Relationship of Neurovascular Elements to Neuron Injury during Ischemia

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Key Words

Ischemic stroke • Neurons • Neurovascular unit • Proteases • Hemorrhagic transformation

Abstract

Occlusion of flow to the brain regions identifies regions of vulnerability within the vascular territory at risk, which coalesce to become the mature ischemic lesion. A large number of unsuccessful clinical trials have focused on neuron and extravascular targets in humans that have shown apparent salvage in preclinical models. However, the observation that microvessel and neuron responses to ischemia occur simultaneously in these regions suggest that the responses could be coordinated. This presentation examines evidence in support of the conceptual 'neurovascular unit' and its application to the setting of acute intervention trials in ischemic stroke. There are no uniform reasons for which nonvascular interventions, as a class, have not been successful in clinical trials, but both the clinical observations and the hypothesis imply the need to understand interactions with the neurovascular unit as a prelude to further neuron protectant trials.

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Introduction

Focal ischemia in the brain results from occlusion of brain-supplying arteries and the abrupt reduction in O₂ and nutrient supply to the neuron and the glial compart-

ments. Within the territory at risk, maturation of the ischemic lesion by 24 h after arterial occlusion passes through the stages of tissue infarction, involving leukocyte infiltration, and tissue 'rarefaction', and ending in gliosis and tissue cavitation. The molecular processes underlying cell damage and repair and the mechanisms involved in the maturation of the lesion leading to brain infarction are complex and not yet completely worked out. Some of these processes are summarized in table 1. Of increasing interest are the alterations in the intimate relationships between neurons and their microvascular supply initiated by focal ischemia. Viewing the multifaceted molecular mechanisms of injury maturation from the perspective of the neuron-vascular interactions provides a framework with which to evaluate proposed cell damage and repair processes, their clinical implications, and potential interventions. This presentation addresses the basis for and implications of this conceptual framework.

The Neurovascular Unit

The control and modulation of regional and local flow in the absence of ischemic injury is dependent upon neurovascular coupling [1–3]. To some degree, neurovascular coupling reflects the presence and form of neuronal activation, and the functional efforts of intact neurons. The proximity of microvascular endothelial cells to the circumferential astrocyte end-feet, and the support of astrocytes for neurons suggest that communication could

Table 1. Processes involved in ischemic injury of the CNS*Metabolic events*

Generation of free radicals
 NO generation
 Protein oxidation
 Lipid peroxidation
Depolarization by neurons in evolving infarction and peri-infarct area
Acidosis (lactic acidosis)
Ca²⁺-mediated cell demise
 Failure of Ca²⁺ exclusion mechanisms
 Increase in cytosolic Ca²⁺
Activation of lipolytic pathways
Alterations in protein phosphorylation
Depletion of ATP stores with depression of glucose metabolism
Impaired mitochondrial function
Proteolysis
Protein synthesis

Cellular events (acute)

Activation of cell signaling pathways
Decreased endothelial cell matrix-adhesion receptor expression
Decreased astrocyte matrix-adhesion receptor expression
Detachment of astrocyte end-feet from vascular basal lamina matrix
Degradation of vascular basal lamina matrix components
Expression of matrix proteases
 Matrix metalloproteinases
 Cathepsins
 Heparanase
 Serine proteases (e.g. urokinase)
Increased microvascular permeability
Astrocyte swelling
Neuron swelling
Release of glutamate from neurons
Depolarization by neurons in evolving infarction and peri-infarct area
Expression of cytokines
Generation of platelet activating factor
Activation and appearance of inflammation
 Activation of endothelial cell leukocyte adhesion receptors
 Activation of PMN leukocytes and monocytes
 Adhesion and transmigration of PMN leukocytes
 Platelet activation
Microglial cell activation, shape alterations, and migration

Tissue injury

Edema formation
Cellular swelling
Focal 'no-reflow' within ischemic regions
Increased microvascular permeability with leakage
Angiogenesis with capillary bud formation
Cell demise
Hemorrhagic transformation
Liquefaction and cavitation

also be directed from microvessels to the neurons they supply [4, 5]. This surmise suggests the hypothesis that neuron-microvascular interactions can be described by a 'neurovascular unit' which consists of microvessels (endothelial cells, basal lamina matrix, astrocyte end-feet and pericytes), astrocytes, neurons, and their axons, in addition to other supporting cells (e.g. microglia and oligodendroglia). This provides a framework for considering bidirectional intercommunication between neurons and their neighboring supply microvessels via the intervening astrocytes. The resilience of the 'unit' to reduction in flow or to flow cessation is unclear, but the processes are likely to be complex as adjacent units would be connected through their common microvessels and through dendritic connections. This affords intercommunication and protected perfusion at the same time. Alterations in microvessel integrity could have follow-on effects within the neurovascular unit, also affecting neuronal function [5].

Microvessel-Neuron Relationships

Overall, the cerebral vascular circuit and the microvasculature contribute to a low-pressure high-flow system. The arrangement or architecture of the microvasculature within the anterior cerebral circulation accommodates dynamic changes in flow and is determined by the organ development and by the regional location. During development, the relative positions of microvessels and neurons to one another involve microvascular growth along matrix paths [6–10]. Capillaries, astrocytes and endothelial cells interact to form the intervening basal lamina barrier and the interendothelial tight junctions as part of the permeability barrier [11–17]. Elegant xenograft experiments have demonstrated that the close apposition of endothelial cells and astrocyte end-feet are required for the appearance of the permeability barrier phenotype [18]. In general, capillaries are located within a mean of 30 μm of the nearest neighboring neuron [19]. Within the corpus striatum, the distribution of distances between neurons and their nearest microvessels is right-skewed, but highly ordered and predictable [19]. The capillary arrangement has branch points at approximately 30- μm intervals [19]. The branching of the capillaries allows diversion of flow to patent capillaries/microvessels to circumvent occluded vessels. In the cerebral gray matter, there is a hierarchical organization of the arterial supply, as stacked hexagonal arrays descending from the pial supply to the white matter border [20–22]. The branching

Table 2. Matrix adhesion receptors of the microvasculature

Adhesion receptors	Cell source	Response to ischemia
Integrin subunit α_1	endothelial cells	decrease
Integrin subunit α_3	endothelial cells	decrease
Integrin subunit α_6	endothelial cells	decrease
Integrin subunit α_v	endothelial cells	increase
Integrin subunit β_1	endothelial cells	decrease
Integrin subunit α_1	astrocyte end-feet	decrease
Integrin subunit β_1	astrocyte end-feet	decrease
$\alpha\beta$ -Dystroglycan	astrocyte end-feet	decrease

within these microvessel arrays also allows diversion of flow around impediments or occlusions within one or more capillary branches. Within the white matter, capillaries are arranged in line with axons and comprise ~10% of the density of capillaries found within the gray matter [23]. The branching pattern within the white matter is not so clearly described. These considerations indicate that there are region-specific arrangements of the microvasculature. These accord with differences in regional cerebral blood flow in which flow is lowest in the striatum and highest in the cortical gray matter.

Vascular Matrix Scaffold and Matrix Adhesion Receptors

The basal lamina of cerebral microvessels provides a scaffold on which the endothelium and the glial compartments interact. The principal components of the basal lamina consist of the extracellular matrix (ECM) proteins laminin 1 (and laminin 5), collagen type IV, and fibronectin of cellular origin [24]. Minor components consist of perlecan, nidogen, and other proteoglycans [25]. In general, the ECM composition of the basal lamina is distinct from that separating cells in the neuropil.

The integrity of the microvasculature depends upon the proximity of astrocyte end-feet to the endothelium: both are required for the formation of the basal lamina matrix and for the formation of the permeability barrier [11, 18, 26]. The permeability barrier was originally attributed to interendothelial cohesion by the tight junction proteins ZO-1, occludin, and claudin-5 [14–17]. Disruption of the permeability barrier has been associated with changes in tight junction protein expression, although those changes are not rapid. Increased perme-

ability of the microvasculature to small molecules (e.g. albumin) can occur as early as 2–3 h following middle cerebral artery occlusion (MCA:O) in relevant cerebral ischemia models [27]. More recently, it has been proposed that adhesion of both cell components to the intervening matrix via integrin receptors and dystroglycan may play a role in preserving this barrier (see below).

The matrix provides the second barrier, which limits the transmigration or leakage of blood cells: erythrocytes during hemorrhage and leukocytes in response to inflammatory stimuli (e.g. the inflammatory phase of ischemia). The integrity of the microvasculature is also affected by the presence of pericytes located within the matrix or vessel wall histiocytes within larger vessels [28].

The ultrastructure and integrity of cerebral capillaries depends upon their location and regional tissue composition. Integrin and dystroglycan receptors appear to bind endothelial cells and astrocyte end-feet to the individual intervening matrix components (table 2). Specifically, $\alpha\beta$ -dystroglycan is expressed by astrocyte end-feet on all cerebral vessels, while integrin $\alpha_6\beta_4$ is expressed on large penetrating vessels of the gray matter and all microvessels in the white matter [23, 29]. β_1 -integrins are expressed by the endothelium of all cerebral microvessels also within both the gray and the white matter [23, 30, 31].

Focal ischemia initiates rapid loss of (a) integrity of the ECM within the microvasculature, and (b) matrix adhesion receptors (or their conformation). Loss of the ECM corresponds to the appearance of hemorrhagic transformation within the ischemic territory [32]. A significant rapid loss of the β_1 -integrin subunits α_1 , α_3 , and α_6 , the astrocyte integrin $\alpha_6\beta_4$, and dystroglycan occurs following MCA:O within the ischemic core [29, 30, 33]. Interestingly, transcription of β_1 -integrin increases on microvessels within multiple adjacent cores, suggesting that the microvascular endothelium actively attempts to generate more β_1 -subunits [34, 35]. This coincides with the up-regulation of integrin $\alpha_v\beta_3$ on the microvasculature in the same regions [36].

Loss of the matrix proteins has been attributed to the rapid generation of members of several protease families. Heo et al. [37] have demonstrated the rapid appearance of the latent matrix metalloproteinases (pro-MMP)-2 and -9 in the ischemic territory following MCA:O (nonhuman primate) [37, 38]. pro-MMP-9 is associated with the appearance of hemorrhagic transformation [37]. pro-MMP-2 generation is directly related to the appearance of neuron injury within the ischemic core regions [37]. Proteases associated with the activation of pro-MMP-2 also appear within this territory in temporal context [38]. Ca-

thepsin L, a member of the cysteine protease family, is generated rapidly in excess in the ischemic regions following MCA:O and accompanies degradation of perlecan and laminin within the microvasculature [39]. Heparanase is also generated in temporal and topographic context [39]. Among serine proteases that appear following MCA:O, plasmin is likely generated by the appearance of urokinase (u-PA) in association with its receptor u-PAR on microvessels and nearby neurons [38]. While these proteases appear within the ischemic core(s) and the ECM of the microvasculature is disrupted within this territory, there is still little direct proof that this is due to the proteases so far identified in their active form. Nonetheless, it is likely that these ECM-degrading proteases do participate in changes within the microvasculature. As one recent example, blockade of MMP-like activity by the general inhibitors GM6001 and 1,10-phenanthroline prevents degradation of β -dystroglycan from murine primary cerebral astrocytes subject to experimental ischemia [23].

Another important feature of the ultrastructural changes in the microvasculature is the observation that proteases that degrade certain ECM components appear simultaneously on the microvessels and neurons in close proximity. These include u-PA, cathepsin L, heparanase, and pro-MMP-2 [37–39]. The mechanisms causing the coincident appearance of these proteases on several elements within the microvascular unit are not known, but they do reinforce the impression that events within different elements of microvascular units within the target territory can be coordinated during focal ischemia.

In summary, (a) microvessel responses and neuron injury occur in the same time frame and the same subregions of ischemic injury; (b) an ordered relationship exists between microvessels and neighboring neurons within the susceptible territory; (c) rapid significant alterations in the matrix of the vascular compartment and in the nonvascular compartment of the ischemic territory occur, and (d) the loss of matrix adhesion receptors that accompanies ECM degradation can be prevented, in part, by inhibition of MMP-like proteases.

These observations suggest a hypothesis that links both neuron and microvascular functions and their responses.

A Hypothesis

The rapid, simultaneous and coincident appearance of matrix proteases by microvessels and their adjacent neurons implies coordinate responses of these elements of

the neurovascular unit to focal ischemia [40]. While it is apparent that cytokine and chemokine generation occurs within the region of the evolving ischemic lesion, the coincident neuron-microvascular events are heterogeneously scattered within the region among uninjured microvessel-neuron pairs early following MCA occlusion [19]. The upregulation of β_1 -integrin transcripts within microvessels in the boundaries of adjacent subregions of injury lends further support to a microvascular component of the evolving ischemic lesion, in which neurons are irreversibly injured [35]. These and related observations provide a basis for a hypothesis that states that focal ischemia can initiate coordinated events within the microvasculature and nearby neurons in which both elements behave as a unit. These are typified by alterations in both microvascular and extravascular ECM, and the rapid coordinate appearance of members of four families of proteases by adjacent microvessels and neurons in the evolving ischemic core.

One of the implications of this hypothesis is that neurons and the microvascular endothelium can communicate in both directions. Rather than a unidirectional dependence of regional microvascular flow and dynamics upon neuron signaling, the proximity of the endothelium to astrocyte end-feet and the bifunctional nature of their matrix adhesion receptors imply that communication is possible across the matrix. Both integrin and dystroglycan receptors can indicate to the cell the nature of its environment, and can also participate in events that involve cell activation. Efforts are underway to understand if this is possible in the cerebral microvasculature.

Focal ischemia in experimental systems alters endothelial cell-astrocyte relationships via its matrix receptors [23, 29, 30, 35]. The implications for ischemic stroke are that (a) efforts to preserve or rapidly re-establish flow through the threatened microvascular bed are likely to reduce neuron injury within a regional network; (b) 'neuron protection' per se may not be sufficient if the astrocyte-endothelial cell relationships are disrupted by ischemia; (c) preservation of the matrix-matrix receptor interactions could contribute to preservation of neuron/neurovascular function, and (d) preservation of astrocyte function would seem essential for maintaining the normal function of the neurovascular unit. Given our natural limitations in detecting small improvements in function clinically, efforts that successfully maintain neurovascular function in the face of focal ischemia may not be detectable in this setting. However, experience with acute interventions so far should give us a view of whether this direction of enquiry has merit.

Another implication of this hypothesis is that events occurring during focal ischemia within cerebral microvessels are linked to the actions of neurons supplied by those microvessels, via the syncytium of astrocytes, and that strategies designed to protect neurons should also protect function within the entire neurovascular unit.

Acute Interventions in Ischemic Stroke and the Neurovascular Unit

In the last two decades, considerable effort has been expended to treat patients acutely after the onset of ischemic stroke when it was shown that neurological benefit could be gained with arterial recanalization procedures. Prospective controlled randomized trials have examined two classes of treatments: (a) 'neuroprotectant' agents, and (b) antithrombotic agents. Together, these compounds include NMDA receptor antagonists (e.g. cerestat, citicholine), free radical scavengers (e.g. tirilazad mesylate, NXY-059), anti-inflammatory immune inhibitors (e.g. enlimomab), select antiplatelet agents (e.g. abciximab), and plasminogen activators (e.g. rt-PA, single-chain urokinase, scu-PA; fig. 1).

Neuron Protectants

Agents with demonstrated ability to decrease neuron demise in either isolated cell culture or in small animal models of focal cerebral ischemia have been termed 'neuroprotectants'. The presumed target of activity of the agents is the neuron, and a few mechanisms subjected to inhibition include Ca^{2+} regulation and transit, neurotransmitter release, and cell demise pathways. While fundamental studies have identified compounds with neuron salvage properties in preclinical model systems, use of these compounds in the clinical setting has, for various reasons, proved unsuccessful. The recently terminated program to develop NXY-059 for acute intervention in ischemic stroke is instructive [41–45]. The SAINT-1 trial, a prospective placebo-controlled randomized multicenter study of NXY-059 on stroke outcome, was equivocal [42], while the larger phase III prospective trial, SAINT-2, did not demonstrate a significant improvement in stroke outcome with NXY-059 [44]. Among the reasons posited, the agent known to quench free radicals did not cross the permeability barrier into the CNS, to the abluminal microvascular interface where free radical generation is known to occur [46]. Other agents have also proven to be unhelpful in the setting of focal isch-

Fig. 1. Relationship of targets of acute intervention for ischemic stroke evaluated by prospective well-controlled clinical trials. **a** General classes of agents (and specific agents within specific classes where applicable) that have been tested with results to date. All have had activity in animal focal ischemia models. **b** Agents that have been shown to have efficacy, and can be applied safely when strict rules are followed. Note that all of the agents directed against neuron-associated targets (e.g. NMDA receptors, glycine antagonists) and some against the generation of reactive oxygen species (free radicals) known to appear at the microvessel-neuropil interface have not been shown to have activity in human patients. Only antithrombotic agents and, in particular, plasminogen activators have shown activity.

emia despite acute intervention. These can be mapped relative to their presumed cellular target(s) in the neurovascular unit (fig. 1). While many agents with putative neuron protectant activity have been shown to reduce neuron injury in vitro or decrease injury volume following MCA:O, their actual targets may be nonneuronal. This raises the concern that data specific for the intended target, the impact of target injury on neuron viability, and the relation of those responses to the tissue responses would be of interest for assessing the relevance of these and related compounds to the clinical setting.

For neuron protection, these considerations raise a number of questions: Why have agents shown to protect neurons in model systems been uniformly unsuccessful

in clinical trials? Are there characteristics of human cerebral responses to ischemia that current brain injury models cannot reproduce? Can restitution of flow through an acutely thrombosed cerebral artery limit injury progression in all patients?

Antithrombotic Agents

The vascular-thrombotic nature of the majority of ischemic strokes draws attention to the potential benefit of antithrombotic agents in treatment, including antiplatelet agents, anticoagulants, and plasminogen activators. Antiplatelet interventions are employed for secondary prevention of recurrent ischemic events after a signal TIA or ischemic stroke, while anticoagulants are employed for primary prevention of thromboembolic events from nonvalvular atrial fibrillation and select prosthetic valve devices. The potential role(s) of antithrombotic agents in microvascular disease, typified by lacunar lesions or neuropsychiatric lupus, has not been defined, but consideration of their use is clinical. For ischemic strokes without clear etiology, well-controlled oral anticoagulation appears to have no advantage over aspirin [47]. Careful management of patients with acute infusion of the plasminogen activator rt-PA provides consistent improvement in their outcome [48]. To date, the prudent use of rt-PA provides the most definitive reduction in neuron injury.

Early experience in the setting of stroke employed urokinase (u-PA) or streptokinase (SK). These agents have since been superseded by the recombinant form of the endogenous tissue plasminogen activator (rt-PA), in single (alteplase) and two-chain (duteplase) forms and other derivatives of rt-PA (including r-PA; tenecteplase, TNK), and other exogenous PAs. Each of the PAs targets fibrinogen and fibrin within the thrombus lattice. t-PA is a 527-amino-acid single chain, that is converted to the two-chain form by plasmin cleavage of the arg275-isoleu276 linkage [49, 50]. Both the single-chain and two-chain forms have similar catalytic efficiencies. The kinetically preferential cleavage of fibrin (in the presence of fibrin-bound plasminogen) over fibrinogen underlies the impression that both forms of rt-PA are fibrin selective. The $t_{1/2}$ of both forms of rt-PA is ~3–8 min in humans following a single infusion, but the biologic $t_{1/2}$ is believed to be somewhat longer, accounting for delayed hemorrhage from hemostatic thrombi [51, 52]. The thrombus selectivity and catalytic efficiency of TNK are very similar to rt-PA [53, 54]. These characteristics allow for a single bolus injection. Additionally, no changes in fibrinogen and a modest decrease in plasminogen accompany bolus TNK.

Recombinant desmoteplase (DS-PA) is derived from the saliva of the vampire bat (*Desmodus* species) and has properties distinct from t-PA. The fibrin-binding of the DS-PAs depends entirely upon the presence of the finger domain; the catalytic efficiency of plasminogen activation by α_1 DS-PA is ~200-fold greater than t-PA in the presence of fibrin [55, 56]. Additional studies suggested enhanced hemorrhage from regions of vascular injury with α_1 DS-PA [57]. Of these PAs, only the rt-PAs have been developed through to the demonstration of reduced injury in preclinical models, and improved neurological outcome in patients.

Recanalization of carotid artery territory occlusions by intravenous infusion techniques has been achieved in 34–59% of patients treated within 8 h of symptom onset [58–61]; but early studies using direct catheter-dependent SK or u-PA delivery reported recanalization of symptomatic carotid artery territory occlusions in 46–90% of patients treated within the same time frame [62, 63]. In those studies, 18–33% of treated patients suffered hemorrhagic transformation within the ischemic territory. Clinical improvement was observed in a substantial number of those patients, although outcomes were not quantitatively assessed.

Acute recanalization of MCA division and branch occlusions occurred more frequently than ICA occlusions [58]. Mori et al. [61] first demonstrated that patients treated with duteplase had improved recanalization and a significantly better clinical improvement at 30 days than those treated with placebo. Hemorrhagic transformation occurred in 29–53% of treated patients in those studies [58–61]. Those studies demonstrated both the feasibility and safety of exposure to PAs and were the first to indicate that re-establishment of flow in the CNS could improve neurological outcome. They suggested, in more contemporary terms, that preservation of flow to/through the microvascular components of the neurovascular unit could preserve neuron function.

The National Institute of Neurological Disorders and Stroke (NINDS)-funded two-part, four-armed, placebo-controlled outcome study of rt-PA in patients entered within 3 h from ischemic stroke symptom onset further supports the possibility that microvascular flow/patency could underlie preservation of neuron function [48]. rt-PA recipients displayed a significant 11–13% absolute increase over placebo in the number of patients with no or minimal disability/deficit in Barthel index, modified Rankin scale (mRS) score, Glasgow outcome scale score, and NIHSS, at 3 and 12 months [48].

Table 3. Efficacy and hemorrhagic transformation: carotid territory ischemia, intravenous delivery

Study	Agent	Patients	$\Delta(T-0)$, h	Outcome	% improved	Hemorrhage			Reference
						HI	PH	% ^a	
Abe	u-PA	57	<720	N ^b	70	0	0		[80, 81]
	C	56			47	0	0		
Atarashi	u-PA	191	<120	N ^b	45	2	0	1.0	[82]
	C	194			44	1	0	1.1	
Otomo	u-PA	176	<120	N ^b	52	2	0	1.1	[83]
	C	188			41	1	0	0.5	
Otomo	rt-PA	171	<120	N ^b	59	2	0	1.1	[84]
	u-PA	184			55	3	0	1.6	
Abe	rt-PA	145	<72	N ^b	66	3	0	2.0	[85]
	u-PA	77			45	6	0	7.8	
Haley	rt-PA	10	<1.5	N ^c	60 ^g	0	0		[86]
	C	10			10	0	0		
	rt-PA	4	1.5–3.0	N ^c	50	0	0		
	C	3			67	0	1		
MAST-E	SK	156	<6.0	M ^d	34 ^g	63	33	21.0 ^g	[87]
	C	154			18	57	4	3.0	
ASK	SK	165	<4.0	M ^d	36 ^g	33	23	13.2 ^g	[88]
	C	163			21	23	5	3.1	
MAST-I	SK	313	<6.0	M ^d	25 ^g	60	21	6.7 ^g	[89]
	C	309			12	27	2	0.7	
ECASS	rt-PA	313	<6.0	D ^f	36 ^g	72	62	19.8 ^g	[64]
	C	307			29	93	30	6.5	
NINDS I	rt-PA	144	0–3.0	N ^c	47	–	13	5.6 ^g	[48]
	C	147			39	–	3	0.0	
NINDS II	rt-PA	168	0–3.0	D ^f /N ^c	48	–	21	7.1 ^g	[48]
	C	165			39	–	81	2.1	
ECASS-II	rt-PA	409	<6.0	N ^c	40	142	48	11.7 ^g	[66]
	C	391			37	141	12	3.1	
ECASS-III	rt-PA	418	3.0–4.5	D ^f	52 ^g	91	22	5.3 ^{g, h}	[90]
	C	403			45	82	9	2.2	

$\Delta(T-0)$ = Interval from onset to treatment; SK = streptokinase; u-PA = urokinase plasminogen activator (urokinase); rt-PA = recombinant tissue plasminogen activator; C = control; N = neurological outcome; M = mortality; D = disability.

^a Percent patients displaying parenchymal hemorrhage. ^b N, neurological outcome. ^c N, neurological outcome according to scoring instrument, e.g. National Institutes of Health Stroke Scales (NIHSS) score. ^d M, early mortality. ^e M, mortality at 3 or 6 months. ^f D, best disability outcome (modified Rankin Scale (mRS) score) at 3 months. ^g Statistically significant differences. ^h ECASS-II definition.

Three further phase III prospective, randomized safety and efficacy studies of intravenous rt-PA (alteplase) broadened these impressions (table 3). The European Cooperative Acute Stroke Study (ECASS) compared rt-PA to placebo within 6 h of symptom onset; although, at 3 months, there was no significant difference between the two groups in disability outcome [64]. Discarding patients who were entered in violation of the inclusion criteria indicated an 11–12% absolute improvement (mRS 0 or 1) in the rt-PA-treated group over the placebo group

[64, 65]. In ECASS-II, a favorable outcome (mRS = 0 or 1) was seen in 40.3% of rt-PA patients compared to 36.6% of placebo patients [66]. Cerebral hemorrhage causing death or deterioration was significantly more frequent in the rt-PA group than the placebo group (11.7 vs. 3.1%). Recent experience from study of patient treatment within a broader acquisition time from symptom onset has been encouraging [67, 90]. Given variations in the outcome differences, the trends (in ECASS and ECASS-II) and the benefits (in NINDS and ECASS-III) are consistent.

These and other studies have also suggested significant contributors to the risk of hemorrhage from intravenous plasminogen activators. Contributors include excessive time from the onset of symptoms to treatment, low body mass index, diastolic hypertension, older age, and the use of rt-PA [48, 58, 68–71]. The appearance of ‘early signs of ischemia’ on the initial CT scan is also associated with an increased risk of hemorrhage and demise [64]. Taken together, the results of the ECASS and NINDS studies indicate the enormous importance of patient selection to reduce the hemorrhagic risk accompanying the use of plasminogen activators in acute stroke.

Evidence with TNK and rDS-PA is conflicting and incomplete. TNK has been examined in an open dose-escalation safety study of patients entered with qualifying strokes compatible with the criteria for the NINDS-sponsored rt-PA study [72]. The escalation was terminated when 2 of 13 patients suffered symptomatic intracerebral hemorrhage in the 0.5-mg/kg tier (compared to no hemorrhages in the lower doses). To date, there are no data regarding the ability of TNK at this or lower doses to achieve recanalization or preservation of neuronal function.

In contrast, the ability of rDS-PA to achieve recanalization and preservation of neuronal function was tested through phase II and phase III formats using magnetic resonance technology to define a target population. Note should be made that no dose-equivalency studies comparing rDS-PA with rt-PA have been performed. The first study (DIAS) was a phase II blinded placebo dose-finding efficacy trial that initially compared fixed doses of 37.5 and 50 mg ($n = 13$), then 25 mg ($n = 17$) of rDS-PA with placebo ($n = 16$) in patients entered from 3 to 9 h after symptom onset if they demonstrated a PWI/DWI ratio of >1.2 . This study was halted for safety reasons when it became apparent that both the lower and upper doses produced excessive symptomatic hemorrhage frequencies of 23.5 and 30.8%, respectively. A proper dose-escalation study comparing 62.5, 90 and 125 $\mu\text{g}/\text{kg}$ ($n = 15$ each) with placebo ($n = 11$) confirmed recanalization in 10 of 15 patients at the 125- $\mu\text{g}/\text{kg}$ dose. A second phase II blinded placebo-controlled three-arm dose-finding efficacy study was then performed (DEDAS) comparing 90 $\mu\text{g}/\text{kg}$ ($n = 14$) rDS-PA, 125 $\mu\text{g}/\text{kg}$ ($n = 15$) rDS-PA, and placebo ($n = 8$) under the same conditions as DIAS. In the intention-to-treat analysis, no hemorrhages were observed and 8 of 15 patients demonstrated recanalization at the 125- $\mu\text{g}/\text{kg}$ dose, supporting the observations of the revised DIAS. The frequency of symptomatic hemorrhage was acceptable. It was claimed that in the target population significant improvement in 90-day ‘good out-

come’ as defined by the investigators was observed at the higher dose ($p = 0.022$). Armed with these data, a phase III blinded three-arm placebo-controlled dose-finding efficacy trial (DIAS-2) was performed comparing 90 $\mu\text{g}/\text{kg}$ rDS-PA, 125 $\mu\text{g}/\text{kg}$ rDS-PA, and placebo under the same conditions as DIAS and DEDAS. On 31 May, 2007, the sponsor reported no efficacy from this trial [73, 74]. The program was halted.

In summary, only rt-PA (alteplase) has been associated with improvement in clinical outcome among ischemic stroke patients treated within 3 h of symptom onset, implying that recanalization could be translated through the microvasculature to preserve neuron function. Otherwise, with the exception of two early small studies, none have correlated documented flow or recanalization with neuron function. Studies of MCA occlusion and restitution of flow in the nonhuman primate were the first to demonstrate that perfusion is associated with decreased injury volume [75, 76].

Another potential clinical test of the microvessel-neuron relationship was attempted by two prospective studies of acute intra-arterial delivery of recombinant single chain u-PA (scu-PA or pro-UK) in patients with proximal MCA occlusions [71, 77]. A prospective double-blind placebo-controlled level I dose-finding study (Prourokinase in Acute Cerebral Thromboembolism, PROACT) compared recombinant scu-PA (6 mg) with placebo in patients treated within 6 h of symptom onset for recanalization of M1 and M2 segment MCA occlusions and safety [71]. Both the significant increase in MCA recanalization and hemorrhage observed in the scu-PA group were heparin-dependent. A follow-on study, PROACT-2, prospectively tested the effect of intra-arterial recombinant scu-PA (9 mg) against no instrumentation for recanalization and disability outcome in an unblinded fashion [77]. Recanalization was marginally significantly increased with recombinant scu-PA (65.7%) compared to no intervention (18.0%), and the frequency of symptomatic intracerebral hemorrhage also increased. Disability outcome measured as mRS = 0–2 improved in the scu-PA cohort, but was not significantly different from the control group when measured as mRS = 0–1. The program was halted after regulatory review requested a third study. While two studies indicated that acute restitution of perfusion of the occluded territory was feasible using intra-arterial infusion, any benefit observed was marginal and has not been improved upon. No prospective properly controlled studies of this problem have been undertaken since.

Hemorrhagic Transformation and Acute Plasminogen Activator Use

Focal ischemia is accompanied by hemorrhagic transformation in the areas of ischemic injury in up to 65% of patients and select focal ischemia models [78, 79]. These hemorrhagic events can be either hemorrhagic infarction (HI, petechial to confluent petechial hemorrhages), parenchymal hemorrhage (PH, hematomas causing mass effect and most often clinical worsening), or a combination [58].

HI appears to represent local leakage of erythrocytes from microvessels within the ischemic core regions when the basal lamina matrix is degraded. The disruption of the microvascular matrix barrier has been suggested to correspond to matrix protease generation by proximity, timing, sources, and the upregulation of specific protease activation systems (fig. 1) [24, 32]. Extravasation of blood elements leads to fibrin generation from fibrinogen within the leaked plasma. Although a large molecule (360 kDa), fibrinogen appears in the extravascular space within several hours of MCA occlusion, indicating that leakage can be initiated rapidly as some microvessels are displaying evidence of occlusion (focal 'no-reflow').

PH has been attributed to degradation of the structure of large microvessels under arterial pressure [78]. Importantly, the risk of detectable hemorrhage appears to depend upon the presence of antithrombotics, and their dose and target. For instance, highest incidences of PH are associated with the use of plasminogen activators. The substrates of plasmin include fibrin(ogen), in addition to the matrix proteins laminin and collagen (as well as myelin basic protein). Hemorrhage could be accentuated when uninhibited plasmin degrades extravascular fibrin, thereby preventing the sustained formation of a fibrin network and hemostatic thrombus [58]. In this setting, clinically, PH frequency depends upon a number of known risk factors (table 4). Coagulation inhibitors, and to a lesser degree antiplatelet agents, accentuate hemorrhage by decreasing fibrin formation or by decreasing the activation of platelets and their participation in thrombus formation, respectively. Hence, the incidence of PH is dependent upon modulation of hemostasis and vascular stability.

PH impedes neuron preservation or salvage. This appears to be at least population dependent. In the NINDS-sponsored study, the frequency of PH was significantly greater among those patients receiving rt-PA (6.4%) than among those who received placebo (0.6%) at 3 months. While mortality was unchanged, intracerebral hemor-

Table 4. Factors affecting hemorrhagic transformation with plasminogen activators

Factor	Agent
Time from symptom onset	rt-PA (d)
Diastolic hypertension	rt-PA
Low body mass	rt-PA
Age	rt-PA
Atrial fibrillation	rt-PA
'Early signs' of ischemia	rt-PA
	rscu-PA

rhage contributed to demise in the rt-PA group [48]. In ECASS, the frequency of PH associated with rt-PA exposure was higher, as was that of the placebo group [64]. Similarly, in ECASS-II PH in both treatment groups exceeded that of the NINDS study, but was intermediate with respect to ECASS [66]. A survey of the CNS hemorrhagic events in recent trials of acute thrombolysis indicates that the increased incidence of PH among patients treated acutely with rt-PA is directly related to the incidence of PH in the placebo population, and that this varies from study to study. Therefore, one might postulate that in the instance of PH the extent of neurovascular unit recovery depends upon attributes of individual patients or patient groups.

The Neurovascular Unit – Reprise

As a framework for understanding acute intervention responses during ischemic stroke, further basic information is required that adequately relates microvascular responses to neuron integrity in mammalian systems. The observations of matrix-matrix receptor interactions within cerebral microvessels and the rapid expression of matrix-sensitive proteases under normoxic and ischemic conditions provide starting points for dissecting the direction and consistency of microvessel-neuron communication. Those ongoing studies do not yet examine how these processes affect neuron excitability and membrane function, but they do indicate how many levels of function within the neurovascular framework are still not understood. One implication of this paucity of information and the clinical trial experience so far is that interventions that rely solely upon 'neuron protection' may be wholly inadequate to achieve clinical benefit. Strategies that extend to structural integrity and astrocyte function

may be revealing. A more fundamental understanding of the cell-cell, and hence tissue functional, interrelationships is necessary.

Conclusions

When distanced from considerations of the intracellular processes within neurons and glia initiated by focal ischemia in the brain, the perspective that a coordinated response by neurons and their microvessels is initiated by occlusion of brain-supplying artery(ies) allows enquiry into the relevance of the intracellular and cellular responses of the entire 'unit' to tissue fate. Cell and tissue model studies indicate the importance of microvessel-neuron relationships in the setting of focal ischemia. Historically, considerations of injury mechanisms have focused on the sensitivity of the neuron to ischemia; but more recent observations indicate that microvessel responses to ischemia and neuron injury occur simultaneously and in the same regions. The coordinate and potentially unitary nature of these relationships is suggested by known matrix responses to focal ischemia. These have been further supported by the integrin receptor and dysglycan responses of microvascular endothelial cells and astrocytes to experimental ischemia (e.g. oxygen-

glucose deprivation). They support the hypothesis that in the CNS events within microvessels are linked to the actions of neurons supplied by those microvessels, via a syncytium of astrocytes, and that strategies designed to protect neurons during focal ischemia should also protect function within the entire neurovascular unit.

The importance of the microvasculature to stroke outcome offers a clinical setting for this hypothesis. Among treatment approaches to ischemic stroke, the management of vascular targets has been the most successful, while other targets within the neurovascular unit have been so far uniformly unsuccessful. Both these clinical observations and the hypothesis imply the need to understand interactions within the neurovascular unit better.

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