Role of M2-type macrophage-specific modulation in a time-dependent manner against post-ischemic brain damage in rat

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Abstract

Background

Cerebral ischemia triggers inflammatory changes, and early complications and unfavorable outcomes of endovascular thrombectomy for brain occlusion promote the recruitment of various cell types to the ischemic area. Although phenotype-specific monocytes/macrophages appear to play a role, the detailed effects remain unclear. To test our hypothesis that post-ischemic phase-dependent modulation of macrophages may represent a potential therapy against ischemic brain damage, we studied the significance of inducing anti-inflammatory M2-type, but not pro-inflammatory M1-type, macrophages after brain ischemia.

Methods

Seven-week-old male Wistar rats subjected to middle cerebral artery occlusion-reperfusion (MCAO-R) were treated for 7 days with an activator of M2-type macrophages, Gc-protein macrophage-activating factor (GcMAF), in the acute (day 0–6) or subacute (day 7–13) phase after ischemia induction and compared with vehicle-treated control rats.

Results

In MCAO-R rats, brain damage expansion elicited by increased mRNA levels of interleukin (IL)-6 and IL-1β abated on day 7. Acute-phase GcMAF treatment augmented anti-inflammatory CD163+ M2-type- and pro-inflammatory CD16+ M1-type macrophages, resulting in no beneficial effects. During days 7–14, GcMAF injection increased only CD163+ M2-type macrophages accompanied by elevated mRNA levels of arginase-1 and IL-4. M2-type macrophages co-localized with CD36+ phagocytic cells led to clearance of the infarct area which was abrogated by clodronate-liposomes. Finally, the expression of survival-related molecules on day 28 was augmented in the infarct border, suggesting that activation of M2-type macrophages in the subacute post-ischemia phase may play a therapeutic role.

Conclusion

Our findings require further studies to assess the time-dependent therapeutic significance of M2-type-macrophages against cerebral ischemia.

Background

Cerebral ischemia triggers inflammatory changes and early complications. Ischemia-reperfusion injury has recently been reported as a major cause of early complications and unfavorable outcomes after
endovascular thrombectomy therapy in patients with acute ischemic stroke (1). As a result, modulation of microglia/macrophage polarization and subsequent inflammatory response may represent a potential adjunctive therapy to recanalization.

In the perivascular space, ischemia and/or reperfusion activate perivascular macrophages and mast cells (2). Mast cell degranulation releases vasoactive mediators such as proteases and tumor necrosis factor (TNF), then activated macrophages release pro-inflammatory cytokines. These pro-inflammatory mediators contribute to the endothelial expression of adhesion molecules and to blood-brain barrier damage that promotes the infiltration of neutrophils, lymphocytes, and monocytes (3). Microglia/macrophages or monocyte-derived macrophages have been implicated in stroke-induced inflammation and injury (4).

Macrophages are mainly classified into M1 and M2 phenotypes (5–7). M1-type macrophages, characterized by increased secretion of pro-inflammatory mediators such as TNF-α, interleukin (IL)-1β, and IL-6, are present in cerebral ischemia as well as in myocardial infarction. M1-type macrophages may exacerbate neuronal death through the release of harmful mediators and nitric oxide (8) and impair axon regrowth. On the other hand, M2-type macrophages (anti-inflammatory macrophages) are thought to be mediated by cytokine IL-4 and arginase-1 (Arg-1) improving long-term neurological outcomes after stroke (9–12). Arg-1 is also associated with the reduction of inflammatory mediators, thereby promoting the survival of cortical neurons under ischemic/hypoxic conditions (12, 13, 14). Arg-1 is the enzyme that hydrolyzes the amino acid L-arginine to ornithine and urea. Deletion of Arg-1 worsens ischemic injury, suggesting a protective role of Arg-1 (15). In addition, M2-type macrophages accelerate axon growth/sprouting in cultured neurons, suggest a critical role in tissue recovery and re-innervation (15, 16). A post-stroke imbalance between M1- and M2-type macrophages may thus be associated with the expansion of neuronal ischemic damage or protective effects. However, the role of phenotype-specific macrophage activation in cerebral ischemia and the timeline in which these effects manifest is not well known. In particular, the therapeutic role of M2-type macrophage-specific modulation in a time-dependent manner against post-ischemic brain damage has not been studied.

Group-specific component Macrophage-Activating Factor (GcMAF) is a mammalian protein with an incredible potency to directly activate macrophages (17). Gc protein is a 53-kDa serum protein belonging to the albumin superfamily and its physiological functions include vitamin D transport and storage, and scavenging of extracellular G-actin (18). Of 120 isoforms of Gc proteins among humans, Gc protein is hydrolyzed by β-galactosidase of an activated B-cell and sialidase of a T-cell, respectively, to produce GcMAF. In an inflammatory response, the effects of GcMAF are that degalactosylated/desialylated bovine colostrum induces macrophage phagocytic activity as an immunostimulatory in clinical settings (19). In our preliminary experimental study, we confirmed that activation of macrophage by GcMAF exerted anti-inflammatory effects and phagocytic activity against mice brain damage subjected to microblood injection to imitate traumatic brain injury.
Given this background, we hypothesized that after cerebral infarction, modulation of M2-type macrophages in a phase-dependent manner under post-ischemia conditions may provide a potential therapy against ischemic brain damage under the right circumstances. To test our hypothesis, we used GcMAF to examine the phenotypic expression profile of macrophages and the changes in the secretion of cytokines over time, and the role of the activation of M2-type macrophages by GcMAF delivered in the acute and subacute phases of ischemia.

**Methods**

**Study approval**

This study was approved by the ethics committee of Tokushima University Graduate School of Biomedical Sciences in compliance with the animal care guidelines of Tokushima University, Tokushima, Japan. All animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use and the ARRIVE guidelines of Laboratory Animals (20). Seven-week-old male Wistar rats obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan) were housed in a temperature- and humidity-controlled room (approximately 23°C and 50%, respectively) under a 12-h light cycle (08:00–20:00) and were allowed free access to food and water. Before all surgical procedures, the animals were anesthetized by 2–4% isoflurane inhalation. Body temperature was monitored and maintained at 37°C using a warming plate (NISSIN, Tokyo, Japan). Investigators involved in all surgical procedures, drug treatments, and end-point assessments were blinded to the group to which each animal had been assigned.

**Focal cerebral ischemia induction**

Middle cerebral artery occlusion (MCAO) was induced with a 4–0 monofilament suture, as described previously (21–24). Blood flow to the region surrounding the MCA was measured with a laser Doppler flow probe (Unique Medical, Tokyo, Japan) to confirm its occlusion. A reduction in regional cerebral blood flow (rCBF) to less than 30% of the baseline indicated successful MCAO. The suture was withdrawn after 120 min to allow reperfusion. Ipsilateral blood flow was restored to approximately 80–100% of the baseline value. The extent of rCBF decrease before and the rCBF increase after reperfusion were identical in all rats with successful MCAO. Rats consistently displayed a circling behavior, decreased resistance to lateral push, forelimb flexion, and shoulder adduction. Rats with incomplete MCAO (approximately 10%) were excluded from further experiments.

**Measurement of infarct volume, assessment of phagocytosis and neurological deficit**

Sliced brain tissues were immersed in a 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution in PBS. The extent of ischemic infarction was traced and the integrated volume was calculated using BZ-X710 equipped image analyzing system (KEYENCE). To reduce artifacts from brain edema, we applied the indirect measurement method based on the contralateral brain volume (25). Infarct, cleaned, and non-
Infarct volumes were calculated as a percentage of the contralateral hemisphere. To determine the size of the cleaned area (%) we used the following formula:

\[
\text{Contralateral hemisphere volume} - \text{Ipsilateral hemisphere volume} \cdot \frac{100}{\text{Contralateral hemisphere volume}}.
\]

To assess the effects of GcMAF against brain damage in rats subjected to 2-h MCAO-reperfusion (MCAO-R), rats were randomly allocated into two groups receiving intra-peritoneal injection of GcMAF (40 ng/kg/day) or saline (vehicle control, VC) for 7 days on days 0–6 or days 7–14 and sacrificed on day 7 or day 14 (GcMAF, n = 6; VC, n = 6). To examine the phagocytic effects of macrophages after cerebral ischemia, rats subjected to 2-h MCAO-R were injected intraperitoneally with 10 ml/kg of clodronate-liposomes (CLOD) (Clophosome®-A, Tribioscience, CA, USA) according to the instructions from the manufacturer (0.2 ml/20–25 g) on day 7 and then received injections of GcMAF (40 ng/kg/day) or saline (CLOD, n = 6; CLOD plus GcMAF, n = 6) and underwent determination of mRNA levels in the peri-infarct area.

Another set of rats in each group was assessed immunohistochemically (n = 5). The other MCAO-R rats were recorded neurological score after treatment during days 7–28 and rats were sacrificed on day 28 (GcMAF, n = 6; VC, n = 6) for immunohistochemical assessment.

Neurological deficits were assessed by an examiner blinded to the treatment the rats had received. According to neurological scoring as described by Huang et al. (26) and Yang et al. (27), we recorded as a score from 0 to 12 after successful MCAO-R.

**Preparation of GcMAF**

GcMAF and biotin-labeled GcMAF were prepared by Uto et al. (18) at the Faculty of Bioscience and Bioindustry, Tokushima University and intraperitoneally injected every day at a dose of 40 ng/kg/day on days 0–6, days 7–14 or days 7–28 after microblood injection into the brain. To examine the distribution of intraperitoneally injected GcMAF in the brain, rats were injected daily with biotin-labeled GcMAF (40 ng/kg/day) starting on the day following 2-h MCAO-R.

**Immunohistochemistry**

Rat brains were transcardially perfused with 4% paraformaldehyde in PBS on ice. After fixing, 10-µm-thick frozen sections were mounted on Matsunami adhesive silane-coated glass slides (Matsunami Glass, Tokyo, Japan). After 30 min blocking with serum-free protein block (Dako Cytomation, Agilent, Tokyo, Japan), slides were incubated with primary antibodies diluted with Canget signal immunostain (Toyobo, Osaka, Japan).

Antibodies were mouse monoclonal antibody against CD16 (sc-52376; Santa Cruz Biotechnology, Texas, USA), CD163 (Abcam, Tokyo. Japan), rabbit monoclonal against CD36 (ab133625) and CD68 (Abcam), rabbit polyclonal against neuronal nucleus (NeuN. Ab104225) and nestin (Chemicon, CA, USA). Antibody against vascular endothelial growth factor (VEGF) (SC-7269; Santa Cruz Biotechnology) was also used, as was rabbit polyclonal antibody against MAP2 (SC-20172; Santa Cruz Biotechnology), goat polyclonal
antibody against glial fibrillary acidic protein (GFAP) (SC-6170; Santa Cruz Biotechnology), and CD36 (SC-7641; Santa Cruz Biotechnology). Sheep polyclonal antibody was used against bromodeoxyuridine (BrdU) (Abcam). was dissolved in 0.9% saline (concentration, 100 mg/ml). To label proliferating cells in the brain, rats received intraperitoneal injections of BrdU (50 mg/kg) at 48, 24, 12, and 4 h before sacrifice. To study progenitor cell proliferation, sacrifice was performed 4 h after the 4th BrdU injection on day 28. To examine the specificity of immunoreactivity, the primary antibody was omitted to provide a nonspecific control. Visualization was achieved with Alexa Fluor 594 donkey anti-mouse immunoglobulin (Ig)G, 594 rabbit anti-mouse IgG, 594 donkey anti-rabbit IgG, 594 donkey anti-goat IgG, 488 goat anti-rabbit-, 488 donkey anti-goat-, or 488 donkey anti-sheep IgG (Molecular Probes, Eugene, OR). The positive area was assessed under BZ-X710 microscope equipped with an image analyzing system (Keyence).

**RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted with MagNA Pure Compact (Roche Diagnostics, Tokyo, Japan). For the reverse transcription of total RNA to cDNA we used a transcriptor first-strand cDNA synthesis kit (Roche Diagnostics). For each sample, qRT-PCR was performed in a Light Cycler 2.0 system (Roche Diagnostics). Light Cycler Fast Start DNA master and SYBR green I (Roche Diagnostics) were used for IL-6, IL-1β, IL-4, Arg-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers were: forward (F), 5’-TCT CAG GGA GAT CTT GGA AAT G-3’; reverse (R), 5’-TAG AAA CGG AAC TCC AGA AGA C-3’ for rat IL-6; (F), 5’-TGC AGG CTT CGA GAT GAA C-3’, (R), 5’-AGC TCA TGG AGA ATA CCA CTT G-3’ for rat IL-1β; F, 5’-CAC CGA GAT GTT ACC AGA-3’, R, 5’-TGA AGC TGC TTT CAA TTG CCA TA-3’ for rat Arg-1; and F, 5’-TAC ACT GAG GAC CAG GTT G-3’, R, 5’-CCC TGT TGC TGT AGC CAT A-3’ for rat GAPDH. PCR conditions were: 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 8 s. The results were normalized to the expression of GAPDH mRNA.

**Statistical analysis**

All data are presented as the mean ± standard deviation (SD). Differences between two groups were examined using Student’s t-test and differences among three groups with the Tukey-Kramer test. Differences of p < 0.05 were considered statistically significant. Statistical analyses were conducted using JMP version 13.2 software (SAS Institute Inc., NC, USA).

**Results**

Infarct area accompanied by an increase in M1-type macrophages and inflammatory cytokine in the early phase of post-ischemia

In rats subjected to 2-h MCAO-R (Fig. 1A), the infarct area was largest on day 1, decreased gradually until day 7, then stabilized (Fig. 1B). On day 3 of post-ischemia, expression of CD16+ was larger than the expression of CD163+ macrophages and both macrophages were significantly elevated compared to
sham rats (Fig. 1C). The mRNA levels of \textit{IL-6} and \textit{IL-1\beta} were significantly higher in MCAO-R rats than in sham-operated rats on day 1; then decreased to baseline on day 7 as in sham-operated rats (Fig. 1D). These findings indicate that the expansion of ischemic brain damage was accompanied by increased pro-inflammatory cytokines in the acute phase, and stabilized on day 7, reflected by the reduced mRNA of \textit{IL-6} and \textit{IL-1\beta} in MCAO-R. In contrast, mRNA levels of \textit{Arg-1} and \textit{IL-4} showed no significant difference between sham and MCAO-R rats during the 7 days (Fig. 1D). These findings suggest that inflammatory cytokines were elevated transiently and accompanied by larger expression of CD16\textsuperscript{+} than CD163\textsuperscript{+} macrophages in the acute phase.

**Activation of M2-type macrophages in the acute phase of post-ischemia led to increased M1-type macrophages**

Next, to examine the role of M2-type macrophage activation in the acute phase, we used GcMAF, an activator of M2-type macrophage. Unfortunately, treatment with GcMAF seemed to enlarge the infarct area on days 1, 3 and 7 (Fig. 2A, B), which significantly increased CD16\textsuperscript{+} M1-type macrophages as well as CD163\textsuperscript{+} M2-type macrophages, compared to VC in the penumbra adjacent the infarct area (Fig. 2C). Both CD16\textsuperscript{+} and CD163\textsuperscript{+} macrophages co-localized with CD36\textsuperscript{+} cells (Fig. 2D). Together, under conditions of high \textit{IL-6} and \textit{IL-1\beta} (Fig. 1D) in the acute phase, CD163\textsuperscript{+} M2-type macrophages induced by GcMAF may transform phenotypes, resulting in an elevation of CD16\textsuperscript{+} M1-type macrophages and leading to expansion of the cerebral infarct area (Fig. 2E). These findings seem consistent with the results from Hu et al. (5) and Caire et al. (28).

Clearance of the infarct area was associated with activation of M2 macrophages by GcMAF in the subacute phase of post-ischemia

We further examined the effects of cerebral infarction on day 14 after treatment with GcMAF during days 7–13 (subacute phase) after ischemia induction using serial slices (Fig. 3A). No difference was seen in size of the non-infarcted area (Fig. 3B), while the disappeared area was significantly larger in all GcMAF-treated rats than in VC rats, resulting in significantly smaller cortical infarct area in all GcMAF-treated rats than in VC rats (Fig. 3B). Immunohistochemically, we found that expression levels of CD16\textsuperscript{+} cells were low and no difference was evident between VC and GcMAF treatment groups (Fig. 3C). On the other hand, CD163\textsuperscript{+} cells were highly expressed on day 14 after GcMAF treatment during the subacute phase (day 7–13) and accompanied the elevation of CD36\textsuperscript{+} phagocytic cells (Fig. 3C). The disappeared cortical infarct area seemed to be associated with phagocytosis via the increased M2-type macrophages resulting from GcMAF administration.

**Phagocytosis activated by GcMAF in the infarct area is attributable to M2 macrophages**
To further confirm the phagocytosis by activation of M2 macrophages on the infarct area, we administered CLOD one day before the delivery of GcMAF in the subacute phase after ischemia induction. CLOD ingested macrophages via endocytosis, fusing with the lysosomes, thereby disrupting and killing macrophages (29, 30). Based on the report that CLOD encapsulated in liposomes at a concentration of 7 mg/ml depletes 90% of macrophages within 24–36 h after systemic administration, CLOD was injected 1 day before treatment with GcMAF (Fig. 4A).

Injection of CLOD abrogated the GcMAF-induced decrease in the cortical infarct area and the increase in the phagocytosed area (Fig. 4B) without affecting the non-infarct area (data not shown). The \( IL-6 \) mRNA level was low and unaffected by CLOD, whereas the mRNA level of \( Arg-1 \) was significantly higher in GcMAF-treated rats than in VC rats; this increase was abolished by CLOD (Fig. 4C).

Immunohistochemically, CLOD abolished the GcMAF-induced increase in CD163\(^+\) M2-type macrophages and CD36\(^+\) cells without affecting CD16\(^+\) M1-type macrophages (Fig. 4D). Together, these findings demonstrated the clearance of cortical infarct area and the elevation of Arg-1 via activation of M2-type macrophages increased by GcMAF.

**Expression of neurogenesis- and angiogenesis-related molecules in the peri-infarct area associated with M2-type macrophage activation**

Finally, to assess whether the activation of M2-type macrophages by GcMAF is associated with the expression of neurogenesis- and angiogenesis-related molecules, we evaluated the expression of MAP2, BrdU, and nestin, a neuronal stem cell marker in rats treated with GcMAF during days 7–28 after ischemia induction. In the cortical peri-infarct area, expressions of MAP2, BrdU, and nestin were significantly higher in GcMAF-treated rats than in VC rats (Fig. 5A). The appearance of neurovascular unit constituents involving VEGF, GFAP and the neuronal cell marker NeuN was also observed in the cortical peri-infarct area of GcMAF-treated rats, but not VC rats (Fig. 5B). However, we could not observe significant neurological improvements in GcMAF-treated rats on day 28 (data not shown). Assessment after a longer duration of treatment with GcMAF may be required.

**Discussion**

Several studies have reported that activation of M2-type macrophages is protective against cerebral ischemia. However, we first demonstrated that the significance of activating M2-type macrophages differed between the acute and subacute phases after cerebral ischemia in rats. Importantly, under conditions of the predominant expression of M1 macrophages with the elevation of IL-6 and IL-\( \beta \) in the acute phase of post-ischemia, specific activation of M2 macrophages by GcMAF did not exert beneficial effects. Unfortunately, GcMAF augmented the inflammatory response, thereby expanding the cortical infarct area. Under the predominant conditions of M1 macrophages accompanied by increased IL-1\( \beta \) and IL-6 cytokines, treatment with GcMAF increased pro-inflammatory M1-type macrophages as well as anti-inflammatory M2 type-macrophages (Fig. 2E). On the other hand, treatment with GcMAF in the subacute
phase of post-ischemia was associated with “cleaning” of the infarcted area by phagocytosis through the activation of M2-type macrophages without affecting M1-type macrophages, leading to high expression of neurogenesis- and angiogenesis-related molecules in the late phase (Fig, 5C). Our findings suggest that phase-specific activation of M2 macrophages post-ischemia may be crucial to the induction of beneficial effects. The role of M2-type macrophage-specific activation after cerebral ischemia may differ depending on the post-ischemic phase. We therefore emphasize that the timing leading to activation of M2-type macrophages should be taken into consideration.

Microglia/macrophages are activated within minutes of ischemic onset and induce the production of inflammatory cytokines, including IL-1β and TNF-α (29). After the rapid activation of resident microglial cells, blood-derived macrophages are recruited into ischemic brain tissue and rapidly mobilized to the site of injury, where they initiate the release of effector molecules and recruit other immune cells (1, 16). Since mRNA levels of IL-6 and IL-1β were higher on day 1 than on day 7 after cerebral ischemia, these increases may be attributable to the high expression of M1 macrophages in our study. In association with the up-regulation of IL-6 and IL-1β, expression of M1 macrophages increased on day 1 and reached baseline levels on day 7. In the early post-ischemic phase, expression of CD16+ M1 macrophages was dominant. As this phenomenon coincided with expansion of the ischemic penumbra in the presence of increased cytokines, the presence of M1 macrophages may be associated with the detrimental effects observed in the early post-ischemic phase. Hu et al. (5) reported that the mRNA level of IL-6 gradually increased in the ischemic core over the course of 14 days. A more recent study documented post-ischemic changes in IL-1β and TNF-α via modulating microglial activation and M1 polarization (29, 30). These findings partly coincide with our findings. Since GcMAF had no beneficial effects under the condition of high expression of M1-type macrophages with IL-6 and IL-1β, the timing of M2-type macrophage activation should be reconsidered.

Despite the decrease in mRNA levels of IL-1β and IL-6 on day 7 after ischemia induction, brain Arg-1 and IL-4 mRNA levels were retained during observation periods for 14 days. Subacute-late phase treatment with GcMAF produced an M2-type macrophage-dominant condition without affecting M1-type macrophages, and an increase in “clearance” through phagocytosis of the infarcted area. We confirmed that the effects were abrogated by CLOD (30). Since CD163+ M2-type macrophages induced by GcMAF were co-localized with CD36+ cells, but not Iba-1+ cells (data not shown), the delivered GcMAF may play a role in the activation of M2-type monocytes/macrophages derived from peripherally to perform phagocytosis.

Phagocytosis promotes the secretion of anti-inflammatory mediators, and thus may contribute to the creation of favorable post-stroke conditions for brain recovery (15). The M2 macrophage-related post-ischemic production of TGF-β, IL-10, and Arg-1 may facilitate tissue repair by promoting the resolution of inflammation and provision of direct cytoprotective effects to surviving cells in the ischemic territory (1, 15). These earlier findings support our observation that the increase in Arg-1 elicited by GcMAF was associated with the expression of survival-related molecules in the peri-infarct area.
Other studies (31, 32) have also suggested that M2-type macrophages play a crucial role in resolving the inflammatory response and participate in debris scavenging, tissue remodeling, and angiogenesis. Consistent with these findings, when M2-type macrophages dominated in the subacute-phase of post-ischemia, phagocytosis may accelerate to remove debris including apoptotic or necrotic cells without affecting the non-infarct area in this study. In addition, the increase in M2-like macrophages was associated with expression of survival-related molecules in the peri-infarct area. Thus, activation of M2-type macrophages by subacute- to late-phase treatment with GcMAF may have beneficial effects (32, 33)

**Study limitation**

This study shows some limitations. How subacute-to late-phase treatment with GcMAF activates M2 macrophages remains unknown. GcMAF is thought to be an activator of macrophage phagocytosis, but does not switch the cell polarization to M1-like macrophages. Uto et al. (17) reported that *in vivo* and *in vitro*, degalactosylated/desialylated bovine colostrum indues macrophage phagocytic activity without affecting the production of inflammatory cytokines such as TNF-α and IL-1β. Desestret et al. (32) showed that in co-cultures of bone-marrow-derived macrophages and in hippocampal slices subjected to oxygen and glucose deprivation, as a condition mimicking cerebral ischemic conditions, M2-type macrophages provided potent protection against neuronal cell loss. When they evaluated the possibility of M2-type macrophage cell therapy for stroke, treatment in the acute phase (day 4) failed to improve stroke outcomes. In IL-4-KO mice with experimental brain ischemia, M2 macrophages were reduced, and treatment with IL-4 resulted in neurological recovery from day 14 through M2-like macrophages. However, IL-4 injection into wild-type mice had no favorable effects (11). We found that M1 macrophages remained potent even 3 days after ischemia induction (data not shown) and the level of *IL-4* mRNA was retained, suggesting that the timing of treatment with M2 macrophages is key to successful cell therapy and that a reduction in the number of M1 macrophages in the early phase (within 7 days) is needed to obtain the beneficial effects of M2-like macrophage cell therapy.

The GcMAF dose delivered in our experimental studies was based on earlier clinical and basic studies (18, 19). In mice and in *in vitro* studies, GcMAF extracted from human plasma promoted phagocytic activity via macrophage activation (17, 18, 19). but the phenotype of the involved macrophages remained to be identified and we do not yet know the detailed mechanisms by which GcMAF activates M2 macrophages. Iadecola and Anrather (2) proposed a process orchestrated by “find me” and “eat me” signals. The “find me” signal involves purines released from injured cells and chemokines that attract microglia and macrophages to the site of injury. The possible mechanisms underlying phagocytosis by M2-like macrophages remain to be elucidated.

**Conclusion**

In the subacute to late post-ischemic phase, but not the acute phase, we offer the first documentation that the increase in M2-type macrophages elicited by treatment with GcMAF was associated with promotion of phagocytosis in the infarct area without affecting non-infarct area and high expression of
survival-related molecules in the peri-infarct area. Our findings may facilitate the development of new therapeutic strategies in a phase-dependent manner for post-stroke individuals.

**Abbreviations**

GcMAF: group-specific component protein-derived macrophage activating factor

MCAO-R: middle cerebral artery occlusion-reperfusion

VC: vehicle control

IL: interleukin

BrdU: bromodeoxyuridine

TNF: tumor necrosis factor

BSA: bovine serum albumin

CLOD: clodronate-liposomes

CBF: cerebral blood flow

TTC: 2,3,5-triphenyltetrazolium chloride

PBS: phosphate-buffered saline

MAP2: microtubule-associated protein 2

NeuN: neuronal nucleus

VEGF: vascular endothelial growth factor

GFAP: glial fibrillary acidic protein

HRP: horseradish peroxidase

qRT-PCR: quantitative real-time polymerase chain reaction

cDNA: complementary deoxyribonucleic acid

mRNA: messenger ribonucleic acid

Arg-1: arginase-1

GAPDH: glyceraldehyde-3-phosphate dehydrogenase
SD: standard deviation

ANOVA: analysis of variance

TGF-β: transforming growth factor β

**Declarations**

**Ethics approval and consent to participate**

All animal experiments were approved by the ethics committee of the Institute of Tokushima University Graduate School, and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

Conception and design of study: YK, KTK

Acquisition of data: TK, KS, KY, TM, MS,

Analysis and/or interpretation of data: KY, KS

Drafting the manuscript: YK, HK

Revising the manuscript critically for important intellectual content: KTK, YT,
Approval of the version of the manuscript to be published: YU, YT

All authors read and approved the final manuscript.

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None

References


Figures
Figure 1

**High expression of CD16\(^+\) but not CD163\(^+\) cells associated with elevation of cytokines IL-6 and IL-1b in the acute phase after cerebral ischemia-reperfusion**

A. Experimental protocol. Rats were subjected to middle cerebral artery occlusion for 2-h and reperfusion (MCAO-R) under anesthesia and compared to sham-operated rats.

B. Changes of infarct area in cortex and basal ganglia

C. The expression of CD16\(^+\) and CD163\(^+\) cells on 3 days after middle cerebral artery occlusion-reperfusion (MCAO-R). \(* p<0.05\) vs. sham-operated rats by Student’s \(t\)-test, \# \(p<0.05\), CD16\(^+\) vs. CD163\(^+\) cells in MCAO-R rats

D. Changes in mRNA levels of IL-6, IL-1b, Arg-1 and IL-4 in the acute phase after MCAO-R. Data are given as mean ± SD (n=6). \(* p<0.05\) vs. sham by Student’s \(t\)-test.
**Figure 2**

**Deleterious effects by M2-type macrophage activation in the acute phase after cerebral ischemia-reperfusion**

A. Experimental protocol. Each cortical infarct area was determined on days 1, 3, and 7 after acute-phase treatment with GcMAF (Days 0–6) and compared to vehicle control (n=6).

B. Changes in infarct area shown as a percentage of the contralateral hemisphere in VC and GcMAF groups. Data are given as mean ± SD (n=6).

C. Expression of CD16⁺ and CD163⁺ cells on day 7 after treatment with VC and GcMAF. Data are given as mean ± SD (n=4). *p<0.05 vs. sham by Student’s *t*-test.

D. Immunohistochemical staining of CD16⁺ and CD163⁺ cells co-localized with CD36⁺ phagocytic cells and CD68⁺ monocyte/macrophages.

E. Schematic diagram; deleterious effects of treatment with GcMAF in the acute phase of post-ischemia.
**Figure 3**

Effects of M2-type macrophages activation during the subacute phase (days 7–13) after MCAO-R

A. Experimental protocol and photographs of brain slices treated with GcMAF on day 14 after MCAO-R.

B. Non-infarct area, cortical infarct area and phagocytosed area shown as a percentage of the contralateral hemisphere. Each data indicates mean ± SD (each, n=6).

C. Expression of CD16\(^{+}\) M1- and CD163\(^{+}\) M2-type macrophages and CD36\(^{+}\) cells assessed immunohistochemically in the peri-infarct region on day 14 of post-ischemia. Data are given as mean ± SD (each, n=4). \(*p<0.05\) vs. VC by Student's t-test.
Effect of treatment with GcMAF in the subacute phase of post-ischemia with or without clodronate-liposome (CLOD) pre-treatment

A. Experimental protocol. CLOD (10 ml/kg, intraperitoneal) was injected one day before treatment with GcMAF during days 7–13 and each assessment was performed on day 14.

B. Cortical infarct area and phagocytosis area on day 14 are shown as percentages of the contralateral hemisphere.

C. The mRNA levels of IL-6 and arginase-1 (Arg-1) determined in the penumbra on day 14. Data are given as mean ± SD (each group, n=7).

D. Immunohistochemical expressions of CD16+ M1-type macrophages, CD163+ M2-type macrophages and CD36+ phagocytes in the peri-infarct region of rats treated with GcMAF. Data are given as mean ± SD (n=4). *p<0.05 vs. VC by ANOVA followed by Scheffe’s test.
Figure 5

**Expression of neuro- and angiogenesis-related molecules after treatment with GcMAF in the subacute to late phase of post-ischemia**

A. Expression of MAP2, BrdU, and Nestin in the ischemic penumbra. Data are given as mean ± SD (each group n=4, *p<0.05 vs. VC by Student’s t-test).

B. Expressions of VEGF, GFAP, and NeuN in the ischemic penumbra of GcMAF-treated rats.

C. Schematic diagrams. Effects of macrophages modulated in the acute or subacute to late phase against brain ischemia and the role of GcMAF.