様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）Development of an improved therapy for stroke based on multiple cell types transplantation

研究課題名（英文）Development of an improved therapy for stroke based on multiple cell types transplantation

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研究成果の概要（和文）：この研究の目的は、脳卒中に対する細胞ベースの改善された治療法を開発することです。 M2 ミクログリア (M2-MG6)次に神経幹細胞 (NSC) を脳卒中モデルに移植しし、構造、機能、および分子の変化が評価された。NSC+M2-MG6 グループでは、ビヒクル グループと比較して、構造的および機能的な改善が観察された。組織学的には、組織損傷と神経膠症の減少、ニューロン数の増加、食作用、血管新生、および移植された NSC が NSC + M2-MG6 グループで見つかりました。したがって、M2 ミクログリアの以前の移植は、損傷した組織を取り除き、脳卒中領域における NSC の生存率を改善しました。

研究成果の学術的意義や社会的意義
脳卒中の病理学における脳卒中領域のミクログリア依存性洗浄の重要性と、その状況で NSC を埋め込むより良い方法であります。
脳卒中は、世界中で死亡および障害の主な原因であり、疾患を完治させる方法は未だ見つかっておりません。この研究は、病気のためのより良い細胞ベースの管理システムを策定するために重要です。

研究成果の概要（英文）：The purpose of this study is to develop a cell transplantation-based improved therapy for stroke We transplanted M2 microglia (M2-MG6) first, then neural stem cells (NSCs) in a stroke model. Structural, functional, and molecular changes were assessed. Stroke volume was decreased, and neurological performances were increased in both NSC-alone or NSC+M2-MG6 groups compared to vehicle group, but no difference between NSC-alone and NSC+M2-MG6 groups. Histologically, cell accumulations and tissue damages were higher in NSC-alone group compared NSC+M2-MG6 group. GFAP+ astrocytes were similar, but Iba-1+ microglia numbers were decreased in NSC+M2-MG6 groups compared to NSC-alone group. Angiogenesis and neuron numbers were increased in NSC+M2-MG6 groups compared to NSC-alone. Moreover, phagocytosis process, and transplanted NSC numbers were increased in NSC+M2-MG6 group. Thus, prior transplantation of M2 microglial cleared the damaged tissues and improved NSC survival in the stroke area.

研究分野：Cell-based therapy for cerebral infarction.

キーワード：Cerebral infarction　cell-based therapy　M2-Microglia　Neural stem cells　phagocytosis

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Stroke is a leading cause of death and disability worldwide. Although we have much understandings about stroke pathology, disease modifying therapy is still illusive. Recently, cell-based therapy is gaining much interest. However, there are some drawbacks: 1. Transplanted cell homing in the lesion area is not efficient. 2. Survival of transplanted cells are inadequate, resulting failure of incorporation to the host tissue. Hence, the beneficial effects of the transplanted cells are often limited. The reason of such limited homing and survival could be a hostile environment in the lesion area caused by M1-microglia that induce inflammation and tissue damages.

M2 type microglia produce anti-inflammatory cytokines, chemokines and growth factors. Also, it can clean the lesion area by phagocytosis. Hence, we hypothesized that early transplantation of M2-microglia in the stroke condition could induce an anti-inflammatory environment by releasing anti-inflammatory cytokines and phagocytic cleaning of damaged tissue, which provides a better microenvironment for engraftment of transplanted NSCs.

２．研究の目的

Purpose of this study is to develop an improved cell-based therapy for stroke. Several studies have been done where mono-cell therapy of neural stem cells (NSCs), mesenchymal stem cells, iPS, embryonic stem cells or microglia have been done in stroke animal models. Each cell type modulates different aspect of the pathology. Although transplantation of different cell types shows some improvement of the condition, homing of the transplanted cells was low, which exist in the lesion area for a limited time. Hence, the aim of this study is to investigate about the effects multi-cell transplantation of M2 type microglia and NSCs sequentially in stroke condition, which could improve the homing and grafting of NSC in the lesion area.

３．研究の方法

1. Cell culture:

   Generation and culture of NSC: NSC was isolated from a wild-type mouse embryo of MRL background, and immortalized by introducing small and largeT antigen. Following media were used for NSC and neurosphere culture; NSC medium: DMEM (high glucose): F12 ham 1:1, 2% FBS, N2 supplement, bFGF 20 ng/ml, EGF 20 ng/ml. Neurosphere medium: DMEM (high glucose): F12 ham 1:1, 1X N2 supplement, 1X B27 supplement, bFGF 20 ng/ml, EGF 20 ng/ml. For neuronal differentiation, following medium was used DMEM (high glucose): F12 ham 1:1, bFGF 20 ng/ml, EGF 20 ng/ml, and the cells were cultured in a poly-L-lysine coated dish for at least 14 days.

   MG6 culture: Microglia cell line, MG6 was a generous gift from Dr. Hiroshi Kitani. MG6 was cultured in DMEM (high glucose), 10% FBS, 100 μM β-mercaptoethanol, and 10 μM insuline. For M2 differenciation, MG6 was stimulated with IL-4 (10 ng/ml) for 24 h. Characterization of NSC and MG6 culture was done by checking the marker expression using real time PCR and immunocytochemistry.

2. Generation of stroke model: Stroke animal model was generated by mechanically occluding right middle cerebral artery (MCAO: middle cerebral artery occlusion) of Wister rats. Adult male rats (n=20; 8 weeks old; CLEA Japan,Tokyo) weighting 250 to 300 g were used in this experiments. Rats were anesthetized with 4% halothane, and anesthesia was maintained

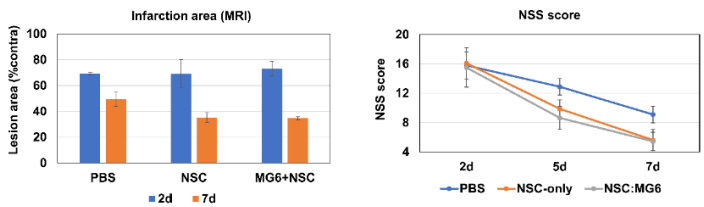
with 1–2% halothane using a face mask. Then right common carotid artery, external carotid artery and internal carotid artery were exposed via a ventral midline incision. The tip of a 4-0 monofilament nylon suture was rounded and coated with silicon, and inserted in the right external carotid artery. The suture was advanced into of the internal carotid artery to block the origin of the right middle cerebral artery. After 90 minutes, the animals were re-anesthetized with halothane and nylon monofilament was removed, and the end of the external carotid artery was tied. The rats were allowed to recovery from anesthesia and returned to the cages.

3. Transplantation of Microglia and NSC: Six hours after MCAO, the rats were evaluated functionally using a neurological severity scoring (NSS) system. Rats of similar NSS, was randomly divided into 4 groups: 1. PBS control group, 2. NSC-alone transplanted group, 3. Mg6-alone group and 4. NSC+MG6 group. Grouping was done in a blinded manner. Then 6 h after MCAO, $3X10^6$ IL-4 treated MG6 was transplanted through tail vein to rats of group 3 and 4. Twenty four h after MCAO, $3X10^6$ IL-4 NSC was transplanted through tail vein to rats of group 2 and 4.
4. Stroke volume measurement: Stroke volume was measured by MRI.
5. Neurological performances: Neurological performance was evaluated using a neurological severity scoring system. NSS was done on day 2, 5 and 7 after MCAO in a blinded manner.
6. Evaluation of histological and molecular pathology: Histological changes was done by hematoxylin and eosin staining, and molecular changes was evaluated by immunostaining.

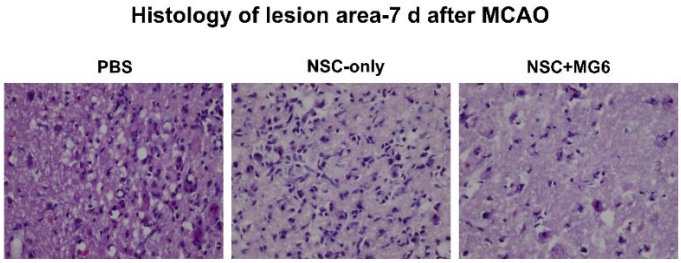
４．研究成果

Characterization of NSC line: First, we characterized the NSC line we have generated. Cell culture data revealed that the NSC line we generated could make neurosphere in neurosphere culture condition. Also, it can differentiate into neurons in appropriate culture condition. Additionally, it showed high expression of NSC specific proteins including nestin and sox2.

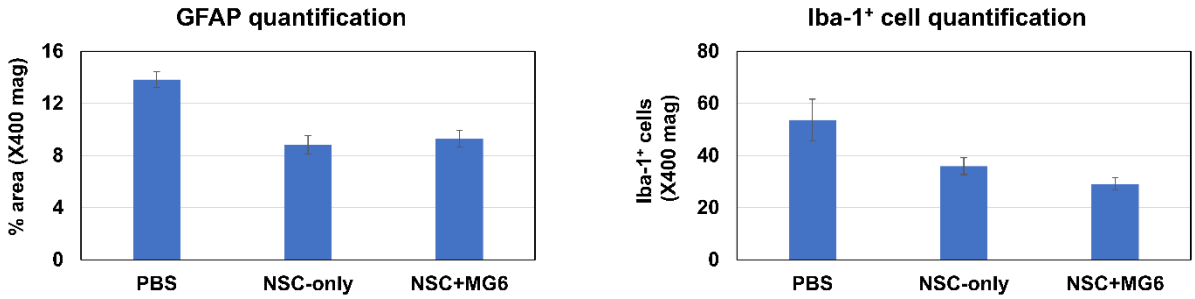
Stroke volume and neurological severity scores: Stroke volume was measured by MRI. MRI results showed that both NSC-alone and NSC+MG6 group showed significantly decreased stroke volume compared to PBS control group. However, the stroke volume was not different among NSC-alone and NSC+MG6 groups. Similarly, Neurological performance was severity improved in NSC-alone and NSC+MG6 groups compared to PBS control as shown by decreased NSS scores.



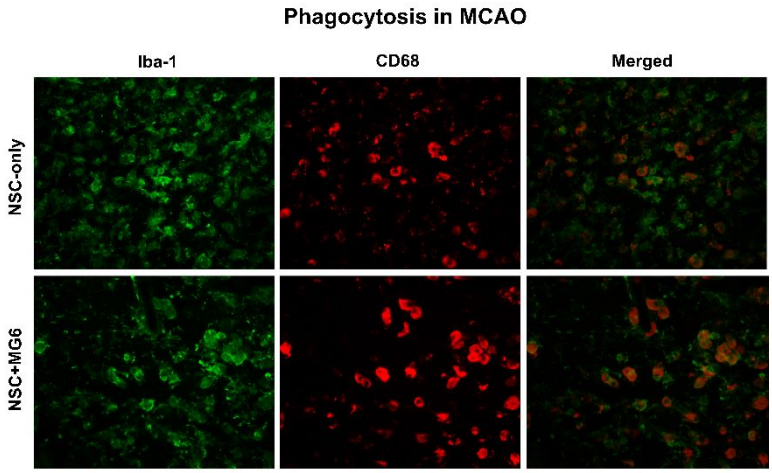
Histological analysis: Histological analysis showed that in NSC-alone group (left picture), cell number in the core region was higher compared to NSC+MG6 group (right picture). Also, tissue damage was decreased, and vessel-like structure was increased in NSC+MG6 group.



Glial reaction: Changes of astrocytes and microglia was evaluated by GFAP and Iba-1 immunostaining, respectively. Immunostaining results showed that GFAP+ astrocytes number was similar between NSC-alone and NSC+MG6 group. But Iba-1+ microglia number was decreased in NSC+MG6 group. Moreover, protoplasmic-type astrocytes and iNOS+ cell number was decreased number was decreased, suggesting a decreased inflammatory condition in NSC+MG6 group.



Phagocytic microglia: Next, we checked the levels of phagocytic microglia levels in MCAO condition. Double immunofluorescence staining was done to identify phagocytic microglia, where microglia were identified using Iba-1 and phagocytic cells using CD68. The results showed that in NSC+MG6 group, phagocytic microglia numbers were higher than NSC-alone group. Such results indicate that phagocytic cleaning of the lesion area could be the reason of decreased inflammation observed in NSC+MG6 group.

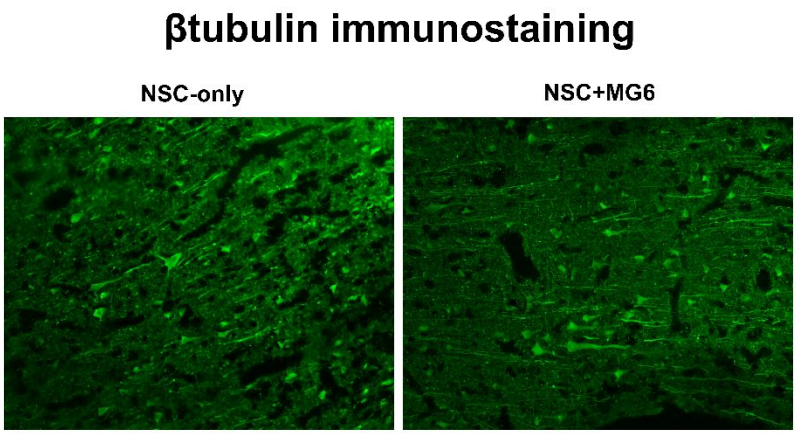


Neural progenitor cell homing in the lesion area: Since, inflammatory condition is detrimental for the survival of neural progenitor cells, and NSC+MG6 group showed decreased levels of inflammation, we checked neural progenitor cell number in the core of MCAO rat brains by nestin immunostaining. The results showed that in the lesion area, nestin+ cell number was increased in NSC+MG6 group compared to NSC-alone group.

Migration of transplanted NSC in the lesion area of MCAO model: Then we checked

accumulation of transplanted NSC in the lesion area. Since, transplanted cells were immortalized by transducing largeT antigen, we identified transplanted cells in the brains by largeT antigen immunostaining. Our immunostaining results showed that largeT antigen+ transplanted cell number was slightly but significantly increased in the lesion area of NSC+MG6 group.

Number and morphology of neurons in the lesion area of MCAO model: Finally, neuronal cell number in the lesion area was evaluated by NeuN immunostaining. After counting the NeuN+ cells in the core of the lesion area, we found that the positive cell number was increased in NSC+MG6 group. We also checked the morphology of neuronal cells in the lesion area by βtubulin immunostaining. The results showed that in both NSC-alone and NSC+MG6 groups, βtubulin+ neuronal cells extended processes. However, in NSC+MG6 group the processes seemed to be well-formed and longer than NSC-alone group.



Larger neuron processes found in NSC+MG6 group

Thus, our results showed that prior transplantation of IL-4 primed microglia cleanup the lesion area in MCAO condition by phagocytosis, which decreased the inflammatory condition without affecting the astrocytic support. Such condition could be beneficial for grafting of transplanted cells, and homing of native neural progenitor cells. Moreover, differentiation of mature neurons could be supported in this condition. Since such sequential transplantation of IL-4 primed microglia and NSC improve homing, grafting and differentiation of neuronal stem cells, such method could be useful for the therapy for stroke condition.

５．主な発表論文等

〔雑誌論文〕　計4件（うち査読付論文　4件／うち国際共著　0件／うちオープンアクセス　1件）

１．著者名 Maniwa K, Yano S, Sheikh AM, Onoda K, Mitaki S, Isomura M, Mishima S, Yamaguchi S, Nabika T, Nagai A.	４．巻 10
２．論文標題 Association between Cystatin C gene polymorphism and the prevalence of white matter lesion in elderly healthy subjects.	５．発行年 2020年
３．雑誌名 Sci Rep.	６．最初と最後の頁 4068
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41598-020-61383-7	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Haque Md. Ahsanul、Sheikh Abdullah Md.、Al Mamun Abdullah、Yano Shozo、Hashimoto Michio、Shido Osamu、Nagai Atsushi	４．巻 15
２．論文標題 A Mesenchymal Stem Cell Line Transplantation Improves Neurological Function and Angiogenesis in Intraventricular Amyloid β-Infused Rats	５．発行年 2018年
３．雑誌名 Current Alzheimer Research	６．最初と最後の頁 1331～1342
掲載論文のDOI（デジタルオブジェクト識別子） 10.2174/1567205015666180911145159	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Sheikh Abdullah Md.、Yano Shozo、Mitaki Shingo、Haque Md. Ahsanul、Yamaguchi Shuhei、Nagai Atsushi	４．巻 311
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３．雑誌名 Experimental Neurology	６．最初と最後の頁 182～193
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.expneurol.2018.10.001	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Matsuda Chikashi、Shiota Yuri、Sheikh Abdullah Md、Okazaki Ryota、Yamada Kazuo、Yano Shozo、Minohata Toshikazu、Matsumoto Ken-ichi、Yamaguchi Shuhei、Nagai Atsushi	４．巻 478
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３．雑誌名 Clinica Chimica Acta	６．最初と最後の頁 1～6
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.cca.2017.12.016	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

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1．発表者名 長井篤・Azad AK・Sheikh AM・長子晴美・堺　弘道・矢野彰三・道川　誠
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4．発表年 2018年

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〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

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７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関