Bone marrow mononuclear cells activate angiogenesis via gap junction mediated cell-cell interaction

Akie Kikuchi-Taura, PhD[1], Yuka Okinaka[1], Yukiko Takeuchi, MSc[1], Yuko Ogawa, PhD[1], Mitsuyo Maeda, PhD[1,2,3], Yosky Kataoka, MD, PhD[2,3], Teruhito Yasui, PhD [4], Takafumi Kimura, MD, PhD [5], Sheraz Gul, PhD[6], Carsten Claussen, PhD[6], Johannes Boltze, MD, PhD[1,7], Akihiko Taguchi, MD, PhD[1*]

[1]Department of Regenerative Medicine Research, Institute of Biomedical Research and Innovation, Kobe, Japan

[2]Multi-modal Microstructure Analysis Unit, RIKEN-JEOL Collaboration Center, RIKEN, Kobe, Japan

[3]Laboratory for Cellular Function Imaging, RIKEN Center for Biosystems Dynamics Research, Kobe, Japan

[4]National Institutes of Biomedical Innovation, Health and Nutrition, Ibaraki, Japan

[5]Japanese Red Cross Kinki Block Blood Center, Ibaraki, Japan

[6]Fraunhofer Institute for Molecular Biology and Applied Ecology IME – ScreeningPort, Hamburg, Germany

[7]School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK.

Address correspondence to: AKIHIKO TAGUCHI, MD, PhD

Department of Regenerative Medicine Research, Biomedical Research and Innovation
2-2 Minatojima-Minamimachi, Chuo-ku, Kobe, Japan, 650-0047
E-mail: taguchi@fbri.org, Phone +81-78-304-5772, Fax +81-78-304-5263

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Background and Purpose: Bone marrow mononuclear cells (BM-MNC) are a rich source of hematopoietic stem cells and have been widely used in experimental therapies for patients with ischemic diseases. Activation of angiogenesis is believed to be one of major BM-MNC mode of actions, but the essential mechanism by which BM-MNC activate angiogenesis have hitherto been elusive. The objective of this study is to reveal the mechanism how BM-MNC activate angiogenesis

Method: We have evaluated the effect of direct cell-cell interaction between BM-MNC and endothelial cell on uptake of vascular endothelia growth factor (VEGF) into endothelial cells *in vitro*. Cerebral ischemia model was employed to evaluate the effects of direct cell-cell interaction with transplanted BM-MNC on endothelial cell at ischemic tissue.

Results: The uptake of VEGF into endothelial cells was increased by BM-MNC, whilst being inhibited by blockading the gap junction. Low molecular weight substance was transferred from BM-MNC into endothelial cells via gap junctions *in vivo*, followed by increased expression of hypoxia inducible factor-1α and suppression of autophagy in endothelial cells. The concentration of glucose in BM-MNC cytoplasm was significantly higher than in endothelial cells and transfer of glucose homologue from BM-MNC to endothelial cells was observed.

Conclusion: Our findings demonstrated cell-cell interaction via gap junction is the prominent pathway for activation of angiogenesis at endothelial cells after ischemia and provided novel paradigm that energy source supply by stem cell to injured cell is one of the therapeutic mechanisms of cell-based therapy.

Introduction

Transplantation of bone marrow mononuclear cells (BM-MNC) is known to robustly activate angiogenesis in ischemic tissue, including human[1, 2]. BM-MNC harboring hematopoietic stem cells can be obtained in an autologous fashion and processed swiftly for re-purposing. This makes them a well-suited source of cell-based therapies and BM-MNC are frequently used in experimental treatment of ischemic diseases, including limb ischemia[1], myocardial infarction[3], and stroke[4]. BM-MNC contain various cells, including lymphoid, myeloid, erythroid, and hematopoietic stem cell populations. Although individual BM-MNC populations were shown to exert different effects[5], a basis for the success of this approach as a therapeutic during BM-MNC transplantation is considered to be, at least in part, due to the substantial increase in angiogenesis. However, the precise mechanism is not fully understood.

Initial studies indicated that differentiation of hematopoietic stem cells into endothelial cells was the major contributory mechanism[6]. However, subsequent studies showed that only a very small number of transplanted cells differentiate into endothelial cells[7], thereby ruling this out as the major contributory mechanism. Secretion of multiple pro-angiogenic factors and cell derived exosomes had been proposed, but its impact on overall effects are in arguments [8]. One important facet which we have investigated is the role of endothelial

cells which are known to communicate with bone marrow cells at the vascular niche in

bone marrow[9]. This interaction is mediated by direct cell-cell contact via gap junctions[10].

The gap junction channels exhibit selectivity based on molecular size and allow the

movement of molecules smaller than 1000 dalton[11]. Small molecules can be directly

transferred freely between cells via gap junctions depending upon the concentration

gradient of the cytoplasm between cells[11]. In this article, we provide evidence that gap

junction mediated cell-cell interaction is the key mechanism for activation of

angiogenesis at ischemic tissue by BM-MNC transplantation.

Methods

All animal experiments were approved by the Animal Care and Use Committee of Foundation for Biomedical Research and Innovation (reference number 16-03-02) and comply with the Guide for the Care and Use of Animals published by the Japanese Ministry of Education, Culture, Sports, Science and Technology. Experiments and results are reported according to the ARRIVE guidelines.

A detailed Methods section is available in the Supplement.

BM-MNC preparation

Bone marrow was obtained from 6-week-old male C57BL/6 (Japan SLC, Shizuoka, Japan) or 6-week-old male CB-17 mice (Oriental Yeast, Tokyo, Japan) for *in vitro* or *in vivo* experiment, respectively, as described previously[12].

Human vascular endothelial growth factor (hVEGF) measurement in culture medium

Human umbilical vein endothelial cells (HUVEC, Kurabo, Osaka, Japan) were cultured with medium, serum and growth factors (HuMedia-EB2, Kurabo) according to manufacturer's protocol. The level of hVEGF in medium before and after co-culture with BM-MNC was measured using Bio-Plex Multiplex System (Bio-Rad, Hercules, CA). To evaluate the effect of cell-cell interaction between HUVEC and BM-MNC, cell culture

insert (1µm pore size: BD Bioscience, MA, USA) was employed to avoid direct cell-cell interaction.

hVEGF uptake into HUVEC

BM-MNC and APC-labeled hVEGF (10 nM in final concentration: R&D systems, MN USA) were added to HUVEC, and incubated for 3 hours at 37 ºC. The level of APC in HUVEC (CD31-positive, CD45-negative and 7AAD-negative) was evaluated on a FACS Calibur fluorescent cell sorter (BD). Confocal microscope (Olympus FLUOVIEW FV1000) was used to obtain the orthogonal confocal view of uptaken hVEGF in HUVEC.

Low molecular weight fluorescence molecular in cytoplasm of BM-MNC

BM-MNC were incubated with 5 µM of BCECF-AM (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester, Dojindo, Kumamoto Japan) for 30 minutes at 37 ºC. BCECF-AM was converted to BCECF (2',7'-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein) at cytoplasm and BCECF loaded BM-MNC were washed twice with PBS before use.

Glucose concentration and transfer assays

The glucose concentration in BM-MNC and HUVEC was measured by glucose assay kit (Biovision, Milpitas, CA). Fluorescence-positive glucose homologue (2-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) (2-NBDG, Peptide institute, Osaka, Japan)

was used to assess the transfer of glucose between BM-MNC and HUVEC.

Induction of focal cerebral ischemia and injection of BM-MNC

A murine stroke model was utilized for this study which offers excellent reproducibility in 7-week-old male CB-17 mice, as we described previously[13].

Immunohistochemistry

To analyze infarcted cortex histochemically, mice were anesthetized with sodium pentobarbital and perfused transcardially with saline followed by 2% paraformaldehyde (PFA). The brain was cut into coronal sections (20 µm) using a vibratome (Leica, Wetzlar, Germany). Quantitative analysis was performed by blinded investigators.

Electron microscopy

Coronal brain sections were cut using a microslicer (Leica) at a thickness of 100 µm. The sections were post-fixed with 1% OsO_4 for 1 hour, dehydrated, and flat-embedded on siliconized glass slides in epoxy resin. Ultrathin sections (70 nm thickness) were cut, mounted on Formvar-coated single slot (2 x 1 mm) grids, and contrasted with 2% uranyl acetate and lead citrate. Electron micrographs were taken at 80 kV on JEM-1400EX+ (JEOL, Tokyo, Japan). The number of autophagosome-like vacuoles in the ischemic lesion, in lesion border cortex and corresponding contralateral cortex were counted by a blinded investigator.

Data analysis

Statistical comparisons among groups were determined using one-way analysis of variance (ANOVA) followed by post-hoc analysis using Dunnett's test. Where indicated, individual comparisons were performed using Student's t-test. In all experiments, the mean ± SD is reported.

Data availability

The data supporting the findings of this study are available from the corresponding author on reasonable request.

Results

BM-MNC promote VEGF uptake into HUVEC through gap junction mediated interaction

VEGF is one of the most prominent pro-angiogenic factors[14]. HUVEC are known to secrete VEGF into culture medium and uptake it in an autocrine/paracrine manner[15]. In order to evaluate the effect of BM-MNC on VEGF autocrine/paracrine of HUVEC, HUVEC were co-cultured with BM-MNC and changes in concentration of hVEGF in culture medium was evaluated. We noted that the concentration of hVEGF in culture medium was significantly decreased after co-culturing with BM-MNC, whereas it was

increased in control (Figure 1A). As the co-culture with BM-MNC separated by a cell culture insert did not reduce the level of hVEGF, our findings suggest that BM-MNC promote hVEGF uptake into HUVEC via a cell-cell interaction mechanism.

To test the hypothesis, allophycocyanin (APC) labelled hVEGF (APC-hVEGF) was added to the culture medium and its uptake into HUVEC was determined by FACS where HUVEC and BM-MNC were clearly distinguished by antibodies against CD31 and CD45 (Figure 1B). We observed a significant increase in APC-hVEGF uptake into HUVEC when co-cultured with BM-MNC (Figure 1C). Gap junctions are known to play critical roles in cell-cell interactions, including between hematopoietic stem cells and endothelial cells in the bone marrow[10]. BM-MNC were pretreated with gap junction inhibitors (1-octanol or carbenoxolone)[16], and APC-hVEGF uptake into HUVEC was evaluated by co-culture with BM-MNC. The co-cultures with BM-MNC treated with gap junction inhibitor did not increase hVEGF uptake into HUVEC (Figure 1C). Furthermore, co-cultures with BM-MNC separated by cell insert did not increase hVEGF uptake into HUVEC (Figure 1C). These findings indicate that BM-MNC promote hVEGF uptake into HUVEC via a gap junction mediated interaction. The localization of uptaken hVEGF was evaluated by confocal microscope. Consistent with previous report that VEGF-VEGF receptor complex, which is formed at cell membrane, internalize into endosomes in

endothelial cells [17], uptaken hVEGF was observed at cytoplasm of HUVEC (Figure 1D).

It should be noted that minimal hVEGF uptake was observed into BM-MNC when cultured either with or without HUVEC (Supplemental Figure Ia-c).

***In vitro* transfer of low molecular weight substance from BM-MNC to endothelial cells through gap junction**

BCECF is a low molecular weight fluorescent substance and known to pass gap junctions[18]. In order to evaluate the gap junction mediated interaction between BM-MNC and HUVEC *in vitro*, BCECF was loaded into the cytoplasm of BM-MNC, co-cultured with HUVEC and the BCECF signal in HUVEC was evaluated by FACS. Although no BCECF signal was observed in HUVEC following co-culture with non-BCECF loaded BM-MNC (Supplemental Figure IIa), BCECF signal was observed in HUVEC after co-culture with BCECF loaded BM-MNC (Supplemental Figure IIb). In contrast, no BCECF signal was observed in HUVEC when BCECF loaded BM-MNC and HUVEC were co-cultured separated by a cell culture insert (Supplemental Figure IIc), and the transfer of BCECF was suppressed when BCECF loaded BM-MNC were pre-treated with a gap junction inhibitor before co-culture (Supplemental Figure IId). To confirm the gap junction mediated interaction between BM-MNC and HUVEC, expression of connexin 37 and 43 was investigated at the cell surface. Although little expression of connexin 37

was observed at cell surface of HUVEC (Supplemental Figure IIe), expression of connexin 43 was observed (Supplemental Figure IIf). Similarly, little expression of connexin 37 was observed at cell surface of BM-MNC (Supplemental Figure IIg) and expression of connexin 43 was observed (Supplemental Figure IIh).

***In vivo* transfer of low molecular weight substance from BM-MNC to endothelial cells through gap junction**

To evaluate the cell-cell interaction between BM-MNC and endothelial cells through gap junctions *in vivo*, cerebral ischemia was induced in mice and the expression of gap junction molecules was evaluated. Although little expression of connexin 37 was observed in cerebral cortex of non-stroke mice, increased expression of connexin 37 was observed in the lesion area at 48 hours after stroke induction (Figure 2A). Similarly, little expression of connexin 43 was observed in the cerebral cortex of non-stroke mice and its increased expression was observed in the lesion area at 48 hours after stroke induction (Figure 2A). BCECF loaded BM-MNC were transplanted intravenously into mice at 48 hours after induction of stroke. Ten minutes after transplantation of BM-MNC, mice were sacrificed, and BCECF localization was evaluated by fluorescence confocal microscopy. BCECF-positive cells were observed in the microvasculature in the lesion area (Figure 2B). Higher magnification image showed the transfer of BCECF transfer from BM-MNC

into endothelial cells across endothelial cell membrane (Figure 2C). The number of BCECF-positive endothelial cells was counted and confirmed that blockade of gap-junction of BM-MNC by 1-octanol significantly suppress the transfer of BCEDF to endothelial cells *in vivo* (Figure 2D)

To confirm gap junction mediated BCECF transfer from BM-MNC into endothelial cells, co-localization of gap junction and transferred BCECF in endothelial cells was investigated at 10 minutes after cell transplantation. BCECF-positive signals were observed in endothelial cells with accumulation of connexin 37 (Figure 3A) and connexin 43 (Figure 3B). It should be noted that transferred BCECF signals were also observed at non-endothelial cell which is located at outside of vasculature (Figure 3C). The number of Cx37/BCECF-positive or Cx43/BCECF-positive endothelial cell was quantified and confirmed that the transfer of the BCECF was significantly suppress by blockade of gap-junction of BM-MNC with 1-octanol (Figure 3D).

Increased Hif-1α expression at microvasculature in the lesion area after BM-MNC transplantation

Hif-1α is known to induce upregulation of VEGF uptake into endothelial cells[19]. To investigate the upstream regulators of VEGF uptake in endothelial cells, the expression of Hif-1α was investigated in the post-stroke brain. Hif-1α is activated by hypoxia but

known to be not activated by severe ischemia, such as at the lesion area after permanent cerebral artery occlusion[20]. As well as in the cerebral cortex of non-stroke mice, little expression of Hif-1α was observed at 3 or 48 hours after induction of stroke at the lesion area (Figure 4A). BM-MNC were intravenously injected at 48 hours after induction of stroke and found the increased expression Hif-1α at vasculature like cells in the lesion area at 1 hour after cell injection (Figure 4A). To investigate the correlation between gap junction mediated small molecule transfer from BM-MNC and increased expression of Hif-1α, BCECF loaded BM-MNC were injected intravenously at 48 hours after induction of stroke and the localization of BCECF and Hif-1α at endothelial cells was investigated by confocal microscope at 10 minutes after cell injection. The co-localization of BCECF and Hif-1α was observed in endothelial cells (Figure 4B, C) which indicates the transfer of low molecular substance from BM-MNC induces Hif-1α expression at endothelial cells in post-stroke brain. In contrast, no Hif-1α/BCECF-positive endothelial cell was observed in mice that received BM-MNC pre-treated with 1-octanol (Figure 4D).

BM-MNC transplantation reduces autophagy in endothelial cells after stroke

Autophagy is known to be induced in brain endothelial cells after cerebral ischemia[21]. To examine the effects of BM-MNC transplantation after stroke on autophagy in endothelial cells, brain sections were stained with the autophagy marker, LC3, and investigated its

expression at the lesion, lesion border and contralateral cortex (Supplemental Figure IIIa).

LC3-positive endothelial cells were observed in both PBS- and BM-MNC treated mice

in the lesion area at 72 hours after stroke induction (i.e., 24 hours after PBS or BM MNC

injection, Supplemental Figure IIIb). However, LC3-positive endothelial cells were rarely

observed in BM-MNC treated mice in lesion border areas, although strong LC3

expression was observed in most endothelial cells of mice that received PBS

(Supplemental Figure IIIc). Furthermore, no LC3-positive endothelial cells were

observed in contralateral cortex of BM-MNC treated mice, although some endothelial

cells were LC3-positive in mice that received PBS (Supplemental Figure IIId). These data

suggested that BM-MNC transplantation suppresses autophagy in endothelial cells after

cerebral ischemia.

To confirm the effect of BM-MNC transplantation on autophagy suppression, specimens

for electron microscopy were prepared and ultrastructural changes were investigated in

endothelial cells after BM-MNC transplantation. Figure 5A shows intact capillary with a

normal blood brain barrier (BBB) in the cerebral cortex of non-stroke mice being

composed of endothelial cells, a basal lamina, pericytes, and astrocytic endfeet.

As expected, degeneration and necrosis of neurons and glial cells were significant in the

lesion area at 72 hours after stroke induction (i.e., 24 hours after PBS or BM-MNC

injection, respectively) (Figure 5A). In PBS-treated mice, a number of autophagosome-like vacuoles were formed in endothelial cells at lesion cortex, whilst sometimes containing amorphous material or a layered structure. Degenerated mitochondria with blurry cristae were also observed in the cytoplasm of endothelial cells. In contrast, less changes were observed in BM-MNC treated mice. At the lesion border cortex, vacuoles of various sizes were formed in the cytoplasm of endothelial cells in mice that received PBS. This further contrasted with mice that received BM-MNC in that less formation of autophagosome-like vacuoles in endothelial cells were observed. It should be noted that some vacuoles in endothelial cells were observed in the contralateral cortex of mice that received PBS, but no such vacuoles were observed in mice that received BM-MNC. Quantification of autophagosome-like vacuoles in each area confirmed the impression that BM-MNC transplantation reduces the formation of autophagosome-like vacuoles in all areas of interest (Figure 5B).

Glucose concentration in BM-MNC and HUVEC

Autophagy is known to be induced by shortage of energy supply at ischemic tissue[22], and glucose is the major energy source in endothelial cells[23]. Hematopoietic stem cells are known to be capable of maintaining an anaerobic metabolism with upregulation of glucose uptake[24]. Since low molecular weight water soluble substances, such as glucose,

can freely pass through gap junctions when a concentration gradient is present, we hypothesized that one of the substances that transfers from BM-MNC to endothelial cells and suppressed autophagy would be glucose. To investigate this, the concentration of glucose in BM-MNC and endothelial cells was compared *in vitro*. We ascertained that BM-MNC contained >10-fold higher concentration of glucose, when compared with HUVEC (Figure 5C). The transfer of uptaken glucose between BM-MNC to HUVEC was evaluated by fluorescence-positive glucose homologue (2-NBDG) [25]. BM-MNC and HUVEC was separately incubated with 2-NBDG and washed twice before co-culture. Significant increase of 2-NBDG level was observed in HUVEC by co-culture with BM-MNC (Figure 5D). In contrast, the level of 2-NBDG in BM-MNC was significantly decreased by co-culture with HUVEC (Figure 5D).

Discussion

In this article, we have demonstrated that BM-MNC accelerate VEGF uptake into endothelial cells and suppress autophagy through gap junction mediate cell-cell interaction. Our findings provide a novel concept that gap junction mediated signaling is a prominent pathway for activation of angiogenesis and support survival of injured endothelial cells after ischemia.

Endothelial cells are known to communicate with bone marrow cell populations at the bone marrow vascular niche, and that this communication is mediated through gap junctions[10]. The gap junctional channels allow the movement of molecules smaller than 1000 dalton promptly according to its concentration gradient in cytoplasm between cells[11]. A gap junction channel between two cells is composed of two identical connexons or a different connexin in each half of the cell pair. In this article we have demonstrated that connexin 37 and 43 expression in brain endothelial cells are upregulated after stroke and about 25% of BM-MNC express connexin 43. As more than 20 connexins had been identified so far[16], further studies are required to identify the contributions of each connexin and its pair to cell-cell interaction between BM-MNC and endothelial cells, beside connexin 37 and 43.

Low molecular weight water soluble substances transfer from transplanted BM-MNC into endothelial cells, and that this transfer increased the Hif-1α content. It should be noted that molecular weight of Hif-1α is about 120k dalton[26] and does not pass through gap junction. Bone marrow cells reside in a niche with relatively low oxygen content[24] and our results revealed that the intracellular glucose concentration in BM-MNC is >10-fold higher than in healthy endothelial cells cultured in glucose containing medium. Furthermore, our results indicated that uptaken glucose-homologue at BM-MNC is

transferred to endothelial cells. As enhanced glucose uptake is known to stimulate Hif-1α

activation[27], our results indicate that one of the small molecules that transfer from BM-

MNC to endothelial cells with activation in Hif-1α would be glucose, though transfer of

other small molecules, such as glycolytic substrate and amino acids, would also be

involved in Hif-1α activation.

Hif-1α is one of the key regulators and known to regulate about 2% of the entire

endothelial cell gene pool[28] and its activation is known to induce upregulation of VEGF

uptake into endothelial cells[19]. Extracellular VEGF binds to cell surface VEGF receptor

followed by internalization of the receptor-ligand complex into endosomes with

activation of angiogenesis[17,29]. Consistent with these findings, uptaken fluorescence

labeled-VEGF were observed at cytosol of HUVEC. Activation of Hif-1α is also known

to enhance phosphorylation of eNOS, which explains our previous finding that BM-MNC

activates eNOS in endothelial cells *in vitro*[30]. Taken together, these findings indicate that

BM-MNC mediated activation of Hif-1α in endothelial cells, followed by activation of

various signals including upregulation of VEGF uptake and phosphorylation of eNOS, is

a key mechanism for cell therapy-mediated angiogenesis.

We further demonstrated that BM-MNC transplantation suppressed autophagy in

endothelial cells. Autophagy provides metabolic substrates to maintain energy charge

under challenging conditions[31]. A number of molecules that block autophagy cascades have been identified[32], but none is known to exist in endothelial cells at sufficient levels, apart from available metabolic substrates themselves. Our results regarding to the concentration gradient in glucose and cell-cell interaction via gap junction indicated that one of the small molecules that suppressed autophagy in endothelial cells would be glucose, although many other small molecules would be transferred to endothelial cells thus suppressing autophagy.

Hypoxia activates Hif-1α in endothelial cells followed by initiation of angiogenesis cascades[33] (Figure 6A). However, a drastic reduction of glucose and oxygen supply by severe ischemia was shown to induce autophagy without Hif-1α activation (Figure 6B), probably because endothelial cells disable to continue regular glucose metabolism. Transplanted BM-MNC, which contains significantly higher glucose concentration in cytoplasm than HUVEC, transferred small molecules to endothelial cells via gap junction followed by activation of Hif-1α and suppression of autophagy (Figure 6C). BM-MNC are a mixture of various cell populations including hematopoietic stem cells. In view of the proposed mode of action, the required cellular properties that can activate Hif-1α and suppress autophagy in endothelial cell would be higher cytoplasmic glucose concentration than endothelial cell and expression of gap junction at cell surface. The pO$_2$

of the bone marrow environment is known to be very low and heterogenous[34] and anaerobic metabolism in hypoxic condition requires high glucose uptake. Hematopoietic and surrounding non-hematopoietic cells are known to communicate each other through gap junction[35] and our results showed that approximately 25% of BM-MNC were connexin 43-positive. Taken together, hematopoietic stem cells fulfill the required properties to transfer energy source via gap junction, but stemness and differentiative potential are not essential for this process. Thus, non-hematopoietic stem cells can possess similar properties. Recently, we have demonstrated clot-derived contaminants in transplanted BM-MNC impair the therapeutic effect and that can explain serious of neutral or negative results observed in clinical trials of BM-MNC transplantation for ischemic diseases[36]. Current study revealed that therapeutic mechanism is not likely to be directly related to the cause of non-responder for BM-MNC transplantation.

Following BCECF loaded BM-MNC transplantation into stroke mice, BCECF-transferred cells were also observed at location outside of the vasculature. The endfeet of astrocyte are one of the major components that compose BBB but no gap junction formation with endothelial cells is reported. In contrast, gap junction between pericyte and endothelial cells is well known and pericytes have significant regulatory roles in neurovascular unit in health and disease[37]. Therefore, small molecule transfer from BM-

MNC, including glucose, might have roles in metabolic support in pericyte after ischemia.

In conclusion, our current findings demonstrated cell-cell interaction via gap junction is a prominent pathway for activation and support survival of injured cells after ischemia.

Disclosures

Dr Taguchi reports grants from Kaneka, Mitsubishi Tanabe Pharma, Suntory, and Asubio Pharma outside the submitted work; in addition, he has a patent to patent pending (PCT/JP2019/008701).

References

1.   Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: A pilot study and a randomised controlled trial. *Lancet*. 2002;360:427-435

2.   Taguchi A, Ohtani M, Soma T, Watanabe M, Kinosita N. Therapeutic angiogenesis by autologous bone-marrow transplantation in a general hospital setting. *Eur J Vasc Endovasc Surg*. 2003;25:276-278

3.   Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, et al. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N Engl J Med*. 2006;355:1210-1221

4.   Taguchi A, Sakai C, Soma T, Kasahara Y, Stern DM, Kajimoto K, et al. Intravenous autologous bone marrow mononuclear cell transplantation for stroke: Phase1/2a clinical trial in a homogeneous group of stroke patients. *Stem Cells Dev*. 2015;24:2207-2218

5.   Yang B, Parsha K, Schaar K, Xi X, Aronowski J, Savitz SI. Various cell populations within the mononuclear fraction of bone marrow contribute to the beneficial effects of autologous bone marrow cell therapy in a rodent stroke model.

Transl Stroke Res. 2016;7:322-330

6. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964-967

7. Taguchi A, Soma T, Tanaka H, Kanda T, Nishimura H, Yoshikawa H, et al. Administration of cd34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. *J Clin Invest*. 2004;114:330-338

8. Qadura M, Terenzi DC, Verma S, Al-Omran M, Hess DA. Concise review: Cell therapy for critical limb ischemia: An integrated review of preclinical and clinical studies. *Stem Cells*. 2018;36:161-171

9. Ramalingam P, Poulos MG, Butler JM. Regulation of the hematopoietic stem cell lifecycle by the endothelial niche. *Curr Opin Hematol*. 2017;24:289-299

10. Foss B, Hervig T, Bruserud O. Connexins are active participants of hematopoietic stem cell regulation. *Stem Cells Dev*. 2009;18:807-812

11. Kumar NM, Gilula NB. The gap junction communication channel. *Cell*. 1996;84:381-388

12. Nakano-Doi A, Nakagomi T, Fujikawa M, Nakagomi N, Kubo S, Lu S, et al. Bone marrow mononuclear cells promote proliferation of endogenous neural stem cells

through vascular niches after cerebral infarction. *Stem Cells*. 2010;28:1292-1302

13. Taguchi A, Kasahara Y, Nakagomi T, Stern DM, Fukunaga M, Ishikawa M, et al. A reproducible and simple model of permanent cerebral ischemia in cb-17 and scid mice. *J Exp Stroke Transl Med*. 2010;3:28-33

14. Kazerounian S, Lawler J. Integration of pro- and anti-angiogenic signals by endothelial cells. *J Cell Commun Signal*. 2018;12:171-179

15. Lee S, Chen TT, Barber CL, Jordan MC, Murdock J, Desai S, et al. Autocrine vegf signaling is required for vascular homeostasis. *Cell*. 2007;130:691-703

16. Nielsen MS, Axelsen LN, Sorgen PL, Verma V, Delmar M, Holstein-Rathlou NH. Gap junctions. *Compr Physiol*. 2012;2:1981-2035

17. Simons M. An inside view: Vegf receptor trafficking and signaling. *Physiology (Bethesda)*. 2012;27:213-222

18. Paradiso AM, Tsien RY, Machen TE. Na+-h+ exchange in gastric glands as measured with a cytoplasmic-trapped, fluorescent ph indicator. *Proc Natl Acad Sci U S A*. 1984;81:7436-7440

19. Gleadle JM, Ratcliffe PJ. Induction of hypoxia-inducible factor-1, erythropoietin, vascular endothelial growth factor, and glucose transporter-1 by hypoxia: Evidence against a regulatory role for src kinase. *Blood*. 1997;89:503-509

20. Bergeron M, Yu AY, Solway KE, Semenza GL, Sharp FR. Induction of hypoxia-inducible factor-1 (hif-1) and its target genes following focal ischaemia in rat brain. *Eur J Neurosci*. 1999;11:4159-4170

21. Wang P, Shao BZ, Deng Z, Chen S, Yue Z, Miao CY. Autophagy in ischemic stroke. *Prog Neurobiol*. 2018;163-164:98-117

22. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. *N Engl J Med*. 2009;361:1570-1583

23. Keaney J, Campbell M. The dynamic blood-brain barrier. *The FEBS journal*. 2015;282:4067-4079

24. Suda T, Takubo K, Semenza GL. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. *Cell stem cell*. 2011;9:298-310

25. Yoshioka K, Takahashi H, Homma T, Saito M, Oh KB, Nemoto Y, et al. A novel fluorescent derivative of glucose applicable to the assessment of glucose uptake activity of escherichia coli. *Biochim Biophys Acta*. 1996;1289:5-9

26. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem*. 1995;270:1230-1237

27. Liu Z, Jia X, Duan Y, Xiao H, Sundqvist KG, Permert J, et al. Excess glucose induces hypoxia-inducible factor-1alpha in pancreatic cancer cells and stimulates

glucose metabolism and cell migration. *Cancer Biol Ther*. 2013;14:428-435

28. Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, et al. Transcriptional regulation of vascular endothelial cell responses to hypoxia by hif-1. *Blood*. 2005;105:659-669

29. Simons M, Gordon E, Claesson-Welsh L. Mechanisms and regulation of endothelial vegf receptor signalling. *Nat Rev Mol Cell Biol*. 2016;17:611-625

30. Taguchi A, Zhu P, Cao F, Kikuchi-Taura A, Kasahara Y, Stern DM, et al. Reduced ischemic brain injury by partial rejuvenation of bone marrow cells in aged rats. *J Cereb Blood Flow Metab*. 2011;31:855-867

31. Guo JY, Teng X, Laddha SV, Ma S, Van Nostrand SC, Yang Y, et al. Autophagy provides metabolic substrates to maintain energy charge and nucleotide pools in ras-driven lung cancer cells. *Genes Dev*. 2016;30:1704-1717

32. White E, Mehnert JM, Chan CS. Autophagy, metabolism, and cancer. *Clin Cancer Res*. 2015;21:5037-5046

33. Zimna A, Kurpisz M. Hypoxia-inducible factor-1 in physiological and pathophysiological angiogenesis: Applications and therapies. *BioMed Res Int*. 2015;2015:549412

34. Spencer JA, Ferraro F, Roussakis E, Klein A, Wu J, Runnels JM, et al. Direct

measurement of local oxygen concentration in the bone marrow of live animals. *Nature*. 2014;508:269-273

35. Gonzalez-Nieto D, Chang KH, Fasciani I, Nayak R, Fernandez-Garcia L, Barrio LC, et al. Connexins: Intercellular signal transmitters in lymphohematopoietic tissues. *Int Rev Cell Mol Biol*. 2015;318:27-62

36. Okinaka Y, Kikuchi-Taura A, Takeuchi Y, Ogawa Y, Boltze J, Gul S, et al. Clot-derived contaminants in transplanted bone marrow mononuclear cells impair the therapeutic effect in stroke. *Stroke*. 2019;50:2883-2891

37. Hill J, Rom S, Ramirez SH, Persidsky Y. Emerging roles of pericytes in the regulation of the neurovascular unit in health and disease. *J Neuroimmune Pharmacol*. 2014;9:591-605

Figure Legends

Figure 1. Increased hVEGF uptake into HUVEC by co-culture with BM-MNC

(A) Significant decrease of the hVEGF concentration in culture medium was observed by co-culture with BM-MNC. In contrast, co-culture with BM-MNC separated from HUVEC by a cell culture insert did not reduce hVEGF levels. (B) HUVEC and BM-MNC could be clearly distinguished by anti-CD31 and anti-CD45 antibodies. (C) APC-labelled VEGF uptake in HUVEC was evaluated using FACS. The mean VEGF uptake (mean fluorescence intensity) in HUVEC and the % of APC-positive HUVEC was significantly increased with co-culture with BM-MNC. Blockade of gap junction (GJ) with 1-octanol or carbenoxolone neutralized the effect. BM-MNC separated from HUVEC by an insert did not increase VEGF uptake in HUVEC. (D) Representative fluorescent confocal image of hVEGF uptake into HUVEC (cytosol; red, nucleus; blue, uptaken hVEGF; green). Orthogonal confocal image (right panel) showed uptaken hVEGF is located in the cytosol. * p<0.01 versus medium, ** p<0.01 versus cell insert, n=3 in each group (A). * p<0.01 versus BM-MNC negative group, n=6 in each group (C). Arrows indicate hVEGF uptake. Orthogonal ZX and ZY views, centered on the white crosshairs. Scale bar, 4 μm (D).

Figure 2. Transfer of BCECF from BM-MNC to endothelial cells via gap junction *in*

vivo

(A) Although negligible expression of connexin 37 was observed in healthy cerebral cortex of non-stroke mice, it was clearly expressed in lesion area at 48 hours after stroke induction. Similarly, little expression of connexin 43 was observed in healthy cerebral cortex in non-stroke mice and increased expression was observed in lesion area after stroke. (B) 10 minutes after intravenous injection of BCECF-loaded BM-MNC. At post-stoke brain, BCECF-positive cells (green) were observed in the cerebral microvasculature (red). Image merging demonstrates the BCECF transfer from BM-MNC into endothelial cell across the CD31-positive endothelial cellular membrane. (C) Higher magnification of merged image with explanation. Transfers of BCECF from BM-MNC cytoplasm were observed across the endothelial cell membrane to endothelia cell nuclei. (D) Quantitative analysis revealed that the BCECF transfer from BM-MNC into endothelial cell is significantly suppressed by blockade of BM-MNC gap junctions. Scale bars, 40μm (A), 20μm (B), 4μm (C). * $p < 0.01$ versus BM-MNC group, n=10 in each group (D).

Figure 3 Localization of gap junction and transferred BCECF in endothelial cells

 (A) Spotty accumulations of connexin 37 (light blue) were observed at cell membrane of endothelial cells (red). Transferred BCECF signals (green) were observed around

connexin 37 positive spots. (B) Similarly, spotty accumulations of connexin 43 (light

blue) were observed at endothelial cells (red) with transferred BCECF signals (green).

(C) Merged image of connexin 43 with explanation. Signals of transferred BCECF were

also observed in non-endothelial cells which were located at outside of the vasculature.

(D) Quantitative analysis revealed that the number of Cx37/BCECF-positive or

Cx43/BCECF-positive endothelial cell was significantly decreased by blockade of gap-

junction of BM-MNC. Scale bars, 5µm (A-C). * p<0.01 versus BM-MNC group, n=5 in

each group (D).

Figure 4. Expression of Hif-1α after BM-MNC transplantation

(A) As well as cerebral cortex of non-stroke mice, little expression of Hif-1α was

observed in lesion area at 3 or 48 hours after stroke induction. However, increased Hif-

1α expression was observed in capillary-like structures at 1 hour after BM-MNC

transplantation. (B) BCECF signals (green) were co-localized with Hif-1α expression

(light blue) in CD31-positive endothelial cells (red). (C) Merged image with explanation.

The expressions of Hif-1α in endothelial cells were limited in cell that received BCECF

from BM-MNC. (D) No Hif-1α//BCECF-positive cell was observed in mice that received

BM-MNC treated with 1-octanol. Scale bars, 40µm (A), 10µm (B,C). * p<0.01 versus

BM-MNC group, n=10 in each group (D).

Figure 5. Ultrastructural changes in endothelial cells suggest reduced autophagy in BM MNC-transplanted animals

(A) Representative picture of a normal capillary composed of endothelial cells, basal lamina, pericytes and astrocyte foot processes in cerebral cortex of non-stroke mice (normal cortex) and representative pictures of capillaries in the lesion cortex, lesion border cortex and contralateral cortex. In lesion cortex, significant number of autophagosome-like vacuoles were observed in the endothelial cells in PBS treated mice. In contrast, despite of the disappearance of astrocyte foot processes, only a few autophagosome-like vacuoles with attached pericytes were observed in endothelial cells in BM-MNC treated mice. In the lesion border cortex, significant numbers of autophagosome-like vacuoles were observed in endothelial cells in PBS treated mice. In contrast, few autophagosome-like vacuoles were observed in BM-MNC treated mice. In the contralateral cortex, autophagosome-like vacuoles were observed in some endothelial cells in PBS treated mice. In contrast, no autophagosome-like vacuoles were observed in BM-MNC treated mice. (B) Quantitative analysis confirmed the impression that BM-

MNC transplantation suppresses the formation of autophagosome-like vacuoles in each area. (C) Glucose concentration in BM-MNC was significantly higher than that in HUVEC. (D) Significant increase of 2-NBDG level was observed in HUVEC by co-culture with BM-MNC. In contrast, the level of 2-NBDG in BM-MNC was significantly decreased by co-culture with HUVEC. Scale bars, 2μm (A). Arrowhead indicates autophagosome-like vacuole (A). *$p<0.05$ versus PBS, n=5 in each group (B). *$p<0.01$ versus BM-MNC, n=3 in each group (C). *$p<0.01$ versus without BM-MNC (left panel) or HUVEC (right panel). n=4 in each group (D).

Figure 6. Schematic illustration of our hypothesis

(A) Hypoxia is known to induce angiogenesis with activation of Hif-1α at endothelial cells. (B) However, drastic reduction of oxygen and glucose supply by severe ischemia dose not activate Hif-1α but induces autophagy. (C) Transplanted BM-MNC transferred small molecules to endothelial cells via gap junction followed by activated Hif-1α and suppressed autophagy at endothelial cells. It should be noted the molecular weight of Hif-1α protein is too large to pass through gap junction.