Neuroprotection after cerebral ischemia

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Cerebral ischemia, a focal or global insufficiency of blood flow to the brain, can arise through multiple mechanisms, including thrombosis and arterial hemorrhage. Ischemia is a major driver of stroke, one of the leading causes of morbidity and mortality worldwide. While the general etiology of cerebral ischemia and stroke has been known for some time, the conditions have only recently been considered treatable. This report describes current research in this field seeking to fully understand the pathomechanisms underlying stroke; to characterize the brain’s intrinsic injury, survival, and repair mechanisms; to identify putative drug targets as well as cell-based therapies; and to optimize the delivery of therapeutic agents to the damaged cerebral tissue.

Keywords: stroke; cerebral ischemia; cerebrovascular disease; neurovascular unit; cell therapy; repair; immune response

Background and perspectives

Following interruption of blood flow to the brain in an ischemic event, cells undergo a series of events, such as loss of ion gradients, including failure of the sodium–potassium pump (Na–K), which leads to cellular swelling and cytotoxic edema. As cells switch from aerobic to anaerobic metabolism, metabolic acidosis ensues. Loss of ion gradients also leads to accumulation of intracellular calcium and excitatory amino acid (EAA) release, with activation of corresponding EAA receptors, leading to further calcium influx, mitochondrial dysfunction, and cell death through both necrotic and apoptotic pathways. Upon reperfusion, injured cells elicit a stress response, characterized by upregulation of immediate early and other stress response genes, which, in turn, leads to in situ production and/or upregulation of immune modulators, such as cytokines, and to trafficking of circulating immune cells into the ischemic brain. Necrotic cells can also release nucleic acids and other molecules that can act as damage-associated molecular patterns (DAMPs) on immune cells, including microglia, leading to immune cell activation and proinflammatory signaling. Proinflammatory molecules can then activate other proteins, such as matrix metalloproteinases (MMP), involved in the disruption of the blood–brain barrier (BBB) and the extracellular matrix. This worsens ischemic injury by causing vasogenic edema and hemorrhage. Reperfusion and immune cell signaling can also lead to astrocyte activation, with elaboration of prosurvival factors, setting the stage for reparative processes, such as neurogenesis and angiogenesis, as well as gliosis. The initial stress response also leads to induction of various survival factors, such as Akt and the cAMP response element–binding protein (CREB).

The “Trans-Pacific Workshop on Stroke” was held at the Wyndham Riverfront Hotel in New Orleans, Louisiana, on October 17–18, 2012, and was organized by Midori A. Yenari and Hiroaki Ooboshi, along with Shobu Namura and Jialing Liu. The workshop was sponsored by the U.S.–Japan Brain Research Cooperative Program and the Japan–U.S. Science and Technology Cooperation Program,
Brain Research Division. Below is a detailed report of the workshop presentations and themes.\(^a\)

Three goals were proposed to develop strong collaborations among investigators in both nations: (1) to exchange increasing knowledge of ischemic stroke, from the basic to clinic level, among researchers in the United States and Japan; (2) to identify barriers and gaps that inhibit complete understanding of the pathomechanisms underlying stroke; and (3) to identify research areas for future study. The scientific sessions were focused on several areas of investigation (Fig. 1): injury, survival, and repair mechanisms; potential drug targets and cell-based therapies; delivery of therapeutic agents, such as manipulating the BBB; and state-of-the-art imaging of neurovascular changes and for tracking delivered therapeutic agents. Speakers were selected from both countries to cover these areas. Participation of students and junior investigators was encouraged; emphasis was given to the diversification of the future workforce in stroke research, particularly in the United States. A total of 30 attendees were invited and presented their studies. A schematic of areas discussed as they relate to cell–cell interactions, signaling, and therapeutics within the neurovascular unit is shown in Figure 2.

**Neuroprotection and clinical studies against ischemic stroke**

The scientific session began with a talk by Kazuo Kitagawa (Osaka University), who has led the field of ischemic tolerance for more than two decades. He presented his recent progress in the understanding of the molecular mechanism underlying the phenomenon.\(^1\) In the cytoplasm of neurons under nonstimulated conditions, SIK2 is highly expressed and phosphorylates and sequesters CRTC1. After ischemic stimuli, CaMK isoforms I/IV

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\(^a\)In this report, attendee presentations were organized thematically. Thus, the order of the report does not necessarily follow the order of the presentations. Discussions related to goals 2 and 3 are summarized below.
phosphorylate SIK2, resulting in degradation of SIK2. Consequently, CRTC1 is dephosphorylated and translocates into the nucleus, binding to the promoter region of CREB. By doing so, this signaling pathway activates CREB-mediated survival genes, such as BDNF, PGC-1α, and Bcl2. Interestingly, this pathway (CaMK–SIK2–CRTC1–CREB) seems to be specifically downstream of synaptic NMDA receptor (NR2A-subunit containing) activation but not of other glutamate receptors. The concept was supported by *in vivo* findings in SIK2 null mice that showed strong resistance to ischemic stroke. Given the robustness of neuroprotection provided by this signaling pathway, further research should explore how to trigger this survival pathway pharmacologically, ideally after the onset of stroke.

Another neuronal survival mechanism was proposed by Shigeru Tanaka (Hiroshima University). Gene transfection of the G protein–coupled receptor (GPR)-3 to cultured neurons conferred resistance to hypoxia. By contrast, GPR3 gene knockdown by siRNA transfection augmented hypoxic neuronal apoptosis. Consistent with these findings, GRP3 knockout mice are more vulnerable to transient focal cerebral ischemia compared with wild-type animals. GRP3 ligands may have a therapeutic value, although it is unknown how the GRP3 activation renders neuronal survival.

The role of autophagy, a cellular mechanism for clearing unnecessary debris, was discussed by Eisuku Dohi (Hiroshima University). He showed that disrupting chaperone-mediated autophagy worsened neuron death due to hypoxia.

The neuroprotective efficacies of docosahexaenoic acid (DHA), a fish oil component, and its derivative neuroprotection D1 (NPD1), were shown by investigators at Louisiana State University. Ludmila Belayev tested post-stroke intravenous injection of DHA (22:6n–3) in rats subjected to two hours of middle cerebral artery occlusion (MCAO). DHA significantly improved behavior outcomes and attenuated brain edema (MRI T2-weighted image) and infarct size up to seven days after stroke. Such improvements were seen when DHA was injected at five hours after onset of stroke (i.e., three hours after reperfusion). Enhanced NPD1 synthesis in the brain penumbra area in DHA-treated animals was demonstrated by lipidomic analysis.
Nicolas Bazan (Louisiana State University) presented his unique approach using the combination of aspirin and DHA. Aspirin alone has been shown to afford beneficial effects against cerebrovascular diseases. Bazan found that aspirin and DHA co-treatment induced the synthesis of aspirin-triggered NPD1 (AT-NPD1) in the brain. Injection of AT-NPD1 sodium salt or methyl ester at three hours after stroke onset was effective in improving outcomes in rats subjected to two hours of MCAO.

Alberto Musto (Louisiana State University) reported protection by NPD1 in a mouse status epilepticus model induced by pilocarpine. Post-status epilepticus NPD1 treatment reduced recurrent seizure frequency and improved electrophysiological outcomes, suggesting that neuroprotection by NPD1 could operate at the postsynaptic level.

Another potential neuroprotectant candidate was proposed by Byron Ford (Morehouse School of Medicine). Ford studied neuregulin-1, which had originally been identified as a growth factor at the neuromuscular junction. Carotid arterial injection with neuregulin-1 showed impressive infarct reductions and neurological improvements in rats.

Zhenfeng Xu, a member of Ford’s laboratory, presented their recent attempt at genomic and transcriptomic approaches using rat brain samples after stroke. Ford also discussed his recent experience with a nonhuman primate model in searching for potential biomarkers for predicting stroke outcomes.

Mami Noda (Kyushu University) discussed her unique neuroprotection approach using molecular hydrogen. She tested the oral administration of hydrogen-containing water in rodent models of Parkinson’s disease and in an ischemic model of the optic nerve. Although the hydrogen level in the brain was not influenced, and although the underlying mechanism remained to be studied, drinking hydrogen-containing water was protective against those pathological conditions in these models. Since inhalation of hydrogen gas has been shown to protect against ischemic stroke in rats, drinking hydrogen-containing water may also protect against stroke.

Findings from recent clinical studies were also presented. Shunya Takizawa (Tokai University) reported the outcomes of a phase I study of intravenous granulocyte colony-stimulating factor (G-CSF) in ischemic stroke patients. The clinical study was based on the previous findings of Takizawa and colleagues, in mice, that hematopoietic cytokines reduced infarct volume with improvements in motor and cognitive functions. According to the phase I study, G-CSF (150 and 300 µg/day) was safe and well tolerated in patients after ischemic stroke. A phase II study is underway testing G-CSF treatment 24 hours after onset.

Koji Abe (Okayama University) presented the outcomes of edaravone (Radicut®) in a retrospective study of 114 consecutive stroke patients who received tissue plasminogen activator (tPA, 0.6 mg/kg) within three hours after onset. Edaravone is a free radical scavenger that was approved for treating acute ischemic stroke in Japan in 2001. Edaravone-treated patients showed a higher recanalization rate after tPA compared to those who did not receive edaravone, with the caveat that edaravone-treated patients had a higher prevalence of cardiogenic embolism and lower NIHSS scores on admission.

Cerebrovasculature and neurovascular protection against ischemic stroke

Study of the cerebrovasculature and its interaction with the neurovascular unit are traditional and indispensable areas confronting stroke research. Recent advances in the pathophysiology of the BBB and novel approaches to cerebrovascular regulation were discussed.

Tetsuro Ago (Kyushu University) discussed the neurotrophic roles of pericytes. He showed that the expression of platelet-derived growth factor receptor β (PDGFRβ) is drastically elevated in pericytes of the peri-infarct areas in mice after MCAO. Heterozygous PDGFRβ gene knockout suppressed the elevation of PDGFRβ with increased infarct formation. In addition, PDGFβ, the ligand for PDGFRβ, elevates the expression of nerve-growth factor (NGF) and neurotrophin-3 (NT-3) in cultured pericytes. These findings suggest that the PDGFβ-mediated NGF/NT-3 production from pericytes may afford neuroprotection against ischemic stroke.

David S. Miller (National Institute of Environmental Health Science) described the importance of nuclear factor E2–related factor 2 (Nrf2) in drug delivery through the BBB. Nrf2 is a redox-sensitive, ligand-activated transcription factor that induces
multiple antioxidant and glutathione-generating enzymes in response to oxidative stress. He assessed the effects of Nrf2 activation at the BBB on drug efflux transporters, including P-glycoprotein. The Nrf2 ligand sulforaphane elevated protein expression of P-glycoprotein as well as the efflux activity in isolated rodent brain capillaries, suggesting that this pathway contributes to restricting drug delivery across the BBB when Nrf2 is activated.

Jeffrey M. Gidday (Washington University) highlighted a discussion of vascular mechanisms involved in ischemic tolerance. He demonstrated that hypoxic preconditioning reduced post-stroke leukocyte adhesion and BBB dysfunction. Downregulation of intercellular adhesion molecules, as well as enhanced integrity of tight junctions (e.g., ZO-1 and claudin-5), has been documented as a common phenotype in vascular ischemic tolerance. A better understanding of the vascular aspects of ischemic tolerance is warranted.

David Busija (Tulane University) summarized his research regarding vascular mitochondria as a therapeutic targets. Transient activation of ATP-dependent potassium channels that are presented on the inner mitochondrial membrane (mitoKATP channels) induces immediate and long-term protection of the cerebral endothelium against subsequent stress. Attenuations in both intracellular calcium elevation and reactive oxygen species production after stress are likely to induce this protection. In addition, many of the molecular consequences of mitoKATP channel activation have the potential to influence cerebrovascular tone. These phenomena are blunted in insulin-resistant Zucker obese rats, suggesting negative impacts of abnormal glucose/lipid metabolism, common comorbidities in stroke patients. Prasad V.G. Katakam (Tulane University) showed that mitochondrial activation also promotes neuronal isotype-mediated nitric oxide (NO) generation, suggesting the existence of a novel link between neuronal metabolism and vasodilation.

A therapeutic approach targeting peroxisome proliferator–activated receptor (PPAR) α in cerebral vessels was discussed by Shobu Namura and Donghui Li (Morehouse School of Medicine). The PPARs are nuclear receptors that act as transcription factor. Fibrates, clinically used drugs for dyslipidemia, are known to activate PPARα. Fibrates improve cerebral blood flow (CBF) in the penumbral area. Fibrate-induced elevation of superoxide dismutase activity in brain microvessels may contribute to maintaining NO bioavailability, with improvement of ischemic cerebral blood flow.

**Immune responses and ischemic stroke**

Immune responses following stroke continue to be an active area of investigation, and both innate and adaptive immune responses in stroke were discussed. Innate immune responses have been more extensively investigated, since acute neurological insults were not traditionally considered in the context of prior antigen exposure. Newer aspects of innate immunity were also discussed, as they relate to acute and long-term effects of stroke. Adaptive immunity may be relevant in the search for a vaccine against stroke and may also help to explain why concurrent infections are detrimental to stroke outcome.

Kyra Becker (University of Washington) reviewed the literature on adaptive immune responses in experimental stroke and presented a new model of adaptive immunity, whereby the systemic administration of lipopolysaccharide at the time of stroke led to Th1 responses and worsened outcome. This model could be likened to the negative outcomes in stroke patients with complicating infections. Interestingly, adoptive transfer of splenocytes primed toward a Th1 response led to a worsened outcome, whereas adoptive transfer of splenocytes primed toward a T<sub>reg</sub> cell response led to a better outcome. These data suggest that interventions preventing Th1 or enhancing T<sub>reg</sub> cell responses may have translational value.

A study of novel immune molecules in experimental stroke was presented by Hiroaki Ooboshi (Fukuoka Dental College). Prior work has shown that lymphocytes contribute to adverse outcomes. However, the role of T lymphocyte subtypes has not been well studied. The γδ T cells can produce IL-17 following stimulation by macrophage-derived IL-23. These cytokines appear to contribute to stroke evolution, as mice deficient in these cytokines are protected. Further, peroxiredoxin (Prx), an endogenous antioxidant, induces IL-23 and leads to worsened stroke outcome through Toll-like receptors-2 and -4 and the MyD88 pathway. These findings show that Prx is a novel damage-associated molecular signal. Inhibiting Prx appears to be protective.
While many of the immune responses described to date are largely detrimental in the acute phase, Midori A. Yenari (University of California, San Francisco) described a newly characterized innate immune receptor that has a potentially beneficial role. This receptor, triggering receptors expressed on myeloid cells-2 (TREM-2), is thought to trigger phagocytosis in microglia and macrophages. Its ligand has been identified in brain cells, and recent work has shown that exposure of neurons to apoptotic insults leads to the activation of TREM-2.\(^{16}\) TREM-2 deficiency decreased phagocytosis of injured neurons. Masahito Kawabori (University of California, San Francisco) then showed that the proportion of microglia with TREM-2 expression appears to be enhanced under conditions of therapeutic hypothermia. Thus, TREM-2 may contribute beneficial effects, such as the clearance of cellular debris.

The link between pain and inflammation is well known, and Nozomi Akimoto (Kyushu University) presented new findings showing how the CCL-1 cytokine enhances nociception and microglial activation.

**Recovery and repair, and modeling of post-stroke fatigue**

Since the discovery of endogenous neural stem cells in the adult brain, and following recent reports that post-stroke systemic administration of mesenchymal stem cells improved outcomes in animals, neuronal repair has rapidly emerged as a potential method for treating stroke. One week before the workshop, the Nobel Prize in Physiology or Medicine 2012 was awarded to Sir John B. Gurdon and Shinya Yamanaka for the discovery that mature cells can be reprogrammed to pluripotency. Presentations concerning this topic were actively discussed.

Koji Abe (Okayama University) discussed his exploration of the potential of the induced pluripotent stem (iPS) cell transplantation as a novel therapy for ischemic stroke. Unexpectedly, intracranially transplanted iPS cells formed teratomatous tumors in the ischemic mouse brains and the clinical recovery from stroke was delayed, despite increases in the number of neuroblasts and mature neurons in the ischemic brains.\(^{17}\) He concluded that iPS cell therapy had a promising potential to provide neural cells after stroke if tumorigenesis could be controlled.

Optimal delivery methods and timing of neural stem cell therapy against stroke were discussed by Raphael Guzman (University Hospital Basel).\(^{18}\) He recommended that intravascular injection was advantageous over intraparenchymal transplantation for achieving a widespread distribution while being minimally invasive and repeatable. Compared to intravenous injection, which often results in entrapment of transplanted cells in the lung, intraarterial injection provides better homing into the brain. With reference to timing of the intra-arterial approach, injection at three days after ischemia resulted in the highest cell engraftment.\(^{18}\) Pretreatment of transplanted cells with BDNF enhanced the therapeutic efficacies.

Another recovery approach targeting angiogenic factor Netrin-1 was discussed by Jialing Liu and Chin Cheng Le (University of California, San Francisco). Netrin-1 gene delivery into the ischemic penumbra not only increased vascular density but also promoted the migration of immature neurons into the peri-infarct white matter, which was accompanied by improved recovery of motor function.\(^{19}\) The enhanced neurogenesis by Netrin-1 likely contributes to neurological recovery, because conditional ablation of neuroprogenitor cells through targeting nestin in adult mice delayed the recovery of cognitive function after stroke without affecting CBF and subsequent lesion size.

Finally, Allison Kunze (University of Washington) presented a novel approach for detecting post-stroke fatigue in popular rodent stroke models. This approach should open up the field to the identification of new treatments for a significant but understudied clinical problem.

**Novel imaging technologies for stroke studies**

*In vivo* optic brain imaging is an emerging area in experimental stroke studies. For instance, as the fluorescence labeling technique advances, two photon–excited microscopy provides enormous potential for repeated documentation not only of neurovascular morphology and hemodynamics, but also of changes at the molecular level, including those in intracellular ion levels.

\(^{b}\)At the time of the conference, Dr. Guzman was affiliated with Stanford University.
Chris Schaffer (Cornell University) discussed his experience in developing microscale stroke models utilizing femtosecond laser pulses. By controlling the amount of delivered energy, either vessel occlusion or rupture can be produced at a precise point. With regard to neocortex microinfarct, the location of the occlusion determines the consequence: in contrast to the sustained perfusion deficit in the distal portions after occlusion of a penetrating arteriole, there was a robust reversal flow from distal branches after occlusion of cortical surface arterioles. On the other hand, rupture of a penetrating arteriole (microhemorrhage) triggered local inflammatory responses without detectable neurovascular pathology, such as dendrite deformation and capillary collapse, in the surrounding tissue. Nozomi Nishimura (Cornell University) showed in a mouse model of Alzheimer’s disease that capillary flow was dramatically stalled, which was accompanied by leukocyte adhesion to the endothelium. The capillary plugging by leukocytes may cause CBF impairment in Alzheimer’s disease.

Jialing Liu and Yusuke Akamatsu (University of California, San Francisco) shared their experience with optical coherence tomography and optical microangiography for measuring cerebrovascular microstructure and flow, and post-ischemia collateral circulation in a mouse model of type 2 diabetes mellitus ($db/db$). Type 2 diabetes mellitus is known to be associated with worse stroke outcomes. Compared with nondiabetic $db/+ $ mice, $db/db $ showed lower regional CBF and lower density of functional blood vessels in the ischemic hemisphere. These new imaging methods are useful for monitoring collateral flow development in mice after stroke.

**Overall summary and future directions**

The meeting was viewed by most of the attendees as a successful beginning to the development of collaborative efforts between the two countries in the investigation of the pathomechanisms of ischemic stroke. The size and format of the meeting were well received, especially by the Japanese participants and junior investigators, who often feel intimidated when asked to speak at large conferences. The location and timing surrounding the Society for Neuroscience meeting was convenient for the U.S. participants, particularly the basic researchers and students.

This meeting clearly demonstrated the need for future meetings that could expand upon the topics covered. For example, edaravone has been used with stroke patients in Japan, and U.S. participants expressed an interest in learning more about the experiences of Japanese clinicians, with an eye toward larger-scale international studies. Neural repair using iPSCs was another actively discussed topic. Because of its accessibility, pluripotency, and autologous nature, iPSC technology has tremendous potential for treating neurodegenerative diseases, including stroke. Future investigations that apply iPSC technology to the treatment of stroke patients may be an important area of collaboration between investigators in the two countries. Establishing standardized preclinical models and unbiased study methods would allow comparison of biological robustness of findings across laboratories, which is important when considering clinical translation. Related to this issue, developing a reproducible non-human primate model of stroke will certainly be useful for testing promising neuroprotectants, such as neuregulin-1, DHA, and NPD1. Collaborations with physicists and chemists are needed, as shown by the examples of in vivo imaging.

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Koji Abe gave the featured lecture, “Current topics on neuroprotection after cerebral ischemia,” which included a brief report of the impact of the earthquake/tsunami in March 2011 on healthcare service in the affected Tohoku pacific coastal areas of Japan. His report reminded the attendees that the
workshop was located in the area hit by Hurricane Katrina seven years before. Local speakers Nicolas Bazan, Ludmila Belayev, and David Busija were featured.

**Conflicts of interest**

The authors have no conflicts of interest.

**References**