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Review

Angiogenesis and stem cell transplantation as potential treatments of cerebral ischemic stroke

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Abstract

Ischemic stroke is a leading cause of human death and disability. Although stroke survivors may gain spontaneous partial functional recovery, they often suffer from sensory-motor dysfunctions, behavioral/neurological alterations, and various degrees of paralysis. Currently, limited clinical intervention is available to prevent ischemic damage and restore lost function in stroke victims. In addition to the extensive research on protective maneuvers against ischemia-induced cell death, increasing attention has been focused on potential strategies of promoting tissue repair and functional recovery in the damaged post-ischemic brain. Angiogenesis, or the growth of new blood vessels, may contribute to cell survival and functional recovery of the area of insult. The study of angiogenesis will increase the understanding of the mechanism underlying post-ischemia neurovascular plasticity and regeneration. Additionally, stem cell transplantation has emerged in the last few years as a potential therapy for ischemic stroke, because of their capability to differentiate into multiple cell types and the possibility that they may provide trophic support for cell survival, tissue repair, and functional recovery.
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Keywords: Angiogenesis; Ischemic stroke; Stem cell transplantation; Apoptosis

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1. Introduction

In the past decade, numerous attempts focusing on neuroprotective strategies have been made to rescue neurons in the ischemic brain. It has been recognized through recent clinical trials that preventing the acute injurious events, that often become irreversible within a few hours of ischemia onset, is a difficult, if not impossible, task in the clinical setting. It appears rational that future investigations should consider more clinically feasible alternatives or additional approaches that repair the damaged brain and promote functional restoration during chronic phases of the disease.

After stroke, the restoration of local blood flow reverses the ischemic environment and is essential for long-term recovery. Recently, transplantation of embryonic and adult stem cells has provided new hope that survival and differentiation of these cells in the host brain may provide a method for tissue repair after ischemic stroke. It is proposed that enhancement of angiogenesis in the penumbra region of the brain may provide an improved environment for regeneration and enhance survival of both exogenous transplanted stem cells as well as the endogenous cells that are involved in repair and recovery.

2. Necrotic, apoptotic, and hybrid cell death in the ischemic brain

Apoptosis occurs during development and in many disease states [1,2]. In addition to the excitotoxic necrotic cell death, apoptosis has been identified as an important mechanism leading to brain damage after ischemic stroke [3–6]. Apoptosis, as a programmed cell death, is controlled by multiple pathways mediated by caspases [7–9] and controlled by genes in the *bcl-2* family [10]. It has also been shown that there is a close relationship between apoptosis and necrosis following cerebral ischemia, recently coined ‘hybrid cell death’ [11]. Our recent data demonstrated that the hybrid death, neither typical necrosis nor typical apoptosis, occurs in the penumbra region and non-ischemic thalamus after cerebral ischemia, represented by unique biochemical and morphological characteristics (Figs. 1 and 2) [12].

The ischemic brain is an unfavorable milieu for transplanted stem cells to survive in view of the robust activation of death signaling cascades. Transplanted cells may die in large numbers. For instance, 70–90% of implanted dopaminergic neurons in the striatum die after transplantation [13,14]. This death after transplantation may present an additional burden to the post-ischemic brain already compromised by a cellular debris load [15,16]. Because immature cells are particularly vulnerable to apoptosis [17–20], transplanted stem cells may likely die from apoptosis in the post-ischemic environment. Thus, anti-apoptotic strategies should be explored to promote the survival of transplanted cells. Consistently, overexpression of Bcl-2 reduces neuronal apoptosis in vitro and in vivo [21–25].

In addition to anti-apoptotic properties, Bcl-2 has been implicated in promoting neural differentiation and maturation [26,27] and in the induction and maintenance of axonal growth [28–31]. Bcl-2 levels decline with aging in most CNS neurons, but remain elevated in peripheral neurons which maintain the ability to regenerate axons [31]. Bcl-2 may be involved in selective survival of neurons during the critical period of innervation in the developing CNS [21]. High levels of Bcl-2 expression also correspond to the entire phase of axonal elongation [32]. Vector induction of human *bcl-2* leads to robust neurite formations when compared with a control vector [33]. The ability of Bcl-2 to promote axonal extension is probably independent of its anti-apoptotic properties. In 6-OHDA lesioned rats, Bcl-2 overexpression in graft neurons did not change cell survival but significantly improved fiber outgrowth [34]. These data indicate that *bcl-2* gene overexpression may be important in the maturation of transplanted stem cells into functional neurons.

3. Angiogenesis and vascular remodeling after focal cerebral ischemia

Vascular growth and remodeling are needed in the development and maturation of organs during early life. Active formation of new vessels is also an integral component in carrying out reproductive and reparative functions in the adult organism [35]. The growth of new blood vessels from the pre-existing vascular tree, also known as angiogenesis, occurs in situations, such as wound healing, arthritis, cardiovascular diseases, cancer, and cerebral ischemia. We and others reported that brain capillary endothelial proliferation can be shown by BrdU incorporation after experimental strokes (Figs. 3 and 4) [36–39]. Another marker of blood vessel formation is the expression of the integrin, $\alpha_v\beta_3$, which is essential for angiogenesis (Fig. 4) [38,40]. We showed that 30 days after cortex ischemia, the diameters and lengths of surface collaterals in the ischemic border grow significantly, and arteriocapillary latencies in the region are shortened compared with those just after arterial occlusion (Fig. 3). These features of angiogenesis are confined to the ischemic border [38,41].

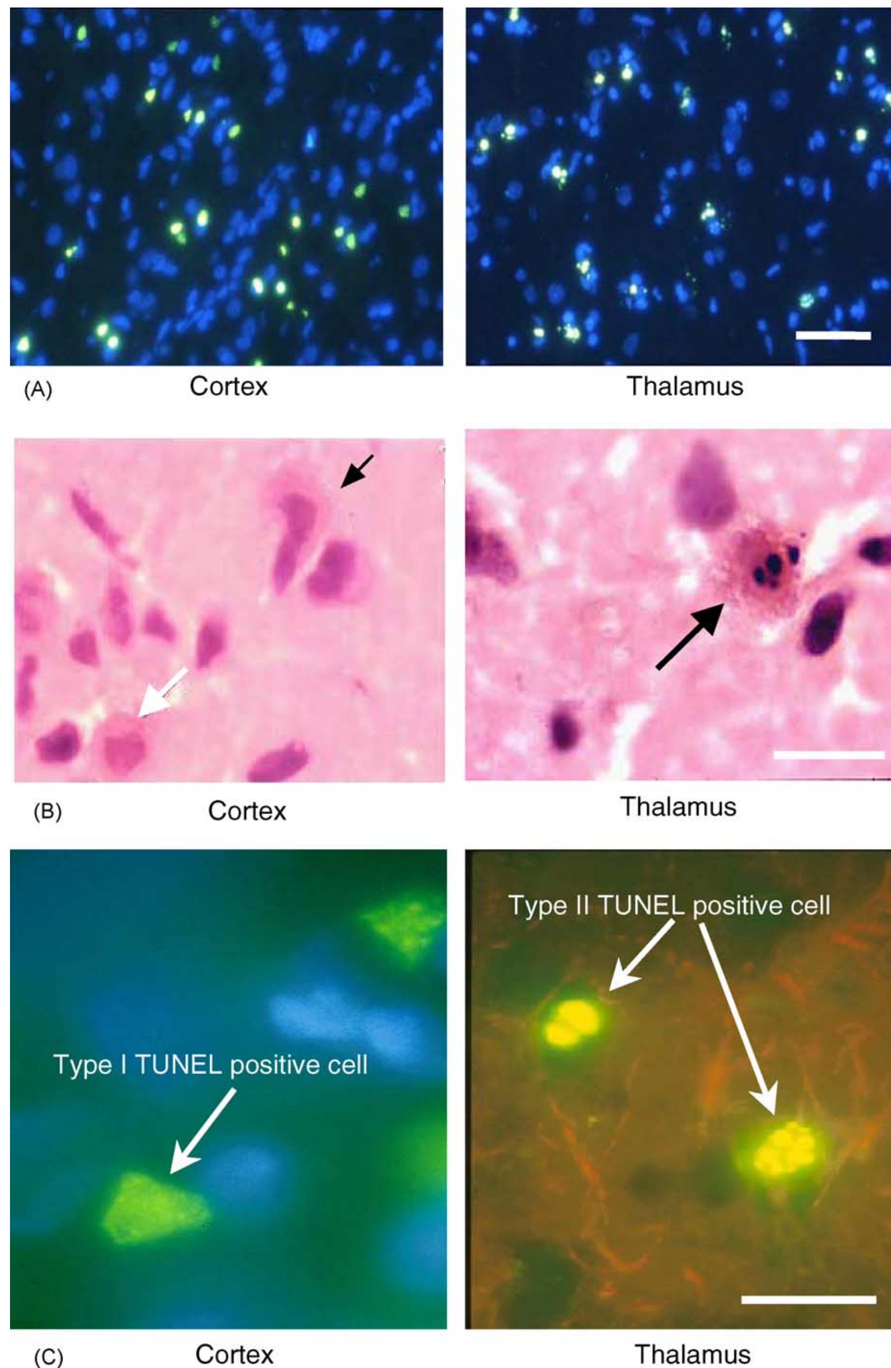
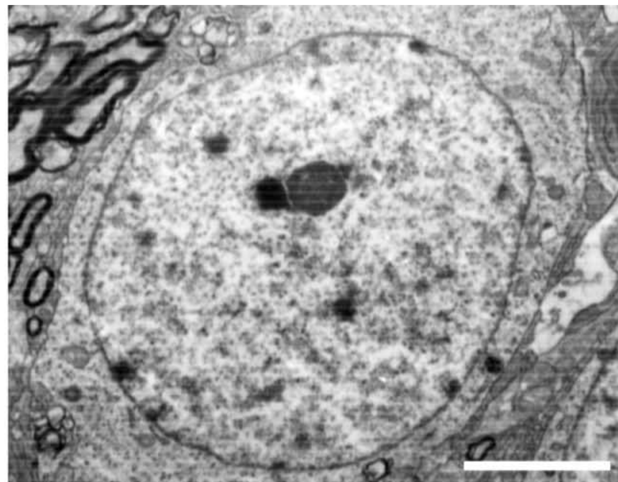
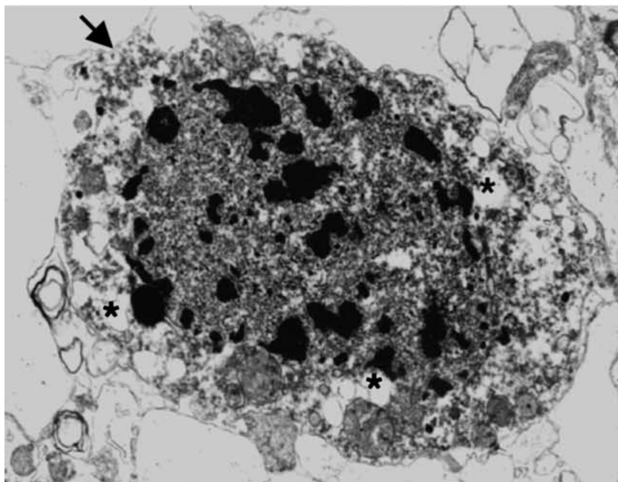


Fig. 1. TUNEL positive cells of different morphology in the barrel cortex and thalamus. (A) TUNEL positive cells (green) 24 h after ischemia in the ischemic barrel cortex and the non-ischemic thalamus; most cells in the cortex still have intact nuclei (named as Type I TUNEL positive cells). On the other hand, TUNEL positive cells in the thalamus showed nuclear condensation and fragmentation (named as Type II TUNEL positive cells). The Hoechst staining (blue) labeled all cells. (B) H&E staining of damaged cortex and thalamus cells 10 days after the cortex ischemia, showing different morphology of nuclei in these cells (arrows point to typical cells in the cortex and thalamus). (C) High magnification of TUNEL positive cells in the ischemic cortex and non-ischemic thalamus 10 days after ischemia. In striking contrast to Type I cell in the cortex, Type II TUNEL positive cells in the thalamus showed distinct feature of nuclear condensation and fragmentation. Bar = 100 μm in A, 20 μm in B and C. From Fig. 4 in [12].

New capillaries in the parenchyma and on the brain surface may be arterialized by the addition of albuminal smooth muscle in response to the increased luminal pressure perfusion [42]. In contrast to cancer therapy, which is benefited by suppressing angiogenesis, interventions that facilitate new vessel growth may be a practical approach to improve blood flow to ischemic organs, such as the heart or brain [38]. Extensive studies in cardiovascular ischemia have shown the therapeutic potential of angiogenesis in the restoration of local blood flow and functional recovery of the heart [43]. The rela-





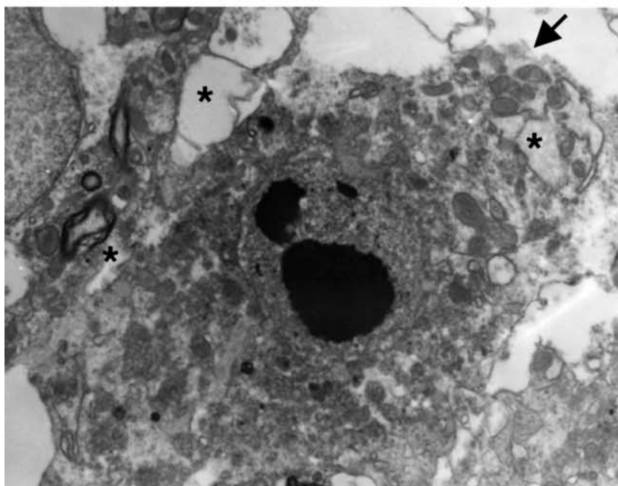


Fig. 2. Electron microscopy of cell injury in the cortex and thalamus. Electron micrographs show ultrastructural alterations in individual neurons in the ischemic barrel cortex and ipsilateral thalamus. (A) The control cortical neuron had a large nucleus, relatively small cytoplasm volume, and intact mitochondria and other organelles. (B) Primarily necrotic alterations appeared in a damaged cortical neuron 3 days after ischemia, featured by swollen cytoplasm, disrupted organelles, formation of vacuoles (*), and deteriorated membranes (arrow). Note that no nuclear condensation took place. (C) The injured thalamic neuron 7 days after the cortex ischemia showed both apoptotic and necrotic features; there was apoptotic nuclear shrinkage, chromatin condensation, and necrotic chaotic injuries in the cytoplasm including volume increase, formation of vacuoles (*), swollen organelles and deteriorated membranes (arrow). Bar = 5 μm. From Fig. 5 in [12].

tionship between angiogenesis and brain function after focal cerebral ischemia has not been studied to the extent seen in cardiovascular research, but available evidence implicates an important role in the brain [38,44,45].

4. Vascular growth factors

A large number of factors have been noted to promote angiogenesis during development and under various pathological conditions. Only a few of these factors have been studied in the brain after ischemia. Prominent among these angiogenic factors are basic fibroblast growth factor (bFGF) [46,47] and vascular endothelial growth factor (VEGF) [48], which play crucial roles in angiogenesis under pathological conditions. We will review select vascular growth factors that may be of relevance in maintaining neuronal viability, restoring function after stroke, or having important roles in angiogenesis.

4.1. Basic fibroblast growth factor

Ischemic brain injury, resulting in low tissue oxygen tension in the brain, often leads to neovascularization to meet metabolic demand [49]. In one study, the extent of angiogenesis was correlated with survival in stroke patients [44]. Altered levels of several polypeptide growth factors known to have angiogenic activity, including bFGF, VEGF, angiopoietin-1 (Angpo-1), and angiopoietin-2 (Angpo-2), have been shown to be involved in repair and maintenance of residual viable tissue after cerebral ischemia. Basic FGF, the most extensively studied of the factors, is a biologically active polypeptide with mitogenic, angiogenic, and neurotrophic properties. Two forms of FGF, acidic and basic, are widely distributed throughout the immature and adult CNS. Both molecules share a sequence homology of about 55% and their activities seem to be mediated through the same receptor, localized in the CNS. Basic FGF protects against hypoxia-ischemic insult in vitro [50] and in vivo [51] and enhances recovery of rat behavior following traumatic brain injury [52]. As such, the role of bFGF may be a restorative neural response to ischemic brain injury. Using a three-vessel cerebral ischemia-reperfusion model to induce ischemia confined to the cortex in the right middle cerebral artery (MCA) territory, Lin et al. [53] showed an increased expression of both bFGF mRNA and bFGF protein, corresponding to a robust angiogenic process in the ischemic right cortex [54].

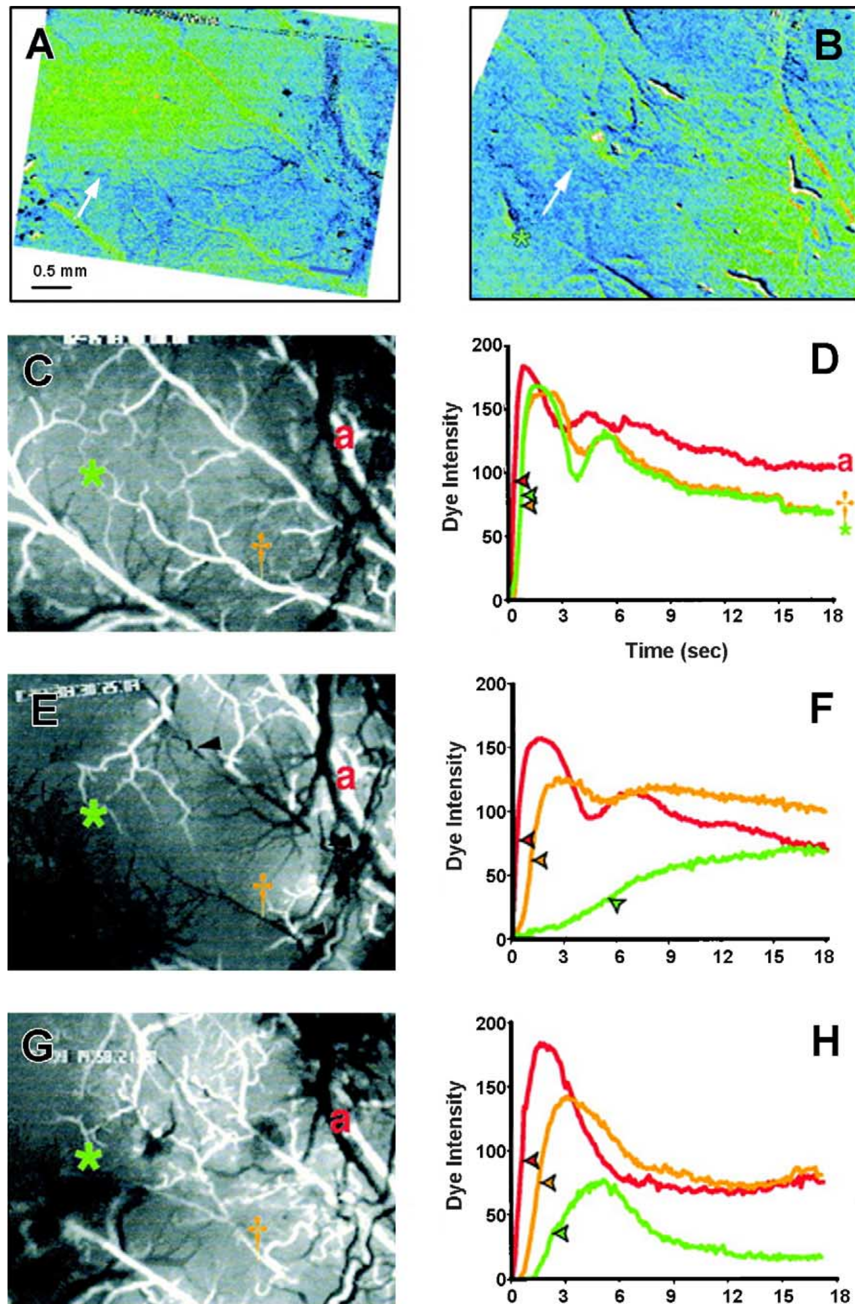


Fig. 3. Angiogenesis after focal ischemia in the whisker–barrel cortex. The whisker–barrel cortex ischemic stroke in rats was induced by selective occlusion to branches of the MCA [191]. (A) Before ischemia, optical signals (IOS) upon stimulating large whiskers on the left face. Activated area represents larger whiskers (arrow, warm colors; midline is up and rostral to the right). Bar = 0.5 mm (applies to B, C, E, and G). (B) IOS 30 days later. The area activated by stimulation of the large whiskers was smaller and rostral to that in panel A (arrow). There is no activation of infarcted cortex (blue; compare with panel G). (C) Arterial phase after FITC injection before ligations. Sites for the arterial time courses (a) and parenchymal fields in the central (*) or the peripheral (†) ischemic border. (D) Time courses of transits after FITC at the three locations. Arrowheads indicate half-maximal dye intensity values that were reached at short latencies after the dye first appeared in the arteriole (a) (<0.5 s). FITC recirculates after 4 s in the artery and the parenchyma. (E) Transit 3 min after ligations (arrowheads) were placed. Collaterals provide delayed, less robust blood flow to the central ischemic border. (F) FITC transit in the central ischemic border, which was considerably delayed and diminished acutely after ligations compared with that at the peripheral ischemic border, which was significantly slowed (compare with panel D). (G) FITC transit 30 days later. Collaterals have increased in diameter, are more tortuous, and have more prominent branches. The infarct is at the lower left. (H) FITC transit after 30 days, which was faster in the central ischemic border but delayed compared with that in the peripheral ischemic border. Transits were still slower than immediately before the ligations (D). From Fig. 2 in [38].

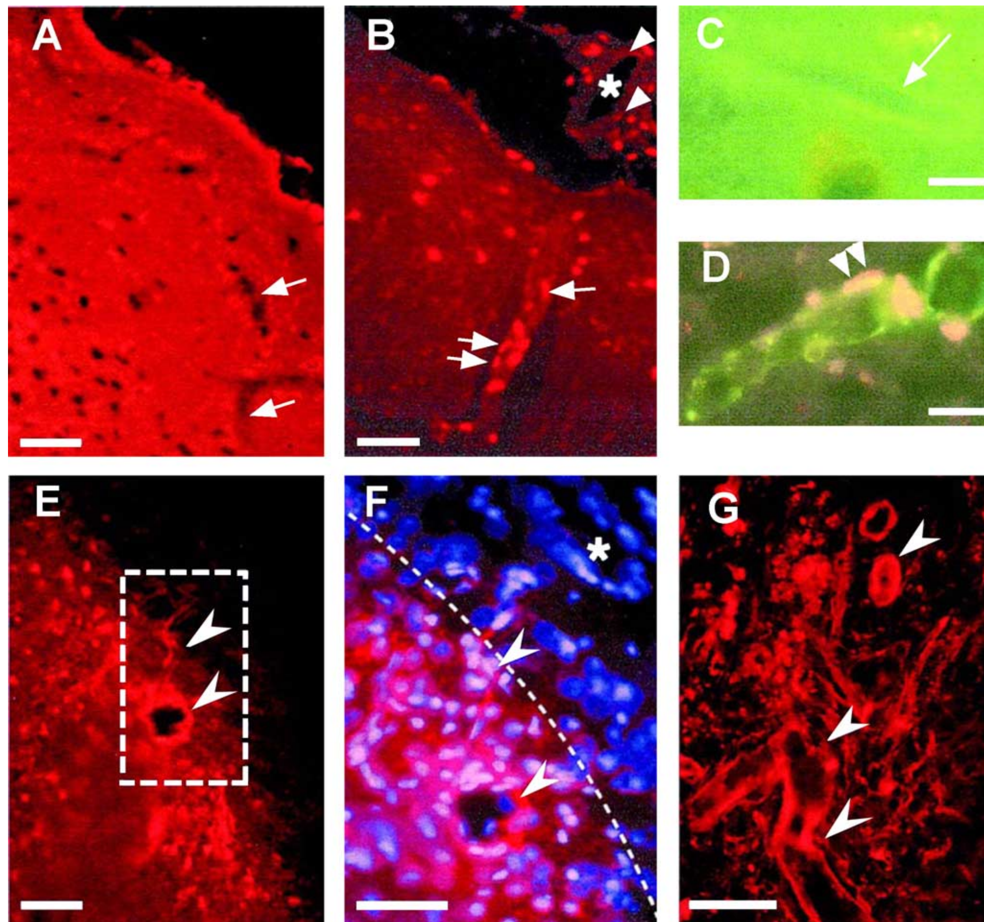


Fig. 4. Angiogenesis after whisker–barrel cortex ischemia. The whisker–barrel cortex stroke in rats was induced by MCA branch ligations [191]. Angiogenesis was indicated by specific labeling for DNA synthesis (BrdU), integrins ($\alpha_v\beta_3$), and capillary endothelium (GLUT-1). (A) At 5 days after a cranial window without arteriolar ligations (sham-operated control), no microvessels (arrows) or surface vessels in the cortex were labeled with BrdU. (B) BrdU-labeled nuclei in the ischemic border 5 days after ligations are shown. A surface arteriole at the upper right (*) shows labeled nuclei in presumed perivascular smooth muscle (arrowheads). A radial intraparenchymal vessel below it has several labeled nuclei (arrows) that are likely endothelial. Other labeled nuclei are not associated with vessels. (C) Endothelium of an intraparenchymal microvessel in a sham-operated control animal is labeled lightly for GLUT-1 (green). (D) Double labeling of BrdU (yellow) and GLUT-1 demonstrates endothelial proliferation in an intraparenchymal capillary in the ischemic border. (E) Antibody staining for $\alpha_v\beta_3$ shows a sharp division between the ischemic border (lower left) and normal unstained cortex (upper right) 7 days after ligation. A large and a small vessel are labeled (arrowheads). (F) Higher power image of the field outlined in panel E shows association of $\alpha_v\beta_3$ with vessels in the ischemic border (bis-benzimide counterstain). Above the dashed line, there is no vascular staining for $\alpha_v\beta_3$ in the normal cortex (*). (G) $\alpha_v\beta_3$ in the ischemic border 10 days after arteriolar ligation outlines vessels (arrowheads). Bars = 100 μm (A, B, and E), 10 μm (C), and 50 μm (F and G). From Fig. 3 in [38].

4.2. Vascular endothelial growth factor

VEGF, also known as vascular permeability factor, is a dimeric glycoprotein that is mitogenic for endothelial cells and has the potential to increase vascular permeability. By alternative splicing, four different isoforms exist in vivo: VEGF206, VEGF189, VEGF165 and VEGF121. The polypeptides VEGF206 and VEGF189 bind well to heparin-containing proteoglycans in the extracellular matrix; however, VEGF165 and VEGF121 do not bind to proteoglycans, and thus act as diffusible factors. VEGF has been shown in our investigations to promote angiogenesis and functional recovery after focal ischemia in the whisker–barrel cortex in mice [55]. Since VEGF may also increase vascular permeability, it may be harmful in cases of ischemic stroke by potentiating brain edema. In this regard, the timing for VEGF administration can be critical for minimizing the edema.

Using simple transient occlusion of the right MCA, Hayashi et al. [56] demonstrated that VEGF mRNA was induced after reperfusion. Two bands of 38 and 45 KDa proteins corresponding to VEGF121 and VEGF165 were detected in the ischemic region. Using the three-vessel MCA stroke model in rats, we have also reported an increase in VEGF mRNA expression, correlating with a post-ischemic angiogenic process [55]. It is possible that VEGF expression may contribute to the increased vascular permeability and resultant edema formation shortly after the ischemic insult as well as the angiogenesis that occurs during the first few days after stroke. Further study is needed to address these issues.

Recent findings have indicated that VEGF also has a direct effect on neural cells and may be involved in neuroprotection as well as angiogenesis. Sun et al. [57] showed that intracerebroventricular VEGF administration 1 day after reperfusion reduces infarct size, improves neurological performance, enhances the survival of newborn neurons in the dentate gyrus and SVZ, and stimulates angiogenesis.

4.3. Angiopoietin-1/angiopoietin-2

In addition to bFGF and VEGF, Angpo-1 and Angpo-2 are also upregulated during angiogenesis. Angpo-1 is the ligand for the Tie-2 receptor on the endothelial cell surface [58]. Angpo-1 contains 498 amino acids, including an N-terminal secretory signal sequence. Mice lacking Angpo-1 display a characteristic vascular defect that is distinguished from that caused by VEGF gene deletion, but they are reminiscent of Tie-2 gene knockout mice [59]. They appear grossly abnormal by embryonic day 11 and die by embryonic day 12.5. Even though the total number of endothelial cells seems to be unaffected, the number of large vessels and their caliber are reduced with fewer and straighter branches than in wild type animals. Angpo-2 is comprised of 496 amino acids with a N-terminal signal sequence and has 60% sequence homology to Angpo-1.

In contrast to Angpo-1, which stimulates phosphorylation of Tie-2 receptors in endothelial cells, Angpo-2 fails to generate the same response. In fact, Angpo-2 blocks the binding of Angpo-1 to Tie-2 receptors, suggesting that a biological role for Angpo-2 may be to antagonize Angpo-1 in its activation of the Tie-2 receptor. Supporting this idea, transgenic embryos overexpressing Angpo-2 die on embryonic day 9.5–10.5, and the defect in these embryos was similar to that found in mice lacking either Angpo-1 or Tie-2 receptors.

An increased expression of Angpo-1 and -2 mRNA in the ischemic right MCA cortex was noted after focal cerebral ischemia-reperfusion, correlating with a robust angiogenic process in the three-vessel ischemic model [60]. During embryogenesis, it has been shown that Angpo-2 may work in concert with VEGF at the front of invading vascular sprouts by blocking the action of constitutively expressed Angpo-1, allowing vessels to remain in a more plastic state in response to the sprouting signal provided by VEGF [61]. The VEGF mRNA induction profile is very similar to that of Angpo-2 mRNA and parallels the evolution of vascular density in the ischemic right MCA cortex [60]. In the absence of VEGF, Angpo-2 inhibition of constitutive Angpo-1 signal may contribute to vessel regression. This intimate interaction between Angpo-2 and VEGF after ischemic insult is similar to that observed during development.

Although Angpo-2 is associated with vessel sprouting, Angpo-1 stabilizes the vasculature. The transient increase in Angpo-1 mRNA expression and depression of Angpo-2 mRNA levels during the early phase of ischemia may reflect an initial attempt of the ischemic region to stabilize vascular integrity. This trend toward Angpo-1 dominance is reversed later by a decrease in Angpo-1 mRNA levels and an increase in Angpo-2 expression, peaking at 24 h, in parallel with VEGF expression. The expression of Angpo-2 and VEGF during the same period may provide a drive for sprouting angiogenesis. Assuming the relative dominance of Angpo-1 and Angpo-2 represents vessel stabilization versus angiogenic sprouting, respectively, the changing Angpo-1/Angpo-2 ratios may reflect the evolution of vascular hemodynamic changes (taking into account a lag in mRNA translation). The Angpo-1/Angpo-2 mRNA ratio may be a useful index for vascular remodeling activity. A decreasing Angpo-1/Angpo-2 mRNA ratio may reflect sprouting capillary remodeling activity or low vascular remodeling activity, whereas a higher Angpo-1/Angpo-2 mRNA ratio is crucial for remodeling into large vessels.

4.4. Tie-1/Tie-2 receptors

Tie-1 and Tie-2 are receptor tyrosine kinases (RTKs) that are expressed exclusively in endothelial cells [62]. The restricted expression in endothelial cells suggests that the signaling pathways mediated by these RTKs play key roles in vascular function. Both Tie-1 and Tie-2 receptors are present in quiescent adult endothelial cells [63,64]. Tie-2 receptors are also present in angiogenic endothelial cells in adult rats [65,66]. The temporal expression of mRNA for Tie-1, Tie-2, as well as Tie-2 ligands Angpo-1 and Angpo-2 was studied following focal cerebral ischemia in rats [54]. The expression of Tie-1 and Tie-2 mRNA increased starting 24 h after reperfusion and remained elevated for up to 2 weeks after ischemia. The temporal profiles of the expression of these Tie/Angiopoetin genes were different from those of other angiogenic genes, such as VEGF and bFGF. Both Tie-1 and -2 expression exhibited a biphasic pattern, corresponding to the evolution of angiogenesis after focal cerebral ischemia [67]. Cellular colocalization experiments revealed that Tie-2 is expressed in proximity to its antagonist, Angpo-2, as well as bFGF and VEGF in cortical layer 1, where active vessel remodeling was noted. Tie-1 protein, to a lesser extent, also colocalized with Angpo-2, bFGF, and VEGF in cortical layer 1. These findings are interesting in view of observations that Tie-1 is more prominent in promoting angiogenic capillary growth and Tie-2 is involved in large vessel remodeling, maintenance of vascular structure, vasculogenesis, and nonsprouting angiogenesis [59,68]. This may explain, at least in part, why Tie-2 was expressed in cortical layer 6b, where vasculogenesis occurred, and both Tie-1 and Tie-2 are expressed in cortical layer 1, where arteriogenesis (vessels grow both in length and width) and sprouting angiogenesis were most robust.

Judging from the fact that numerous angiogenic growth factors can induce robust neovascularization after focal cerebral ischemia-reperfusion, it will be imperative to explore the individual signal transduction mechanisms involving various growth factors in angiogenesis after ischemia. In Section 5, we will present a cascade driven by tumor necrosis factor-

alpha (TNF-α), a pro-inflammatory cytokine expressed in the ischemic cortex.

5. The TNF-α cascade in angiogenesis

5.1. TNF-α and its receptors in ischemic brain injury and recovery

The inflammatory cytokine TNF-α activates the TNF receptor superfamily that contains a large number (>20) of structurally related proteins activated by different ligands [67]. Two distinct receptors, p55 (TNFR1) and p75 (TNFR2), are expressed in most cell types, including neurons and glial cells, and mediate diverse activities [69–73]. The TNFR1 is linked to signal transduction pathways, which activate either the transcription factor nuclear factor-kappa B (NF-κB) [74,75] or the caspase cascade [76]. The transduction pathway of TNFR2 interacts directly with TNF-α-associated factor 2 which then complexes with TRAF1; TRAF2-dependent signals also activate NF-κB [75].

5.2. Injury-induced TNF-α elevation and its dual effects on cell survival and nervous system injury

Ischemic and other insults can induce increases in TNF-α levels in the human brain [77–80], in plasma, and cerebrospinal fluid [81–83]. Acute increases (1–6 h) in TNF-α protein and mRNA expression are observed in brain regions after experimental brain injury in the rat [84–88]. After permanent MCA occlusion, elevations of TNF-α mRNA may persist for 5 days, suggesting lasting effects of this cytokine [89].

Many studies suggest that TNF-α is a mediator of tissue damage and that its inhibition confers protection [78,86,87,90]. Supporting this contention, exogenous TNF-α exacerbates focal ischemic injury in a dose-dependent manner [91] and neutralization of endogenous TNF-α by means of a soluble TNF-α receptor or TNF-α antibody can decrease cell death [92–95]. Consistently, TNF-α may lead either to necrotic or apoptotic cell death [96–99].

Paradoxically, increasing evidence suggests that TNF-α can also prevent cell death in vitro [73,100–103] and in vivo after administration of excitotoxins [104,105], peripheral nerve injury [106] and cerebral ischemia [103,107]. TNF-α has also been associated with the regulation of tissue remodeling, gliosis, and scar formation [108–110]. Acute production of TNF-α in conditions of tissue "stress" may also represent an important regulatory mechanism promoting tissue defense and repair [78,111]. In contrast to its proinflammatory effects, a recent study showed that TNF-α might also possess some anti-inflammatory effects in autoimmune-mediated demyelination [112].

Direct evidence for the neuroprotective effect of TNF-α comes from mice lacking TNFR1 or TNFR2. Enhanced injury observed in TNFR1 deficient but not in TNFR2 deficient mice following cerebral ischemia suggests that TNFR1 receptor signal transduction confers a neuroprotective effect [104]. Neuroprotective effects of TNFR1 and injurious actions of TNFR2 have also been reported in retinal ischemia [113]. Recently, enhanced caspase activation, apoptotic death, and spinal cord lesion size were shown in mice lacking TNFR1 and correlated to a reduction in NF-κB activation [114].

Scherbel et al. [115] showed that significant motor deficits in brain-injured TNF-$\alpha^{-/-}$ mice persisted for up to 4 weeks, and trauma-induced cortical cell loss was markedly exacerbated at both 2 and 4 weeks in the TNF-$\alpha^{-/-}$ mice, while wild-type mice recovered to pre-injury levels by 2–3 weeks post-injury.

How can one reconcile the experimental data supporting a neuroprotective effect of TNF-α with those supporting a neurotoxic role for the same cytokine? One possibility is that low concentrations of TNF-α may be trophic or protective, but higher concentrations may potentiate neuronal injury. This idea is supported by in vitro and in vivo evidence that excessive production of TNF-α may induce the proinflammatory and cytotoxic effects, while low concentrations of this cytokine may be neuroprotective [116]. The enhanced ischemic injury in TNFR1 deficient mice also suggests that basal activity of this cytokine receptor is essential for brain cell survival. Alternatively, the timing of TNF-α production may be an important factor related to its neuroprotective effects. The prolonged presence of unbound TNF-α may also induce pathologic cellular changes in a receptor-independent fashion [117,118]. Finally, the receptor with which TNF-α interacts may play a role in its divergent actions.

5.3. Differential roles of TNFR1 and TNFR2 in cell injury

TNFR1 and TNFR2 share some homology in their extracellular regions; however, no homology is seen in the intracellular regions of the proteins [119], suggesting that the two receptors may activate distinct signaling pathways. In cultured neurons, a neuroprotective effect of TNF-α against NMDA toxicity was mediated by TNFR1 but not TNFR2 [120]. In mice lacking TNFR1 ($p55^{-/-}$) there is an ischemia enhanced tissue injury compared to wild-type mice; this was not seen in TNFR2 deficient ($p75^{-/-}$) mice [95,104]. However, TNFα had little effect on hippocampal neurons in $p55^{-/-}$ mice, whereas neurons from $p75^{-/-}$ mice are vulnerable to TNFα, suggesting that TNFR1 mediated an injurious effect in this paradigm [73].

The role of TNFR2 in cell death has remained controversial, and its activity has been proposed to be independent of signal transduction. TNFR2 may regulate the rate of TNF-α association with TNFR1 [121]. In PC60 cells, the presence of both receptor types is required to induce apoptosis following either specific TNFR1 or TNFR2 triggering, pointing to a mechanism of receptor cooperation [122,123]. In human neuronal SH-SY5Y cells, although TNFR2 may not be re-

quired for normal cell viability, it may play a protective role following injury [124]. Collectively, TNFR1 is often associated with protective effects while the role of TNFR2 remains obscure.

5.4. TNF-α, angiogenic growth factors, and the angiopoietin/Tie-2 system in angiogenesis

TNF-α modulates the expression of several growth factors, such as VEGF and bFGF [125]. Messenger RNA levels of VEGF, bFGF, interleukin-8 (IL-8), and their receptors increased after human microvascular endothelial cells (ECs) were exposed to TNF-α [66]. Inhibition of VEGF production and several other growth factors blocked TNF-α induced angiogenesis. Administration of NF-κB antisense oligonucleotides almost completely inhibited TNF-α-dependent IL-8 production and partially abrogated TNF-α-dependent VEGF production; an anti-IL-8 or anti-VEGF antibody also blocked TNF-α-induced neovascularization in the rabbit cornea in vivo. Thus, TNF-α-induced angiogenesis appears to be modulated through angiogenic factors, such as VEGF [66].

In vitro, TNF-α inhibits EC proliferation, whereas in vivo it often stimulates blood vessel growth [126,127]. Furthermore, high doses of mouse recombinant TNF-α inhibit angiogenesis, whereas low doses of TNF-α induce angiogenesis [126]. After cerebral ischemic insults, TNF-α-induced and bFGF-induced angiogenesis can be beneficial for subsequent neurovascular remodeling [66]. TNF gene knockout mice demonstrate that TNF-α may modulate arteriogenensis via the TNFR1 [128]. Studies in our lab indicate that after focal cerebral ischemia there is reduced angiogenesis of the penumbra region in $p55^{-/-}$ mice compared to wild type mice as seen by EC BrdU incorporation [129]. We have also seen that $p55^{-/-}$ mice are unable to upregulate expression of the angiogenic factors VEGF, bFGF, and Angpo-2 after ischemic insult in comparison to the wild type mice. Not only do $p55^{-/-}$ animals suffer increased injury after focal cerebral ischemia, but they also appear to be unable to repair or remodel their vasculature when compared to wild type animals. Our results support the idea that TNF-α signal transduction through TNFR1 is essential in microvascular plasticity.

Angiopoietins, ligands of the tyrosine kinase receptor Tie-2 [61,130], have been identified and shown to have an important role in angiogenesis and vascular formation [131]. Exposure to hypoxic conditions (1% O_2) led to a significant time-dependent rise in Tie-2 protein levels in human microvascular ECs (1.7–3.2-fold increase within 24 h) in a time-dependent and dose-dependent fashion [66]. Both Angpo-1 and Angpo-2 enhanced VEGF-induced neovascularization in mice, indicating that angiopoietins may potentiate the effects of other angiogenic cytokines [132]. VEGF and/or hypoxia can upregulate Angpo-2 in bovine microvascular ECs [133] and human endometrial ECs [134]. TNF-α has been shown to increase Tie-2 protein levels (one- to three-fold) in ECs [66], and Angpo-2 mRNA and protein levels in human umbilical vein ECs [135], implicating a link between TNF-α and the angiopoietin/Tie-2 receptor system. The functional relationship between TNF-α and angiopoietin/Tie-2 expression and their role in post-ischemic angiogenesis warrants further investigation.

5.5. NF-κB as a mediator in the TNF-α signaling pathway

In parallel with the up-regulation of TNF-α, an increase in NF-κB activity in brain tissue has been described following focal or global ischemia in rodents [136–138]. In addition, rapid and delayed (on the order of days) increases in NF-κB activation have been described following focal ischemia-reperfusion [139] and kainic acid injection [140]. Indeed, TNF-α exerts its neuroprotective action via activation of NF-κB [141,142]. Binding of TNF-α to TNFR1 induces activation of NF-κB, which prevents apoptosis in various cell cultures and in vivo models [100,101,104]. The anti-apoptotic action of TNF-α can be reproduced by treatment with IκB antisense oligonucleotides, which stimulates NF-κB activation [101]. Treatment of neurons with NF-κB decoy DNA, which selectively blocks NF-κB activity, abolished the cytoprotective effect of TNF-α [142]. NF-κB decoy DNA also increased kainite-induced neuronal death within the CA1 and CA3 regions of the hippocampus [73].

Further evidence demonstrated that expression of a non-functional mutant form of IκB in cultured hippocampal neurons increased their vulnerability to hypoxia-induced cell death [143]. Studies using an NF-κB peptide inhibitor introduced into sympathetic neurons provided evidence that NF-κB prevents apoptosis of these cells [143]. NF-κB may also play a role in the anti-apoptotic actions of Bcl-2 [143]. TNF-α may increase Bcl-2 and Bcl-X_L levels in cultured hippocampal neurons and the increases can be blocked by expression of a dominant-negative IκB [143]. Alternatively, levels of NF-κB activity were increased in cultured myocytes that overexpress Bcl-2 [144]. Activation of NF-κB may also indirectly protect neurons by inducing the expression of neurotrophic factors and cytokines. In addition, NF-κB may modulate the expression of proteins involved in the regulation of cellular Ca^{2+} homeostasis [100,102].

In mice lacking TNF-α receptors, NF-κB and MnSOD expression was reduced following traumatic brain injury [145] and traumatic spinal cord injury [114]. Mice lacking the p50 subunit of NF-κB exhibited increased injury to hippocampal pyramidal neurons and attenuated increases in levels of TNF-α and MnSOD following kainite administration. Cultured cells from these mice exhibited increased elevations of $[Ca^{2+}]_i$ following exposure to glutamate and were more vulnerable to excitotoxicity than were neurons from wild-type mice [73]. Infusion of a proteosome inhibitor of NF-κB induced DNA fragmentation in several brain regions [146]. NF-κB may have an anti-apoptotic role in non-neuronal cells. Agents that prevent activation of NF-κB such as microinjection of IκBα, an inhibitory subunit, prevent apoptosis [73]. Collectively, these and other data suggest an extensive anti-

apoptotic role for TNF-α and NF-κB in neurons and other cells.

5.6. PI_3/Akt pathway

Increasing evidence suggests that the downstream PI_3/Akt pathway may play a central role in TNF-α-mediated signaling. Akt, also referred to as protein kinase B, is well documented in multiple cell systems to be a critical anti-apoptotic factor in controlling the balance between survival and apoptosis [147,148]. Activated by a number of growth factors and cytokines, Akt is a critical regulator of PI3 kinase-mediated survival, by inhibiting apoptosis through the phosphorylation and inactivation of several pro-apoptotic targets including Bad, forkhead transcription factor, and caspase 9 [149–155]. Although the antiapoptotic activity of Akt is well established in vascular endothelial cells, it also serves as a multifunctional protein kinase to regulate other aspects of cellular function, including cell migration, glucose metabolism, protein synthesis, and angiogenesis [156]. Recent evidence suggests that VEGF-mediated cell survival in endothelial cells is modulated by the VEGFR2-PI3/Akt pathway [148]. In addition to its anti-apoptotic effects, VEGF stimulates Akt-mediated eNOS phosphorylation, leading to an increased NO production in endothelial cells, which contributes to cardiovascular homeostasis and vessel integrity [148]. Furthermore, some studies have shown that VEGF enhances endothelial cell migration and capillary-like structure formation in vitro in a PI3-Akt dependent fashion [157].

6. Angiogenesis and cell transplantation

An angiogenic environment is essential for tissue repair and functional recovery after an ischemic insult, and an intact and functional vasculature is needed to deliver oxygen and nutrients to the transplanted cells to ensure that they survive and become functional. Therefore, angiogenesis in the post-ischemic brain is proposed to be vital for successful stem cell transplantation.

It is well known that ischemic stroke causes active angiogenesis, particularly in the ischemic penumbra [38,44]. This contribution of angiogenesis, however, may not be sufficient to support the brain plasticity required for functional recovery [158]. Hypoxic preconditioning induces tolerance to neuronal injury, and has been investigated to enhance the angiogenic response. A sublethal exposure to hypoxia alters gene expression and activation of different intracellular signaling pathways. Following hypoxic preconditioning, hypoxia-inducible factor 1-α (HIF1α) expression is upregulated. This transcription factor is upstream of many other factors, including VEGF. Preliminary data in our laboratory shows that hypoxic preconditioning enhances angiogenesis in the ischemic neonatal rat brain [159]. This enhanced environment may be favorable for long-term survival of transplanted stem cells.

Additionally, transplanted cells may release angiogenic and/or trophic factors; this property may be enhanced by gene-modification with angiogenic factors. Angiogenic factors can also be administered to the transplantation site to expedite the growth of new blood vessels, thereby enhancing the integration of transplanted cells into the existing environment. Systemic administration of human cord blood-derived $CD34^+$ cells to immunocompromised mice subjected to stroke 48 h earlier stimulated angiogenesis with specific neovascularization around the cortical degeneration site [160]. Endothelial progenitor cells (EPCs) are being engineered ex vivo to overexpress angiogenic growth factors. The gene-modified EPCs rescue impaired neovascularization in an animal model of limb ischemia [161]. Chen et al. [162] have recently shown that treatment with bone marrow stromal cells enhances angiogenesis by increasing endogenous levels of VEGF and VEGFR2. They previously demonstrated that administration of recombinant human VEGF165 to rats 48 h after stroke significantly increased angiogenesis in the penumbra and improved functional recovery [163]. Understanding the regulation of angiogenesis and its relationship to transplanted cells are necessary so that a controlled angiogenic response results in appropriate formation of a functional vascular network in the place needed and at the desirable time.

7. Cell transplantation and brain repair

Neurons and oligodendrocytes are the two neural cell types that are most susceptible to ischemic/hypoxic injury and are least likely to regenerate spontaneously. Although recent evidence shows neurogenesis activities occur in the adult and/or injured brain, the capacity and capability of this endogenous compensatory repair is limited [164]. Cell replacement therapy utilizing multipotential cells derived from embryos, fetuses, or even adult tissues is thus an alternative approach to repair the damaged brain tissues [164]. The survival of embryonic brain tissue grafted into the cortical regions of animals following diffuse hypoxic injury was first demonstrated by Polezhaev and Alexandrova [165]. In subsequent experiments using the permanent occlusion of the MCA, neocortical cells were shown to survive in the infarcted areas [166]. Grafted donor fetal cells and neural progenitor cells survived in the ischemic hippocampus and striatum and developed the properties of normal neurons, and were able to establish connections with the host brain, release transmitters, and contribute to functional recovery [167–171]. Experiments using hippocampal, striatal, and cortical grafts demonstrate that fetal cells/tissues, immortalized cells, and engineered cell lines can not only survive grafting into the ischemic adult brain, but can also correct neurotransmitter release, establish both afferent and efferent connections with the host brain, and restore functional and cognitive deficits after ischemic stroke or neurodegenerative diseases [164,172,173]. More recently, several investigators

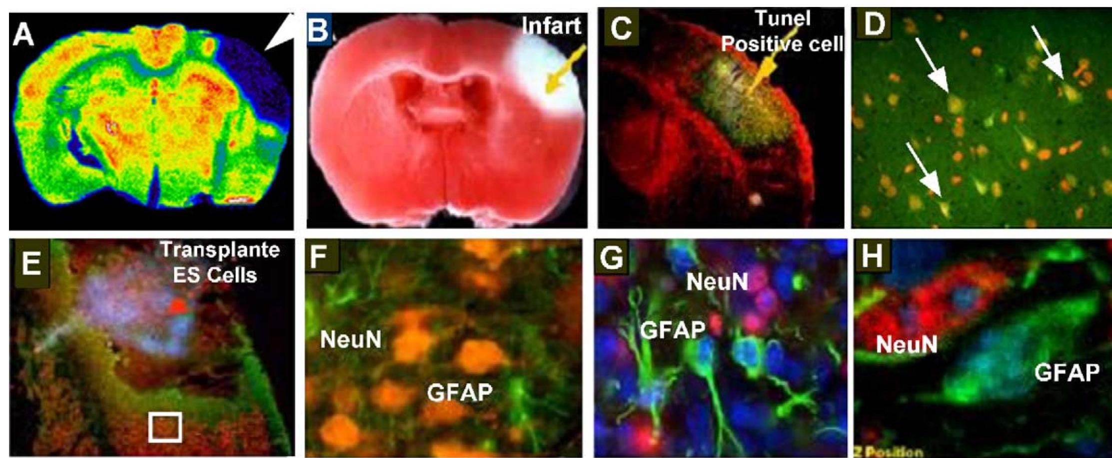


Fig. 5. ES cell transplantation after focal ischemic stroke. The whisker–barrel cortex ischemic stroke in rats was induced by selective occlusion to branches of the MCA [191]. (A) Cerebral blood flow measured by ^{14}C-IAP autoradiography was substantially reduced in a discrete cortical region 10 min after ligation of MCA branches and occlusion of the ipsilateral common cerebral artery. (B) Infarct formed in the ischemic core revealed by TTC stain. (C) TUNEL positive cells (yellow) in the ischemic cortex, especial layer IV, 24 h after ischemia. Red denotes GFAP positive cells (astrocytes). (D) Activated caspase-3 positive cells (arrow) co-localization with PI staining in the ischemia cortex. (E) ES cells pre-labeled with Hoechst (blue) in the ischemic cortex 7 days after transplantation. Green represents GFAP positive cells and red NeuN positive neurons. (F–H) Enlarged images from E. (F) Native neurons (NeuN positive, red) and astrocytes (GFAP positive, green) in the non-ischemia region (frame in D). (G) Differentiated neurons derived from ES cells (NeuN positive, red) and astrocytes (GFAP positive, green) in the transplantation site of ischemic core. The overlap of these markers with pre-labeled Hoechst (blue) demonstrates their ES cell origin. (H) Confocal triple labeling demonstrates NeuN-positive neurons (red), GFAP-positive astroglia (green), and Hoechst (blue) labeled nuclei indicating ES cells origin. Modified from Fig. 2 in [184].

have utilized adult progenitor cells derived from bone marrow or human umbilical cord blood to improve functional outcome after ischemic stroke in animal models [174–177]. Still other studies have used embryonic stem (ES) cells for the treatment of heart or brain ischemia and spinal cord injury [165,178,179].

Stem cells, especially ES cells, are capable of proliferation and differentiation into progenitor cells and ultimately mature cells. Transplanted stem cells may aid in the restoration of lost functions by integrating into functional synaptic networks within host tissues or contributing trophic support for neurogenesis and angiogenesis in and around damaged areas in both animal models and human studies [167,180]. ES cells following exposure to retinoic acid in vitro differentiate into neural progenitor cells [181]. Studies from our group and others showed that neurally differentiated ES cells transplanted in animal models of spinal cord injury and focal ischemia could survive, migrate, differentiate, myelinate, and contribute to functional recovery [182–184]. For example, after focal cerebral ischemia, ES cells transplanted 7 days after ischemia differentiated into neuron-like cells as identified by specific markers 10 days later (Fig. 5) [184]. Using ES cells overexpressing Bcl-2, we observed that Bcl-2 not only increases ES cell survival but may also promote neuronal differentiation of these cells [184], which is consistent with previous observations [26,27]. In our experiments of 7 days after transplantation (i.e. 14 days after ischemia), some transplanted ES cells in the post-ischemic cortex and striatum stained positively for the astrocyte marker GFAP, the oligodendrocyte precursor marker NG-II, and the mature oligodendrocyte marker APC. In addition, some ES cells were immunolabeled for Glut-1, a marker for differentiated endothelial cells [184]. These cells often appeared in tubular shapes, consistent with their organization into microvessels [12].

8. Conclusion and remarks

Understanding the mechanisms leading to angiogenesis following ischemic stroke may lead to the identification of therapeutic targets for enhancing recovery. Available data suggest that specific angiogenic growth factors and their corresponding receptors may play important roles in post-ischemic angiogenesis. It is likely that the intricate interplay of these various factors and receptors results in the sequence of events that comprise angiogenesis. For example, the Angpo-1/Angpo-2 ratio may be crucial for remodeling of vessels into large vessels or capillaries. Likewise, Tie-1 and Tie-2 may regulate capillary density versus vasculogenesis and nonsprouting angiogenesis. Our preliminary understanding of the signal transduction mechanisms suggests that NF-κB and the PI_3/Akt pathway may regulate multiple critical steps in new vessel formation.

It is unclear what may direct the differentiation and migration processes of transplanted stem cells in the ischemic brain. Previous studies showed that grafted neurons can establish functionally appropriate connections in the adult brain, and this capacity is much increased when the host circuitry is damaged, suggesting that mechanisms regulating neuronal differentiation and connectivity during development may be reactivated by lesions or degenerative changes [185–187].

Thus, in and around the injured area, endogenous local signals may try to shift differentiation of transplanted cells to compensate for neuronal cell death. Identification of these signals will improve the efficacy of stem cell transplantation. On the other hand, measures to evaluate and prevent a potential long-term risk of tumorigenesis from the transplanted stem cells need to be considered.

In addition to rebuilding damaged structures through cellular replacement, an enhanced local trophic environment may play an important role. In this regard, the enhanced cell survival promoted by Bcl-2 overexpression is likely an optimizing factor for more trophic support and fewer burdens from dead cells.

Recent studies suggest that cell transplantation may produce beneficial increases in host plasticity [188,189]. In rats with spinal cord injury, neural stem cell transplantation after the insult promotes host axonal growth, at least in part by secreting growth factors [190]. On the other hand, it was noticed in our study that axonal growth among transplanted NeuN positive cells was not well organized, in contrast to the parallel axonal distribution in the non-ischemic cortex [184]. Further study is important and necessary to delineate signals that may direct new axons to grow towards the correct targets for appropriate synaptic connections.

It is expected that understanding the mechanism underlying angiogenesis and its role in post-ischemic recovery coupled with the perfection of stem cell transplantation may ultimately help in achieving optimal therapeutic outcome after cerebral ischemia in stroke patients.

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