Proteolytic Extracellular Matrix Fragments Following Ischemic Stroke: New Insights to Potential Therapeutic Targets

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Abstract: Stroke is the leading cause of long-term disability and the third leading cause of death in the United States. The sudden disruption of blood flow that occurs following ischemic stroke ultimately leads to neuronal death, breakdown of the blood brain barrier, and proteolytic processing of the surrounding extracellular matrix (ECM). Within 3 days after ischemic stroke, new blood vessels begin to form around (ischemic penumbra) the site of injury by a process known as angiogenesis. Angiogenesis, in turn is regulated by a number of growth factors and by processed biologically active fragments of the ECM. Unfortunately, current experimental stroke therapies that might enhance post-stroke angiogenesis, including growth factors, pharmaceuticals, and stem cells may engender significant and unacceptable risks. Furthermore, while most other stroke therapies have focused on neuroprotection during the acute phase of stroke injury, we hypothesize that exploiting brain self-repair mechanisms, including ECM matrix fragment generation, may provide better therapies. In this mini-review we will discuss the importance of the ECM following stroke, including the potential role of its proteolytic fragments in modulating angiogenesis and neurogenesis.

Keywords: Perlecan, extracelluar matrix, laminin, fibronectin, stroke, ischemic stroke, neurovascular unit.

INTRODUCTION

Stroke is the leading cause of long-term disability and the 3rd leading cause of death in the United States. Eighty percent of patients who are affected by stroke suffer from the ischemic version while the remainders are hemorrhagic in nature. However, while incremental advances have been made in acute stroke treatment, our understanding of the mechanisms underlying brain self-repair after stroke remains poor. Therefore, the problem of brain repair and stroke rehabilitation is an emerging research priority [1], with the underlying goal of *identifying and improving brain reparative process*.

In order to identify and improve brain reparative processes, one must appreciate the concept of the neurovascular unit. This unit consists of several cell types within the brain including endothelial cells, astrocytes, pericytes and neurons, all of which are closely knit together by the extracellular matrix (ECM). The ECM, in turn, plays many important roles in cell biology by regulating cell morphology, control of cell fate, scaffolding, and more importantly, regulating cell-cell interactions [2]. Within the past two decades, research has focused on investigating the cryptic fragments that are released from the extracellular matrix when exposed to active proteases such as matrix metalloproteinases (MMPs), and cathepsins. There is an elevation of these proteases within the stroke ischemic core (central region of irreversible neuronal injury) and penumbra that leads to degradation of the extracellular matrix (ECM). This in turn can lead to the generation of these cryptic fragments and affect the surrounding neurovascular unit.

This mini-review will discuss the biology of ischemic stroke and its current therapies but will focus primarily on the potential significance of the generation of ECM fragments following stroke. Specifically, we will discuss how these fragments might play a role in post-stroke brain repair, including the processes of neuronal regeneration (neurogenesis) and blood vessel regeneration from pre-existing vasculature (angiogenesis).

ACUTE AND CHRONIC ISCHEMIC STROKE

An ischemic stroke can generally be defined as an area of brain tissue that has sufficiently lost the supply of oxygen and blood flow to cause cell death. Typically, in ischemia, this occurs when a thrombus in an artery has formed. Then oxygen and glucose supplies for downstream tissues become inadequate and necrosis begins. The area of severe ischemia, where irreversible neuronal injury occurs, is defined as the ischemic core. The area surrounding the ischemic core, the ischemic penumbra (Fig. **1**), is also at risk for cell death. The penumbra is still viable several hours following ischemic injury allowing for neuroprotective therapies to rescue neuronal injury and cell death within this area of tissue.

Immediately following an ischemic insult, a cascade of events take place. First, there is a disruption in oxygen levels that leads to ATP-reliant ion transport pumps to fail. Once this happens there are an influx of calcium ions within the cell which leads to cell stress and the release of glutamate. The release of glutamate, acting on AMPA receptors, causes neighboring cells to uptake more calcium. Cells then begin to undergo apoptosis. These dying cells affect neighboring

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cells by the release of other toxins and proteases. The ischemic core continues to grow due to this process by infringing upon the ischemic penumbra.

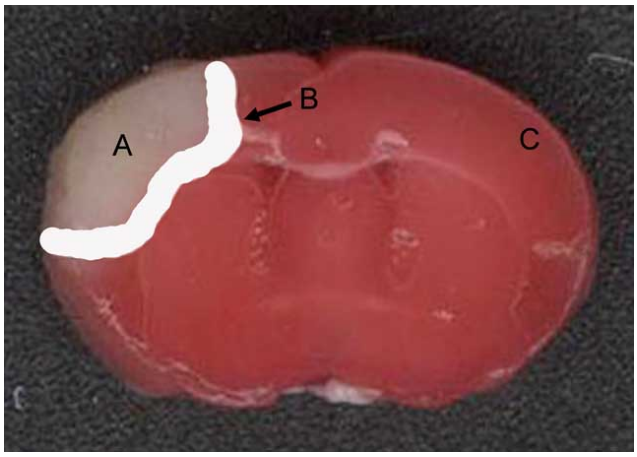

Fig. (1). TTC-derived mouse brain slices following 24 h middle cerebral artery (MCA) occlusion. (**A**) Ischemic core; (**B**) Ischemic penumbra/peri-infarct brain shaded in grey; (**C**) contralateral cortex. TTC stains oxygenated tissue red, leaving stroked brain tissue white, as labeled as (**A**).

The penumbra is able to reverse this process by reparative angiogenesis and neurogenesis. Both of these processes occur in close proximity, i.e. in a neurovascular niche, [3-5] which affords mutually supportive growth factor-mediated neuron-endothelial cell cross-talk [6-8]. For angiogenesis, endothelial cell proliferation can occur as early as 12 to 24 h post-stroke [9]. These newly generated endothelial cells then migrate towards ischemic brain regions in response to a number of endothelial cell mitogens such as vascular endothelial cell growth factor (VEGF) and platelet derived growth factor (PDGF) and form new blood vessels in peri-infarct cortex after 3-7 days [9]. Angiogenesis then continues for at least 21 days.

Previous and newly formed angiogenic blood vessels serve as a physical scaffold for new neurons to migrate towards the ischemic core, even in the absence of blood flow [3]. For post-stroke neurogenesis in rodents, neuronal progenitor cell proliferation is significantly enhanced in the subventricular zone (SVZ) and the hippocampal dentate gyrus (DG) as early as 2-3 days post-stroke correlating with a specific expression pattern of cytokines, chemokine and vascular growth factor signaling [9], and peaks after 1-2 weeks, returning to control levels by 3-4 weeks [10]. Once generated, these progenitor cells continue on for approximately 2-3 weeks in the DG but only for a week in the SVZ [11]. Instead, the SVZ progenitor cells largely migrate as far as the striatal stroke penumbral area and there differentiate into mature striatal neurons and astrocytes [12]. Consequently, relatively few neural progenitors migrate into stroked tissue and those few that complete the trip usually fail to become mature neurons for unknown reasons [13]. Collectively, niche neurovascular coupling appears to represent an important means of post-stroke brain repair that could be therapeutically exploited [14].

BRAIN SELF REPAIR

In the neurovascular niche, diffusible factors help afford cross-talk between the closely associated brain endothelial cells and the neuronal precursor cells in a fashion that is consistent with the developmental association of neurogenesis and vasculogenesis [15]. For example, the newly produced vasculature promotes neurogenesis and neuroblast migration by production of angiopoietin 1 (Ang1) and stromal-derived factor 1 (SDF1) [16, 17] which act on the neuroblast Tie2 and CXCR4 receptors, respectively [6, 7]. Erythropoietin (EPO) also increases the number of immature neurons in the peri-infarct tissue [18]. Neuroprotection, neuronal migration and neural stem cell renewal is also afforded by vascular production of brain-derived neurotrophic factor (BDNF) binding to neuronal TrkB receptors [8, 19, 20] although this may not be the case in the adult mouse and rat subventricular zone [21]. A recently defined example of neurovascular cross-talk occurs in brain endothelial cell-neural stem cell co-cultures in which neural stem cell Nitric Oxide (NO) reportedly induces brain endothelial cell release of BDNF and VEGF, which in turn induce endothelial cell angiogenesis *via* VEGFR2 and TrkB receptors, as well as having neuronal stem cell renewal affects [20]. The cumulative effect of this niche interaction between brain endothelial cells and immature neurons is to afford a protected means by which these neuroblasts reach the peri-infarct cortex and migrate towards the infarcted tissue [3, 22].

CURRENT THERAPIES AND EXPERIMENTAL TREATMENTS FOR ISCHEMIC STROKE

Recent experimental stroke treatments, including pharmaceuticals, stem cell replacement therapies, and growth factors have attempted to capitalize on the emerging understanding on the neurovascular niche to promote functional stroke recovery [23-27]. However, drug and growth factors raise the questions of potentially serious side effects, drug interactions, and contra-indications, while cell-based therapies raise the important safety issue of "where do the cells go?" once injected and concerns of administered cells become undesirable tumors or other abnormal ectopic tissue. Furthermore, growth factors can have vastly different positive or negative consequences for stroke depending on when they are administered relative to the stroke injury. For example, vascular endothelial growth factor (VEGF) both worsens already disrupted blood brain barrier permeability, a hallmark of early stroke pathology, and enhances angiogenesis and neurogenesis more chronically [28, 29].

Tissue plasminogen activator (tPA), the only FDA approved drug treatment for ischemic stroke, works by dissolving blood clots or thrombi that cause ischemic strokes. However, tPA must be administered to the patient within 4.5 hours following the onset of stroke symptoms to be effective without excessive bleeding risk [30]. As the signs and symptoms of stroke are not always obvious, and the confirmation of an ischemic *vs* hemorhagic stroke takes time, this brief therapeutic window is quite often missed.

Kozak *et al*. have recently demonstrated that the anti-hypertensive drug Candesartan, given immediately after reperfusion in a rat transient middle cerebral artery (MCA) model, resulted in an increase in brain tissue and

cerebrospinal fluid VEGF (as well as matrix metalloprotease, (MMP)), increased striatal vascularization, and improved neurobehavioral test results [24]. Yao *et al*. have demonstrated that administration of the herbal extract cornel iridoid glycoside (CIG) 3 hours after MCA occlusion in rats resulted in increased brain VEGF expression, increased post-stroke angiogenesis, neurogenesis and improved neurological function [25]. Chen *et al*. have demonstrated that the cholesterol lowering drug Simvastatin increases Ang1/Tie2 expression after stroke in rats, thereby reducing blood brain barrier leakage, stabilizing vasculature, and promoting formation of the neurovascular niche [23]. Furthermore, the same group demonstrated that cholesterol lowering drug Atorvastatin, improved functional recovery in stroked mice due to increased VEGF, VEGFR2 and BDNF [26].

In addition to treatment with drugs, stem cell therapies might also function to improve stroke outcome through promoting the neurovascular niche. These cells might integrate into areas of cell loss to repair circuitry and restore function, or release factors that support the neurovascular niche and subsequent brain repair [27]. For example, Deng *et al*. have shown that 24 hr post- rat MCA stroke administration of bone marrow-derived mesenchymal stem cells resulted in improved neurological function, reduced neuronal apoptosis and the promotion of neuronal proliferation *via* the release of VEGF [31]. Many other examples of cell-based stroke therapies exist [32] prompting the Stems Cell Therapies as an Emerging Paradigm in Stroke (STEPS) [27].

A third treatment method is to directly administer the growth factor or hormone. For example, Wang *et al*. have shown that treatment of rats 24 hours after embolic stroke with recombinant EPO significantly increased post-stroke angiogenesis, neurogenesis by increasing VEGF and BDNF, and improved functional stroke outcome [33]. Unfortunately, the results of a recently completed human multicenter efficacy study of recombinant human erythropoietin in acute ischemic stroke are unclear. However, the cumulative message of these studies is that promoting the neurovascular niche could be a viable stroke therapeutic paradigm.

In addition to the apparent difficulties in translating successful animal therapies to successful human stroke therapies, the above mentioned therapies present other problems. Pharmacologic based therapies have distinct disadvantages; drugs are artificial substances to the body subject to all of the problems associated with such foreign substances including side effects, tolerability, toxicities, etc.

EXTRACELLULAR MATRIX PROTEOLYSIS

In stroke, dying and infiltrating inflammatory cells release MMPs and cathepsins which disturb the blood brain barrier and proteolytically process the ECM [34]. The cerebral ECM consists of the proteins laminin-1, collagen type IV, fibronectin, and perlecan. Roughly 60% of these ECM proteins are lost in the ischemic core within 24 hrs post MCA occlusion [35]. The two major systems responsible for ECM proteolysis following brain injury is plasminogen activator (PA) and the MMPs. The expression, activity, and roles of these proteases are actively being investigated to better define their importance following brain injury. MMP and PA expression are up regulated following stoke and overproduction of MMPs can result into cell death and inflammation and inhibitors of the MMPs can reduce edema and infarction size [36]. The source of these proteases may be endothelial cells, astrocytes, neurons, and microglia [37]. The generation of these proteases can be induced by cleaved fragments from the ECM interacting with cell receptors known as integrins. This initiates a feedback loop whereby ECM fragments produce proteases which in turn can produce more ECM fragments.

Although the initial processing and degradation of ECM is largely thought of as a negative consequence of acute stroke, one additional consequence of matrix proteolysis is the generation of bioactive matrix fragments [38]. Indeed, many matrix components are known to harbor bioactive matrix fragments in their C-terminal regions that can inhibit angiogenesis outside of the central nervous system [39, 40], but their capability of affecting angiogenesis remains uncharacterized in the brain.

During brain development, fibronectin is the most abundant ECM component along with endothelial cell expression of its main receptor, the $\alpha_5\beta_1$ integrin [41]. Fibronectin's positive effect on cell survival and proliferation is mediated through the $\alpha_5\beta_1$ integrin [42]. Reports demonstrate that vasculogenesis (the denovo production of blood vessels) and angiogenesis depend primarily on fibronectin and the $\alpha_5\beta_1$ integrin [43]. Interestingly, although the developing CNS expresses high levels of fibronectin and $\alpha_5\beta_1$ integrin, this expression is down regulated following maturation of the CNS in favor of the $\alpha 6\beta 1$ integrin and laminin [44]. More recently, Milner *et al*. have demonstrated a pro-angiogenic "switch" following ischemia in which the vasculature reverts to a developmental, proangiogenic environment consisting of fibronectin and $\alpha_5\beta_1$ integrin [44].

Another important ECM component involved with neuronal development, cell proliferation and growth is perlecan. Perlecan, a heparan sulfate proteoglycan containing a multi-domain protein core and 3 glycosaminoglycan chains at its N-terminus, has been shown to play a role in both neurogenesis and angiogenesis. It affects neurogenesis in the developing telencephalon, can be found in newly formed blood vessels, and plays a major role in vascular response to injury *in vivo* [45-47]. Del Zoppo has investigated ECM sensitivity to proteolysis following ischemia and concluded that perlecan is the most sensitive (when compared to collagen or laminin) to proteolysis in the ischemic core two hours post middle cerebral artery occlusion. They also concluded that perlecan degradation is sensitive to MMP-9 and cathepsin-L, proteases that are increasingly active post-stroke [34]. More recently, Caihier *et al*. have demonstrated that the biologically active C-terminal portion of perlecan, domain V (DV), is cleaved when treated with cathepsin L [45]. Could this suggest that DV is generated post-stroke?

POSSIBLE INVOLVEMENT OF ECM FRAGMENTS IN BRAIN REPAIR

Currently, the generation and role of biologically active ECM fragments in ischemic stroke is poorly understood (see Fig. (**2**) for possible flow chart of post-stroke ECM

generation). Most of these fragments have been isolated from the ECM of tumor microenvironment and have been shown to inhibit angiogenesis processes. At least nine ECM derived inhibitors of angiogenesis have been reported [48]. Proteolysis of fibronectin can produce a fragment called anastellin (c-terminal) that has antimetastic activity [49], while collagen type IV proteolysis generates three anti angiogenic fragments, arresten canstatin and tumstatin, depending on the alpha chain composition [39]. Endostatin, the C-terminus of the heparan sulfate proteoglycan collagen type XVIII is also anti-angiogenic [48]. Importantly, these angiogenesis inhibitors have been characterized primarily, but not exclusively [3] outside of the central nervous system.

Arresten, derived from the C-terminal of the type IV collagen alpha1 chain, inhibits migration, tube formation of stimulated endothelial cells and the positive proliferative effect of basic fibroblast growth factor (bFGF) stimulated endothelial cells [39]. The mechanism in which arresten induces its angiogenic effect is likely due to interaction with the α1β1 integrin and subsequent blockade of MAPK signaling [50, 51] which has been shown to inhibit hypoxia inducible factor-1α (HIF-1α) an upstream transcription factor of VEGF [36]. A mutation in arresten has been linked to patients suffering from intracerebral hemorrhaging [52]. This gives evidence that arresten is present in the brain. Currently there is no evidence that the collagen type IV alpha2 or alpha3, parent molecules canstatin or tumstatin, respectively, are present in the brain. Endostatin has been shown by Tian *et al*. to be rapidly upregulated following ischemic stroke with unknown consequence [38]. This increase is maximal 2 hours post-stroke and gradually fades by 48 hours post-stroke, the last recorded time point. More recently, we have demonstrated that by post-stroke day 7 in rats, endostatin is undetectable (Lee *et al.*, submitted manuscript) suggesting that endostatin is created rapidly but transiently after stroke. Importantly, the release of endostatin following ischemia could have positive results. Endostatin has been documented to play a role in stabilizing cell-cell and cell-matrix adhesions this could potentially play an important role in stabilizing the BBB and decrease its permeability following ischemia [36].

TIMING AND LOCATION ARE EVERYTHING

As mentioned previously, post-stroke angiogenesis does not occur in force until post-stroke day 3-7. Why such a delay? Could this in fact be due to the acute generation of anti-angiogenic matrix fragments that might serve to stabilize the blood-brain barrier and acutely inhibit the angiogenic response? Indeed, the acute but transient generation of endostatin would seemingly support this argument. It also raises the question as to whether exogenous administration of endostatin during the acute stroke period might further promote blood-brain-barrier stability and thereby improve outcome. Unfortunately, this same endostatin, if given during the more chronic phase of stroke recovery, might hinder recovery. Indeed, the same time dependence would be true of any modulator of angiogenesis (see Lo *et al*. Nature Medicine, for an excellent review of this concept [53]). However, the angiomodulatory role of a particular ECM-derived matrix fragment should not be assumed based on a role characterized outside of the CNS, as endothelial cell heterogeneity, could account for endothelial cells in different vascular beds responding differently to angiomodulatory factors. This may be due to differences in respective microenvironments, differences in expressed receptors, or differences in signal transduction components. Examples of angiogenic differences between brain and nonbrain endothelial cells have been reported. Wnt/β-catenin signaling is required for CNS, but not non-CNS angiogenesis [54], platelet-derived pro-angiogenic sphingosine-1-phosphate (S1P) is anti-angiogenic in brain endothelial cells due to their lack of MT1-MMP expression [55]. Therefore, the activity of potential ECM-derived modulators of angiogenesis in the post-stroke brain is far from certain.

CONCLUDING REMARKS

Our understanding of the mechanisms underlying the brain's response to ischemic stroke is limited. Ultimately, angiogenesis and neurogenesis following ischemic stroke are key areas of research that may allow investigators a link from "bench to bedside". Currently research suggests that the post-stroke brain does start to repair itself and in the process may generate biologically active matrix fragments that influence this repair. The pathophysiological role of these ECM fragments is largely underexplored but is likely to yield an improved understanding of brain repair mechanisms and potentially new ischemic stroke therapies.

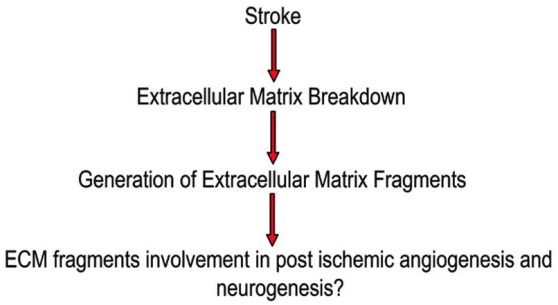

Fig. (2). Flow chart of post-stroke ECM fragment generation and speculative effect.

Following ischemic stroke, ECM fragments are produced and are in close proximity to cells within the neurovascular niche. These ECM fragments have the potential to play a key role in modulating how the brain self repair process is done by either inhibiting angiogenesis/neurogenesis acutely or increasing angiogenesis/neurogenesis chronically.

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