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EDITORIAL REVIEW

Inflammation After Stroke: Mechanisms and Therapeutic Approaches

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Abstract Reperfusion of ischemic brain can reduce injury and improve outcome, but secondary injury due to inflammatory mechanisms limits the efficacy and time window of such treatments for stroke. This review summarizes the cellular and molecular basis of inflammation in ischemic injury as well as possible therapeutic strategies.

Keywords Stroke · Ischemia · Inflammation · Inflammatory cells · Cytokines · Cell adhesion molecules · Eicosanoids

Introduction

Ischemic stroke occurs when blood flow to the brain is interrupted resulting in immediate deprivation of nutrients and oxygen needed to support the brain's metabolic requirements. Restoration of perfusion, if done very early after the onset of ischemia, can reduce or mitigate damage from stroke, but the efficacy of reperfusion is limited by secondary injury mechanisms. This complex series of events leads to the activation of noxious cycles of inflammation, oxidant stress, and apoptosis that ultimately result in delayed death of neurons even when the brain is successfully reperfused [1]. Increasing evidence, from both clinical as well as experimental studies, suggests a major role of inflammation in this secondary injury that occurs after reperfusion [2]. This review addresses the cellular and molecular mediators of inflammation and describes experimental therapeutic strategies that may improve outcome after stroke.

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Inflammatory Cells

Leukocytes/Neutrophils

Increased infiltration of peripheral leukocytes into the brain has been observed within hours to days after the ischemic insult in human cases of stroke [3–6]. Generally, neutrophils are first among the leukocyte species to accumulate in the brain after stroke, and their number has direct correlation with infarction volume [7]. Price and colleagues, using selective labeling of neutrophils, reported that neutrophils are recruited to the brain within 24 h of the ischemic insult [8]. In a rat model of cerebral ischemia, neutrophils may remain more than 3 days or longer in the ischemic brain; however, their presence becomes less evident after 3 days, obscured by the massive accumulation of activated microglia/macrophages.

Several studies have shown that inhibition of neutrophil infiltration reduces cerebral ischemic injury and improves outcome [9]. In the rat model of cerebral ischemia, neutrophil inhibitory factor, a 41kD recombinant glycoprotein derived from hookworm, showed neuroprotective effects by reducing the number of infiltrated neutrophils and infarct volume [10]. High doses of the protein adminis-

tered for 7 days exhibited maximum neuroprotection in a temporary focal ischemia model but was ineffective in a permanent cerebral ischemia model [11]. In another study involving rats, RP-3 monoclonal antibodies that selectively reduce leukocytes in the rat by 90% to 95% showed a significant reduction in both neutrophil accumulation and infarct size [12].

Microglia/Macrophages

Microglia are the resident macrophages of the brain and spinal cord. Microglia act as the main form of active immune defense in the central nervous system and have phagocytic properties, serving as scavenger cells in the event of injury and infection. Once activated, microglia can undergo morphologic transformation into phagocytes, making them virtually indistinguishable from circulating macrophages [13, 14]. Price and colleagues demonstrated significant microglial activation after 72 h, extending to 30 days, in core infarction areas, contralateral hemisphere, and the peri-infarct zone of stroke patients, although only minimal activation of microglia is seen before 72 h [8]. The mechanisms by which microglia are activated following ischemia have not been entirely elucidated, but involve activation of CD14 receptors in microglia [15].

Microglia are activated after ischemia and release a variety of substances, many of which are cytotoxic and/or cytoprotective [16–18]. In the four-vessel rat model of cerebral ischemia, increased staining of microglia, consistent with activation, are found in the dentate hilus and CA1 of ipsilateral hippocampus starting as early as 20 min after reperfusion. The strongest microglial activation was observed 4–6 days after reperfusion when reactive microglia were abundant throughout all laminae of CA1 and the dentate hilus [16]. In transient middle cerebral artery occlusion (MCAO), phagocytic microglia are found in the cerebral cortex of the ischemic hemisphere [19, 20].

Cytokines and Chemokines

Cytokines

There is a large literature demonstrating an increased production of cytokines after stroke. Several clinical studies have shown an increase in expression of IL-6, but other studies show increases in expression of tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1; or CCL2) and interleukins (IL)-1β, IL-8, and IL-10. Differential expression of cytokines occurs after stroke and depends on the time of sampling and preexisting levels of peripheral inflammation [21]. Many studies have reported increases in plasma levels of cytokines after stroke that correlated with poor outcomes. The increase in IL-6 concentration starts as early as 24 h up to 7 days [4, 22–24]. Tarkowski and colleagues found that increased cerebrospinal fluid (CSF) cytokine levels but not serum IL-6 correlated with stroke outcome. Furthermore, increased IL-1β levels were observed in CSF [25] but not in serum [26]. Similar results were obtained from patients at 4 h after stroke onset, who had increases in serum IL-6 [27]. Serum and CSF levels of TNF-α are increased within the first 24 h after stroke [28] but may not be correlated with lesion size or neurological impairment [29].

Interleukins

Compelling evidence from experimental studies suggests a prominent role for interleukins in ischemic injury [3, 9, 13]. IL-1β mRNA expression has been shown to increase in cerebral cortex, striatum, thalamus, hippocampus, and olfactory bulb after transient brain ischemia in rats within minutes of ischemia and increases are sustained until 7 days [30–33]. Exacerbation of ischemic brain injury caused by exogenous IL-1β administered into the brain has been observed [34, 35]. Mice deficient in IL-1α/ β exhibited dramatically reduced ischemic infarct volumes compared with wild-type; however, mice lacking either IL-1α or IL-1β alone did not have significantly altered infarct volumes [36]. IL-1 receptor null mice had smaller infarcts compared with wild-type controls and overexpression of IL-1ra antagonist reduced ischemic injury [37, 38]. Similar results were obtained in a rat stroke model using the IL-1ra antagonist [39, 40]; mice lacking IL-1ra exhibited a dramatic increase in ischemic damage [41]. Among the two receptors IL-1R1 and IL-1R2 for IL-1, only IL-1R1 is reported to have role in mediating ischemic injury [42]. Inactivating or knocking out IL-1R1 decreased the extent of damage caused by a hypoxic–ischemic (H/I) insult and preserved neurological function [43].

TNF-α

TNF-α is also upregulated in the brain after ischemia and plays a vital role in the execution of inflammatory cascade after ischemia [3, 9, 13]. TNF-α protein and message is increased within a few hours of middle cerebral occlusion in rats [44]. Protein expression is initially observed in neurons and later in glia [44, 45].

There are conflicting data as to whether TNF-α exacerbates ischemic injury. Several studies support a role for TNF-α in exacerbating injury: TNF-α overexpressing rats had larger Infarcts than wild-type at 24 h and 7 days after cerebral ischemia [46]. Inhibition of TNF-α using anti-murine TNF-α antibody also reduced ischemic brain injury in mice [47]. Application of recombinant TNF-α

protein after stroke onset worsens ischemic brain damage by non-neuronal mechanisms [48]; tumor necrosis factor binding protein was protective against stroke in mice [49]. However, other studies have not supported a role for TNF-α in ischemic injury: Murakami et al. found a difference in hippocampal cell death after global ischemia between wild-type and mice bone marrow transplanted-chimeric–TNF-α gene-deficient [50]. TNF-α may also protect the brain from ischemic insult by invoking ischemic tolerance probably via TNF receptor 1 upregulation [51]. TNF-R1 is upregulated after ischemia [52] and TNF-R1 receptor null mice had larger infarcts after temporary focal ischemia [53]. Conflicting studies about the role of TNF-α might be due in part to its two receptors, TNF-R1 and TNF receptor 2 (TNF-R2), which may have opposing effects upon cell death. Most of the studies have highlighted the role of TNF-R1 in cell death or cell survival. However, there seems to be no role for TNF-R2 in ischemia [54].

Chemokines

Chemokines are a family of regulatory polypeptides with roles in cellular communication and inflammatory cell recruitment in host defense, such as regulating the migration of leukocytes in inflammatory and immune responses. Chemokines operate via G protein-coupled receptors and can be divided into four groups: C, CC, CXC, and CX3C based on the positions of their cysteine residues [55]. MCP-1, the major chemokine in mammalian systems, may play a crucial role in ischemic injury. High levels of MCP-1 have been observed in CSF as compared with controls [56]. Several studies have found increased concentrations of MCP-1 in the serum of stroke patients compared with healthy controls [57–59]. Another study [60] reported that CXCL1, a potent neutrophil chemoattractant, was significantly higher in the CSF of stroke patients as compared with controls and that these levels correlated positively with the volume of brain CT hypodense areas.

Monocyte Chemoattractant Protein-1

MCP-1 is expressed in ipsilateral cortex and striatum after MCAO in rats [57, 61] beginning 12 h after ischemia in neurons and 2 days after ischemia in astrocytes [62]. MCP-1 deficiency is protective in a mouse stroke model, probably because of IL1-β in ischemic tissue [63]. Overexpression of MCP-1 in the brain exacerbates ischemic brain injury and is associated with recruitment of inflammatory cells [64]. Administration of MCP-1 significantly enhanced the permeability of blood brain barrier (BBB) *in vivo* and *in vitro* [65].

Fractalkine, a neuronally expressed chemokine, acts through its G protein-coupled receptor CX3CR1. It is a transmembrane protein possessing adhesion properties and exists in two forms with intrinsically different spatial properties and biologic functions [66–70]. Interestingly, fractalkine is expressed in neurons while its receptor is found predominantly on resident microglial cells, suggesting coordination between neurons and microglia [13, 71–73]. In animal stroke studies, fractalkine expression was increased in injured cortical neurons that peaked at 48 h and returned to basal levels by 7 days after ischemia. Upregulation of fractalkine was also detected in endothelial cells of the infarcted area at 48 h and 7 days after ischemia. Additionally, fractalkine receptor CX3CR1 expression was detected in the activated microglia in infarcted tissue at 24 and 48 h after ischemia and became strongly upregulated in 7 days. These data suggest that fractalkine may participate in the activation and chemoattraction of microglia into the infarcted tissue and contribute to the control of leukocyte trafficking from blood vessels into the injured area [74]. In addition, fractalkine-deficient mice have smaller infarct volumes and lower mortality after transient focal cerebral ischemia [75].

Adhesion Molecules

Cell adhesion molecules (CAMs) are membrane-bound proteins that are involved in many important processes of the cell. CAMs act as a molecular link between the outside and inside of the cell environment and have a role in cell–cell communication. CAMs facilitate migration of inflammatory cells to the site of injury. The major CAMs are selectins (P-, E-, and L-subtypes), integrins (LFA-1, Mac-1), intercellular adhesion molecule-1 (ICAM-1), and vascular adhesion molecule-1 (VCAM-1) [13, 76–78]. Strong precedent exists in the literature regarding the importance of CAMs in stroke-related brain injury [79–81]. CAMs play a central role in the infiltration of leukocytes into the brain parenchyma after stroke and may represent important therapeutic targets. Activated leukocytes result in further damage of ischemic lesions through reperfusion or secondary injury mechanisms [13, 82, 83]. Thus, CAMs may represent important therapeutic targets.

Selectins

The selectin family of adhesion molecules mediates the initial rolling and tethering of leukocytes to endothelium. L-selectin is expressed constitutively on lymphocytes; E-selectin is expressed by activated endothelial cells, and P-selectin is expressed by activated platelets and endothelial cells [84]. In patients, there is an early increase of E-selectin levels in plasma in the early hours after stroke [111]. Post-mortem samples of brain from patients who died of cardiac arrest or focal infarction revealed a robust expression of P-selectin

and ICAM [85], and patients with severe periventricular white matter lesions demonstrated increased levels of P-selectin [86]. Soluble P-selectin and E-selectin were significantly elevated in plasma in patients with acute stroke [87].

In experimental stroke models, expression of P- and E-selectins is also increased, and there is compelling data that suggests that selectin expression exacerbates ischemic injury [88, 89]. P-selectin-overexpressing mice had larger infarcts as compared with wild-type, and treatment with antibodies or inhibitors against P- and E-selectin was associated with improved neurological outcome [89, 90]. P-selectin immunoreactivity was observed in post-capillary venules of the cerebral cortex and caudate in the MCA territory with a peak at 8 h to 1 day after cerebral ischemia in rats [91]. Treatment with anti-P-selectin monoclonal antibody significantly reduced ischemic damage in transient [92] and permanent cerebral ischemia [93] by improving blood flow and reducing infiltration of leukocytes [93] Additionally, P-selectin knockout animals had smaller infarctions and improved survival compared with wild-type mice. A post-ischemic blockade with monoclonal antibody raised against P-selectin also improved early reperfusion and stroke outcome [94]. Pharmacologic inhibition of both P- and L-selectin with fucoidin significantly reduced infarct size and improved neurological function in experimental stroke in rats [84]. L-selectin does not appear to be important in exacerbating cerebral ischemic injury. Treatment with L-selectin antibody proved ineffective in a rabbit model of stroke [95].

CAM and VCAM

ICAM-1 (CD54) is constitutively expressed in low levels on cell membranes of endothelial cells, leukocytes, epithelial cells, and fibroblasts, and its expression increases upon stimulation by cytokines. VCAM-1 (CD106) is an endothelial cell membrane receptor, whose expression is also induced by cytokines. The roles of ICAM-1 and VCAM-1 in stroke have been addressed in a number of studies [1, 3, 9, 13, 96].

ICAM-1 expression in brain is increased within hours after cerebral ischemia and peaks at about 12–24 h [97–100]. Pharmacological blockade or genetic ablation of ICAM-1 has protected against ischemic damage in rodents [3, 13]. Ablation of the ICAM-1 gene in mice led to smaller infarcts, improvement of neurological deficits, and increased blood flow to infracted areas as compared with wild-type mice after stroke [101, 102]. ICAM-1 deficiency attenuates necrosis but not apoptosis after cerebral ischemia [103, 104]. In mice, rosuvastatin, a 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitor, protected mice from ischemia/reperfusion injury by suppression of post-ischemic ICAM-1 expression [105]. Blockade of ICAM-1 with antibodies [106–108] and antisense oligonucleotides against ICAM-1 mRNA protected against stroke-related injury and behavioral deficits in rats [109].

Clinical studies have shown that there is an increase in serum, plasma, or CSF levels of ICAM-1 and VCAM-1 within the first 24 h after stroke [110–113], and these levels were dependent on leukocyte response [110]. Expression of ICAM-1 and VCAM-1 was observed in post-mortem brains of patients who died of stroke [85, 114]. Treatment with a murine anti-ICAM-1 antibody (enlimomab) was investigated in patients with acute ischemic stroke in the Enlimomab Acute Stroke Trial (EAST). The trial had to be halted after it was observed that the antibody led to significantly more adverse events than placebo [115]. However, the outcome of this trial may have been influenced by the use of a murine antibody in humans.

Blood concentrations of VCAM-1 are also increased in stroke patients compared with healthy controls [114, 116], and its expression was observed in cerebral endothelium after stoke [117]. Cervera and colleagues have shown that heparin-induced neuroprotection involves decreased VCAM-1 expression along with other inflammatory mediators in rats after cerebral ischemia [118]. But, other studies have not confirmed a role of VCAM-1 expression in ischemic injury. Antibodies targeted against VCAM-1 had no apparent effect on stroke outcome in rats and mice [119].

Arachidonic Acid Metabolites

Arachidonic acid and its metabolites are important inflammatory mediators in ischemic injury. Arachidonic acid (AA) is an unsaturated fatty acid that is released by activation of phospholipase A2s (PLA2s), particularly cytoplasmic PLA2 from the membrane phospholipid of the cell. Thereafter, it may be metabolized by at least two cyclooxygenase (COX) isoforms to prostaglandins and related compounds, via lipoxygenases to leukotrienes and via p450-catalyzed metabolism to epoxyeicosatrienoic acids (EETs) [120].

Cyclooxygenase Pathway

Arachidonic acid, by the action of cyclooxygenases, is metabolically degraded into prostaglandins that have been shown to have both pro- and anti-inflammatory roles in mammalian systems besides serving in other physiological functions. Cyclooxygenases exist in two isoforms, COX-1 and COX-2, sharing the same catalytic functions but having different physiological roles. COX-1 is present in most cells constitutively and is involved in normal housekeeping functions while COX-2 is the predominant isoform expressed in neurons, and its expression is induced by excitatory amino acids and spreading depression [121–123].

Clinical studies have shown that COX-2 protein is expressed in infarcted human brains and is present in both neuronal and glial cells throughout the brain [124]. Furthermore, Iadecola and colleagues observed its expression in infiltrating neutrophils, vascular cells, and neurons in the peri-infarct zone [125]. Strong precedent exists in the literature highlighting a key role for COX-2 in cerebral ischemic injury in animal models [2, 126–128]. Disruption of the COX-2 gene provides protection against ischemic brain injury in rodents [129, 130] while COX-2 overexpressing mice had larger infarcts after experimental stroke [131]. Selective pharmacologic inhibition of COX-2 activity has proven to be a potential therapeutic target against stroke in animal models [132, 133].

COX-1 is thought to be involved in housekeeping functions in brain such as regulation of blood flow. Mice deficient in COX-1 were more susceptible to stroke, possibly due to COX-1's role in vasodilatation [134]. Conflicting results have been reported [135] in transient global cerebral ischemia, wherein pharmacologic inhibition of COX-1 with valeryl salicylate increased the number of healthy neurons in the CA1 region of hippocampus.

Prostaglandin E2 and D2 are two major prostanoids formed by the cyclooxygenase pathway and have been implicated in ischemic injury in brain. Prostaglandin PGD2 and PGE2 levels are increased in brain after ischemia [126, 132, 133, 136–138]. After concerns about the safety of COX-2 inhibitors were raised in 2004, most of the research related to role of the cyclooxygenase pathway in stroke was focused on the receptors of PGE2 and PGD2. PGE2 receptors, which elicit their actions through four G protein-coupled receptors (EP1-4), are mediators of stroke-induced injury, proving to have some paradoxically protective effects depending on which receptor is activated [139]. It has been demonstrated that the prostaglandin EP1 receptor signals through phospholipase C and phosphatidylinositol turnover and prompts excessive of release of intracellular calcium through a G_i-coupled mechanism. EP2 and EP4 receptors stimulate adenylyl cyclase and increase intracellular levels of cAMP via a G protein-coupled receptor mechanism [140]. The EP3 receptor has multiple isoforms and signals via the activation of several pathways, leading to increased cAMP levels, calcium mobilization, and activation of phospholipase C [140, 141].

Accumulating evidence suggests that EP1 receptors are mediators of COX-2 toxicity in stroke and excitotoxicity that is executed through its effects on intracellular calcium. EP1 receptor knockout animals or its pharmacological inhibition results in reduced susceptibility to cerebral ischemia as compared with wild-type controls [142–144]. Inhibition of the EP1 receptor pathway in cerebral ischemia has a long therapeutic window [145], involves the PI3K/AKT signaling cascade [146], and modulates cerebral blood flow [147].

The EP2 receptor, through a cAMP-dependent pathway, is protective in cerebral ischemia and excitoxicity [148–150]. Pharmacologic activation of EP3 receptors abrogated neuronal injury [151] while its disruption or genetic deletion prevented stroke-induced damage in mice [152] 72 h after ischemia. However, a conflicting study was reported by Li and colleagues [153], who observed no differences between EP3-deficient mice and their control littermates. The EP4 receptor has been implicated in cerebral ischemia [153], and its pharmacological activation protected NMDA-induced brain lesions in mice [154].

Prostaglandin D2 is the most abundant prostaglandin in brain and has pronounced effects on ischemic injury by activating its DP1 and DP2 receptors. It has been observed that mice lacking in the DP1 receptor have increased infarcts after ischemia [155] and that selective stimulation of DP1 receptors afforded protection against cerebral ischemia in mice [156] after 72 h. The DP1 receptor was also protective against excitoxicity in a cAMP-dependent manner [155, 157]. DP1 receptor activation also protected hypoxic/ischemic injury in mice while DP2 gene deletion did not increase infarct size [158]. Cyclopentenone prostaglandin metabolites of PGD2 such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), have been shown to exhibit neuroprotective effects in experimental stroke, by reducing infarct volume and improving neurologic deficit through a peroxisome proliferator-activated receptor gamma (PPARγ)-dependent mechanism, inhibiting NFκB signaling and neutrophil infiltration [159–161]. 15d-PGJ_2 can be protective or toxic depending on the dose [162].However, prostaglandin J_2 induced toxicity and expression of inflammatory mediators in neuronal cells through the p38MAPK pathway [163].

5-Lipoxygenase Pathway

Arachidonic acid is also metabolized to 5-hydroperoxyeicosatetraenoic acid by 5-lipoxygenase (5-LOX), which is further metabolized to leukotriene A4, a precursor of cysteinyl leukotrienes. There is some evidence that 5-LOX has a role to play in stroke. It had been observed that 5-LOX translocates from the cytosol to the membrane fraction after ischemic damage [164]. Tomimoto and colleagues have observed 5-LOX immunoreactivity in perivascular monocytes in autopsied human brains of stroke victims [165], and 5-LOX was expressed in animal brains after excitoxicity and cerebral ischemia [166–168]. In animal studies, 5-LOX inhibitor, AA861, abrogated brain edema, cell death, and LTC4 levels [166, 169]; additionally caffeic acid, which inhibits 5-LOX, attenuated OGD-induced death of PC12 cells [170]. Elevated levels of LTC4 along with arachidonic acid after ischemia lead to membrane permeability, resulting in BBB dysfunction, edema, and ultimately to neuronal death [171]. However, genetic deletion of 5-LOX proved to

be ineffective in transient and permanent cerebral ischemia in mice [172]. In contrast to this study, pharmacological inhibition and/or genetic deletion of 5-LOX inhibited rosiglitazone-induced neuroprotection and down-regulation of inflammatory gene expression, LXA [4] synthesis, and PPARγ transcriptional activity in rodents after cerebral ischemia [173]. Some 5-lipoxygenase metabolites, such as lipoxin A4, may protect the brain from ischemic injury [174].

Other Lipoxygenase Pathways

Less is known about the role of the other lipoxygenase pathways in cerebral ischemic injury since there are fewer available specific inhibitors. Non-specific lipoxygenase inhibitors such as BW755C decrease leukocyte migration, prevent post-ischemic hypoperfusion, and decrease infarct volume after middle cerebral artery occlusion in rats [175]. More recent studies suggest an important role for 12/15 lipoxygenase in ischemic injury. The natural product baicalein is an inhibitor of 12/15-lipoxygenase and reduces infarct volume in mice after temporary focal ischemia. Furthermore, 12/15-lipoxygenase null mice have smaller infarction after temporary focal ischemia compared with wild-type controls [176].

20-HETE and EETs

Arachidonic acid may be metabolized by cytochrome P450 to produce the potent vasoactive metabolites, 20-hydroxyeicosatetraenoic acid (20-HETE) and EETs. 20-HETE is a potent vasoconstrictor produced by metabolism of AA by the 4F isoform of the P450 enzyme. Inhibition of synthesis of 20-HETE with HET0016 decreases infarction volume after temporary focal ischemia and increases post-ischemic regional cerebral blood flow [177]. EETs are produced in astrocytes by the P450 2C11 isoform, have vasodilatory effects on the cerebral circulation, and may play an important role in coupling local cerebral blood flow to metabolic demand [178].

Conclusion

A wealth of data from both human and animal studies indicates that inflammation mediated by inflammatory cells, cytokines, cell adhesion molecules, and eicosanoids occurs after ischemic injury and may exacerbate ischemic injury. The development of a variety of approaches to prevent this post-ischemic inflammation shows great promise to augment therapies aimed at reperfusion. As yet, no such therapies have been translated to clinical application, but the treatment of inflammation in stroke remains a viable target for drug development.

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