevated in mice brain tissue of the IVH + UC-MSCs group. BDNF and HGF have been reported to be able to permeate across blood brain barrier (BBB). In addition, it has been reported that IVH induces BBB disruption and increased BBB permability. Therefore IVH surgery in my study might induce increased permeability of BBB followed by elevation of BDNF and HGF although I couldn't detect them in all mice treated with UC-MSCs in my hands. As for mechanisms that UC-MSCs relieved gliosis and hypomyelination, BDNF has been reported to improve hypomyelination via Erk phosphorylation or TrkB signaling. And also HGF has been reported to influence the development and growth of oligodendrocytes, as well as the proliferation of myelin-forming Schwann cells. In addition, it has been reported HGF reduces gliosis by suppressing MCP-1 induction. These mechanisms of BDNF and HGF might alleviate gliosis and hypomyelination observed after IVH.

Finally I investigated whether HGF and BDNF secreted from UC-MSCs contribute to neurogenesis, proliferation and viability in injured nerve system *in vitro*. I firstly confirmed secretion of HGF and BDNF from UC-MSCs from three different UCs, and inhibition of them by neutralizing antibodies. In order to investigate the effect of HGF and BDNF alone or both of them secreted from UC-MSCs on neurogenesis, I examined GAP-43 expression in injured neurons after oxygen glucose depletion (OGD). The results revealed that co-culture with UC-MSCs increased GAP-43-positive immature neurons and restored neurite outlength. However, addition of neutralizing antibodies of HGF and BDNF attenuated this improvement. Next I investigated the effect of HGF and BDNF on cell proliferation of cortical neurons after OGD. Proportion of Histone H3-positive cells, and incorporation

of BrdU significantly decreased after OGD, and co-culture with UC-MSCs improved them. Addition of neutralizing antibodies of HGF and BDNF attenuated the cell proliferation. Interestingly the effects of expression of GAP-43, neurites outlength and cell proliferation were reverted partially by the addition of neutralizing antibodies, and there was no synergistic effect by the addition of them. I also evaluated caspase activity in cortical neurons after OGD, using fluorescent-labeled inhibitor of caspases (FLICA), and the results exhibited the ratio of the number of apoptotic and necrotic cells to the total number of cells exhibited that co-culture with UC-MSCs significantly improved apoptosis and necrosis of cortical neuron after OGD, whereas addition of neutralizing antibodies increased both of apoptotic and necrotic cells. These results indicate that HGF and BDNF secreted from UC-MSCs play an important role for neurogenesis, cell proliferation and viability after OGD, but they are not all secreted from UC-MSCs which contribute to cell proliferation and viability, and there are also other factors secreted from UC-MSCs which can contribute to neurogenesis. Both of HGF and BDNF are multi-functional growth factor expressed in both of adult and fetal central nervous system, and are reported to play an important role for anti-apoptosis and neurogenesis via activating phosphatidylinositol 3-kinase/Akt pathway and MAP-kinase pathway. Consistently, in our study, inhibition of HGF and BDNF decreased the extent of neurogenesis and cell viability. The results in our study indicate that HGF and BDNF secreted from UC-MSCs contribute to neurogenesis, cell proliferation and survival in injured nerve system, and that there are also other factors secreted from UC-MSCs which contribute to neurogenesis, proliferation and viability of neurons.

Further studies are needed to examine the elevation of cytokines after IVH and determine the dose protocol of UC-MSC administration. The development of IVH results in an inflammatory response, and it has been reported that UC-MSCs have immunomodulatory properties. Therefore, UC-MSCs may play a key role in management of these inflammatory cytokines. In addition, inhibition experiment *in vitro* suggested the necessity of further study to prove the presence of other important neurotrophic factors secreted from UC-MSCs. As another problem, the concentrations of secreted HGF and BDNF demonstrated variability that was dependent on UC-MSC lot. Lot-to-lot variation in these secreted neurotrophic factors is an important issue considering their potential for clinical application.

These results suggest that UC-MSCs ameliorate neuronal injury followed by functional improvement by secretion of neurotrophic factors such as BDNF and HGF rather than neuronal differentiation and eternal cell replacement, and that intravenous injection of UC-MSCs may be feasible treatment for neonatal brain injuries.