Mini Review

Peroxiredoxin triggers cerebral post-ischemic inflammation

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Post-ischemic inflammation is an essential step in the progression of ischemic stroke. However, it has not been sufficiently clarified how post-ischemic inflammation begins. Brain is a sterile organ, but Toll-like receptor (TLR) 2 and TLR4 have a pivotal role in the initiation of inflammation. Some endogenous danger associated molecular patterns (DAMPs) are released from injured brain cells. Among them, high mobility group box 1 (HMGB1) and peroxiredoxin (Prx) family proteins are key initiators of post-ischemic inflammation. HMGB1 induces blood brain barrier breakdown at the hyperacute phase; on the other hand, Prxs directly activate infiltrating macrophage through TLR2/4 and induce inflammatory cytokines production at the acute phase. Thus, there is a time lag as well as functional differences between HMGB1 and Prxs. Novel neuroprotective treatment could be developed through more detailed elucidation of the regulation of post-ischemic inflammation.

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Introduction

Stroke is a major cause of death and disability in Japan, and about 70 % of all cases are ischemic stroke. Post-ischemic inflammation is an essential step in ischemic brain injury and is correlated with patient's prognosis. More detailed understanding of inflammatory processes enables us to develop a more effective treatment with a prolonged therapeutic time window[1, 2].

Brain infarction is a brain tissue death caused by insufficient blood supply (ischemia) which is due to severe stenosis or occlusion of cerebral artery. The deprivation of oxygen, glucose, and other nutrients caused by ischemia results in the failure to maintain the neuronal microenvironment which consists of glial cells, endothelial cells, and neurons. The dysfunction of cerebrovascular units leads to blood-brain barrier (BBB) breakdown and subsequent inflammation caused by the infiltration of hematopoietic immune cells[3].

Hematopoietic cells, such as macrophages and T cells are implicated in post-ischemic inflammation and play a

role as effectors of innate immunity[4]. To prevent the primary and secondary progression of an infarct lesion, it is indispensable to clarify the detailed mechanism of post-ischemic inflammation evoked by these immune cells. Despite intensive studies, the complexity of the brain inflammation mechanisms has thus far remained insufficiently clarified.

The initiation of inflammatory process in ischemic brain injury

In the acute phase of brain ischemia, infiltrating macrophages produce various cytokines and promote inflammation. However, circulating intravascular blood cells do not produce inflammatory cytokines much. These hematopoietic immune cells should be activated by the infiltration into the ischemic brain.

Toll-like receptor (TLR) is one of the most important molecules for the activation of macrophages. Originally, TLR functions as a pathogen sensor which recognizes the lipoprotein, lipopolysaccharide, and nucleotides derived from bacteria and virus. It has been reported that TLR2 and TLR4 are implicated in post-ischemic inflammation, although there are no pathogens in both the normal and ischemic brain[5, 6]. It remains clarified what molecule stimulates TLR in ischemic brain.

Recently, it has attracted attention that some endogenous molecules released from the injured tissues activate infiltrating immune cells. Such endogenous molecules are called danger-associated molecular patterns (DAMPs). Heat shock proteins (HSPs), β-amyloid (Aβ), hyaluronic acid, formyl peptides, oxidized low-density lipoproteins (oxLDL) have been considered as possible DAMPs in the ischemic brain[7-9]. However, it has been insufficiently elucidated what molecule triggers post-ischemic inflammation in brain.

Potential DAMPs in ischemic brain

Among these DAMPs, high mobility group box 1 (HMGB1) is an important DAMP[10]. HMGB1, which is localized in cell nuclei in normal condition, translocates into the cytosol under ischemic condition, and is released into the extracellular compartment. The extracellular release of HMGB1 occurs within 2 to 4 hours after the stroke onset and induces the breakdown of blood brain barrier (BBB)[11]. The administration of HMGB1 neutralizing antibody improves the vascular permeability and attenuates ischemic brain damages. Thus, HMGB1 is an essential DAMP in the hyperacute phase of ischemic brain injury.

The number of infiltrating macrophage increases from day 1 to 3 after stroke onset; on the other hand, the extracellular release of HMGB1 seems too early to have significant effects on infiltrating macrophages directly[12-14]. It is possible that another DAMP in the ischemic brain triggers post-ischemic inflammation by activating immune cells and such a molecule may be one of the ideal therapeutic target for ischemic stroke.

We have investigated other potential DAMPs in brain homogenate lysates and determined peroxiredoxin (Prx) family proteins as an essential initiator of cerebral post-ischemic inflammation[12]. We have discovered that brain homogenate lysate has an inflammatory cytokines-inducing activity in cultured dendritic cells. Because such an activity was almost completely diminished by heat or proteinase treatment, some specific proteins were presumed to function as DAMPs. With various fractionation methods including sucrose density gradient centrifugation, we have detected high activity to induce inflammatory cytokines in 15-25 kDa fractions. After further analysis by LC/MS and the recombinant protein assay, we have identified Prx family proteins (Prx1, Prx2, Prx5, and Prx6) as strong inducers of inflammatory cytokines in cultured dendritic cells.

Peroxiredoxin as a previously unknown DAMP

Compared with HMGB1, it may take more time for Prxs to function in the ischemic brain. Prxs expression is induced by ischemic stress and becomes evident 12 hours after stroke onset[12]. The highly expressed Prx is released into the extracellular compartment around injured brain cells and co-localizes with the membranes of infiltrating macrophages. Such an extracellular release of Prxs directly activates infiltrating macrophages. In fact, the neutralization of extracellular Prxs decreases the expression of inflammatory cytokines in infiltrating inflammatory cells, and thereby attenuates ischemic damage in mice. Interestingly, an increased amount of Prx proteins in the extracellular fluid of the ischemic brain has been also reported in rats and stroke patients[15, 16]. Thus, the extracellularly released Prxs from injured brain cells appears to activate infiltrating macrophages and initiates post-ischemic inflammation.

Prxs have been well-known as ubiquitous anti-oxidant enzymes, which are more abundant in brain than in other

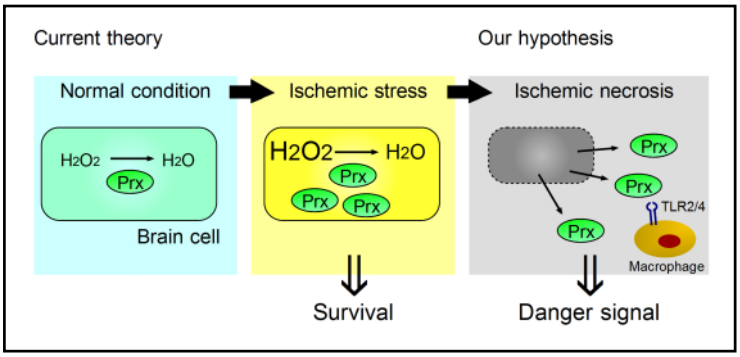

Fig.1 Two functions of Prx, one inside and one outside brain cells

The expression of Prxs within brain cells is induced by ischemic stress. Intracellular Prxs contribute to the survival of brain cells by catabolizing reactive oxygen species (ROS). When ischemic phenomena finally result in necrosis, intracellularlly expressed Prxs are released into the extracellular compartment. Such extracellular Prxs function as strong TLR2 and TLR4 stimulators (DAMPs) and activate infiltrating macrophages in ischemic brain.

tissues. Prx proteins contain one (1-cysteine (1-Cys): Prx6) or two (2-Cys: Prx1-5) cysteine residues to scavenge ROS in cooperation with thioredoxin (Trx)[17]. Intracellular induction of Prxs are observed in various brain tissue injuries and are considered to diminish ROS and protect from tissue damages[18]. However, another function of extracellularly released Prxs as DAMPs has not been reported. We have demonstrated that Prx family members are strongly induced inside the injured ischemic cells to improve their survival. Thereafter, they are released into the extracellular compartment once the cells are about to die (ischemic necrosis), and such an extracellular release of Prxs activates infiltrating macrophages directly and triggers the production of inflammatory cytokines. Thus, Prxs have two opposing functions, one inside and one outside the brain cells (Fig.1).

To investigate the common structure of Prxs for the induction of inflammatory cytokines, we have developed mutant recombinant proteins of Prxs and elucidated that the cysteine residues which are important for anti-oxidant activity do not function as DAMPs. On the other hand, if α3 helix region of Prxs are deleted, their cytokine-inducing activity is diminished[12]. The α3 helix region of Prxs exists in the relatively sequestered position on the molecular surface and is located almost in the opposite side from the location of anti-oxidant activity, according to the previous crystal structure analysis[19-21]. Targeting this region of Prxs, a specific anti-inflammatory therapy may be developed for ischemic stroke.

TLRs as DAMPs receptors

TLR2 and TLR4 have been recently reported to contribute to non-infectious sterile organ injury, including ischemic brain injury. Indeed, HMGB1 and Prxs can stimulate TLR2 and TLR4[10, 12]. The activation of macrophages and T cells through TLR pathway induces strong inflammatory responses. The clinical relevance of TLR2 and TLR4 in stroke patients has been also demonstrated[22].

Both leukocytes and brain cells express TLRs and this fact makes it difficult to fully understand the complexity of inflammatory mechanisms in ischemic brain. The function of TLRs on brain has not been sufficiently clarified. Brain cells, which are activated by ischemic injury probably via TLRs, also contribute to the initiation of post-ischemic inflammation by producing inflammatory mediators. TNF-α, IL-1β, nitric oxide, and MMPs which are produced from brain cells regulate the cerebrovascular permeability and exaggerate brain edema[23, 24]. Microglia, resident macrophage in the brain, is also activated via TLRs, and not only acts as an inflammatory mediator by producing neurotoxic cytokines, but also produces neurotrophic factors for tissue repair[25].

We have tried to investigate the function of TLRs in brain cells by using bone marrow (BM) chimeric mice[12]. Although microglia, which are radiation-resistant, are a rapid effector in ischemic brain, TLR2- or TLR4-deficient microglia transferred with wild-type BM reveals no improvement in ischemic brain injury[26]. Mice lacking MyD88, which is the adaptor protein required for almost all TLR signaling cascades (other than TLR3), showed either no improvement or exaggeration[27]. These results have indicated that the effect of TLRs is dependent of the cell types in ischemic brain and the timing of their activation. Due to such a complexity, more detailed knowledge about TLRs function in ischemic brain is needed for the development of the therapeutic potential of this strategy.

Inflammatory cytokines and infiltrating immune cells

Macrophages are the main inflammatory effectors. The

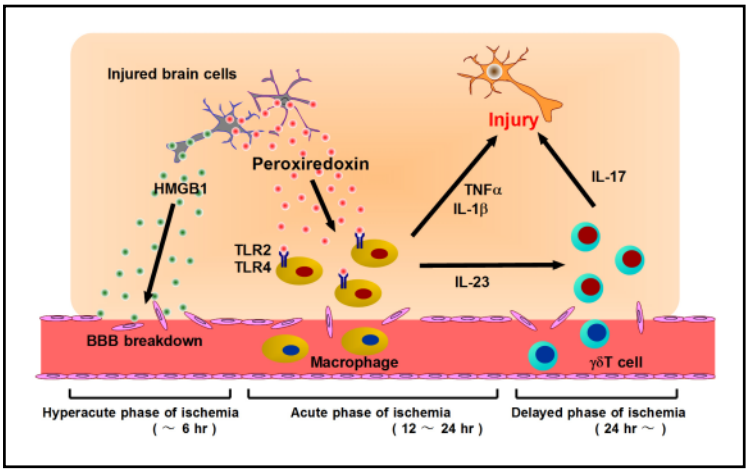

Fig.2 Post-ischemic inflammation is triggered by DAMPs

At the hyperacute phase of brain ischemia (within 6 hours after stroke onset), high mobility group box 1 (HMGB1) is released from injured brain cells and promotes BBB breakdown. Thereafter, circulating macrophages begin to infiltrate into the ischemic brain during the acute phase of ischemia (12 to 24 hours after stroke onset). In this phase, the expression of Prxs is induced within the wounded brain cells by ischemic stress for their survival. When Prxs are released into the extracellular compartment from necrotic brain cells, extracellularly released Prxs induce the various inflammatory cytokine expression from infiltrating macrophages through TLR2 and TLR4. IL-1β and TNF-α directly promote neuronal cell death. On the other hand, IL-23 acts on $\gamma\delta$T cells, which infiltrate during the delayed phase (more than 24 hours after stroke onset) and induces the IL-17 production which enhances ischemic brain damages.

infiltration of macrophages becomes evident from 12 hours to 24 hours after stroke onset and produces various inflammatory cytokines, such as IL-1β and IL-23. These cytokines induces the subsequent inflammation caused by T cells (Fig.2).

T cells are also important effectors in the delayed phase of brain ischemia. The number of infiltrating T cells increases over 24 hours after stroke onset and reaches the peak around day 3[28, 29]. T cells appear to be localized to the infarct boundary zones. IL-23 produced by infiltrating macrophages induces IL-17 production from $\gamma\delta$ T cells in the delayed phase[4]. This IL-23/IL-17 inflammatory axis is implicated in the infarct growth in the delayed phase (from days 1 to 4) in mice. Targeting IL-23- or IL-17-producing $\gamma\delta$T cells may be a promising therapeutic strategy for ischemic stroke[30].

Conclusion

Recent research has gradually shed light on the mechanisms of cerebral post-ischemic inflammation mediated by immune cells. More detailed understanding of this process and tissue repair could lead to the development of specific treatment for ischemic stroke.

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Conflict of interest

None

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