Adventures in the Pathophysiology of Brain Ischemia: Penumbra, Gene Expression, Neuroprotection

The 2002 Thomas Willis Lecture

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Background—The pathophysiology of cerebral ischemia is well studied in small-animal models, which offer reproducibility and control of confounding variables—factors essential to hypothesis-testing. This presentation first highlights insights into the ischemic penumbra enabled by a multimodal experimental approach; second, discusses gene expression in ischemia; and third, confronts the challenges of neuroprotectant therapy.

Summary of Review—The ischemic penumbra: Transient (2-hour) middle cerebral artery suture-occlusion in anesthetized rats gives rise to highly consistent neurological and histopathological sequelae. Autoradiographic local cerebral blood flow (LCBF) studies at 2 hours of occlusion define the penumbra as a region of intermediate CBF depression (20% to 40% of control) surrounding the densely ischemic core (5% to 20% of control) and constituting one half of the entire lesion. Local glucose metabolic rate in the acute penumbra is not reduced despite the critical CBF reduction, so that the penumbral metabolism/blood flow ratio is markedly elevated. In contrast, following 1 hour of recirculation, glucose metabolism throughout the previously ischemic hemisphere has become markedly depressed, and the metabolism/flow ratio has pseudonormalized. By correlating these data with histopathology using multimodal image analysis, the probability of infarction is shown to be highly determined by the degree of antecedent CBF reduction. These animal data agree strikingly with published results in patients with acute stroke studied by positron emission tomography. This remarkable correspondence belies the assertion that data from lower species may not be relevant to human stroke. Gene expression: Perfusion gradients also determine differential patterns of gene expression in ischemia. This can be demonstrated by correlating in situ hybridization autoradiographs for gene expression with autoradiographic LCBF data and histological infarct maps derived from replicate series. In other studies, DNA microarray technology is used to screen for thousands of expressed genes. In the 2-hour middle cerebral artery occlusion model with 3-hour recirculation, we have identified 28 known ischemia-hypoxia response genes that are upregulated and 6 that are downregulated, together with 35 upregulated and 41 downregulated genes newly connected with ischemia. These findings underscore the enormous complexity of ischemic biology and suggest possible novel mechanisms for future exploration. Neuroprotection: A desirable neuroprotectant would, in theory, antagonize multiple injury mechanisms. We have explored 2 such therapies of particular promise. Mild brain hypothermia (32°C target temperature, for 5 hours) is highly neuroprotective even when initiated at the onset of recirculation. Another highly protective agent is human albumin, administered in doses of 1.25 to 2.5 g/kg—a therapy that reduces infarct volume in this ischemia model by 60% to 65%, markedly diminishes brain swelling, and has a therapeutic window extending to 4 hours.

Conclusion—The careful study of rodent ischemia models can yield valuable, clinically relevant insights into the pathophysiology of ischemic stroke. (Stroke. 2003;34:214-223.)

Key Words: cerebral blood flow ■ gene expression ■ glucose utilization ■ neuroprotection ■ penumbra
TABLE 1. Expressions of Skepticism

- “Unfortunately, some conclusions reached may be specific to the animal model and not generalizable to spontaneous disease states encountered in nature.”
- “… An over-reliance on [animal] models may impede rather than advance scientific progress in the treatment of this disease. The complexities of creating a truly representative model for human ischemic stroke go far beyond developing ways to occlude a cerebral artery in a given animal.”
- “It is obvious that there can never be one model of ‘stroke’ that will mimic all ‘strokes.’” … models of cerebral embolism and thrombosis … are the only models … that closely mimic human ischemic stroke.21

reminding him that that the real purpose of our work is the alleviation of human disease.

Small-Animal Models of Cerebrovascular Disease

Various expressions of skepticism voiced in our own journal (and others) over the years have challenged us with questions as to the relevance and utility of small-animal models of human disease19–21 (Table 1). A moment’s reflection, however, provides the appropriate response: Human disease is often random, sporadic, and variable in its occurrence, and, beyond a certain point, its biochemical and molecular complexities are simply inaccessible without turning to animal models, which offer reproducibility, replicability, and control of confounding variables—factors essential to scientific hypothesis-testing. The rodent genome—now largely elucidated—is strikingly similar to the human,22 and murine mutants now permit the exquisitely targeted dissection of the roles played by individual gene products.

The discussion that follows draws on the author’s own studies of ischemic stroke and has a 3-fold objective: first, to illustrate insights into the pathophysiology of the ischemic penumbra enabled by a multimodal experimental approach; second, to discuss gene expression in ischemia; and third, to confront the challenges of neuroprotectant therapy.

Experimental Focal Ischemia: Pathophysiology of the Ischemic Penumbra

The author’s laboratory has devoted extensive attention to an exceptionally reproducible and, hence, useful model of transient (2-hour) middle cerebral artery occlusion (MCAO) produced in halothane-anesthetized Sprague-Dawley rats by insertion of an intraluminal suture coated with poly-L-lysine to enhance its adhesiveness to the surrounding vessel.23 (Only male rats are used, as estrogen-mediated gender effects influence the outcome of cerebral ischemic insults.24) Animals are awakened during the period of occlusion and are tested on a standard behavioral scale to verify the presence of a neurological deficit. Essential to the reproducibility of this and other models is full physiological monitoring and control of blood pressure, arterial blood gases, and rectal and cranial temperatures; brain temperature, in particular, is a powerful modulator of ischemic outcome.25–27 Because this model is highly reproducible, it can be applied to correlative studies in which complementary methods such as radioisotopic autoradiography,28 in situ hybridization for gene expression,29 histopathology,30 MRI,31 and other techniques are used in matched groups. Pixel-based computerized image-averaging greatly facilitates the analysis of these data.32,33 A key prerequisite for the latter was the development of an algorithm termed disparity analysis, which permits image data sets derived from replicate animal studies to be co-mapped into the same anatomic template and arithmetically manipulated.34 The average brain retains the anatomic fidelity of the individual images and permits pixel-based statistical analysis of group behavior.

This approach has been particularly fruitful in facilitating a multimodal exploration of the ischemic penumbra by using replicate animal series to define its topography and time course. Local cerebral blood flow (LCBF) studied autoradiographically after 2 hours of MCAO reveals, in a series of coronal levels from the averaged data set, an obvious marked reduction of blood flow in the ischemic hemisphere.28 By image-thresholding, it is possible to identify gradients of perfusion within the ischemic hemisphere (Figure 1). Thus, it becomes heuristically useful to define the penumbra in hemodynamic terms, namely as a tissue region within which LCBF is reduced to an intermediate degree, ie, to 20% to 40% of control (contralateral) values. The ischemic core, by definition, consists of those portions of the ischemic zone in which LCBF is more severely reduced, to 0% to 20% of control. From inspection of averaged LCBF image data at multiple coronal levels, it is apparent that the ischemic core predominates within the midportion of the forebrain but is less prominent at the anterior and posterior poles of the ischemic lesion (Figure 1, upper row). By contrast, the ischemic penumbra is present throughout the rostrocaudal extent of the ischemic lesion and tends to surround the ischemic core (Figure 1, middle row). The pixel-based approach permits the volume of the ischemic penumbra to be readily computed. Surprisingly, the volume of the penumbra is virtually equivalent to that of the ischemic core; that is, the early penumbra constitutes one half of the entire ischemic lesion.28

Having defined the blood-flow topography of the ischemic penumbra, one can then study a matched group of animals to investigate the behavior of local glucose metabolism within the penumbra as so defined, using the autoradiographic 2-deoxyglucose method.35 By so doing, one observes that, despite the critical decline of perfusion in the penumbra, its level of local glucose metabolism tends to be maintained at normal levels.28 By applying image-division algorithms to derive an averaged image data set depicting the local glucose metabolism/blood flow ratio—a measure of metabolism/flow uncoupling36—one observes that the acute ischemic penumbra is the site of a marked elevation of this ratio, ie, metabolism/flow uncoupling (Figure 1, lower row). Indeed, the extent of elevation of the metabolism/flow ratio in the acute penumbra is, on average, approximately 4- to 5-fold above normal resting levels.

The maintenance of a normal rate of glucose phosphorylation in the acute penumbra in the face of critically diminished perfusion signals severe metabolic stress to the tissue. The nature of this stress becomes more evident by recording the direct-current (DC) potential from the cortical penumbra. One typically observes so-called peri-infarct depolarizations;
these bear some resemblance to normal spreading depression but, in contrast to the latter, originate from critically hypoperfused tissue.36,37 These DC potential shifts, representing episodic cellular depolarizations, are associated with potassium efflux from cells, sodium and calcium influx, and the need for metabolic energy to restore these ionic gradients. Tissue PO2 measurements taken from the penumbra substantiate that tissue deoxygenation occurs during these depolarizations,38 and biochemical measurements confirm episodic adenosine triphosphate depletion.39 Eventually, the penumbra becomes irreversibly depolarized, at which point tissue adenosine triphosphate levels are depleted and the tissue has become irreversibly damaged.

The fate of the ischemic penumbra subsequent to 2-hour MCAO was explored by applying autoradiographic methods to matched series of animals studied at 1 hour following the institution of recirculation (produced by withdrawal of the occluding intravascular suture). CBF studies at this time point reveal an absence of tissue regions perfused at core levels (ie, 0% to 20% of control CBF), whereas some regions of the previously ischemic brain continue to show CBF within the 20% to 40% range characteristic of the former penumbra. Thus, postischemic recirculation is incomplete.28

A more striking observation emerges from measurements of local glucose metabolic rate at the 1-hour recirculation time point in matched animals. These brains exhibit a marked confluent depression of glucose metabolic rate throughout the previously ischemic hemisphere (Figure 2).28 This is in marked contrast to the preservation of normal glucose phosphorylation in the ischemic penumbra during the antecedent period of MCAO just 1 hour earlier. Following 1-hour recirculation, the metabolism/flow ratio has now pseudonormalized.28

By using multimodal image analysis to co-map these physiological data sets with quantitative histological maps of the resulting infarction, it becomes possible to correlate these physiological measures with histopathology.30 Brains are harvested by perfusion-fixation after a 3-day survival period to permit maturation of the infarct and are paraffin-embedded, sectioned at multiple standard coronal levels, and stained with hematoxylin and eosin to permit computerized analysis of infarct topography. The disparity-analysis algo-
Glucose Metabolic Rate at 1-h Recirculation

Figure 2. Averaged autoradiographic images (n=5 brains) depicting local cerebral glucose utilization measured by the 2-deoxyglucose method at 1-hour recirculation following 2-hour MCAO by intraluminal suture and shown at 3 representative coronal levels (anterior, middle, and posterior). Local cerebral glucose utilization is markedly depressed throughout the previously ischemic hemisphere. (Data reproduced with permission from Belayev et al.40)

To summarize, important features of the ischemic penumbra include its substantial initial size, amounting to about one half of the entire early ischemic lesion; the fact that the penumbra lies within a narrow range of perfusion and thus is precariously dependent on small perfusion pressure changes; that the penumbra is electrophysiologically dynamic and undergoes recurrent energy-consuming depolarizations; and finally, that it is metabolically unstable—the site of severe metabolism/flow dissociation. Becoming subsumed within (ie, indistinguishable from) the ischemic core.

In patients with acute ischemic stroke, positron emission tomography (PET scanning) permits measurements of regional blood flow and metabolism in a fashion similar to the autoradiographic animal studies presented above. In practice, however, relatively few sequential correlative PET studies have been carried out in series of patients with acute stroke, owing chiefly to the formidable logistic challenges posed by studying these acutely ill patients over many hours. One study of this type has been recently published, however, which provides data suitable for comparison with the animal results presented above.40 In that study, 10 ischemic stroke patients underwent early PET scans for regional CBF (as well as for flumazenil binding, a marker of neuronal integrity), and they were subsequently studied by MR imaging at 3 weeks for final infarct size. The various images were then coregistered, yielding maps that permitted pixel-based correlation of the final infarct to zones of diminished early perfusion. As in the rat, early CBF measurements in this human series clearly distinguish those tissue areas destined to infarct from those tissue regions that escape infarction, with the demarcation lying at approximately 45% to 50% of normal CBF. Graphical results of that study are shown in Figure 4. The probability of infarction was high, ie, greater than 95%, when early CBF fell below 25% of control; and the likelihood of infarction was low (ie, probability less than 5%) when early CBF remained above 50% of control.40 Between those 2 lines is a zone of incremental risk of infarction (Figure 4). From these results, one may compare the predictions derived from the rat28,30 and human.40 As shown in Table 2, there is close agreement between the 2 series. This remarkable correspondence belies the assertion that data obtained in lower species may not possess relevance for human stroke.

These studies establish that brain infarction is a highly deterministic event, precisely predictable on the basis of local perfusion levels at the time of MCAO. In similar fashion, these studies also reveal that the early postischemic decline of local glucose utilization observed at 1-hour recirculation is also a precise predictor that the affected tissue regions will eventually succumb to infarction.40 Taken together, these results clearly show that the acute penumbra is, in fact, evanescent and that it progressively deteriorates over just a few hours after the onset of focal ischemia, eventually becoming subsumed within (ie, indistinguishable from) the ischemic core.
Figure 3. Upper left, representative hematoxylin-and-eosin-stained section of perfusion-fixed brain with 2-hour MCAO and 3-day survival. A large cortical and subcortical (striatal) infarct is apparent. Upper right, computer-generated histological frequency map (based on computer-averaging of histological data in 5 replicate brains), showing the likelihood of infarction at each pixel location (side-reversed with respect to upper left panel). Lower panel, pixel-based graphical plot of LCBF measured at 2-hour MCAO versus the probability of histological infarction at the corresponding pixel location following 3-day survival. LCBF ranges corresponding to ischemic core and penumbra are shown by colored rectangles. (See text for further details.) (Data taken with permission from Zhao et al.30)
cause of these factors, the penumbra clearly has a limited life span and appears to undergo irreversible injury within a few hours unless reperfusion is initiated and/or neuroprotective therapy administered.

Gene Expression in Cerebral Ischemia
A multimodal approach also permits gene expression in ischemia to be evaluated. Sharp and colleagues have proposed the existence of multiple “molecular penumbras,” that...
TABLE 2. Thresholds of Infarction: Comparison of Rat and Human

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<td>70% of infarct occurs</td>
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<td>CBF below which</td>
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<td>90% of infarct occurs</td>
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*Data from Belayev et al.30
†Data from Heiss et al.40
CBF indicates cerebral blood flow.

is, zones within which differential patterns of gene expression are determined on the basis of differential perfusion levels. We have explored this idea by correlating in situ hybridization autoradiograms for gene expression with autoradiographic LCBF data sets and histological infarct maps derived from replicate animal series— an approach requiring the development of algorithms for in situ hybridization normalization.33 This type of analysis reveals that the expression of individual genetic messages after ischemia not only exhibits distinct temporal patterns but also is highly spatially determined by the degree of antecedent ischemia.

The ability to survey thousands of genes for their differential expression under ischemic conditions has become possible with the advent of DNA microarray technology. In a recent study, we conducted differential measurements of messenger RNA from the brains of rats with focal ischemia using the Mouse UniGene 1 microarray (Incyte Genomics, Inc), which contains more than 9000 unique annotated genes or expressed sequence tag clusters, of which more than 3000 are assignable to specific protein function hierarchies.42 Our survey specifically compared gene expression in brains with 2-hour MCAO plus 3-hour recirculation against sham controls. By using established criteria for differential expression (≥1.7 or ≤–1.7-fold) and requiring consistency on at least 2 of the triplicate runs, we identified the differential expression of multiple genes, which could be divided into 2 broad categories: those already known to be regulated by ischemia-hypoxia; and those annotated genes not previously so recognized, termed newly connected genes. Twenty-eight ischemia-hypoxia response genes were upregulated and 6 were downregulated; these consisted of immediate early genes, heat shock proteins, antioxidative enzymes, trophic factors, and genes involved in RNA metabolism, inflammation, and cell signaling. In addition, 35 newly connected genes were also upregulated and 41 were downregulated.42 These findings, taken together, underscore the enormous complexity of ischemic biology and direct attention to potentially novel mechanisms for future exploration.

Neuroprotection in Cerebral Ischemia: Hypothermia and Human Albumin

Replicate studies in highly reproducible animal models not only help to elucidate pathophysiology but also can provide insights into the potential for neuroprotective therapy. There are several factors to be considered as to why many clinical trials of putative neuroprotective agents in ischemic stroke have either failed or yielded inconclusive results.43 Chief among these is that the trial may have been designed in a manner that did not replicate the conditions under which efficacy was demonstrated in the animal laboratory, eg, wrong timing (treatment begun too late) or wrong dose (therapeutic plasma levels not achieved). Unanticipated adverse events also contributed to failed clinical trials. Finally, some agents were taken to clinical trial despite only lackluster evidence of efficacy in animal studies.

Many biochemical and molecular pathways contribute to ischemic injury.44 Thus, a desirable neuroprotectant would, in theory, be one that antagonized multiple injury mechanisms. Two such therapies of particular promise have interested us for several years: mild-to-moderate hypothermia, and high-dose human albumin therapy.

Hypothermia

Abundant experimental studies have shown that even mild-to-moderate degrees of brain temperature reduction are neuroprotective against ischemic and traumatic brain injury.45 This is exemplified by a recent study conducted in the 2-hour MCA suture-occlusion model, in which normothermia was compared with a 5-hour period of moderate brain hypothermia (32°C), initiated either during the period of MCAO or at the onset of the recirculation period (ie, 2h after onset of ischemia).33 Both 32°C cooling regimens led to an equivalent marked reduction of mean infarct volume (Figure 5), and increased depth of cooling (27°C) conferred no additional benefit.33 The protective effect of hypothermia demonstrated here is not surprising in view of its multitude of established mechanistic effects. Studies using intracerebral microdialysis, for example, have shown that the massive glutamate release triggered by normothermic global ischemia is completely blunted by imposing a 3°C brain temperature reduction during the ischemic insult.46 By using the dihydroxybenzoic acid spin-trap technique in conjunction with microdialysis, one can also demonstrate a 2-fold increase in hydroxyl radical production with normothermic ischemia, compared with an absence of hydroxyl radical formation under conditions of hypothermia, and a marked 12-fold increase in hyperthermic ischemia.47 The latter finding calls attention to the important point that moderate hyperthermia is markedly deleterious to the ischemic or traumatized brain.48 Indeed, our studies have shown that, even 1 day following ischemia, a 3-hour period of hyperthermia induces the subsequent development of a large infarct in the previously ischemic territory that would otherwise have been largely spared.49

Human Albumin Therapy

Another strategy that has proven to be extremely promising is human albumin therapy—the administration of 1.25 to 2.5 g/kg of 25% albumin solution. By so doing, one can reduce infarct volume in our model of focal ischemia by 60% to 65% and markedly reduce the extent of brain swelling, with a therapeutic window extending to 4 hours.31,50,51 This protective effect is readily demonstrable on infarct frequency maps and can be substantiated by pixel-based statistics (Figure 5). In our view, this marked protective effect is not mediated by hemodilution alone but rather is based on the multiple specific actions of the albumin molecule in binding to free fatty acids, inhibiting oxygen radical production, and supporting endothelial function. Our recent studies have shown that
Figure 5. Neuroprotective strategies in rat model of 2-hour MCA suture-occlusion. Left column, hypothermia (5-hour cooling to target brain temperature of 32°C, instituted at the end of 2-hour MCAO period). Right column, human albumin therapy, 1.25 g/kg IV, administered 1 hour after onset of recirculation. Upper row, control (vehicle) groups. Middle row, treated groups. Lower row, Fisher exact tests applied on a pixel-by-pixel basis to compare treated versus control groups. Colored pixels denote $P<0.05$. (Reproduced with permission from Huh et al.\textsuperscript{27} and Belayev et al.\textsuperscript{51})
albumin therapy markedly improves postischemic venular perfusion when studied in vivo by laser-scanning confocal microscopy.52

Concluding Comments

The careful investigation of rodent ischemia models yields valuable, clinically relevant insights into the pathophysiology of ischemic stroke. The studies described here are highly interdisciplinary, and their success was dependent on a dedicated team of collaborative investigators and research associates trained in complementary disciplines.

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References


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