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Role of circulating immune cells in stroke and preconditioning-induced protection

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Summary

Stroke activates an inflammatory response that results in the infiltration of peripheral immune cells into the ischemic area contributing to the exacerbation of tissue damage. However, recent evidence indicates that the inflammatory cell infiltration can also promote neuroprotection through regulatory immune cells that mitigate injury. These immune regulatory cells may also be important mediators of neuroprotection associated with preconditioning, a phenomenon whereby small exposure to a potential harmful stimulus is able to induce protection against a subsequent ischemic event. The elucidation of mechanisms that allow these immune cells to confer neuroprotection is critical to developing new therapeutic strategies against acute stroke. In the present review we discuss the dual role of peripheral immune cells in stroke related brain injury and neuroprotection. Furthermore we report new data from our laboratory that supports the important role of peripheral cells and their interaction with the brain endothelium for the establishment of the protective phenotype in preconditioning.

Keywords

stroke; inflammatory cells; preconditioning; neuroprotection

The good and the bad of immune cell activation in stroke

Brain injury due to ischemic stroke is characterized by complex spatial and temporal events evolving over several days. Inflammation plays a key role. The inflammatory response is a complex regulatory process that begins early and lasts for days and even weeks after ischemia and involves many different cell types, inflammatory mediators and extracellular receptors. The sudden loss of oxygen and glucose causes cells in the ischemic area to be rapidly killed by lipolysis and proteolysis due to total bioenergetic failure. As cells die, they release intracellular components that activate neighboring cells to produce pro-inflammatory mediators such as cytokines and chemokines, and promote transmigration of inflammatory cells from the periphery to the ischemic brain area resulting in the exacerbation of damage [1]. The critical role of inflammatory cells in stroke-induced brain injury has been

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Conflict of interest statement

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demonstrated by the attenuation of ischemic damage in mice that are unable to mount an inflammatory response [2, 3]. However certain aspects of the inflammatory response are critical to remove dead cells and promote regeneration after ischemic injury [4]. Thus, the inflammatory cascade can be beneficial or damaging depending on the stage of tissue injury, the magnitude of the response and whether the inflammatory response also activates neuroprotective pathways [4].

Recent studies indicate that brain injury activates populations of inflammatory-related cells that can limit ischemic damage. For example, subpopulations of regulatory lymphocytes are induced after stroke that *suppress* inflammation, thus promoting protection against injury [5, 6]. In addition peripheral inflammatory cells have been implicated in the induction of protection associated with preconditioning [7]. Preconditioning is a phenomenon whereby exposure to a small but potentially harmful stimulus is able to induce protection against a subsequent ischemic event. These studies point to a role for inflammatory cells prior to an ischemic event in mediating the evolution of injury.

This review discusses the role of circulating immune cell activation in the pathology of stroke injury and neuroprotection, and reports data from our laboratory supporting the role of circulating cells in mediating preconditioning.

Peripheral immune cells contribute to ischemic injury following stroke

Following ischemia, neutrophils, macrophages and lymphocytes have been shown to infiltrate the brain [8]. Neutrophils are the most abundant cell population present at the site of injury with a peak influx between 1 and 3 days after ischemia [8, 9]. They have been widely considered key contributors of inflammatory brain injury since they are a main source of free oxygen radicals that can cause direct neuronal death. In addition neutrophils cause endothelial cell dysfunction and blood brain barrier disruption through release of matrix metalloproteinase 9 [10]. Multiple studies using both neutrophil depleted animals and antagonists or blocking antibodies support neutrophils as key players in inflammatory induced ischemic injury [11–16]. However other studies report no effect on infarct size when neutrophils are depleted or blocked [2, 17]. Some of these differences may be attributed to the particular experimental model system employed, with those models involving reperfusion more likely to demonstrate a role for neutrophils in the exacerbation of injury. Clinical trials reflect these differences with slight improvement seen with an antagonist for CD11b, an integrin highly expressed on neutrophils, used in combination with tissue plasminogen activator, an inducer of reperfusion [18]. However, other clinical trials have shown no effect on stroke outcome when neutrophil infiltration is blocked [19, 20]. These results suggest that neutrophils may not be the only contributors to ischemic injury, but instead a complex interaction of multiple cell populations may mediate inflammatory damage.

Resident microglia and infiltrating macrophages also represent a significant immune population identified in the ischemic tissue following stroke. These cells appear as early as 12 hours after ischemia and peak between 3 and 7 days [8, 9]. Macrophages/microglia produce reactive oxygen species (ROS), nitric oxide, and other cytokines that can have

direct toxic effects on brain tissue following stroke. In addition macrophages and microglia amplify the inflammatory response through recruitment of other immune cells such as lymphocytes. The involvement of macrophages/microglia in the development of infarct damage after stroke was recently demonstrated by Tang et al [21]. In this study attenuation of proliferation and infiltration of microglia/macrophages was obtained by knocking down the chemokine receptor 1 (CX3CR1), which is highly expressed on resident brain microglia and peripheral macrophages. Knockout animals demonstrated a significant decrease in the ischemic lesion, number of apoptotic cells and post-ischemic brain inflammatory responses (including ROS and pro-inflammatory cytokine production), indicating that suppression of macrophage/microglia activation reduces ischemia induced inflammation and neurotoxicity.

The importance of lymphocytes (B and T cells) to the inflammatory response following ischemia has become increasingly evident. Although lymphocyte infiltration in the brain after ischemia peaks around day 3 [8, 9], several studies have found lymphocytes, both B and T cells in the brain as early as 4–6 hr after ischemia, suggesting a role in the development of inflammation in the ischemic area [2, 9]. Severe combined immunodeficient (SCID) mice lacking both T and B cells showed significant reduction of ischemic volume and suppression of post-ischemic induction of inflammatory mediators in the brain, demonstrating the important role of T and B cells in the development of the ischemic damage [3].

Protective role of peripheral immune cells responding to stroke

In addition to the damaging affects of peripheral immune cells, recent studies have shown that inflammatory cells also play a role in protecting the brain from ischemic injury. Downes et al [22] showed that hematopoietic cells have a critical role in mitigating the extent of injury following stroke. In particular, they showed that hematopoietic cells exhibit a neuroprotective function after stroke that is mediated by myeloid differentiation factor-88 (MyD88), the adaptor protein for Toll-like receptors (TLRs) and IL-1 signaling cascades. They demonstrated that mice lacking MyD88 signaling on hematopoietic cells had exacerbated ischemic volume, indicating that particular peripheral cell populations can promote protection against brain injury.

Microglia/macrophages have also been implicated in neuroprotection after stroke. These cells phagocytize debris and dead cells, and microglia can produce neurotrophic factors that can promote neuronal growth and survival, both mechanisms that are important in resolution of inflammation and tissue regeneration after injury. Selective ablation of proliferating microglia has been shown to exacerbate ischemic injury [23], indicating an important role of microglia in modulating protection against ischemia. In addition, exogenous microglial cells administered peripherally 24 hr before or after global ischemia, have been shown to reach the ischemic area, and protect against neuronal injury by releasing neurotrophic factors [24].

B cells may represent another hematopoietic population with potential to confer protection against ischemic injury. Two conflicting reports using B cell deficient mice have defined a neutral or protective role for B cells in ischemic injury. Yilmaz et al [2] reported no difference in infarct size in mice deficient in B cells while Ren et al found exacerbation of

injury in B cell deficient mice, supporting a protective role for B cells [25]. Ren et al were also able to reduce the infarct size in their model by adoptively transferring B cells into the deficient mice [25] further supporting a protective role for B cells in ischemic injury. Further work in this model has defined IL10 secreting B cells (B regulatory cells; Bregs) as the subset of B cells mediating protection [6, 26].

Regulatory lymphocytes represent a small percentage of the infiltrating B and T lymphocytes—referred to as Bregs and Tregs, respectively. They are characterized by their ability to control immune responses, regulate the function of effector T cells and regulate the activity of antigen-presenting cells. The anti-inflammatory cytokine, IL10, is the primary effector of regulatory lymphocytes. A growing amount of evidence points to the importance of these regulatory cells in modulating the inflammatory response after stroke leading to suppressed inflammation and added protection against ischemic injury. Similar to the protective role of Bregs, Liesz et al [5] found that depletion of Tregs significantly increases brain infarct volume and worsened functional outcome, indicating a protective effect of Tregs. Protection was mediated through Treg induction of IL10 and the subsequent suppression of inflammatory cytokines and modulation of the invasion and activation of lymphocytes and microglia in the ischemic brain [5].

It is not clear whether regulatory cells need to infiltrate the brain to induce the protective phenotype. Tregs, for example, have been detected in the brain 3 days after stroke, while their effect on modulating cytokines levels in the brain is already evident 6 hr after ischemia [5], therefore, it is more likely that Treg are able to monitor and regulate the inflammatory response from the periphery. Understanding the mechanisms by which peripheral cells communicate with the central nervous system in the context of injury as well as how they may mediate protection may be critical to the development of new therapeutic strategies against stroke.

Involvement of peripheral immune cells in preconditioning induced neuroprotection

Recently peripheral immune cells have been implicated as contributors to the induction of neuroprotection associated with preconditioning. Preconditioning involves mild treatment with an otherwise harmful stimulus in order to reprogram the cellular response to injury thereby leading to a reduction of damage. A particularly effective means of inducing systemic preconditioning against cerebral ischemia occurs through activation of the innate immune response with ligands for TLR. We have shown that prior systemic administration using one of several TLR agonists (i.e. lipopolysaccharide, CpG oligonucleotides) induces robust neuroprotection against subsequent cerebral ischemia in a mouse stroke model [27–30]. We have also shown significant protection with the TLR9 agonist, CpG, in a non-human primate model of cerebral ischemia [31]. TLR9 is expressed on multiple cells both in the periphery (i.e. leukocytes) and in the brain (i.e. neurons, astrocytes, microglia, endothelium). We have published that effective preconditioning and neuroprotection with CpG stimulation requires TLR9 expression on at least two distinct cell populations, one of hematopoietic origin and one of non-hematopoietic origin [32]. We generated bone marrow chimeric mice by irradiating WT mice and repopulating their leukocytes with cells from

TLR9 deficient mice. Thus, these animals lacked TLR9 expression on leukocytes, while still expressing TLR9 on parenchymal cells such as endothelial cells, astrocytes, microglia and neurons. We found that mice lacking TLR9 expression of leukocytes were unable to be protected by CpG preconditioning, demonstrating that TLR9-mediated leukocyte responses are required to confer CpG-induced neuroprotection. In addition, irradiated TLR9KO mice repopulated with leukocytes from WT mice also failed to be protected by CpG preconditioning, demonstrating that TLR9 expression on leukocyte was not sufficient for CpG-induced neuroprotection. These data indicate a strict requirement for TLR9-mediated responses on both leukocytes and parenchymal cells for protection induced by systemic CpG preconditioning and suggests a need for cross-talk between these compartments to achieve neuroprotection [32].

Systemic administration of CpG allows direct contact with brain microvascular endothelial cells belonging to the blood-brain barrier (BBB). The BBB is the interface between the blood and the brain and strictly regulates the passage of molecules and cells between the periphery and the CNS compartment [33]. Stroke dramatically impairs BBB integrity leading to increased permeability and expression of endothelial adhesion molecules resulting in increased leukocyte adhesion and transmigration [1]. We have found using an *in vitro* BBB model consisting of a co-culture of primary brain microvascular endothelial cells (BMECs) and mixed glial cells (Fig. 1A), that CpG preconditioning can signal and protect the endothelium from ischemic injury. Cell cultures were preconditioned with CpG 24 hr prior to modeled ischemia consisting of 5 hr oxygen-glucose deprivation (OGD). CpG significantly attenuated the drop in trans-endothelial-electrical resistance (TEER) (Fig. 1B) and the increase of BBB permeability to Na-fluorescein induced by OGD (Fig. 1C). These results indicate that CpG preconditioning stabilizes the BBB perhaps by affecting endothelial interactions with circulating immune cells which could alter the inflammatory response to ischemia. In support of this, we have found using *in vivo* 2-photon microscopy, that CpG preconditioning induces leukocyte rolling and adhesion to brain micro-vascular endothelium prior to an ischemic event that may contribute to neuroprotection (Fig. 2).

It remains to be elucidated which leukocyte populations are required for CpG induction of neuroprotection. Bregs offer potential as they are known to be immunosuppressive and CpG has been reported to drive the differentiation of B cells into Bregs [34]. In line with a protective role for Bregs prior to stroke, Bodhankar et al recently showed that adoptive transfer of Bregs 24 hr before cerebral ischemia reduces infarct size [26]. Mice receiving IL-10-secreting B cells showed an increase of regulatory cell populations in the periphery and reduced infiltration of T cells and proinflammatory cytokines levels in the ischemic hemisphere. These results show that the increased presence of Bregs before stroke can modulate the inflammatory response potentially contributing to protection against subsequent ischemia. In further support, Monson et al [7] found that repetitive hypoxic preconditioning (RHP) induces an immunosuppressive phenotype in resident B cells prior to stroke and results in decreased infiltration of leukocytes in the brain following-stroke. It appeared that RHP reprograms B cells to downregulate genes involved in T cell differentiation and B-T cell interactions [7].

Conclusions

These studies clearly demonstrate the complex nature of the inflammatory response associated with stroke. The interplay between damaging and protective affects is a delicate balancing act that determines the extent of injury. Understanding this interplay and developing ways to modulate the immune cell effectors could greatly advance the therapeutic treatment of ischemic brain injury, including stroke. Recent data suggests that endogenous mechanisms engaged by preconditioning include modulation of immune cells, highlighting the effectiveness of targeting these responses in mitigating cerebral ischemic injury.

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Figure 1. CpG protects the BBB in an *in vitro* model of ischemic injury

(A) Schematic diagram of *in vitro* BBB model consisting of brain microvascular endothelial cells (BMECs) co-cultured with glial cells. Pretreatment with CpG attenuates OGD-induced decrease of TEER (B) and increase of permeability for Na Fluorescein (C) in an *in vitro* BBB model of ischemic injury. Values are group means $\pm$ SEM; * p <0.001 versus CTR and $^{\circ}$ p <0.01 versus OGD.

Figure 2. CpG-induces leukocyte-endothelial interactions

(A) Representative images of cortical vessels in mice acquired by *in vivo* two-photon microscopy 24h after treatment with saline or CpG (0.8mg/kg). Leukocytes stained in red. (B) Quantification of leukocytes per mm of vessel from 5 separate video clips $\pm$ SEM of an individual mouse for each treatment.