ABSTRACT

Old age is associated with an enhanced susceptibility to stroke and poor recovery from brain injury, but the cellular processes underlying these phenomena are uncertain. Therefore studying the basic mechanism underlying functional recovery after brain ischemia in aged subjects is of considerable clinical interest. The available evidence indicates that (i) compared to young rats, middle aged rats develop a larger infarct area, as well as a necrotic zone characterized by a higher rate of cellular degeneration, and a larger number of apoptotic cells; (ii) in both old and young rats, the early intense proliferative activity following stroke leads to a precipitous formation of growth-inhibiting scar tissue, a phenomenon amplified by the persistent expression of neurotoxic factors; and (iii) the regenerative potential of the rat brain is largely preserved up to 20 months of age but gene expression temporally displaced, has a lower amplitude, and is sometimes of relatively short duration. Whether endogenous neurogenesis contributes to spontaneous recovery after stroke has not yet been established.

If the neurogenesis from endogenous NSCs will be used as therapeutic approach, it will require individual approach to assess the possible extent of neurogenic response and possibilities to alter this response for functional improvement or prevention of further progression of the disease.

KEY WORDS stroke, aging, recuperation; glial scar; degeneration; apoptosis; microglia activation; cytoproliferation; regeneration; neurogenesis.

Introduction

Brain ischemia in aged animals

Age-related brain injuries including stroke, are a major cause of physical and mental disabilities for which no satisfactory treatment exists. Therefore studying the basic mechanism underlying functional recovery after brain stroke in middle aged subjected it is of considerable clinical interest.

Stroke models using aged animals are clinically more relevant than stroke models in young animals

Aging is associated with a decline of locomotor, sensory and cognitive performance in humans [1] and animals [2-4] part of which are due to age-related functional decline of the brain. Studies of brain ischemia in experimental animals have demonstrated the neuroprotective efficacy of a variety of interventions, but most of the strategies that have been clinically tested failed to show benefit in aged humans. One possible explanation for this discrepancy between experimental and clinical studies may be the role that age plays in the recovery of the brain from insult. Indeed, age-dependent increase in conversion of ischemic tissue into infarction suggests that age is a biological marker for the variability in tissue outcome in acute human stroke [5].

Although it is well known that aging is a risk factor for stroke [6,7], the majority of experimental studies of stroke have been performed on young animals, and therefore may not fully replicate the effects of ischemia on neural tissue in aged subjects [8-11]. In this light, the aged post-acute animal model is clinically most relevant to stroke rehabilitation and dementia cellular studies, a recommendation done by the STAIR committee [12] and more recently by the Stroke Progress Review Group [13].

Recent data indicate that neurological function in aged rats was more severely impaired by ischemia than in young rats including a limited recovery of neurological function. Furthermore, aged rats compared to young rats show an accelerated infarct development in the first week post-stroke as compared to the young counterparts [14] All these factors together certainly complicate any therapeutic strategy in the elderly. Indeed, two recent studies have shown that co-administration of a plasminogen activator inhibitor type 1 derived peptide, EEIIIMD, with tissue plasminogen activator, tPA, a drug currently used for thrombolysis in stroke units, showed no improvement in total infarction volume, edema formation, or functional outcome in aged rats [15]. Moreover, attenuation of oxidative stress with apocynin, which is effective in young rats in improving tissue and function restoration, in the
Recuperation from stroke is governed by a complex cytological response

Poor recovery reflects the balance of factors leading to infarct progression (neuronal degeneration, apoptosis, phagocytosis), factors impeding tissue repair (astroglial scar, neurite inhibitory proteins), and factors promoting brain plasticity and repair. Both the timing and the magnitude of these phenomena are dysregulated in the post-ischemic, aged rat brain. Following infarction, sensorimotor function was impaired in most animals, but the young rats began to recover after a brief period of only 1-2 days. In contrast, behavioural recovery in the aged animals did not commence until 2-4 days post-infarct.

Furthermore, young animals fully recovered after 10-15 days, whereas the aged rats only recovered to about 70% of pre-stroke sensorimotor functionality during the same period.

Figure 1 Summary of the time course of behavioral recovery (A) and infarct progression after stroke (B) in young vs. old rats. Time course of changes in cellular events contributing to infarct development in young and old post-stroke rat brains, including the number of degenerating cells (C); the number of TUNEL-positive cells (D) as well as microglia activation (E), is shown.

No further recovery was noted after day 15 post-stroke [17,18](Fig. 1A). It has been hypothesized that rapid infarct development in aged rats, together with the premature appearance of proliferating, degenerating and apoptotic cells, contributes to the long-lasting performance deficits seen in aged rats [14]. The pathogenesis of tissue damage is due mainly to inflammatory interactions involving cytokines, chemokines and leukocytes, as well as neurotoxic factors such as the C-terminal fragment of the β-amyloid precursor protein (βAPP) [9,19-21]. Collectively, this will result in dysfunctional cell-cell signaling in the neurovascular unit [22].

Perturbed cellular response to stroke in aging

Infarct development and neuronal degeneration are accelerated in aged animals

Several interconnected factors may contribute to the rapid development of the infarct in aged animals: (i) early neuronal death; (ii) apoptosis; and (iii) an early, fulminate phagocytic activation of brain macrophages which are removing the remnants of dead neurons and other cellular debris [19,23]. A related consideration is that early disruptions of the blood-brain barrier may contribute to exacerbated neuronal damage and prolonged functional recovery following stroke in aged rats [24]. It should be noted that the vulnerability of brain tissue to traumatic injury [25], and to oxidative stress in particular, also increases with age [26,27].

Measurement of the infarct volume at day 3 post stroke has indicated that 15% of the total ipsilateral cortical volume was devoid of NeuN-immunoreactive neurons in young animals. NeuN immunoreactivity is a marker of neuronal viability which delineate sharply the infarct border [14,18,19]. The degeneration of neurons in the young group continued to progress such that, at one week, the infarcted area had stabilized at approximately 37% of the total volume of the ipsilateral hemisphere (Fig. 1B).

In contrast to young animals, on day 3, the necrotic zone of aged rats lacked NeuN immunopositivity in 28% of the ipsilateral cortical volume. The infarcted area continued to expand, and by day 7 reached 41% of the ipsilateral cortical volume. Thus, the development of the infarct was more rapid in aged rats, but by day 7, the cortical infarcts were similar in size in both age groups, i.e. 37±5.1 % of total cortical volume in young rats and 41±3.9 % in aged rats (Fig. 1B).
The age difference in infarct development after stroke is made evident by a mild episode of cerebral ischemia that causes moderate neuronal degeneration in young animals. In contrast, aged, ischemic rats showed a high degree of degeneration already at day 3.

Fluoro Jade B-staining confirmed that young rats had few obviously degenerating neurons in the infarcted area at day 3. The number of degenerating neurons then increased rapidly through day 7 and reached a maximum at days 7-14. Fluoro Jade B-staining showed that aged rats had an unusually high number of degenerating neurons in the infarct core already on day 3 (3.5-fold vs. young rats). Interestingly, thereafter the number of degenerating neurons did not rise further in aged animals, although the infarcted area continued to expand, so that by day 7 the numbers of degenerating neurons were almost the same in both age groups (Fig. 1C).

Post-ischemic apoptosis is accelerated in aged rats

Another cellular event that contributes to early infarct development in aged rats is augmented apoptosis [28]. Cerebral ischemia triggers two general pathways of apoptosis: the intrinsic pathway, originating from mitochondrial release of cytochrome c and associated stimulation of caspase-3; and the extrinsic pathway, originating from the activation of cell surface death receptors, resulting in the stimulation of caspase-8. Recently, the simplistic concept that stroke-induced apoptosis occurs predominantly in neurons and is caspase-dependent has been challenged. Accumulating evidence indicates that apoptosis is prevalent in nonneuronal cells and that caspase-independent mechanisms also play a key role [14,29].

Aging increases the susceptibility of the central nervous system, to apoptotic events [30]. Apoptotic cells in young rats began to increase in number by day 3 in the infarct core to reach a maximum at day 7 post-ischemia (Fig. 1D). By day 14, the number of apoptotic cells was very low. The aged rats had a 2-fold increase over young rats in the number of apoptotic cells in the infarct core already at day 3. At day 7, however, the ratio was reversed, i.e. the apoptotic cells in aged rats were outnumbered by the apoptotic cells in young rats by 1.7-fold. Thereafter the number of apoptotic cells decreased progressively from day 7 to day 14. From day 14 on, the number of apoptotic cells fluctuated around basal levels in both age groups [14] (Fig. 1D). These results suggest that aging-related increase in the number of apoptotic cells might be related to age-related increase apoptosis of newborn neurons after stroke [31].

Early, fulminant phagocytic activity of brain macrophages in the post-ischemic aged rat brain

The aging brain is characterized by a shift from the homeostatic balance of inflammatory mediators to a proinflammatory state. Basal mRNA expression of CD11b and Iba1, markers of activated microglia, is higher in aged brain as compared to the adult [32,33]. These conditions make the aged brain susceptible to producing an exaggerated response to injuries like traumatic brain injury and stroke [19,32,34].

Ischemic injury causes a local inflammatory reaction by activated microglia and infiltrated inflammatory cells that further release proinflammatory cytokines and ROS within the injured site. Microglial cells also display an age-associated augmentation of reactivity in a variety of mammalian species [35,36]. In young rats, after an episode of mild ischemia the process of microglia activation was rather slow with microglial cells being fully activated at day 14. The situation was quite different in aged animals. A great number of microglia-like cells are fully activated at day 3 post-surgery and the process reached a maximum at day 7. Thereafter the intensity of the microglial reaction diminished progressively with time, but was still evident even at day 28 [19] (Fig. 1E).

In most cases of acute injury depositions of tissue debris is due to cell death and is removed by macrophages. In the CNS, the debris are made up mostly of myelin which contains several growth inhibitory molecules such as Nogo A, which exhibit inhibitory effects on axonal regrowth [37]. Therefore delaying myelin clearance in the CNS after acute injury may contribute to the failure of axonal regeneration.

Microglia in aged rodent and human brains are subject to replicative senescence [38]. Importantly, in young animals myelin debris are removed more effectively than in older animals [39-41]. Furthermore, older rats compared to young rats showed delayed recruitment of phagocytic cells and less clearance of myelin after a toxin-induced demyelination lesion [42], which correlates with the slower remyelination in older animals [43]. In light of these findings it is questionable if a stronger inflammatory reaction in old rats in response to stroke is sufficient to remove cellular debris and create conditions for
Post-stroke tissue restoration and in particular axonal growth and remyelination.

**Post-ischemic cellular proliferation is prematurely increased in middle aged rats and contributes to an early scar build-up**

An important cellular event associated with reduced structural and functional recovery after stroke in aged animals is the early formation of a scar in the infarcted region that impairs subsequent neural recovery and repair (Fig.2A).

**Figure 2**

*Capillary-derived nestin-positive cells delimit the infarct in young rats.*

In young rats the nestin-positive cells begin to accumulate around the infarct core at day 3 post-stroke (A). The inset shows activated nestin cells in the perinfract at a higher magnification. At 2 wks post-stroke, however, the infarct was sharply delimited by nestin processes (B). The inset shows the processes in the scar tissue at higher magnification. (C): By day 3, there were 15% co-localized BrdU- and nestin-positive cells in the corpus callosum, some of them being in a mitosis-like state (arrowheads) (inset shows a 3D reconstruction of nestin- and BrdU-positive cells within a ramified blood vessel). (D): In cross-sectioned blood vessels of the corpus callosum, nestin occupied the inner layer of the vascular wall, and GFAP occupied the outer layer, most likely the endophytic/pericytic cell layer and GFAP marking the outer layer, most likely the astrocytic endfeet [47] (Fig. 2D). In cross-sectioned capillaries whose basal laminae were labelled by anti-laminin antibodies, nestin-positive cells having BrdU-positive nuclei on the verge of leaving the capillary wall could be visualized (Fig. 2E, arrowheads), while other co-labelled cells had already left the capillary. In the infarcted area, nonetheless, there was a heterogeneous population of cells, some of them having a mixed nestin-GFAP phenotype, whereas others were solely GFAP- or nestin-positive [47].

Aged rats had fewer nestin-BrdU double-labelled cells in the corpus callosum and periinfarcted area than did young animals, indicating that the proliferative potential of nestin cells in aged rats is reduced relative to that of young rats. Paradoxically, then, despite a lower number of proliferating nestin cells in aged rats, these cells envelope the infarct site in greater numbers very soon after the ischemic event. A likely explanation for this phenomenon is that the steep upregulation of nestin mRNA shortly after stroke in aged rats led to increased nestin that compensated for the lower proliferation rate of nestin-positive cells. This latter interpretation is supported by data showing that nestin is expressed in astrocytes forming the glial scar in the plaques of multiple sclerosis [47,54].

The vascular origin of nestin-positive scar cells is supported by previous data showing that nestin immunoreactivity is increased after stroke [49], and that the upregulation of the protein persists for up to 13 months after damage to the spinal cord [45]. Additionally, among the early vascular changes following stroke is the up-regulation of the proliferative cell nuclear antigen [55], a general marker of cell division, whereas adult blood vessels give rise, upon transplantation (that is, under initially hypoxic conditions) to hematopoietic cells that incorporate BrdU [56].

The cause of the early accumulation of nestin-positive cells in the lesioned hemisphere of aged rats remains unknown. We hypothesize that a decreased plasticity of the cerebral vascular wall owing to, for instance, age-associated hypertension [57,58] could be one essential factor. Likewise, the increased fragility of aged blood
vessels due to loss of distensible components of the microvessels such as elastin [59], as well as the loss of smooth muscle cells [60] may lead, upon ischemic stress, to rapid fragmentation of cerebral capillaries and rapid release of pericytes from the vascular wall. The post-ischemic cellular proliferation is illustrated in Fig. 3.

**Figure 3 Cellular proliferation after stroke. Hypothetical drawing of proliferating cells migration in the infarcted area.**

Precipitous and persistent expression of the neurotoxic C-terminal fragment of βAPP in the infarcted area of middle aged rats

Cerebral ischemia promotes conditions that are favorable to the focal accumulation of neurotoxic factors such as Aβ, especially in the middle aged brain [9,20]. In middle aged rats, the neurotoxic C-terminal fragment of β-APP steadily accumulated over time and reached a maximum at 14d in the perinfarcted area of aged rats as compared to young rats. In contrast, the N-terminal fragment of β-APP was incorporated into the developing astroglial scar [20].

Evidence derived from mice expressing the 100-amino-acid carboxy terminal fragment of βAPP indicates that this fragment may promote synaptic degeneration and neuronal death [61] and impair learning [62]. It seems that, in general, an overexpression of βAPP695 in postmitotic neurons results in neuronal degeneration due to intracellular accumulation of this isoform [63,64].

**Phenotyping of proliferating cells**

The increased fragility of aged blood vessels due to decreases in distensible components of the microvessels such as elastin [59] may lead, upon ischemic stress, to fragmentation of cerebral capillaries that would promote the leakage of hematogenous cells into the infarct area [35,65]. In young rats, BBB opening may increase the permeability for large molecules such as dextran but the leakage of hematogenous cells into the infarct area is negligible [24].

Recent data show that not only are cells dying earlier in the infarct zone of aged rats, but there are also more newly generated cells at this time. Pulse-labeling with BrdU shortly before sacrifice revealed a dramatic increase in cells undergoing mitosis and/or repair [66] that appeared to be migrating through the cerebral vessels in the infarcted area [67]. Significantly, the number of BrdU-positive cells in the infarcted hemisphere of aged rats at days 3 and 7 vastly exceeded that of young rats. In young rats, transiently labeled, mostly non-neuronal cells in the infarcted hemisphere peaked at day 7 post-stroke, in accordance with a previous report [67,68]. Similarly, bromodeoxyuridine-positive cell counts were significantly higher with severe global ischemia achieved by eight-vessel occlusion than with intermediate ischemia (four-vessel occlusion) or in sham-operated animals, respectively [69].

With double-labeling techniques, the proliferating cells in the aged rat brain after stroke were identified either as reactive microglia (45%), oligodendrocyte progenitors (17%), astrocytes (23%), CD8+ lymphocytes (4%), or apoptotic cells of indeterminate type (<1%) [70] (Popa-Wagner et al., 2006b)(Table 2).

**Table 2. Phenotype of BrdU-positive cells after stroke**

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<tr>
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<th>Young Rats</th>
<th>Aged Rats</th>
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<td>Cells analyzed</td>
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<tr>
<td>ED1</td>
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<tr>
<td>GFAP</td>
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<td>NG2</td>
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<tr>
<td>TUNEL*</td>
<td>58</td>
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<td>CD4</td>
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<tr>
<td>CD8</td>
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<tr>
<td>CD11b</td>
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<tr>
<td>CD45</td>
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<td>MPO</td>
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<td>PMN</td>
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*Apoptotic cells: question mark means "barely detectable"*

If the surgical procedure was properly performed on the sham controls, i.e. there was not excessive injury of tissue, then there is quite limited cell infiltration via the leptomeninges

The regenerative potential of the brain appears to be competent in middle aged rats

The discovery that neural stem cells (NSC) exist in the adult brain and that neurogenesis in the dentate gyrus of the hippocampus and subventricular zone (SVZ) persists throughout the whole life of mammals triggered the development of the concept that this feature of adult brain could be utilized for treatment of neurodegenerative
diseases. The fact that progressive neurodegenerative diseases such as Parkinson’s and Alzheimer’s are age related, raised the question whether or not neurogenesis is altered in the aged brain. Very first studies on this topic have demonstrated that NSC proliferation and neuroblast formation in SVZ are decreased in aged rodents [71,72]. Moreover, behavioral studies demonstrated that in aged mice there is a deficit in olfactory discrimination, which is most likely caused by reduced SVZ neurogenesis [72]. Several explanations have been forwarded to explain the age-dependent decline of neurogenesis. Among others, decreased proliferation and growth factor signaling [71-73], elevated levels of corticosteroids [74-76], and senescence of neural progenitors [77]. The data regarding the decreased number of NSCs in the aged dentate gyrus are conflicting [73, 78,79]. Similarly, in the old SVZ, the number of NSCs was described to be unchanged [71], but data on proliferation, neurosphere formation and ultrastructure in aged brain [72,80,81] have indicated decreased number of NSCs.

Importantly, the brain’s ability to response to the insult with increased neurogenesis is still maintained in the aged animals. The neurogenesis can be increased or even restored to adult levels by enriched environment [82], infusion of growth factors [83], stroke [84,85], or seizures [86] (Gray et al., 2002). It was also demonstrated that the density of dendritic spines in the new neurons is similar to that observed in young adult animals despite the significant decline in the neurogenesis [87]. Recently, it was shown that when isolated in vitro, the NSCs from SVZ of aged animals have capacity for proliferation and multilineage differentiation, including production of functional neurons, similar to that of NSCs in adult mice, albeit with lower efficacy [88]. These findings suggest that the age-related mechanisms leading to reduced neurogenesis do not affect the morphological or electrophysiological properties of the new neurons. If the neurogenesis from endogenous NSCs in the future will be developed as therapeutic tool for human neurodegenerative disorders the ability to generate new functional neurons becomes very important feature of the aged brain [89].

Some symptoms of classical age-related disease such as Alzheimer’s could partly be due to impaired neurogenesis in the dentate gyrus. The important factor for the alteration of neurogenesis in the Alzheimer’s disease seems to be disease severity. While there is a compensatory increase of NSC proliferation in the early stages of the disease, there is decreased proliferation and survival with advanced pathology [90,91]. It has been also shown that the formation of immature hippocampal neurons is increased in senile Alzheimer’s patients [92] but neurogenesis is not changed in the presenile cases [93]. Later studies have indicated deficient maturation of new neurons in Alzheimer’s disease brains [94]. Interestingly both β amyloid and α synuclein, proteins known to misfold and accumulate in Alzheimer’s and Parkinson’s diseases, have been shown to have detrimental effects on neural stem/progenitor cells and neurogenesis [95-97].

Another neurodegenerative disease that is highly increased in aged patients and in which stimulation of endogenous neurogenesis could have therapeutic value is stroke [89,98,99]. In response to stroke, proliferation of SVZ progenitors is increased and they generate new neuroblasts, which migrate to the damaged area in the striatum during several months, differentiate into striatal neurons [100-102], and most importantly morphologically [103], functionally [104] integrate into the existing network. Stroke-induced neurogenesis is maintained in the aged rat brain [85] and recent studies on post-mortem human brains show the evidence that there might be SVZ cell proliferation and neuroblast formation after stroke even in aged patients [105-107]. Whether endogenous neurogenesis contributes to spontaneous recovery after stroke has not yet been established. Neurogenesis consists of several steps such as cell proliferation, migration, differentiation, survival, and integration into the existing neuronal network. Many factors including environmental and genetic strongly influence each of these steps. In addition, age, physical condition of patient and severity of the disease could substantially influence these steps and, therefore, the outcome of this process. In neurodegenerative diseases, the degree of the disease and pathological consequences are very variable.

Conclusions

The available evidence indicates that the middle aged brain has the capability to mount a cytoproliferative response to injury, but the timing of the cellular and genetic response to cerebral insult is deregulated in middle aged animals, thereby further compromising functional recovery. Elucidating the molecular basis for this phenomenon in the aging brain could yield novel approaches to neurorestoration in the elderly. Whether endogenous neurogenesis contributes to spontaneous recovery after stroke has not yet been established. If the neurogenesis from endogenous
NSCs will be used as therapeutic approach, it will require individual approach to assess the possible extent of neurogenic response and possibilities to alter this response for functional improvement or prevention of further progression of the disease.

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