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Increase of galectin-3 expression in microglia by hyperthermia in delayed neuronal death of hippocampal CA1 following transient forebrain ischemia

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ABSTRACT

The ischemic damage in the hippocampal CA1 region following transient forebrain ischemia, delayed neuronal death, is a typical apoptotic response, but the underlying mechanisms are not fully understood. We have reported that mild hyperthermia (38 °C) accelerates DNA fragmentation of the gerbil CA1 pyramidal neurons following transient forebrain ischemia. Recently, we reported that galectin-3, a β-galactosidase-binding lectin, is spatio-temporally expressed only by activated microglial cells located within CA1 region following transient forebrain ischemia in gerbils. Furthermore, expression of galectin-3 and Iba-1 (a specific microglial cell marker) are strongly reduced by hypothermia during ischemic insult. To further elucidate the effect of hyperthermia on the expression of galectin-3 by micloglia in delayed neuronal death, we examined immunohistochemical expression of galectin-3 and Iba-1, in situ terminal dUTP-biotin nick end labeling of DNA fragmentation (for determination of cell death) and hematoxylin and eosin staining (for morphological observation). We observed that between 37 °C and 39 °C, there was a temperature-dependent enhancement of galectin-3 expression in microglial cells in the CA1 region following transient ischemia. Apoptotic DNA fragmentation, detected by TUNEL staining, was observed in CA1 region in normothermia. This TUNEL staining was enhanced by hyperthermia at 37.5 °C and 38 °C, but not at 39 °C. Ischemia-induced neuronal degeneration in CA1 region in gerbil hippocampus subjected to hyperthermia (37.5 °C, 38 °C and 39 °C) observed by HE staining is similar to that in normothermic gerbils. These findings imply that galectin-3 expression in microglia may influence the survival of CA1 pyramidal neurons in cases such as hyperthermia-related neuronal injury.

Transient cerebral ischemia initiates a process of cellular events that leads to delayed neuronal degeneration of several brain regions both in humans and in animal models [19,20]. Furthermore, a brief episode of ischemia causes "delayed neuronal death" in the CA1 region of the hippocampus [13]. Accumulating evidence has indicated that the post-ischemic DNA fragmentation in the hippocampal CA1 area in experimental ischemic models is a key phenomenon for the delayed neuronal death, and is considered as apoptosis [8–10]. Although many studies have suggested some of the underlying mechanisms that contribute to delayed neuronal death after transient ischemia, exact mechanisms are not yet fully understood.

Galectin-3 is a 30 kDa β-galactosidase-binding lectin, that binds IgE and glycoconjugates on mammalian cell surfaces. Galectin-3 distributes in epidermal cells of various organs, Kupffer cell, dendritic cell, and various inflammatory cells such as macrophage and microglia. The expression of galectin-3 is enhanced by inflammation and cell differentiation. Galectin-3 is also important in cell proliferation and apoptotic regulation [15]. It has been reported that galectin-3 is expressed by resident microglial cells in experimental models of adult stroke [23,25]. However, exact role of galectin-3 in ischemic brain injury is still contradictory. We recently reported that a transient increase of galectin-3 expression was observed following transient ischemia in CA1 region, which was maximal 96 h after reperfusion, and this expression was predominantly localized within the CA1 region and observed only in microglial cells [21]. This evidence indicates that galectin-3 might exert its effect by modulating the neuronal damage in delayed neuronal death.

Hyperthermia during transient forebrain ischemia exacerbates ischemia-induced neuronal death in hippocampal CA1 neurons

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[2,7,12]. Production of reactive oxygen species [12], hydroxyl free radicals [5], calcium influx into neurons [1] and vascular permeability [3] has been reported to be enhanced by hyperthermia in neurons of hippocampal CA1 neurons. We previously evaluated the effects of temperatures (37–39 °C) on specific DNA fragmentation in gerbil CA1 pyramidal neurons following transient forebrain ischemia. The results indicated that mild hyperthermia (38 °C) but not severe hyperthermia (39 °C) accelerated DNA fragmentation in the gerbil CA1 pyramidal neurons [8]. This evidence indicates that there is precise temperature-dependent enhancement of DNA fragmentation following transient forebrain ischemia.

It has been reported that hypothermia reduced production of interleukin (IL)-6, IL-10, and nitric oxide (NO) by LPS-activated microglia, while hyperthermia specifically increased IL-10 production in these cells [16]. Recently, we reported that expression of galectin-3 in microglia was strongly reduced by hypothermia during ischemic insult [21]. This suggests that hyperthermia-enhanced delayed neuronal death following transient forebrain ischemia might be related to hyperthermia-induced microglia activation. To test this hypothesis, we evaluated the expression of galectin-3 in microglia within hippocampal CA1 region following transient brain ischemia during a stepwise increase in temperature from 37 to 39 °C, comparing the expression to Iba-1 expression in microglia and DNA fragmentation in CA1 pyramidal neurons.

Male Mongolian gerbils, weighing 65–75 g, were subjected to severe forebrain ischemia as described previously [8,17]. Briefly, the bilateral common carotid arteries were isolated through an anterior mid-cervical incision and occluded with microclips. After 5 min of forebrain ischemia, the clips were removed. Rectal temperature was maintained at 37 ± 0.3 °C using a heating pad, from the induction of anesthesia until 3 h following ischemia (except for hyperthermia experiments, see below). Sham-operated animals underwent the same surgical manipulation, but without occlusion of bilateral common carotid arteries. Five animals per group were used for each experiment. At 96 h after ischemic insult, animals were anesthetized with pentobarbital and perfused transcardially with saline and then with phosphate-buffered 10% formalin. Brains were removed and processed for paraffin embedding. Three micrometers coronal sections were cut at the level of the dorsal hippocampus and then used for hematoxylin and eosin (HE) staining, immunohistochemical staining and TUNEL method. The animals were housed on 12 h light/dark cycles at 22 °C, and were given free access to food and water. We fully complied with the 'Guidelines Concerning Experimental Animals' issued by the Japanese Association for Laboratory Animal Science and exercised due consideration so as not to cause any ethical problems.

Regulation of body temperature was conducted according to the method previously described [7]. Rectal temperatures of 37 °C, 37.5 °C, 38 °C or 39 °C, maintained using a heating pad with a circulating system, were adjusted just before, during and just after ischemic insult. The rectal temperature was monitored and recorded continuously. If the recording showed that body temperature was not maintained at the pre-defined temperature, the animal was excluded from the study.

Anti-mouse galectin-3/Mac2 [Rat IgG 14-5301] and anti-Iba-1 antibody [rabbit immunoaffinity purified immunoglobulin G (IgG), 019-19741] was purchased from Bay bioscience (Hyogo, Japan) and Wako Pure Chemical (Osaka, Japan), respectively. The paraffinized sections were blocked to endogenous peroxidase activity by incubation in distilled water containing 3% hydrogen peroxide for 5 min. Antigen retrieval was performed, using a 0.01 M citrate buffer (pH 6.0) for both anti-Iba-1 and anti-galectin-3 antibodies by the PascalR heat-induced target retrieval system (DAKO Japan, Tokyo, Japan,). Non-specific binding sites were blocked in 0.01 M phosphate-buffered saline (PBS), pH 7.4 containing 2% bovine serum albumin (BSA; Wako Pure Chemical) for 60 min.

Table 1
The level of nuclear DNA fragmentation and immunohistochemistry in CA1 subjected to 37, 37.5, 38 and 39 °C during forebrain ischemia.

Temperature	TUNEL	Iba-1	Galectin-3
Sham	−	±	−
37 °C	+	+	+
37.5 °C	++	+	+
38 °C	++	+	++
39 °C	+	++	++

The level of nuclear DNA fragmentation and immunohistochemistry for Iba-1 and galectin-3 in hippocampal CA1 was evaluated as follows: TUNEL and galectin-3 staining intensities were divided into three grades, negative (−), positive (+) and strongly positive (++). Iba-1 staining intensity was divided into three grades, weakly positive (±), positive (+) and strongly positive (++). Sham operated animals subjected to 39 °C showed negative TUNEL, weakly positive Iba-1 and negative galectin-3.

Anti-Iba-1 and anti-galectin-3 antibodies used at a dilution of 1:1000 and 1:100, respectively, in 2% BSA/PBS were added on the slides and incubated overnight at 4 °C. Iba-1 and Gal3 were detected with a biotinylated anti-rabbit IgG (1: 250 KPL-16-15-06) and biotinylated anti-rat IgG (1:200 DAKO E0468), respectively for 30 min, followed by incubation with avidin-coupled peroxidase (Vectastain ABC Kit, Vector Laboratories, Burlingame, USA) for 30 min. The peroxidase binding sites were detected by staining with 3,3′-diaminobenzidine (DAB) in 50 mM Tris–EDTA buffer, pH 7.6. Finally, counterstaining was performed using Mayer's hematoxylin.

TUNEL method was performed as described previously [6] with some modifications to the method by Gavrieli et al. [4]. After incubation with 20 μg/ml proteinase K (Sigma–Aldrich, Tokyo, Japan), The serial sections used for hematoxylin and eosin staining were immersed in terminal deoxynucleotidyl transferase (TdT; Roche Diagnostics, Basel, Switzerland) buffer (30 nM Trizma base, pH7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TdT (Boehringer Mannheim) and biotinylated dUTP (Boehringer Mannheim) were diluted in TdT buffer at a concentration of 0.15 e.u./μl and 0.8 nmol/μl, respectively. The solution was placed on the sections and then incubated at 37 °C for 60 min. The section was covered with streptavidin peroxidase (DAKO) and stained with DAB as a substrate for the peroxidase. Finally, counterstaining was done using Mayer's hematoxylin.

Results of DNA damage detected by TUNEL staining and immunostaining for galectin-3 and Iba-1 in hippocampal CA1 region subjected to 37, 37.5, 38 and 39 °C during forebrain ischemia 96 h after transient forebrain ischemia is summarized in Table 1.

In ischemic gerbils, HE staining indicated that almost of all nuclei of CA1 pyramidal neurons were pyknotic both in normothermia and in hyperthermia (Figs. 1 and 2). On the other hand, no morphological change of CA1 pyramidal neurons was observed in sham-operated gerbils in normothermia (data not shown) or hyperthermia (39 °C) (Figs. 1 and 2). Apoptotic DNA fragmentation detected by TUNEL staining was observed in CA1 sector in normothermia. This TUNEL staining was enhanced by hyperthermia at 37.5 °C and 38 °C but not at 39 °C (Fig. 2 and Table 1). No TUNEL staining was observed in sham operated gerbils in normothermia (data not shown) and hyperthermia (39 °C) (Figs. 1 and 2 and Table 1).

Morphological changes of resident microglia expressing Iba-1 were evaluated by immunostaining. In the sham-operated group, ramified Iba-1-positive microglia with fine processes, known as resting microglia, were evenly distributed throughout the CA1 region (Figs. 1 and 2). Increased Iba-1 immunoreactivity, mostly in hypertrophied cell processes, was observed in microglia within hippocampal CA1 area 96 h after ischemic insult in normothermia (37 °C) and hyperthermia (37.5 and 38 °C). Strongly positive immunoreactivity of Iba-1was observed in microglia with hyperthermia at 39 °C.