Original article

Folia Neuropathologica

Effects of hypothermia on *ex vivo* microglial production of pro- and anti-inflammatory cytokines and nitric oxide in hypoxic-ischemic brain-injured mice

Tomohiro Matsui[1], Hiroyuki Kida[2], Takuya Iha[1], Tabito Obara[1], Sadahiro Nomura[3], Tatsuya Fujimiya[4], Michiyasu Suzuki[3]

[1]Department of Laboratory Sciences, Yamaguchi University Graduate School of Medicine, [2]Department of Systems Neuroscience, Yamaguchi University Graduate School of Medicine, [3]Department of Neurosurgery, Yamaguchi University Graduate School of Medicine, [4]Department of Legal Medicine, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan

Folia Neuropathol 2014; 52 (2): 151-158 DOI: 10.5114/fn.2014.43786

Abstract

Introduction: *Activated microglia produce neurotoxic factors, including pro-inflammatory cytokines and nitric oxide (NO), in response to neuronal destruction. Therapeutic suppression of microglial release of these factors by various approaches including hypothermia is considered to be neuroprotective after severe brain damage. We examined the effects of hypothermic culture on the production of pro- and anti-inflammatory cytokines and NO in ex vivo microglia that were derived from mice with hypoxic-ischemic (HI) brain injury, through the stimulation of toll-like receptors (TLRs) that play significant roles in the pathological processes underlying a sterile central nervous system injury.*
Material and methods: *Two-day-old mice underwent the right common carotid artery ligation followed by 6% oxygen for 30 min, and thereafter were placed at 37°C for 24 h, after which microglia were isolated and then cultured with TLR2 and TLR4 agonists at 33°C and 37°C. Cytokine and NO levels in culture supernatants were measured.*
Results: *Compared with 37°C, hypothermia (33°C) reduced the production of tumour necrosis factor-alpha (TNF-α: a pro-inflammatory cytokine) at 6 h and interleukin-10 (IL-10: an anti-inflammatory cytokine) and NO at 48 h.*
Conclusions: *In TLR-activated microglia that were derived from mice with HI brain injury, hypothermia reduced the production of TNF-α, IL-10, and NO temporally, a clinically relevant finding suggesting that neuroprotection conferred by therapeutic hypothermia is related to attenuation of early-phase and late-phase inflammatory factors as well as that of late-phase anti-inflammatory factor(s) released from microglia.*

Key words: *hypothermia, hypoxic-ischemic brain injury, microglia, pro-inflammatory cytokine, anti-inflammatory cytokine, toll-like receptor, nitric oxide, ex vivo setting, temporal change.*

Introduction

Increased levels of several pro-inflammatory cytokines, such as interleukin-1 (IL-1) and interleukin-6 (IL-6), and nitric oxide (NO) in the cerebrospinal fluid (CSF) have been reported after severe head injuries in humans [6,13,32]. These potentially neurotoxic factors are produced by activated microglia when neurons are destroyed following ischaemia or trauma [38,48], and they are associated with secondary brain dam-

Communicating author:
Tomohiro Matsui, Department of Laboratory Sciences, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-kogushi, Ube, Yamaguchi 755-8505, Japan, phone/fax: +81-836-22-2865, e-mail: giants@yamaguchi-u.ac.jp

age [3,21,40]. Therapeutic suppression of microglial release of the above-mentioned factors by various approaches including hypothermia is considered to be neuroprotective after severe brain damage such as that occurring after traumatic brain injury (TBI) and cardiac arrest [1,7,23,32]. Previously, we and other groups have demonstrated a reduced production of tumour necrosis factor-alpha (TNF-α: another pro-inflammatory cytokine that is associated with neuronal injury [3,40]), IL-6, interferon-beta (IFN-β, which is known to be associated with neuronal cell death [15]), interleukin-10 (IL-10: an anti-inflammatory cytokine), and NO in hypothermic culture conditions [20,31,33-36,42]. In particular, we proposed that these effects were temporally related to the reduction of early-phase and late-phase inflammatory factors as well as that of late-phase anti-inflammatory factor(s) [33-36].

In the present study, to further explore the relationship between hypothermia and microglial responses in a more clinically relevant manner, we examined the effects of hypothermic culture on the temporal production of TNF-α, IL-10, and NO as representative pro- and anti-inflammatory factors in *ex vivo* microglia derived from mice with hypoxic-ischemic (HI) brain injury, through the stimulation of the toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4). An *ex vivo* setting was chosen as it more closely reflects *in vivo* conditions compared with cell-culture systems *in vitro* [18,44], and the cultures of *ex vivo* microglia obtained directly from the central nervous system (CNS) show functional and temporal similarities in the responsiveness of these cells *in vivo* to a stimulus [12,51]. Moreover, while TLRs are major sensors of pathogen-associated molecular patterns that mediate innate immunity and are involved in the adaptive immune response [2], they can also be stimulated by the non-physiological appearance or unusual concentrations of certain endogenous molecules [24], which may be produced and released by damaged cells in CNS. Thus, TLRs play a significant role in the pathological processes underlying sterile CNS injury. For example, both TLR2- and TLR4-deficient mice exhibit reduced cerebral ischaemia-induced CNS injuries [10,27]. Both microglial TLR2 and TLR4 establish mechanisms by which innate immunity perpetuates CNS inflammation and neuronal damage by responding to endogenous compounds [5,22,28]. Therefore, an understanding of TLR-driven neuroinflammation in microglia derived from a brain injury model, along with their responses to hypothermia, was of particular significance in this study.

Material and methods

All protocols in this study were reviewed and approved by the Animal Care Committee of Yamaguchi University School of Medicine.

Animal model

HI brain injury was induced in the Institute of Cancer Research (ICR) mice on postnatal day 2 (P2). Pups of either sex were anesthetized with halothane (4% for induction, 2% for maintenance) in a 75 : 25 mixture of N_2O and O_2, after which the right common carotid artery was isolated and ligated. The procedure was completed within 5 min. After the procedure, the pups recovered for 1 h in a temperature-controlled incubator. They were then placed in a chamber perfused with a humidified gas mixture (6% oxygen in nitrogen) for 30 min. The temperature in the incubator and that of the water used to humidify the gas mixture were kept at 37°C. The operated pups were placed without the dam in a chamber submerged in a water bath at a stable temperature of 37°C for 24 h, where they entered the P3 stage. Control animals were anesthetized, but not subjected to HI.

To identify the extent of the brain injury induced by HI followed by 24 h at 37°C, hematoxylin staining was performed. In brief, at the end of the 24-h period after the HI, the animals were deeply anesthetized with an overdose (40 mg/kg) of pentobarbital and perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde in phosphate buffer (PB). The brain was removed for post-fixation in 4% paraformaldehyde in PB for 4-12 h. It was then embedded in paraffin, cut into 4-µm coronal sections with a microtome, and attached to silane-coated glass slides. After slides were deparaffinized in xylene and hydrated to distilled water, hematoxylin staining was conducted. In the injured animals, mild laminar disruption was observed in the ipsilateral hemisphere to the injury, while cortical organization in the contralateral hemisphere was normal (Fig. 1). We used the brains from the injured animals in this study.

We are aware that P7 rats have become a widely used model for the study of HI brain injury [17,19,29, 46,49]. However, the overall increases in the severity

of cerebral lesions on the side of the common carotid artery ligation are similar between P2-P3 and P7 rats, while HI brain injury produces age-dependent and region-specific injuries [45]. In addition, we chose to examine mice and not rats in this study because the only commercially available kit separates mouse, but not rat, microglia (see below for the details of the method used to separate microglia).

In preliminary experiments, we first examined the possibility that microglia taken from injured brains may have become primed and accordingly responded more robustly to subsequent TLR2 and TLR4 challenges for 6 h at 37°C (see below for the culture method details). To do this, we compared microglial TNF-α production in mice with P3 HI brain injury with that from day-matched (P3) control animals; however, the TNF-α levels were very similar in injured and control animals. Next, we compared microglial TNF-α production from the ipsilateral hemisphere to the injury with that from the contralateral hemisphere in P3 HI brain-injured mice in the same culture system and, again, found a very little difference between the two groups. Based on these preliminary experiments, we decided to use both hemispheres of P3 HI brain-injured mice for the extraction of microglia. This was consistent not only with the same model used for determining the markers of apoptosis and necrosis [39] but also with another model of focal ischemic brain injury that has been used for determining the expression of several inflammatory factors [41]. In contrast, we are aware that *in vivo* pro-inflammatory cytokine levels and histological microglial activation in the ipsilateral hemisphere of HI brain-injured rats were higher than those in the contralateral hemisphere, albeit in a time-dependent manner [17,43].

Magnetic cell sorting of microglia using CD11b MicroBeads

Both cerebral hemispheres from P3 HI brain-injured pups (4-7 pups for each experiment) were removed and converted to a single-cell suspension by enzymatic degradation using a neural tissue dissociation kit and a gentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. In brief, brain tissues were weighed before mincing, enzyme mixes were added to the tissue pieces, and the mixture was dissociated both by mechanical trituration and by agitation at 37°C. The single-cell suspension was then

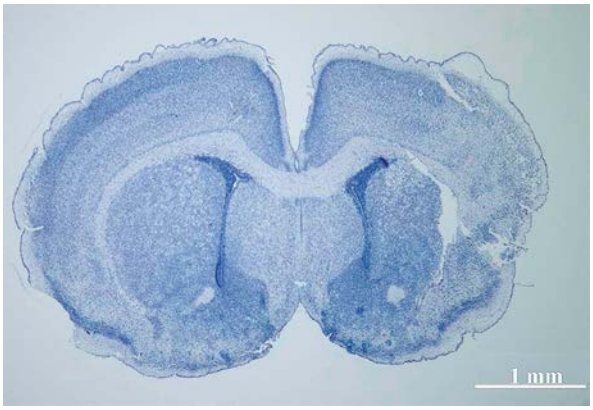

Fig. 1. Representative photomicrograph of hematoxylin staining of the brain after hypoxic-ischemic injury followed by 24 h at 37°C, demonstrating mild laminar disruption in the ipsilateral (right) hemisphere to the injury and normal cortical organization in the contralateral (left) hemisphere. White scale bar = 1 mm.

poured into a 70-µm cell strainer. The resulting cells were processed immediately using MACS MicroBead separation as follows. To separate the microglia, the CD11b-positive cells were magnetically labelled with CD11b (microglia) MicroBeads (Miltenyi Biotec). The cell suspension was loaded onto a MACS column (Miltenyi Biotec), which was placed in the magnetic field of a MACS separator (Miltenyi Biotec). The magnetically labelled CD11b-positive cells were retained within the column. After removing the column from the magnetic field, the magnetically retained CD11b-positive cells were eluted as the positively selected cell fraction. These procedures were completed within 3.5 h. The purity of the separated microglia was confirmed at > 91% by double staining with CD11b and CD45 antibodies (Miltenyi Biotec) using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). The microglia in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% foetal bovine serum (FBS) were allowed to stabilize for 1 day in a 5% CO_2 incubator at 37°C before the cells were stimulated in subsequent experiments.

Microglial cell culture

Microglia (4×10^4 cells/well in untreated 96-well plates (Becton Dickinson)) were cultured with or without 10 ng/ml Pam_3CSK_4 (*N*-palmitoyl-*S*-(2,3-bis (palmitoyloxy)-(2R,S)-propyl)-(*R*)-cysteinyl-seryl-(lysyl) 3-lysine: a TLR2 agonist) (Imgenex, San Diego, CA,

USA) or 1 μg/ml lipopolysaccharide (LPS: a TLR4 agonist) (Sigma-Aldrich, St Louis, OH, USA) to measure TNF-α, IL-10, and NO production. Culture was performed in DMEM containing 10% FBS in a 5% CO_2 incubator at temperatures of 33°C (hypothermia) and 37°C (normothermia) for 6 h for TNF-α assays and 48 h for IL-10 and NO assays. In our preliminary investigations, the doses of the TLR agonists (for Pam_3CSK_4; 10 and 100 ng/ml and for LPS; 0.001, 0.01, 0.1, and 1 μg/ml) for TNF-α production for 6 h were tested. Accordingly, the Pam_3CSK_4 dose of 10 ng/ml and LPS dose of 1 μg/ml were determined because there were very little differences in TNF-α production (pg/ml) between the two doses of Pam_3CSK_4 (10 ng/ml; 94 ± 6 and 100 ng/ml; 101 ± 8, n = 3) and dose-dependent increases in LPS (0.001 μg/ml; 87 ± 6, 0.01 μg/ml; 156 ± 19, 0.1 μg/ml; 190 ± 27, and 1 μg/ml; 258 ± 37, n = 3). These doses were in accordance with our previous *in vitro* studies including those of IL-10 and NO [33,36]. Because the present study was designed to exploit our previous *in vitro* findings in terms of the temporal change of TNF-α, IL-10, and NO production [33,35,36], we selected each culture period above for the cytokines/NO as representative time points.

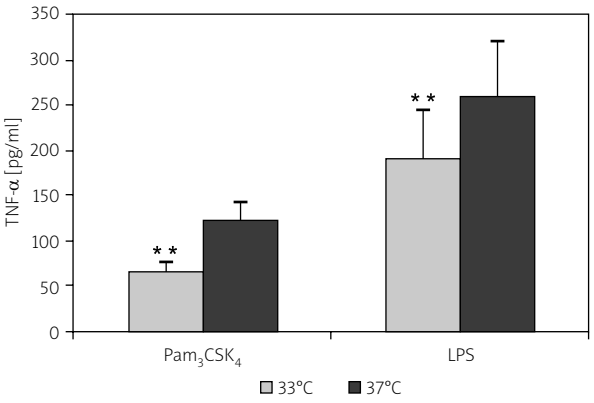

Fig. 2. Effects of hypothermic culture on TNF-α production by TLR2- and TLR4-stimulated *ex vivo* microglia derived from mice with hypoxic-ischemic brain injury. Microglia (4×10^4 cells/well) were cultured with 10 ng/ml Pam_3CSK_4 (a TLR2 agonist) or 1 μg/ml LPS (a TLR4 agonist) in hypothermic (33°C) and normothermic (37°C) conditions for 6 h. TNF-α levels in culture supernatants were measured by ELISA. Data are expressed as mean ± SEM (n = 5 for Pam_3CSK_4 stimulation and n = 6 for LPS stimulation). $^{**}p < 0.01$ compared with 37°C.

Cytokine assay

Concentrations of TNF-α and IL-10 in microglial culture supernatants were measured in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, Camarillo, CA, USA), according to the manufacturer's instructions.

NO assay

NO production was quantified as nitrite (NO_2^-), a relatively stable metabolite of NO, accumulating in the culture medium. A colorimetric assay with Griess reagent (Sigma-Aldrich) was performed, as described in our previous reports [33-36].

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Although the number of samples (n) in this study is modest, each experiment required microglia pooled from 4-7 brains as mentioned above. Differences in values between two groups were analyzed using the paired t-test (StatFlex ver. 5.0, Arteck, Osaka, Japan). $P < 0.05$ was considered to indicate a significant difference.

Results

Effects of hypothermic culture on TNF-α production

TNF-α was virtually undetectable in microglia without TLR agonists after 6 h of culture (basal conditions). Application of TLR agonists to microglia elicited significant TNF-α production, and this effect was greater for TLR4 stimulation than for TLR2 stimulation (Fig. 2). For both types of TLR stimulation, microglial TNF-α production was significantly reduced at 33°C compared with 37°C (Fig. 2).

Effects of hypothermic culture on IL-10 production

IL-10 was virtually undetectable in microglia without TLR agonists after 48 h of culture (basal conditions). Application of TLR agonists to microglia increased IL-10 production, and this effect was greater for TLR4 stimulation than for TLR2 stimulation (Fig. 3). For both types of TLR stimulation, microglial IL-10 production was significantly reduced at 33°C compared with 37°C (Fig. 3).

Effects of hypothermic culture on NO production

NO_2^- was virtually undetectable in microglia without TLR agonists after 48 h of culture (basal conditions). Application of the TLR4 agonist to microglia increased NO_2^- production (Fig. 4), while application of the TLR2 agonist did not (*i.e.*, NO_2^- remained virtually undetectable). Compared with 37°C, this increase was significantly reduced at 33°C (Fig. 4).

Discussion

With the aim of elucidating the possible mechanisms underlying the neuroprotective effects of therapeutic hypothermia, we have previously shown that hypothermia reduces the production of early-phase and late-phase inflammatory factors as well as that of late-phase anti-inflammatory factor in primary microglia *in vitro* [33-36]. We considered that an approach using microglia removed directly from injured brains may yield more clinically relevant information. Hence, in this study, we utilized *ex vivo* microglia with high purity that were derived from mice with HI brain injury. With this approach, we demonstrated that hypothermia (33°C) reduced the production by these cells of TNF-α in the early phase (6 h) and that of IL-10 and NO in the late phase (48 h) after the stimulation of TLR2 and/or TLR4. To the best of our knowledge, this is the first report to describe the direct responses of microglia derived from injured animals to hypothermia.

The finding that hypothermia reduced the TLR2- and TLR4-activated microglial release of pro- and anti-inflammatory cytokines and NO in *ex vivo* conditions was consistent with the findings of reports of the same stimuli *in vitro* [33,36]. In *in vivo* studies, therapeutic hypothermia has also been shown to attenuate the increases in the CNS levels of pro-inflammatory cytokines and NO after brain injury [1,32,50], and this has been associated with a favourable outcome compared with normothermia [1,32]. Furthermore, hypothermia during severe perinatal asphyxia prevents increases in 3',5'-cyclic monophosphate, which is a marker of NO, in the rat brain. In this study, 100% of the hypothermic rats survived, whereas 70% mortality was observed in the normothermic group [30]. Therefore, the present findings strongly supported the idea that a reduction in the microglial production of pro-inflammatory cytokines and NO is an important neuroprotective effect of

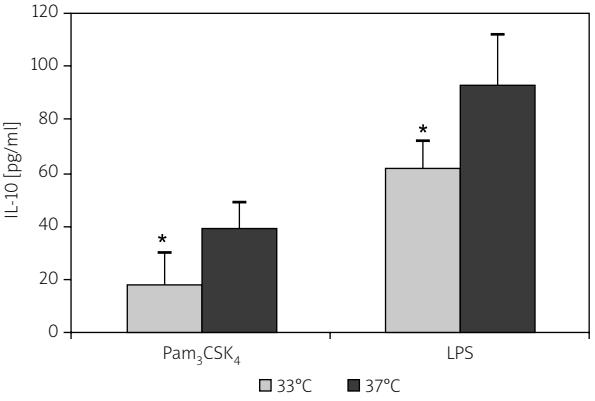

Fig. 3. Effects of hypothermic culture on IL-10 production by TLR2- and TLR4-stimulated *ex vivo* microglia derived from mice with hypoxic-ischemic brain injury. Microglia (4×10^4 cells/well) were cultured with 10 ng/ml Pam_3CSK_4 (a TLR2 agonist) or 1 µg/ml LPS (a TLR4 agonist) in hypothermic (33°C) and normothermic (37°C) conditions for 48 h. IL-10 levels in culture supernatants were measured by ELISA. Data are expressed as mean ± SEM (n = 4 for Pam_3CSK_4 stimulation and n = 5 for LPS stimulation). *$p < 0.05$ compared with 37°C.

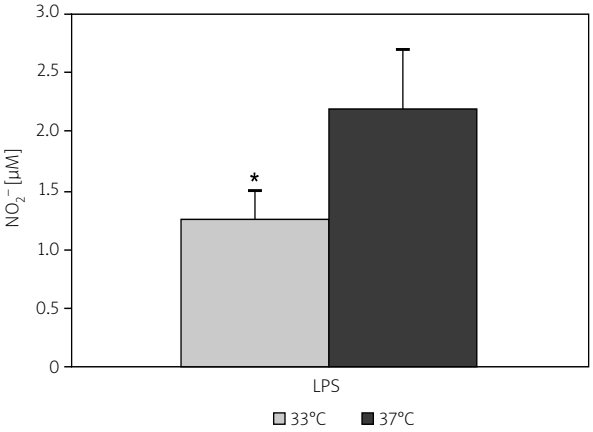

Fig. 4. Effects of hypothermic culture on NO production by TLR4-stimulated *ex vivo* microglia derived from mice with hypoxic-ischemic brain injury. Microglia (4×10^4 cells/well) were cultured with 1 µg/ml LPS (a TLR4 agonist) in hypothermic (33°C) and normothermic (37°C) conditions for 48 h. Nitrite (NO_2^-, a relatively stable metabolite of NO) levels in culture supernatants were measured by colorimetric assay with Griess reagent. Data are expressed as mean ± SEM (n = 5). *$p < 0.05$ compared with 37°C.

therapeutic hypothermia [33-36]. IL-10 has been considered to be neuroprotective because it reduces the production of pro-inflammatory cytokines [8,26]. In contrast, the CSF levels of IL-10 have been shown to increase after severe TBI and to be independently associated with increased mortality [6], indicating a detrimental *in vivo* effect of increased levels of IL-10 in CNS. Microglial activation by TLR2 and TLR4 has been found to lead initially to the synthesis of pro-inflammatory cytokines and later to the synthesis of IL-10 [33,36]. As noted, microglial IL-10 production was reduced by hypothermia in the late phase. Taken together, our findings further suggested that the neuroprotective effects of therapeutic hypothermia are related to the attenuation of the production of early-phase and late-phase inflammatory factors as well as that of late-phase anti-inflammatory factor(s) by microglia. This mechanism may also be applicable to treatment for HI brain injury because in the CSF of patients with neonatal HI encephalopathy (HIE), pro-inflammatory cytokines and NO levels have been shown to be increased, and their concentrations correlate with the degree of injury [4,16], although the involvement of IL-10 in this mechanism is unknown. Interestingly, the concept that we proposed here and in our previous study [36] of the temporal modulation of cytokine production and/or inflammation (neuroinflammatory response) by hypothermia is associated with its neuroprotective effect is supported by the findings of two recent studies: one in animals and one in humans. In an animal model of CNS injury that was induced by cerebral ischaemia, reducing the brain temperature attenuated the early-phase (8 h) production of interleukin-1 beta (IL-1β) in the brain, and this coincided with a reduced infarct size and improved functional outcome [11]. In patients with ischemic and hemorrhagic stroke, the beneficial effects of low temperature on functional outcomes occurred within the first 24 h after the stroke onset, whereas the harmful effects of high temperatures that are associated with inflammation occurred later (in the first 48 h) [9]. Such time- and inflammation-targeted therapeutic interventions may be worth considering in the future.

With the aim of determining a possible pathophysiological significance of the reduced production of TNF-α, IL-10, and NO by microglia for hypothermic neuronal protection, we have recently demonstrated that they individually induced the death of neuronal PC12 cells in a concentration-dependent manner [35,37]. Taking these findings and the facts that their elevated levels in the CNS after brain injury *in vivo* [6,13,47,50] together, these results further support the conclusion that a decrease in the levels of TNF-α, IL-10, and NO during hypothermia contributes toward the protection of neurons.

In animals with HI brain injury, hypothermia has been shown to reduce the mRNA expression of interleukin-18 (IL-18: another pro-inflammatory cytokine), TNF-α, and IL-6 in the brain [19,49]. However, in this model, the sources of these cytokines were unclear, and the effects of hypothermia on IL-10 expression were unknown. Moreover, an investigation of the responses of microglia derived from brain-injured animals to TLR stimulation, along with their responses to hypothermia, is of particular significance because both microglial TLR2 and TLR4 play an important role in triggering immediate responses and/or enhancing reactions to tissue injury and inflammation [5,22,28]. In addition, an *ex vivo* culture of microglia isolated from CNS preserves the *in vivo* phenotype of microglia [18,51]. Importantly, as mentioned above, in terms of the temporal changes of neuroinflammatory responses, our present *ex vivo* findings, as well as our previous *in vitro* findings [33-36], appear to be similar to the *in vivo* findings for brain injury [9,11], as well as to those regarding IL-10 *in vivo*, which indicate that the levels in the brain show a peak during the first days (24-48 h) after brain injury [6,14,25]. Temporal similarity between the *ex vivo* and *in vivo* expressions of a signalling molecule in microglia has also been reported [12,51]. Thus, our *ex vivo* experiments with TLR stimulation may also allow us to study microglial function in pathophysiological states and therefore be useful in examining the effects of hypothermia.

In conclusion, we demonstrated that it is possible to rapidly isolate and functionally culture active microglia from mice with HI brain injury and that hypothermia (33°C) reduced the temporal production of pro- and anti-inflammatory cytokines and NO from TLR-activated *ex vivo* microglia derived from these animals. We were able to further explore the relationships between hypothermia and microglial responses using a brain-injured model.

Acknowledgements

We would like to thank Dr. Jinyao Liu (Department of Legal Medicine, Yamaguchi University Grad-

uate School of Medicine) for his kind support in performance of microglial separation. This research was supported in part by a Grant-in-Aid for Young Scientists (B), No. 22791435, from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to T.M., and a grant from Yamaguchi University Foundation to T.M.

Disclosure

Authors report no conflict of interest.

References

1. Aibiki M, Maekawa S, Ogura S, Kinoshita Y, Kawai N, Yokono S. Effect of moderate hypothermia on systemic and internal jugular plasma IL-6 levels after traumatic brain injury in humans. J Neurotrauma 1999; 16: 225-232.
2. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006; 124: 783-801.
3. Allan SM, Tyrrell PJ, Rothwell NJ. Interleukin-1 and neuronal injury. Nat Rev Immunol 2005; 5: 629-640.
4. Aly H, Khashaba MT, El-Ayouty M, El-Sayed O, Hasanein BM. IL-1beta, IL-6 and TNF-alpha and outcomes of neonatal hypoxic ischemic encephalopathy. Brain Dev 2006; 28: 178-182.
5. Babcock AA, Wirenfeldt M, Holm T, Nielsen HH, Dissing-Olesen L, Toft-Hansen H, Millward JM, Landmann R, Rivest S, Finsen B, Owens T. Toll-like receptor 2 signaling in response to brain injury: an innate bridge to neuroinflammation. J Neurosci 2006; 26: 12826-12837.
6. Bell MJ, Kochanek PM, Doughty LA, Carcillo JA, Adelson PD, Clark RS, Wisniewski SR, Whalen MJ, DeKosky ST. Interleukin-6 and interleukin-10 in cerebrospinal fluid after severe traumatic brain injury in children. J Neurotrauma 1997; 14: 451-457.
7. Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, Smith K. Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. N Engl J Med 2002; 346: 557-563.
8. Bethea JR, Nagashima H, Acosta MC, Briceno C, Gomez F, Marcillo AE, Loor K, Green J, Dietrich WD. Systemically administered interleukin-10 reduces tumor necrosis factor-alpha production and significantly improves functional recovery following traumatic spinal cord injury in rats. J Neurotrauma 1999; 16: 851-863.
9. Blanco M, Campos F, Rodríguez-Yáñez M, Arias S, Fernández-Ferro J, Gómez-Sánchez JC, Castillo J. Neuroprotection or increased brain damage mediated by temperature in stroke is time dependent. PLoS One 2012; 7: e30700. doi: 10.1371/journal.pone.0030700.
10. Cao CX, Yang QW, Lv FL, Cui J, Fu HB, Wang JZ. Reduced cerebral ischemia-reperfusion injury in Toll-like receptor 4 deficient mice. Biochem Biophys Res Commun 2007; 353: 509-514.
11. Ceulemans AG, Zgavc T, Kooijman R, Hachimi-Idrissi S, Sarre S, Michotte Y. Mild hypothermia causes differential, time-dependent changes in cytokine expression and gliosis following endothelin-1-induced transient focal cerebral ischemia. J Neuroinflammation 2011; 8: 60.
12. Clark AK, D'Aquisto F, Gentry C, Marchand F, McMahon SB, Malcangio M. Rapid co-release of interleukin 1beta and caspase 1 in spinal cord inflammation. J Neurochem 2006; 99: 868-880.
13. Clark RS, Kochanek PM, Obrist WD, Wong HR, Billiar TR, Wisniewski SR, Marion DW. Cerebrospinal fluid and plasma nitrite and nitrate concentrations after head injury in humans. Crit Care Med 1996; 24: 1243-1251.
14. Csuka E, Morganti-Kossmann MC, Lenzlinger PM, Joller H, Trentz O, Kossmann T. IL-10 levels in cerebrospinal fluid and serum of patients with severe traumatic brain injury: relationship to IL-6, TNF-alpha, TGF-beta1 and blood-brain barrier function. J Neuroimmunol 1999; 101: 211-221.
15. Dedoni S, Olianas MC, Onali P. Interferon-β induces apoptosis in human SH-SY5Y neuroblastoma cells through activation of JAK-STAT signaling and down-regulation of PI3K/Akt pathway. J Neurochem 2010; 115: 1421-1433.
16. Ergenekon E, Gücüyener K, Erbaş D, Aral S, Koç E, Atalay Y. Cerebrospinal fluid and serum vascular endothelial growth factor and nitric oxide levels in newborns with hypoxic ischemic encephalopathy. Brain Dev 2004; 26: 283-286.
17. Feng Y, LeBlanc MH. Effect of agmatine on the time course of brain inflammatory cytokines after injury in rat pups. Ann N Y Acad Sci 2003; 1009: 152-156.
18. Frank MG, Wieseler-Frank JL, Watkins LR, Maier SF. Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: immunophenotypic and functional characteristics. J Neurosci Methods 2006; 151: 121-130.
19. Fukui O, Kinugasa Y, Fukuda A, Fukuda H, Tskitishvili E, Hayashi S, Song M, Kanagawa T, Hosono T, Shimoya K, Murata Y. Post-ischemic hypothermia reduced IL-18 expression and suppressed microglial activation in the immature brain. Brain Res 2006; 1121: 35-45.
20. Gibbons H, Sato TA, Dragunow M. Hypothermia suppresses inducible nitric oxide synthase and stimulates cyclooxygenase-2 in lipopolysaccharide stimulated BV-2 cells. Brain Res Mol Brain Res 2003; 110: 63-75.
21. Guo MF, Yu JZ, Ma CG. Mechanisms related to neuron injury and death in cerebral hypoxic ischaemia. Folia Neuropathol 2011; 49: 79-87.
22. Hoffmann O, Braun JS, Becker D, Halle A, Freyer D, Dagand E, Lehnardt S, Weber JR. TLR2 mediates neuroinflammation and neuronal damage. J Immunol 2007; 178: 6476-6481.
23. Hypothermia after Cardiac Arrest Study Group. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. N Engl J Med 2002; 346: 549-556.
24. Johnson GB, Brunn GJ, Platt JL. Activation of mammalian Toll-like receptors by endogenous agonists. Crit Rev Immunol 2003; 23: 15-44.
25. Kamm K, Vanderkolk W, Lawrence C, Jonker M, Davis AT. The effect of traumatic brain injury upon the concentration and expression of interleukin-1beta and interleukin-10 in the rat. J Trauma 2006; 60: 152-157.
26. Knoblach SM, Faden AI. Interleukin-10 improves outcome and alters proinflammatory cytokine expression after experimental traumatic brain injury. Exp Neurol 1998; 153: 143-151.
27. Lehnardt S, Lehmann S, Kaul D, Tschimmel K, Hoffmann O, Cho S, Krueger C, Nitsch R, Meisel A, Weber JR. Toll-like receptor 2

mediates CNS injury in focal cerebral ischemia. J Neuroimmunol 2007; 190: 28-33.
28. Lehnardt S, Schott E, Trimbuch T, Laubisch D, Krueger C, Wulczyn G, Nitsch R, Weber JR. A vicious cycle involving release of heat shock protein 60 from injured cells and activation of toll-like receptor 4 mediates neurodegeneration in the CNS. J Neurosci 2008; 28: 2320-2331.
29. Lin S, Rhodes PG, Cai Z. Whole body hypothermia broadens the therapeutic window of intranasally administered IGF-1 in a neonatal rat model of cerebral hypoxia-ischemia. Brain Res 2011; 1385: 246-256.
30. Loidl CF, De Vente J, van Ittersum MM, van Dijk EH, Vles JS, Steinbusch HW, Blanco CE. Hypothermia during or after severe perinatal asphyxia prevents increase in cyclic GMP-related nitric oxide levels in the newborn rat striatum. Brain Res 1998; 791: 303-307.
31. Maekawa S, Aibiki M, Si QS, Nakamura Y, Shirakawa Y, Kataoka K. Differential effects of lowering culture temperature on mediator release from lipopolysaccharide-stimulated neonatal rat microglia. Crit Care Med 2002; 30: 2700-2704.
32. Marion DW, Penrod LE, Kelsey SF, Obrist WD, Kochanek PM, Palmer AM, Wisniewski SR, DeKosky ST. Treatment of traumatic brain injury with moderate hypothermia. N Engl J Med 1997; 336: 540-546.
33. Matsui T, Kakeda T. IL-10 production is reduced by hypothermia but augmented by hyperthermia in rat microglia. J Neurotrauma 2008; 25: 709-715.
34. Matsui T, Motoki Y, Inomoto T, Miura D, Kato Y, Suenaga H, Hino K, Nojima J. Temperature-related effects of adenosine triphosphate-activated microglia on pro-inflammatory factors. Neurocrit Care 2012; 17: 293-300.
35. Matsui T, Motoki Y, Yoshida Y. Hypothermia reduces Toll-like receptor 3-activated microglial interferon-β and nitric oxide production. Mediators Inflamm 2013; 2013: 436263. doi: 10.1155/2013/436263.
36. Matsui T, Tasaki M, Yoshioka T, Motoki Y, Tsuneoka H, Nojima J. Temperature- and time-dependent changes in TLR2-activated microglial NF-κB activity and concentrations of inflammatory and anti-inflammatory factors. Intensive Care Med 2012; 38: 1392-1399.
37. Matsui T, Yoshida Y, Yanagihara M, Suenaga H. Hypothermia at 35°C reduces the time-dependent microglial production of pro-inflammatory and anti-inflammatory factors that mediate neuronal cell death. Neurocrit Care 2014; 20: 301-310.
38. Minghetti L, Levi G. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. Prog Neurobiol 1998; 54: 99-125.
39. Ohmura A, Nakajima W, Ishida A, Yasuoka N, Kawamura M, Miura S, Takada G. Prolonged hypothermia protects neonatal rat brain against hypoxic-ischemia by reducing both apoptosis and necrosis. Brain Dev 2005; 27: 517-526.
40. Ott L, McClain CJ, Gillespie M, Young B. Cytokines and metabolic dysfunction after severe head injury. J Neurotrauma 1994; 11: 447-472.
41. Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, Iwaki T, Okada Y, Iida M, Cua DJ, Iwakura Y, Yoshimura A. Pivotal role of cerebral interleukin-17-producing gammadelta T cells in the delayed phase of ischemic brain injury. Nat Med 2009; 15: 946-950.
42. Si QS, Nakamura Y, Kataoka K. Hypothermic suppression of microglial activation in culture: inhibition of cell proliferation and production of nitric oxide and superoxide. Neuroscience 1997; 81: 223-239.
43. Sizonenko SV, Kiss JZ, Inder T, Gluckman PD, Williams CE. Distinctive neuropathologic alterations in the deep layers of the parietal cortex after moderate ischemic-hypoxic injury in the P3 immature rat brain. Pediatr Res 2005; 57: 865-872.
44. Stein VM, Czub M, Hansen R, Leibold W, Moore PF, Zurbriggen A, Tipold A. Characterization of canine microglial cells isolated ex vivo. Vet Immunol Immunopathol 2004; 99: 73-85.
45. Towfighi J, Mauger D, Vannucci RC, Vannucci SJ. Influence of age on the cerebral lesions in an immature rat model of cerebral hypoxia–ischemia: a light microscopic study. Brain Res Dev Brain Res 1997; 100: 149-160.
46. Vannucci RC, Connor JR, Mauger DT, Palmer C, Smith MB, Towfighi J, Vannucci SJ. Rat model of perinatal hypoxic-ischemic brain damage. J Neurosci Res 1999; 55: 158-163.
47. Vitarbo EA, Chatzipanteli K, Kinoshita K, Truettner JS, Alonso OF, Dietrich WD. Tumor necrosis factor alpha expression and protein levels after fluid percussion injury in rats: the effect of injury severity and brain temperature. Neurosurgery 2004; 55: 416-424.
48. Woodroofe MN, Sarna GS, Wadhwa M, Hayes GM, Loughlin AJ, Tinker A, Cuzner ML. Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production. J Neuroimmunol 1991; 33: 227-236.
49. Xiong M, Yang Y, Chen GQ, Zhou WH. Post-ischemic hypothermia for 24h in P7 rats rescues hippocampal neuron: association with decreased astrocyte activation and inflammatory cytokine expression. Brain Res Bull 2009; 79: 351-357.
50. Yamaguchi S, Nakahara K, Miyagi T, Tokutomi T, Shigemori M. Neurochemical monitoring in the management of severe head-injured patients with hypothermia. Neurol Res 2000; 22: 657-664.
51. Yip PK, Kaan TK, Fenesan D, Malcangio M. Rapid isolation and culture of primary microglia from adult mouse spinal cord. J Neurosci Methods 2009; 183: 223-237.