**Review**

Alternate approach to understanding the molecular mechanisms of stroke-induced injury

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**Summary.** Research in the area of stroke has not yielded any new treatments, besides tissue plasminogen activator. New findings are suggesting that the therapeutic window of providing neuroprotection is wider than once thought. Moreover, the role of the peripheral immune system in abetting neurodegeneration is being elucidated, but it appears this reaction occurs 2-3 days after the stroke. This mini-review examines this new evidence about the molecular mechanisms leading to stroke-induced neuronal death, which suggests new therapeutic approaches to its treatment.

**Key words:** Ischemia, Microglia, Neuronal death

**Introduction**

Stroke is the third leading cause of death and the leading cause of disability not only in this country, but worldwide. This major health problem disorder has remained as the third overall cause of death for over one hundred years with only one FDA-approved treatment. One of the major problems with drug discovery for this disorder is finding a reliable experimental model to test novel agents. Another difficulty relates to the interpretation of the data in the context of the generally accepted strategies for treatment.

Currently, tissue plasminogen activator (TPA) is the only FDA approved agent for stroke treatment and it is useful only for embolic stroke (85% of all strokes), not the hemorrhagic type. This treatment is only effective within 3 hours of onset and only treats the symptoms by dissolving the clot. Only 2-3% of stroke patients are able to use this agent because of its narrow therapeutic window. Other experimental treatments have been aimed at targeting putative neurotoxic substances, intracellular calcium, glutamate and free radicals. These monotherapies have shown negligible or no effectiveness in treating stroke although at least one antioxidant is currently in clinical trials showing minimal efficacy up to 6 hour post-stroke (Jonas et al., 2001). These unsuccessful efforts suggest that strategies that broadly target both promotion of neurosurvival and reduction of the inflammatory response are necessary to treat stroke at delayed timepoints.

Recent studies have shown that at least in the rat middle cerebral artery occlusion model of stroke, the therapeutic window extends up to 48 hours after surgery. Systemic injection of human umbilical cord blood cells (HUCBC) at 48 hours post-stroke reduces infarct volume by 85% and enhances behavioral recovery such that performance did not differ from sham-operated rats. Moreover, markers of neurodegeneration appearing at 48 hours post-stroke also decline by 85% after HUCBC treatment (Newcomb et al., 2006). Another recent study shows that systemic administration of the sigma receptor agonist, 1,3-diotoylguanidine (DTG), is able to reduce infarct volume by 80-85% when given at 24 hours post-stroke in rats (Ajmo et al., 2006). These studies demonstrate that the window of therapeutic intervention is much wider than is conventionally thought.

Both of these treatments share two properties in common, neuroprotection and anti-inflammation. While HUCBC were considered a “stem” cell treatment that replaces damaged neurons, it has come to light that HUCBC secrete substances to reduce neurodegeneration (Vendrame et al., 2005). These cells migrate to the infarct and the spleen to change the cellular environment of this organ from a pro-inflammatory to an anti-inflammatory one (Vendrame et al., 2004). While DTG has not been as well-studied as HUCBC treatment, it and other sigma receptor agonists have long been used as agents to treat stroke and a number of them have potent anti-inflammatory properties (Bourrie et al., 1995, 2002; Gannon et al., 2001). However, the novelty lies in using...
Markers of neurodegeneration

To determine if an agent is capable of treating stroke, it must reduce neurodegeneration and enhance behavioral recovery, although some treatments have shown increased behavioral recovery without a reduction in neurodegeneration in the penumbra. A standardized methodology is crucial to consistently measure neurodegeneration and be able to compare results from one drug study to another.

The quantification of neurodegeneration in the field of stroke research has relied heavily on the use of 2, 3, 5-triphenyltetrazolium chloride (TTC) (Liszczak et al., 1984; Bederson et al., 1986; Lundy et al., 1986). The TTC produces a red reaction product when metabolized by respiratory enzymes located in mitochondria, while infarcted tissue, lacking these enzymes, appears white. This is a quick and easy method to analyze damaged brain tissue on a gross scale in experimental stroke models.

Fluoro-Jade is a fluorescent dye that is supplanting other detection methods in visualizing degenerating neurons (Schmued et al., 1997). This method can detect individual degenerating neurons making it superior to TTC staining (Duckworth et al., 2005). Moreover, Fluoro-Jade staining can still demarcate the infarct area days to weeks after the occurrence of stroke. TTC is only used up to 36 hours post-stroke (Liszczak et al., 1984). The ease of use and reproducibility are other positive attributes of this staining. While the mechanism for Fluoro-Jade staining is unknown, as a basic dye, it may be attracted to degenerating neurons since they are undergoing acidification.

Apoptosis is the putative mechanism for the delayed neuronal death in the penumbra. Death by apoptosis is highly regulated and leads to an organized demise that does not induce inflammation. Activated caspase-3 and TUNEL staining, a determination of the orderly fragmentation of DNA, are two common methods to detect apoptotic processes. However, the question arises whether all of the above mentioned methods are absolute determinants that a neuron is or will be dead. Recent report shows that 85% of the neurons labeled with these markers can be rescued up to 48 hours post-stroke but not any later (Newcomb et al., 2006). Without blood flow supplying oxygen, neurons may revert solely to glycolysis to survive as long as possible. Gene array of hypoxic-exposed neuronal-like cell cultures show that many up-regulated genes are associated with glycolysis, which is not dependent on mitochondrial function (Butler and Penney, 2005). In the absence of oxygen, mitochondrial oxidative phosphorylation would shut down; this would account for the lack of TTC staining. Glycolysis would promote an acidified environment leading to labeling by Fluoro-Jade.

Apoptotic markers do not necessarily determine death since many prosurvival signals, such as inhibitors of apoptosis and Bel proteins, can block the progression of organized neuronal degeneration. Analysis of gene expression shows that both survival and death related genes are both up-regulated after stroke indicating that some neurons are probably expressing both groups simultaneously (Lu et al., 2003). Thus, the interpretation of neurons detected by these stains should be that they are compromised, which without therapeutic intervention, will lead to death.

Microglia and Stroke-induced Injury

Microglia are the endogenous macrophage of the brain and share many characteristics of macrophages (Streit et al., 1988). In fact, they cannot be distinguished immunologically from macrophages and are developmentally derived from the mesoderm. The brain is an immune-privileged organ and microglia are essential to removing extracellular debris. Microglia alter their morphology from a resting ramified form to an amoeboid-like cell that is associated with an activated state. Several immune-associated markers are often used to define the state of activation.

The role of microglia in the brain injury response is controversial but many reports blame them for exacerbating the neurodegeneration in many neurological disorders, including stroke (Morioka et al., 1993; Streit et al., 2004). In stroke models, microglia are activated very rapidly and remain activated for days (Morioka et al., 1993). Microglia once activated can produce nitric oxide and noxious cytotoxic cytokines (Gregersen et al., 2000). The discovery of IL-1β production in microglia was the first example of these cells producing putative cytotoxic substances (Giulian and Lachman 1985; Giulian et al., 1986) which initiated a scientific consensus that these cells play a deleterious role in brain injury and neurological disease states.

Other reports have suggested that microglia are actually neuroprotective in injurious situations. A great deal of work has been performed on facial motor nucleus axotomy resulting in loss of the innervation. In this injury model, microglia become activated and are neuroprotective by pruning injured neurons and releasing neurotrophic factors (Streit, 2002). In fact, exogenous application of microglia to the brain (Kitamura et al., 2004) or brain slices (Neumann et al., 2006) have been shown to protect neurons exposed to...
ischemic conditions. Moreover, activated microglia have been detected with no concomitant neurodegeneration. For example, in several mouse models of Alzheimer’s disease activated microglia are observed surrounding the plaques with no sign of neurodegeneration (Irizarry et al., 1997). Other studies have shown that blocking the microglial response to injury does not affect neurodegeneration (Sriram et al., 2006a). Moreover, generally regarded deleterious substances are not strictly cytotoxic but play multiple roles in a cell-specific manner. For example, TNF-α has been shown to be a growth factor in the hippocampus but is neurotoxic to striatal neurons (Sriram et al., 2002, 2006b).

In conclusion, a large body of evidence suggests that microglia are not the noxious cells that they are purported to be. It is more likely that there are two phases of microglial activation—one that occurs early in a disease process or after an injury and is neuroprotective and a later inflammatory response that if left unchecked can be deleterious. This is consistent with the results of a study by Stromberg et al. (2005). Rats maintained on diets enriched with antioxidants and anti-inflammatory phytochemicals that have been found to be neuroprotective were subjected to 6-hydroxydopamine treatment to induce a dopamine lesion. Those animals on the special diets had a greater microglial response at 1 week post-lesion. However, lesion size was smaller in these treated animals than in the untreated controls. Even though the microglial response in the untreated animals was less at 1 week post-lesion, it was greater at one month post-lesion. Clearly, the early microglial response was neuroprotective or neuroreparative while later responses were destructive. Much more research is needed to determine the mechanisms of action of microglia in brain after injury.

Peripheral immune system and stroke

When ischemic stroke occurs, the body’s inflammatory response triggers the opening of the blood brain barrier permitting entry of immune cells. Many different types of immune cells including neutrophils, T cells, B cells and macrophages have been detected in the stroke-injured brain (Schroeter et al., 1994). There are a number of chemokines whose expression increases after a stroke. Interleukin 8 (IL-8) was the first chemokine identified and is critical for migration of neutrophils. In the rat, GRO/CINC-1 is the equivalent of human IL-8. Expression of this cytokine is elevated at 4 hours post-stroke (Newman et al., 2005). Similarly the stromal derived factor 1 (SDF-1), while constitutively expressed in the brain (Lazarini et al., 2003), is upregulated after stroke in the adult (Hill et al., 2004). In addition, macrophage inflammatory protein -1α (MIP-1α) is elevated and peaks in the brain between 24 and 48 hr post-stroke (Kim et al., 1995). Another of the major beacons that attract these cells to the site of injury is monocyte chemotactrant protein-1 (MCP-1). MCP-1 is released from neurons and astrocytes at the site of injury and its expression peaks at 2 days post-stroke (Che et al., 2001; Sakurai-Yamashita et al., 2006). Eliminating the expression of MCP-1 significantly reduces the stroke-induced infarct (Hughes et al., 2002) while over expressing this chemokine greatly enhances neurodegeneration in an experimental model of stroke (Chen et al., 2003). Moreover, an influx of macrophages at 3 days post-stroke correlates with the timing of the MCP-1 expression and these macrophage are responsible for inflammation in the injured hemisphere (Jander et al., 1995; Schroeter et al., 1997; Weston et al., 2006). This time point also corresponds with microglia acquiring physiologic features of macrophages (Weston et al., 2006). These findings support the idea that stroke can be treated up to 48 hours because the peripheral immune response, primarily in the form of macrophages is the source of most of the neurodegeneration.

Recently, the spleen has been implicated in an inflammatory response to brain injury (Offner et al., 2006a; Vendramine et al., 2006) and is a reservoir of peripheral macrophages in the body. The spleen has been shown to decrease in size in response to stroke and neurotoxic injury (Benner et al., 2004; Vendramine et al., 2005; Offner et al., 2006b), while treatment of stroke using systemic administration of HUCBC maintains the size of the spleen (Vendramine et al., 2006). In response to stroke, the spleen produces inflammatory cytokines. Treatment with HUCBC not only blocks this inflammation but changes the response to an anti-inflammatory one as demonstrated by the increase in the anti-inflammatory cytokine, IL-10. HUCBC treatment significantly reduces the number of macrophage/microglia in the injured brain (Vendramine et al., 2005). There is an increase in circulating macrophages and a reduction in B cells in the spleen in response to stroke (Offner et al., 2006a). Thus, these recent results show that the spleen plays a major role in the physiological response to stroke-induced injury and more research is needed to define its role in promoting inflammation.

Concluding remarks

These recent reports indicate a need to revise our current view of stroke treatment. Strong evidence from experimental models of stroke demonstrates that the therapeutic window for treatment is much wider than the current 3-6 hours. The function of the spleen’s response to brain injury requires more research since it may be a pivotal organ to target with post-stroke therapy.

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