学 位 論 文 の 要 旨

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学　位　論　文　名　Human Mesenchymal Stem Cell-Transplantation Changes Proinflammatory Gene Expression Through NF-κB-Dependent Pathway in a Rat Focal Cerebral Ischemic Model

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論 文 内 容 の 要 旨

INTRODUCTION

In cerebral ischemia, macrophage/microglia accumulate in the lesion area, and play important roles by producing cytokines, growth factors, reactive oxygen species and phagocytosis of dead tissue. The overactivated macrophage/microglia increases production of proinflammatory cytokines, which deteriorate neuroinflammation and increase of apoptotic neuronal loss.

In our previous reports, we have demonstrated that transplantation of human mesenchymal stem cells (B10 MSC) decreased lesion size and neurological deficits in transient cerebral ischemic rats. These changes were accompanied by decreased macrophage/microglia accumulation and expression of proinflammatory factors, suggesting that transplanted B10 cells play a key role in regulation of the activation status of macrophage/microglia. However, the underlying mechanisms of such regulation are still elusive.

Transcription factor, such as nuclear factor κB (NF-κB) has been reported to express in cerebral ischemia where it can modulate the expression of several proinflammatory genes including inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), cyclooxygenase-2 (Cox-2) and monocyte chemoattractant protein-1 (MCP-1). Our previous studies demonstrated that B10-transplantation inhibits iNOS expression in macrophage/microglia. As iNOS was the downstream factors of NF-κB signaling pathway, we

hypothesized that B10-transplantation might regulate these proinflammatory genes through the modulation of NF-κB signaling in macrophage/microglia in cerebral ischemic rats.

MATERIALS AND METHODS

Transient focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) in adult male Sprague-Dawley rats (8 weeks old). After 24 hours, vehicle (PBS) or human MSCs (B10) were transplanted intravenously. The neurological deficits were monitored by modified neurological severity score system (mNSS), and the infarct volume was monitored by MRI. The rats were sacrificed 3 and 5 days after MCAO. Transplanted B10 cells (human origin) were identified by immunofluorescence staining of human nuclei. The accumulation of macrophage/microglia was analyzed by immunofluorescence staining of ionized calcium binding adaptor molecule 1 (Iba-1) and CD68 (ED1). The transcription factors including C/EBPβ and NF-κB were quantified by Western blotting, and their localizations were analyzed by double immunofluorescence staining using ED1 as a macrophages/microglia marker. The ED1-positive macrophage/microglia in the core and the Iba-1-positive macrophage/microglia in penumbra were microdissected by laser capture microdissection, and their mRNA expressions were analyzed by real time-PCR. The localizations of TLR2 and CD40 were analyzed by double immunofluorescence staining of CD40 and ED1, or TLR2 and ED1. To analyze the signaling pathway, double immunofluorescence staining of NF-κB and CD40, or NF-κB and TLR2 were employed.

RESULTS AND DISCUSSION

The evaluation of infarct volume and neurological deficits revealed that they were decreased in the B10 group at Day 5 ($p < 0.05$). The immunofluorescence results revealed that transplanted B10 cells migrated to the ischemic core and the penumbra, which were in the vicinity of macrophage/microglia. The number of B10 cells peaked at Day 3 and gradually decreased. The activated morphology and increased number of macrophage/microglia were displayed in the ischemic region. In the penumbra, only Iba-1-positive macrophage/microglia was found, which had ramified morphology, and the cell number was decreased in the B10 group ($P < 0.05$). In the core, a plentiful of both ED1-positive and Iba-1-positive macrophage/microglia were found, which had round shaped morphology. Moreover, the 77.7% of Iba-1-positive cells were positive for ED1 in the PBS group, whereas that was decreased to 42.7% in the B10 group. The ED1-positive cells include blood-borne macrophages and phagocytic microglia. Hence,

these results indicated that the B10 cells inhibited the recruitment and the transformation of the phenotype of macrophage/microglia.

The Western blotting results revealed that the transcription factor NF-κB protein level was selectively decreased in the core region in the B10 group at Day 3 and Day 5 ($p < 0.05$). In the core region, the NF-κB was localized mainly in macrophage/microglia, and consistent with the Western blotting results, the number of NF-κB-expressing macrophages/microglia was decreased. Then we analyzed the mRNA levels of NF-κB dependent gene in macrophage/microglia in the core and the penumbra of MCAO rat brains, and found the IL-1β, TNFα, iNOS and MCP-1 were decreased in ED1-positive and Iba-1-positive cells in the B10 group. On the other hand, the mRNA of the cytokines that inhibits NF-κB signaling, such as IL-4 and IL-10 were increased in ED1-positive cells in B10 group, therefore, the B10-transplantation might induce an autocrine inhibitory effect on NF-κB signaling. Taken together, our results suggested that B10-transplantation causes changes in the activation of the macrophages/microglia, rendering it to acquire an anti inflammatory phenotype.

As cell surface receptors including TLR2 and CD40 play a crucial role in the activation of NF-κB signaling pathway in the early periods of MCAO, the expression of TLR2 and CD40 were analyzed. Our immunofluorescence results revealed that TLR2 and CD40 were abundantly expressed in ED1-positive macrophage/microglia in the core area. The percentage of TLR2-positive and CD40-positive cells in macrophage/microglia population was significantly decreased in the B10 group at Day 5 ($P < 0.05$). Moreover, the percentage of NF-κB-positive cells in TLR2-positive or CD40-positive cells population was significantly decreased in the B10 group at Day 5 ($P < 0.05$). As a result of decreased expression of the receptors that activate NF-κB, the possibility of such pathway activation is reduced.

Therefore, B10 could affect NF-κB signaling at least three levels, a) at receptor level by inhibiting the expression of TLR2 and CD40, b) at activation level by decreasing proinflammatory cytokines expression and increasing anti-inflammatory cytokines expression, and finally, c) by inhibiting the expression of NF-κB itself through inhibiting its activator such as IL-1β and TNFα.

CONCLUSION

In conclusion, our study clearly demonstrates the inhibitory effects of B10 MSC on NF-κB signaling in the cerebral ischemic condition, and decreased proinflammatory gene expression. This inhibitory effect might be a key feature that ensues the beneficial effects of B10 MSC transplantation.

論文審査及び最終試験又は学力の確認の結果の要旨

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論文審査の結果の要旨

マクロファージ・ミクログリアは虚血脳損傷部位に集積し、サイトカイン分泌や壊死組織の貪食などにより、損傷の回復あるいは増悪に重要な役割を果たしていることが知られている。間葉系幹細胞（Mesenchymal stem cells：MSC）の脳梗塞再生医療への応用が期待される中、未だ脳梗塞モデルラットへのMSC移植が症状を改善する作用機序は不明である。そこで申請者は、移植MSCがマクロファージ・ミクログリアに及ぼす影響について検討した。中大脳動脈一過性閉塞モデルラットを作製して、24時間後にヒト由来MSC（B10）を静注移植し、その効果を3－5日後に評価した。神経重症度やMRIによる評価により、MSC移植は症状改善と梗塞量の減少に繋がることを認めた。免疫染色により、梗塞中心部へ遊走したMSCが、マクロファージ・ミクログリア近傍で観察された。加えて、円形の活性化マクロファージ・ミクログリアであるED1陽性細胞の著しい減少が、MSC移植ラットの梗塞巣で確認された。次に、MSC移植がマクロファージ・ミクログリアの活性化に影響を及ぼす機序として、転写因子の変化を調べたところ、マクロファージ・ミクログリアでNF-κBレベルの低下が認められた。さらに、NF-κBシグナルの下流にある炎症性サイトカインIL-1β、TNFα、iNOS、MCP-1のmRNA発現は減少し、抗炎症性サイトカインIL-4、IL-10は増加していた。また、NF-κBシグナルに深く関与するTLR2やCD40の発現がMSC移植により減少しており、これらの表面抗原の陽性細胞におけるNF-κB陽性率も減少した。従って、MSC移植によるTLR2やCD40を介したNF-κBシグナルの低減が、マクロファージ・ミクログリアの形質変化を導いて活性化やリクルートメントを減少させ、その結果、脳梗塞巣の炎症が低下して梗塞量の減少や症状改善に繋がったことが強く示唆された。

以上より、本研究の成果は臨床応用への可能性を示し、学位授与に値すると判断した。

最終試験又は学力の確認の結果の要旨

申請者は、脳梗塞再生医療を目標に、ヒト間葉系幹細胞を脳梗塞モデルラットへ静注移植して、脳梗塞量の減少と症状改善の要因の一部を細胞および分子レベルで詳細に解明した。予備審査および公開審査では的確に質疑応答し、臨床面での展望も述べ、関連知識も豊富であることから、学位授与に値すると判断した。（主査：中村守彦）

申請者は、ラットの中大脳動脈閉塞モデルにヒト由来の培養間葉系幹細胞B10を投与することで脳梗塞病変を縮小できることを発見し、そのメカニズムが梗塞部位でのミクログリア活性の抑制を介するユニークな機構に基づくことを明らかにした。学位審査における質疑応答も適確で背景の知識も充分に備えており、学位授与に値すると判断した。（副査：並河　徹）

申請者は、脳虚血ラットモデルにヒト間葉系幹細胞（B10）を静脈内移植し、B10移植がCD40, TLR2を介してマクロファージ・ミクログリア内のNF-κB活性ならびに炎症誘発性遺伝子発現を抑制し、脳梗塞の進展を緩和することを明らかにした。公開審査時の質疑応答も適切で、関連分野の知識も豊富であり、学位授与に値すると判断した。（副査：田島義証）

（備考）要旨は、それぞれ４００字程度とする。