学位論文の要旨

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学位論文名　A Human Neural Stem Cell Line Provides Neuroprotection and Improves Neurological Performance by Early Intervention of Neuroinflammatory System

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論文内容の要旨

INTRODUCTION

Stroke is a leading cause of death and disability worldwide. It is caused by cerebral blood flow disruption, which leads to necrotic death of brain tissue. Soon after necrosis, a neuroinflammatory system is activated, marked by sequential infiltration of granulocytes and macrophages. Although these infiltrating cells show favorable effects including clearance of dead tissues, their excessive activation is proved to increase the lesion size by inducing apoptosis in the circulation compromised area around the necrotic core, so called penumbra. Hence, a therapy that replaces the dead tissue, controls neuroinflammation and provides neuroprotection might be good for stroke management.

Recently, transplantation of various types of stem cells including Mesenchymal stem cells, neural stem cells (NSC), embryonic stem cells and induced pluripotent cells are demonstrated to provide beneficial effects in stroke condition. Among the stem cell types, neural stem cell (NSC)-based therapy could be important, because it can differentiate to neurons, enhances angiogenesis and induces endogenous neurogenesis, and modulates neuroinflammatory system. Previously, a neural stem cell line (HB1.F3) is shown to differentiate to neurons and astroglial cells in stroke condition and provide beneficial effects. In this study, we investigated its immune modulatory and neuroprotective effects. Most of the stem cell transplantation studies extensively investigated the immune modulatory effects at sub-acute stage. However, immune modulation during very early phase might also be important, because during this time the

events of neuroinflammation and other pathological aspects of stroke are different than that of sub-acute phase of the disease. We transplanted HB1.F3 in a rat stroke model at an early time point, and found that it modulates the initial events of neuroinflammation at the level of cell infiltration and pro-inflammatory gene expression, provide neuroprotection and consequently improves functional performance.

MATERIALS AND METHODS

The experimental protocol and procedures were approved by the Ethical Committee of the Shimane University School of Medicine. HB1.F3 NSC was generated by infecting primary telencephalon cells with an amphotropic, replication incompetent retroviral vector-containing v-myc, and cultured in DMEM containing 5% horse serum. Cerebral ischemia model was generated by middle cerebral artery occlusion (MCAO) in healthy adult male Wister rats. HB1.F3 cells were transplanted through jugular vein 6 h after MCAO. Neurological performance was tested at 6 and 48 h after MCAO using a neurological severity scoring (NSS) system. The rats were sacrificed at 24 or 48 h after MCAO. Necrosis and tissue damage were evaluated by Haematoxylin and Eosin (HE) staining, and apoptosis by TUNEL assay. Granulocytes and macrophage/microglia infiltration was analyzed by cell-specific marker immunofluorescence staining. Proinflammatory factors including COX-2 and iNOS were evaluated by immunofluorescence staining, and their localization were determined by double immunofluorescence staining with cell type specific markers. The gene expression of growth factors and cytokines in HB1.F3 at basal culture condition was evaluated by real time PCR using gene specific primers. The numerical data are presented as mean values ± SD. Statistical analysis was done by one-way ANOVA, followed by Scheffe's post hoc test or paired *t*-test, and significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

Compared to a selective COX-2 inhibitor (NS-398)-treated, or PBS-treated rats, HB1.F3 transplanted rats showed improved neurological performance, and decreased TUNEL positive apoptotic cell number in the penumbra 48 h after MCAO. However, it did not affect necrosis, as revealed by HE staining and RIPK1 immunostaining. Apoptotic neuronal death in cerebral ischemia is influenced by local inflammatory condition, which can be altered by modulation of that inflammatory condition. To elucidate possible underlying mechanism of improvement, we checked the infiltration of inflammatory cells 24 h after MCAO. Immunostaining of cell type specific markers demonstrated that both granulocytes and macrophage/microglia infiltration were decreased in the core region of HB1.F3 transplanted group, but not in NS-398 group. Neutrophils and macrophage/microglia are shown to play a great role in determining the lesion size and

disease outcome of stroke. Hence, regulation of inflammatory cell infiltration might be one of the main features of HB1.F3 transplantation-induced modulation of neuroinflammation in this condition.

Immunohistochemical analysis further demonstrated that iNOS and COX-2 expressing cell number were decreased in the core and penumbra, respectively, in both HB1.F3-transplanted and NS-398 rats. Double immunofluorescence results revealed that iNOS was mainly expressed in granulocytes and macrophage/microglia in the core region, and COX-2 in neurons and endothelial cells the in the penumbra. A few granulocytes were also shown to be positive for COX-2. As the number of granulocytes and macrophage/microglia, the principal cells that express iNOS, was decreased by HB1.F3 transplantation, the decrease of iNOS positive cell number might be due inhibition of cell infiltration, not due to inhibition of iNOS production. Analysis of the percentage of iNOS expressing cells revealed that indeed, the percentage of iNOS positive granulocytes and macrophage/microglia was similar between HB1.F3 transplantation and PBS-control rats. However, NS-398 treatment decreased the percentage of iNOS expressing granulocytes and macrophage microglia. The number COX-2 expressing neurons and vessel was decreased in both HB1.F3 transplanted and NS-398 treated rats. These results are suggesting that HB1.F3 transplantation affected only COX-2 expression, and reduction of iNOS was due to inhibition of iNOS-producing cell accumulation.

To understand further about the role of HB1.F3 on stroke pathology, we analyze the mRNA expression of growth factors and cytokines in basal culture condition. Our results showed that brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (βFGF) and bone morphogenic protein (BMP)-4 expression was high in cultured HB1.F3 cells. Previous studies showed that these growth factors can provide neuroprotection, and affect neuronal and astroglial differentiation.

Therefore, considering its effect on inflammatory cell infiltration and proinflammatory gene expression in vivo condition, and growth factor expression at basal culture condition, the beneficial effects of HB1.F3 transplantation in stroke condition might be the combined effects of modulation of neuroinflammation and growth factor mediated neuroprotection.

CONCLUSION

Thus, early transplantation of HB1.F3 in stroke could be beneficial through regulation of neuroinflammation and neuroprotection from early phage, and subsequently replacing damaged tissue by differentiation into neurons and astroglia. Such early intervention might be a good strategy for the therapy of stroke.

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論文審査の結果の要旨

脳梗塞の病態に炎症が関与していることが近年報告され、抗炎症作用を持つとされる間葉系幹細胞をはじめとする組織幹細胞を用いた治療応用が検討されている。しかし脳梗塞急性期における神経幹細胞移植の抗炎症作用に関する研究は十分になされていない。申請者はラット脳虚血モデルを用いてヒト神経幹細胞株（HB1.F3）の経静脈的投与による治療を行い、HB1.F3 による抗炎症効果の研究を行った。60 分間のラットの一過性中大脳動脈閉塞モデルに対し、PBS 投与群（Sham 群）、選択的 COX-2 阻害剤（NS398）治療群、HB1.F3 治療群を作成し、炎症性細胞の浸潤と炎症関連物質発現の比較検討を行った。結果、HB1.F3 治療群は神経症状の有意な改善を示した。また虚血コア領域への白血球やミクログリア・マクロファージの浸潤を抑制した。これらの細胞には iNOS の発現が認められたが、iNOS 発現率は HB1.F3 治療群では改善を認めなかった。ペナンブラ領域では神経細胞や血管内皮細胞の COX-2 発現抑制が認められた。HB1.F3 は培養下において BDNF・βFGF といった神経栄養因子や BMP-4、炎症性作用と抗炎症性作用の両面をもつ IL-6 の mRNA の発現を認めた。これらの結果より、HB1.F3 投与の治療機序として神経栄養因子を介した神経保護効果や神経幹細胞を BMP-4 でアストロサイトに分化誘導することによる神経保護や抗浮腫・抗炎症効果、IL-6 を介した TNF-α や IL-1 の抑制による抗炎症効果が推察された。本研究はヒト神経幹細胞株移植の脳梗塞急性期における抗炎症性効果という新たなストラテジーを示唆する研究であり、博士（医学）の学位授与に値すると判断した。