It is an honor to receive the Thomas Willis Lecture Award this year on behalf of the research program that we established 3 decades ago. This award recognizes the work of Thomas Willis, regarded as the father of neuroscience and modern neurology, the influential 17th century physician–scientist and cofounder of the Royal Society of London. Early members of the Royal Society included Marcello Malpighi (1628–1694), trained in anatomy at the University of Bologna, who was among the first to describe the capillary structure in a number of articles submitted to the Royal Society, beginning in 1668. Approximately 300 years later, in 1977, the Ferrier Lecture, given triennially at the Royal Society, was on the topic of the neuron network of the cerebral cortex.1 The proposed architecture of the cortical neuron array summarized morphological work to that point on the relative positions of individual neuron subsets of the cortex. Singularly absent from that depiction of the cortex were the microvascular supplies of the neuron networks they serve.

The Cerebral Microvasculature

The microvasculature of the central nervous system represents 1 network among networks that serve the neuron.2 The separate networks of the neurons, of astrocytes, and of the microvasculature are juxtaposed and also have tight interactions morphologically and functionally.

The cerebral microvasculature is complex. Depending on their location, cerebral capillaries consist of the endothelium, the subtending basal lamina, pericytes and histiocytes within the vessel wall, and astrocytes, whose interactions and functions are still incompletely understood. Whether as arterioles, postcapillary venules, or capillaries, these vessels are unique in being a part of the brain substance, communicating directly with neurons and with those cells that support neurons. Here, astrocytes in their own network and as part of the microvasculature play central roles in supporting neurons while supporting functions (eg, the permeability barrier) of the capillary endothelium.

Experimental Context

Pari passu with clinical studies of acute recanalization, we have had the privilege to pursue work on the effects of injury on the microvasculature. The context of the times in which this work began is important. In the 1970s, stroke patients were relegated to the quiet dark eddies of medicine, with few therapeutic options and a medical attitude that was considered nihilistic. This was set against a keen interest in the physiological effects of global and focal ischemia, particularly on vascular flow, in the brain. The experimental realization and conceptualization of the “penumbra” developed by Symon, Astrup, Siesjö, Branston, Strong, and others used an adult nonhuman primate (Papio sp.) model of focal ischemia as a human-relevant surrogate.3–6 During that time, clinical trials with aspirin were underway and oral anticoagulation was being explored. Computed tomography scan technology was limited in availability. In the late 1970s, fibrinolytic agents were contraindicated in patients with completed stroke and there was fear of the consequences of their use.7 Acute intervention was theoretical, although work had begun to test feasibility by the early 1980s.8

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Given the known condition that neurons dictate regional cerebral blood flow, it was reasonable to consider microvessel function as the product of neuron function and to extend this to the setting of ischemic injury. Cerebral microvessels were considered inert conduits. However, given negative feedback systems known to operate in the rest of the body, it was of interest to examine neuron–microvessel relationships from the perspective of the microvessel as a participant in feedback loops from the microvasculature to the neuron.

**Paths of Enquiry**

Two paths of enquiry were pursued by our group. In the late 1970s, plans were made to test the feasibility and relative safety of acute recanalization strategies using infusion of plasminogen activators in stroke patients. In 1982, the refinement of these plans led to formal applications for funding, and work was initiated with a refined model of middle cerebral artery occlusion in the nonhuman primate (Papio anubis/ cynocephalus). Clinical collaborations began for feasibility studies. With occlusion of brain-supplying arteries, one could presumably reconstitute flow acutely in the stricken territory by the use of a plasminogen activator, with potential injury reduction if the interventions were sufficiently acute. Overall, intracerebral hemorrhage was not increased by acute intervention. Furthermore, there were patients who appeared to improve with direct-infusion urokinase plasminogen activator. Our group was the first to use tissue plasminogen activator acutely in thrombotic stroke, derived from melanoma cells. The atmosphere by the mid-1980s was one of excitement because it now seemed possible to think in terms of patient improvement.

Our second path pursued the consequences of ischemia and arterial reperfusion to the downstream microvasculature. The microvasculature (vessels <100 μm in diameter) is of interest because it represents 96% of the vascular volume in any organ system and contains the sites of nutrient exchange. Occlusion within cerebral vascular beds leads to reversal of flow in this low-pressure, high-flow microvessel network. The arrangement of intersecting capillaries and microvessels in the basal ganglia, for instance, would predict that this system of vascular protection (reversal of flow) could break down if microvascular occlusions occurred in even 20% of the microvessels.

**Cerebral Microvascular Beds**

Capillaries within the central nervous system consist of the endothelium, basal lamina, and astrocytes (via their end-feet), that connect with neurons. The interactions of these 3 microvascular elements, and the other cellular compartments during normoxia and ischemia, were largely unknown in the early 1980s but were likely to be complex. Physiological studies in larger vessels had demonstrated significant capacity for reactivity, and studies of isolated microvessels had already demonstrated site-dependent relevant metabolic features in the cerebral microvascular network.

In addition to its enormous clinical importance, we also used focal ischemia as a “wedge” to elucidate relationships within the microvasculature, and potentially to the neurons they serve. Based on precise clinical observations translated into the acute setting in the high-quality nonhuman primate model of focal ischemia, also used in the plasminogen activator studies by our group, acute observations of microvessel responses in vivo were reduced to and recapitulated in primary murine cell systems. This has allowed a dialogue between experimental systems and the patient, with direct application to patients in clinical trial at many stages along the way. This approach also has provided tissue evidence of injury responses and evidence of exact cellular contributions to ischemic injury development, suggesting precise targets for intervention. Instead of an ascending phylogenetic approach, this approach to translation has used a sophisticated and historically human-relevant model system to generate acute observations applicable in both human and simpler systems.

Although some important differences between the gyrencephalic brain of Papio sp. and the human brain as described by Willis exist, the major vascular and microvascular features are the same. The transorbital approach to middle cerebral artery occlusion provides a closed system, allowing studies to be performed in the awake animal without harm or apparent discomfort. Other relevant advantages include a hemostatic system that is similar to that of the human, an inflammatory system that also bears relationship, and the opportunity to use human immunoprobes that cross-react in ≈90% of settings.

**Cerebral Microvessel Responses to Focal Ischemia**

With the availability of the acute clinical studies, a human-relevant model, and a variety of molecular biology techniques, the journey to examine microvessel responses to focal ischemia was possible, beginning with the blood–endothelial interactions (Figure 1).

**Intravascular Events**

Within 90 minutes after ischemia onset, the microvessel endothelium reacts by presenting P-selectin, a receptor known to slow leukocyte rolling before firm attachment and transmigration. Soon after, intercellular adhesion molecule-1 and E-selectin appear on the luminal surface of the postcapillary venule endothelium, receptors that promote leukocyte attachment. This sequence of events exactly mirrors that seen in human peripheral inflammation and suggests that peripheral inflammatory processes are involved in the evolution of brain injury after ischemia. Adhesion of polymorphonuclear leukocytes is 1 factor contributing to the phenomenon of focal “no-reflow,” the reduction in microvessel patency in the ischemic regions (“territory-at-risk”) that occurs when ischemia is aborted by reperfusion of the supply artery. Occlusion of capillaries and postcapillary venules by degranulated platelets, fibrin, and/or polymorphonuclear leukocytes suggested the possibility that blockade of their adhesion and activation could abort the focal no-reflow phenomenon and recover tissue function. For instance, acute use of a humanized antibody against the leukocyte β₂-integrin receptor (the counter-receptor for intercellular adhesion molecule-1) significantly increased microvessel patency compared with controls. The antibody–receptor interaction was immediate.

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diameters. Simultaneously, \( \text{al}_{24} \) demonstrated a significant reduction in the matrix protein constituents of the basal lamina across all microvessel diameters. Simultaneously, pro-matrix metalloproteinase (MMP)–2, whose active form is known to degrade collagen and laminin, is generated and released immediately after ischemia onset, as demonstrated by high-quality biochemical techniques. \( ^{25} \) Tissue expression of this protease in the nonhuman primate is directly related to the number of neurons injured within the 7-day timeframe of the experiment and to the size of tissue injury. \( ^{25,26} \) Other proteases generated simultaneously, including urokinase plasminogen activator from the endothelium, are not related to neuron injury. Pro-MMP-2 and urokinase plasminogen activator are associated with both microvessels and neurons. \( ^{26} \) In addition, the activation apparatus for pro-MMP-2, including MT1-MMP and MT3-MMP, as well as plasmin, appear simultaneously in the regions of injury as well. \( ^{27} \)

Pro-MMP-9 is significantly associated with hemorrhagic transformation. This has raised the notable issue of species differences in response to focal ischemia between humans/primates and rodents for cerebral function and responses, \( ^{28} \) Other groups have described the generation of pro-MMP-9 in rodent models of ischemic injury. \( ^{29–32} \) \( ^{33–35} \) \( ^{37} \) recently showed graphically the very similar phenomenon of MMP-9 appearance and the loss of basal lamina electron density in rodents.

In addition, detachment of the astrocyte end-feet from the basal lamina was observed even within several hours from the onset of ischemia, supporting the same finding during ischemia in the primate that Garcia \( ^{36} \) had described previously. The loss of select matrix proteins and of matrix density and the detachment of the glial end-feet had no concordant explanation until recently. The roles that matrix proteases might play have remained an active interest here (because they reappear at several levels in the injury of ischemia). Their specific impact on neuron viability remains an open area of research.

A systematic and extensive evaluation of the cerebral microvessel and neuron-associated expression of serine proteases in the primate basal ganglion demonstrated the immediate appearance of plasminogen activator inhibitor-1, urokinase plasminogen activator, and urokinase plasminogen activator receptor, but not tissue plasminogen activator. \( ^{26} \) Hence, microvessels did not increase tissue plasminogen activator content/release during ischemia, suggesting that endogenous thrombolysis was not an active response. \( ^{26,27} \) Two other enzyme families affecting matrix proteins appear acutely during focal ischemia: cysteine proteases and heparanase. \( ^{35} \) Cathepsin-L degrades the matrix substrate, perlecan. \( ^{37} \)

A hypothesis has emerged from the observations of matrix protease expression and microvessel matrix degradation that states that microvessel responses and neuron injury during focal ischemia are linked (Figure 2). This hypothesis implies that even local ischemia can produce potentially irreversible microvessel and, therefore, neuron injury. It also suggests that it is necessary to know the effects of ischemia on cell-matrix adhesion and how and which cell components within the microvasculature and neuropil are responsible for neuron and microvessel integrity or injury. The distribution of possible matrix-altering enzymes and proteases so far observed supports this concept and allows future investigations of cell–cell communication and their specific pathways.
Matrix Adhesion Receptors

Endothelial cells and astrocytes adhere to the basal lamina by specific matrix adhesion receptors. Integrins are αβ heterodimeric transmembrane proteins whose intracellular portion mediates signaling and whose extracellular portion serves the structural role of matrix-binding (the behavior of these molecules is reviewed in 2 recent publications38,39). Microvessel endothelial cell β1-integrins (ie, α4β1, α6β1, and αvβ1) decrease significantly within several hours after focal ischemia.40,41 Similarly, the astrocyte integrins αvβ3 and αvβ5, found on a limited proportion of these cells, decrease significantly.42 These observations have been recapitulated in vitro. Abumiya et al43 demonstrated, in contrast, that integrin αβ3, a signal for angiogenesis, increased significantly in astrocytes, in concert with vascular endothelial growth factor and proliferating cell nuclear antigen, when β1-integrin receptor expression decreased. Notably, the endothelium responds with increased β1-integrin expression in contiguous “minicores” of the ischemic regions,44 emphasizing the heterogeneity of the injury process within the confines of the microvessel supply.

Simultaneously, the nonintegrin matrix receptor αβ-dystroglycan that holds astrocyte end-feet to the basal lamina decreases by ≥60%. This change corresponds to the detachment of astrocyte end-feet from the basal lamina that is seen in vivo in the primate.36 We have recapitulated this observation in vitro, showing that the decrease in αβ-dystroglycan can be reversed, in part, by inhibitors of MMP-like activities.45 These effects also appear to require the presence of serum. It is currently unclear whether the β1-dystroglycan proteolytic activity is generated by astrocytes alone, appears from the plasma, or both.

Hence, immediately after ischemia onset, detectable changes in endothelial cell and astrocyte adhesion receptors that maintain microvessel structure (and signaling) occur. It appears that some of these changes are inhibitable. Whether their inhibition would preserve the relationship of the astrocytes to the endothelium within the microvessel is currently being studied.

Tight Junctions and the Permeability Barriers

These events occur in temporal relation to the postischemic increase in microvessel permeability that leads to plasma leakage.9,18 This generates serum on the tissue side of the microvessels. Among structures that constitute the barrier are tight junction proteins and the basal lamina matrix. The protein complexes that constitute the tight junction include claudin-5, occludin, and zona occludens-1, in addition to junctional adhesion molecule, E-cadherin, and others. Interendothelial tight junctions are bound to the actin cytoskeleton within the endothelial cells and constitute the “horizontal” component of the permeability barrier.46

The second barrier is formed by the basal ganglia matrix, which impedes the movement of leukocytes through the wall and also prevents erythrocyte flux, as hemorrhage. From studies already described, endothelial integrin-matrix-astrocyte αβ-dystroglycan interactions constitute the “vertical” component of the microvessel portion of the permeability barrier.46

We recently have shown that β1-integrin adherence to matrix by the endothelium is connected to the interendothelial tight junction proteins and that β1-integrin and tight junction proteins may share a common fate. When β1-integrin function is blocked, claudin-5 disaggregates and disappears from the interendothelial sites. This leads to a significant increase in permeability to 40-kDa and 150-kDa molecules, sizes that are compatible with the loss of IgG, observed in vivo during focal ischemia.47 Loss of β1-integrin function disturbs tight junction fidelity. The tight junction proteins occludin and zona occludens-1 also decrease. The mediation by F-actin is suspected. We posit that these adhesion receptor matrix interactions are responsible for a third form of barrier interaction.

The condition and responses of the cerebral microvascular endothelium appear to vary along the vascular axis, as initially suggested by Spatz and colleagues,14 and may depend upon the potential contributions of the subjacent matrix and astrocytes. Furthermore, astrocytes

Figure 2. Graphic representation of potential consequences to the neurovascular unit of focal ischemia, with the coordinate expression of membrane-type matrix metalloproteinases (MT-MMP), pro-MMP-2, urokinase plasminogen activator (u-PA), urokinase plasminogen activator receptor (u-PAR), cathepsin-L, and heparanase on microvessels and neurons in the nonhuman primate basal ganglia. Microglia express pro-MMP-9 and cathepsin-L. HSPGs, heparan sulfate proteoglycan (perlecan); HS, heparan sulfate side chains. The hypothesis depicted here states that the alterations of matrix components of the microvasculature and the pericellular matrix during focal ischemia lead to structural and functional alterations within the neurovascular unit. Details of protease system release, activation, and matrix degradation are not provided because these are yet to be worked out specifically in the context of the experiments summarized here.
can modulate endothelial cell responses, as in hemostasis.\textsuperscript{48,49} With postischemic increase in microvessel permeability, plasma extravasates to the abluminal face, generating thrombin via the interaction of factor VIIa with tissue factor at the glial interface, and the activation of hemostatic components in the plasma. This implies an inter-relationship between the endothelium and astrocytes within the microvessel. Recent studies have examined the impact of astrocytes in astrocyte–endothelial cell coculture with and without serum exposure on endothelial cell (eg, \(\beta_1\)-integrin) responses to ischemia. These studies open the way to understand the effects of ischemia on barrier integrity, how signaling takes place within endothelial cells and astrocytes, how these 2 compartments communicate, and how astrocytes contribute to this and perhaps to neuron stability. They offer new targets for intervention. This example of translation from the human to the nonhuman primate to the cell informs acute observations in the patient about microvesel responses at levels not observable in real-time because of limitations of spatial and temporal resolution in the human.

Astrocytes and Beyond

There is 1 more set of observations on this theme that I relate here. The events initiated by focal ischemia within brain tissue outside microvessels are potentially very relevant to both microvessel structure and neuron function. A hint comes from hemorrhagic transformation during focal ischemia.

Several clinical and laboratory research groups have suggested that pro-MMP-9 is increased during cerebral hemorrhage, and that it is responsible for the hemorrhage. Geo et al.\textsuperscript{25} first demonstrated that (pro-)MMP-9 was associated with intracerebral hemorrhage accompanying focal ischemia in the nonhuman primate. Subsequently, it had been suggested that MMP-9 is also associated with injury by cerebral ischemia, edema formation, and aneurysmal hemorrhage. We postulated that edema formation, extravasation of plasma elements into the neuropil as consequence of barrier leakage, could be a common theme. Approximately 10 years ago, we had demonstrated that pro-MMP-9 was generated by cerebral tissue in the regions of hemorrhagic transformation, in excess of the hemorrhage itself; however, the cell sources have been uncertain.\textsuperscript{25} The presence of hemorrhage implies the presence of plasma elements, including circulating matrix proteins.

Activation of microglia occurs in the marginal regions of ischemia and hemorrhage.\textsuperscript{50,51} In cultures devoid of serum (and hence circulating MMP-2 or MMP-9), astrocytes exposed to fibronectin and vitronectin (circulating matrix proteins) increase secretion of pro-MMP-2 under conditions of normoxia.\textsuperscript{51} Under similar conditions, microglia exclusively generate pro-MMP-9, primarily in response to vitronectin. When the cells also are further subjected to experimental ischemia, microglial cells will further increase the amount of pro-MMP-9 by as much as 20\% to 80\%, but not pro-MMP-2. In this case, it is the combination of ischemia and fibronectin from the plasma that further pushes the microglia forward. Astrocytes, however, are quite sensitive to ischemia and produce a modest reduction of pro-MMP-2 generation. Under no circumstances in these conditions are the cells lost. This implies that for microvessel responses to ischemia, microglia stand as sentinels in the tissue and can be activated to generate matrix proteases when they are triggered by specific matrix proteins from the plasma.

This is compatible with the situation in the brain tissue peripheral to the core, but near the hemorrhage. Other studies have examined the effects of these conditions on cathepsin-L and heparanase generation, and define those cell sources as well.

To put this in clinical context, we have shown that microglial cells produce pro-MMP-9 in the setting of plasma/serosum exposure. Astrocytes generate pro-MMP-2. These cells interact with each other and also produce other proteases that are specific for their cell type under conditions of ischemia. This increasingly toxic milieu is all generated within 1 to 2 hours after ischemia onset, reinforcing the need to bring the patient to hospital as quickly as possible.

The Neurovascular Unit

We have translated acute observations in patients to the nonhuman primate, and to primary cell cultures, with implications for stroke patients. These studies suggest that there is an intimate interaction among the microvessel wall, circulating plasma constituents and cellular elements, and elements outside the microvessel that are revealed under the circumstances of ischemia. What is not known is how these interactions take place in real time and the communications that exist between the endothelium and the astrocytes, and onward to neurons.

The changes that take place in both the neurons and their supply microvessels suggest that the early events occur in concert. Furthermore, considerations that focus on the neuron in isolation of the microvasculature do not reflect the structural or the functional interactions between these elements. For this reason, and to aid in the exploration of intervention strategies that might preserve cerebral tissue from injury, we and others have suggested an intellectual scaffold for examining the impact of microvessel on neuron function. The “neurovascular unit” represents a conceptual framework that links microvessel and neuron function and the responses of these compartments to injury.\textsuperscript{22,53} It also suggests that this framework should apply under normal circumstances. The linkage in simple terms involves the astrocyte. It does not exclude the involvement of other cell types, but includes them.

Conclusions

This research/scientific journey has seen the confirmation that acute intervention with plasminogen activators in ischemic stroke can provide benefit to select patients. Microvessel responses under these circumstances are as rapid as neuron responses—these include endothelial cell activation, alterations in microvessel matrix architecture, and the rapid changes in endothelial cell and astrocyte adhesion receptor expression. These correspond to the appearance of proteases with matrix-degrading capability. Important is the early heterogeneous distribution of these events. In the endothelium, the permeability barrier, tight junction proteins, and matrix adhesion by specific receptors are connected.

The identification of the cellular sources of the matrix proteases to the acute microvessel responses to ischemia
and their relation to these events imply an impact of the tissue on the microvasculature. These events also are related to the regions of neuron injury and suggest the unitary hypothesis in which microvessel and neuron injury are connected. Ultimately, this hypothesis will have implications for signalling events, potential treatment targets, and clinical outcomes. There is need for continuous high-quality dialogue between clinical observation and experiment. This and related work extend the notion that microvessel and neuron responses to ischemia are simultaneous and related, and suggest that microvessel events relating to ischemia may contribute to vascular cognitive impairment, Alzheimer dementia, and other vascular disorders of the central nervous system.

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Disclosures
None.

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Gregory J. del Zoppo

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