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

Tomohiro Matsui1, Hiroyuki Kida2, Takuya Iha1, Tabito Obara1, Sadahiro Nomura3, Tatsuya Fujimiy4, Michiyasu Suzuki3

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

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-
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 N₂O and O₂, 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 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 FACS Calibur (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% CO2 incubator at 37°C before the cells were stimulated in subsequent experiments.

**Microglial cell culture**

Microglia (4 × 10⁴ cells/well in untreated 96-well plates (Becton Dickinson)) were cultured with or without 10 ng/ml Pam3CSK4 (N-palmitoyl-S-(2,3-bis (palmitoyloxy)-(2R,3S)-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% CO₂ 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 Pam3CSK4; 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 Pam3CSK4 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 Pam3CSK4 (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.

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 (NO₂⁻), 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).
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Effects of hypothermic culture on NO production

NO\textsubscript{2}\textsuperscript{–} was virtually undetectable in microglia without TLR agonists after 48 h of culture (basal conditions). Application of the TLR4 agonist to microglia increased NO\textsubscript{2}\textsuperscript{–} production (Fig. 4), while application of the TLR2 agonist did not (i.e., NO\textsubscript{2}\textsuperscript{–} 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 × 10⁴ cells/well) were cultured with 10 ng/ml Pam\textsubscript{3}CSK\textsubscript{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\textsubscript{3}CSK\textsubscript{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 × 10⁴ 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\textsubscript{2}\textsuperscript{–}, 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 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.

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