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Zusammenfassung

Altersbedingte Hirnverletzungen einschließlich Schlaganfall sind ein Hauptgrund für physische und geistliche Behinderungen. Dafür sind Untersuchungen an Ratten von mittlerem Alter zu den grundlegenden Mechanismen der funktionellen Erholung nach einem Schlaganfall von bedeutendem klinischem Interesse.

Die Ergebnisse von Verhaltenstests aus unseren Labor deuten auf eine stärkere Schädigung durch einen Schlaganfall im Vergleich zu Jungtieren hin. Zudem zeigten die älteren Ratten eine verminderte funktionelle Erholung. Die Infarktgröße unterschied sich nicht signifikant. In der zytologischen Antwort auf den Schlaganfall zeigten sich kritische Unterschiede, vor allem in einer altersabhängigen Beschleunigung bei der Bildung der glialen Narbe. Die frühe Phase des Infarktes in älteren Ratten ist mit einer vorzeitigen Zunahme an BrdU-positiven Mikrogliazellen und Astrozyten, Aktivierung von Oligodendrozyten, eine Intensivierung der Neurodegeneration und damit assoziierter Apoptose, verbunden. Neuroepithelial-positive Zellen wurden in älteren Ratten schnell in die Glianarbe eingebaut, aber diese Zellen lieferten keinen signifikanten Beitrag zur Neurogenese im Infarkt-betroffenen Kortex von jungen oder älteren Tieren. Schlaganfall geht mit einer starken Entzündungsreaktion im Hirngewebe einher. Wir vermuteten dass eine milde systemische Entzündungsreaktion vor dem Schlaganfall, möglicherweise einen neuroprotektiven Effekt im Rattenmodell für fokale Ischämie auslößt. Um diese Hypothese zu überprüfen, wurde für drei Wochen eine marginale Paradontitis, die eine milde systemische Inflammation hervorruft, in BB/LL Wistar-Ratten induziert. Zwei Wochen nach dem Beginn der Paradontitis wurde eine fokale zerebrale Ischämie durch einen reversiblen Verschluss der mittleren zerebralen Arterie erzeugt. Nach sieben Tagen wurden die Rattenhirne analysiert. Zusätzlich wurden Marker für systemische Entzündung in einer weiteren Gruppe von Tieren 14 Tage nach dem Auslösen von Paradontitis untersucht. Wir fanden heraus, dass Ratten mit einer milden systemischen Entzündung ein reduziertes Infarktvolumen und eine signifikante Reduzierung der Anzahl von Hirn-Makrophagen in dem vom Infarkt betroffenen Areal, haben.

Fazit: Die vorliegenden Ergebnisse lassen vermuten, dass das ältere Hirn noch die Fähigkeit hat eine cytoproliferative Antwort auf eine Verletzung zu bilden. Jedoch ist die zeitliche Abfolge der zellulären und genetischen Antworten auf den zerebralen Insult in den älteren Tieren dereguliert und damit die weitere funktionelle Erholung behindert. Weiterhin fanden wir heraus, dass eine milde systemische Entzündung vor dem Schlaganfall einen neuroprotektiven Effekt in Ratten durch die Reduktion des Infarktvolumens und der Gewebezerstörung durch Hirn-Makrophagen hat.

Summary

Age-related brain injuries including stroke, are a major cause of physical and mental disabilities. Therefore studying the basic mechanism underlying functional recovery after brain stroke in middle aged rats subjected it is of considerable clinical interest.

Data from our lab and elsewhere indicate that, behaviorally, middle aged rats were more severely impaired by stroke than were young rats, and they also showed diminished functional recovery. Infarct volume did not differ significantly in young and middle aged animals, but critical differences were apparent in the cytological response to stroke, most notably an age-related acceleration of the establishment of the glial scar. The early infarct in older rats is associated with a premature accumulation of BrdU-positive microglia and astrocytes, persistence of activated oligodendrocytes, a high incidence of neuronal degeneration, and accelerated apoptosis. In middle aged rats, neuroepithelial-positive cells were rapidly incorporated into the glial scar, but these neuroepithelial-like cells did not make a significant contribution to neurogenesis in the infarcted cortex in young or middle aged animals.

Stroke is accompanied by a strong inflammatory reaction in the brain. We hypothesized that a mild systemic inflammatory reaction as caused by periodontal disease prior to stroke onset, may exert a neuroprotective effect in a rat model of focal ischemia. To test this hypothesis, marginal periodontitis was induced in BB/LL Wistar rats for 3 weeks. Two weeks after periodontitis initiation, focal cerebral ischemia was produced by reversible occlusion of the right middle cerebral artery. After a survival time of 7 days after ischemia, rat brains were analyzed. In addition, markers of systemic inflammation were determined in a different group of laboratory animals at 14 days after the onset of periodontitis. We found that rats with a mild systemic inflammation had a significantly reduced infarct volume and a significant reduction in the number of brain macrophages in the infarcted area. *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. In addition we found that that mild systemic inflammation elicited prior to stroke onset may have a neuroprotective effect in rats by reducing the infarct volume and tissue destruction by brain macrophages.

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Review

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Cellular and Molecular Events Underlying the Dysregulated Response of the Aged Brain to Stroke

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Key Words
Stroke-related mental disabilities · Stroke-related physical disabilities · Cytological response

Abstract
Background: Age-related brain injuries, including stroke, are a major cause of physical and mental disabilities. ***Objective:*** Therefore, studying the basic mechanism underlying functional recovery after brain stroke in aged subjects is of considerable clinical interest. ***Methods:*** This review summarizes the effects of age on recovery after stroke in an animal model, with emphasis on the underlying cellular mechanisms. ***Results:*** Data from our laboratory and elsewhere indicate that, behaviorally, aged rats were more severely impaired by stroke than young rats, and they also showed diminished functional recovery. Infarct volume did not differ significantly between young and aged animals, but critical differences were apparent in the cytological response to stroke, most notably an age-related acceleration in the development of the glial scar. Early infarct in older rats is associated with premature accumulation of BrdU-positive microglia and astrocytes, persistence of activated oligodendrocytes, a high incidence of neuronal degeneration and accelerated apoptosis. In aged rats, neuroepithelial-positive cells were rapidly incorporated into the glial scar, but these neuroepithelial-like cells did not make a significant contribution to neurogenesis in the infarcted cortex in young or aged animals. The response of plasticity-associated proteins like MAP1B, was delayed in aged rats. Tissue recovery was further delayed by an age-related increase in the amount of the neurotoxic C-terminal fragment of the β-amyloid precursor protein (A-β) at 2 weeks poststroke. ***Conclusion:*** The available evidence indicates that the 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 dysregulated in 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.

Introduction

Age-related brain injuries, including stroke, are a major cause of physical and mental disabilities. Therefore, studying the basic mechanism underlying functional recovery after brain stroke in aged subjected is of considerable clinical interest.

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Stroke Models Using Aged Animals Are Clinically More Relevant than Stroke Models in Young Animals

Aging is associated with declines in locomotor, sensory and cognitive performance in humans [1–4]. Many of these changes are due to an age-related functional decline of the brain.

Studies of stroke 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 the 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 [6–9], 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 [10–13]. In this light, the aged post-acute animal model is clinically most relevant to stroke rehabilitation and cellular studies, a recommendation made by the STAIR committee [14] and more recently by the Stroke Progress Review Group [15].

Stroke Models for Aged Rats

Over the past 10 years, several suitable models for stroke in aged rats have been established. All are based on the permanent [12, 16, 17] or transient occlusion of the middle cerebral artery (MCAO). Transient ischemia was accomplished for 30–120 min by means of a thrombus [18], by intraluminal filament occlusion [15, 19, 20] or by means of a hook attached to a micromanipulator [11]. Long-term hypoxia-ischemia could also be induced by unilateral common carotid artery occlusion [21].

Aged Rats Have Higher Mortality Rates but Not Necessarily Larger Infarcts

Generally, the mortality rate in aged rats is higher than that of young rats. The age difference in mortality is greatest if occlusion is produced by means of an embolus (47 vs. 9%) [18]. In comparison, the intraluminal filament method and photothrombosis produce lower (20–24%) poststroke mortality rates in aged rats [11, 15, 20].

In humans, there is no difference in infarct size with age [21, 22]. Some studies in rats found that cerebral infarct in aged rats was the same size as in young [11, 16–18, 23], while others found that the older rats had larger infarct areas [19, 20].

Aged Animals Recover More Slowly and Less Completely than Young Animals

Aging is associated with a declines in locomotor, sensory and cognitive performance in humans [1] and animals [2–4]. These declines are due in large part to an age-related functional decline of the brain.

Aged persons do not recover from stroke as well as younger persons do [24]. Rehabilitation aims at improving the physical and cognitive impairments and disabilities of patients with stroke. Therefore, studies on behavioral recuperation after stroke in aged animals are necessary and welcome. Various experimental settings have been use to assess the recovery of sensorimotor functions, spontaneous activity and memory after stroke in aged rats [13, 15, 16, 23]. Overall, the results indicate that aged rats have the capacity to recover behaviorally after cortical infarcts, albeit to a lesser extent than the young counterparts [12, 13, 15, 20, 23]. It should be kept in mind, however, that before stroke aged rats are already impaired compared to young animals and show significantly decreased performance in a variety of tests, such as spontaneous locomotor activity [23] and the Morris water maze [25].

As shown schematically in figure 1 (based upon work in our laboratory), all rats had diminished performance on the first day following MCAO, some of which was attributable to the surgery itself. Although recovery did occur in aged rats, its onset was delayed by up to 3 or 4 days depending on the difficulty of the testing [16, 24, 26, 27]. Similar findings have been reported recently for poststroke recovery of mice prone to accelerated senescence [28].

The extent of recovery was also dependent on the complexity and difficulty of the test. For example, aged rats had difficulties in mastering complex tasks such as our neurological status test (which measures a complexity of motor, sensory, reflex and balance outcomes), the rotarod or the adhesive removal test (which are measures of somatosensory dysfunction) and the Morris water maze [18, 23, 25]. However, the recovery of aged rats on simpler tasks, such as the foot-fault test and the corner test is equivalent to that of young rats. Another factor influenc-

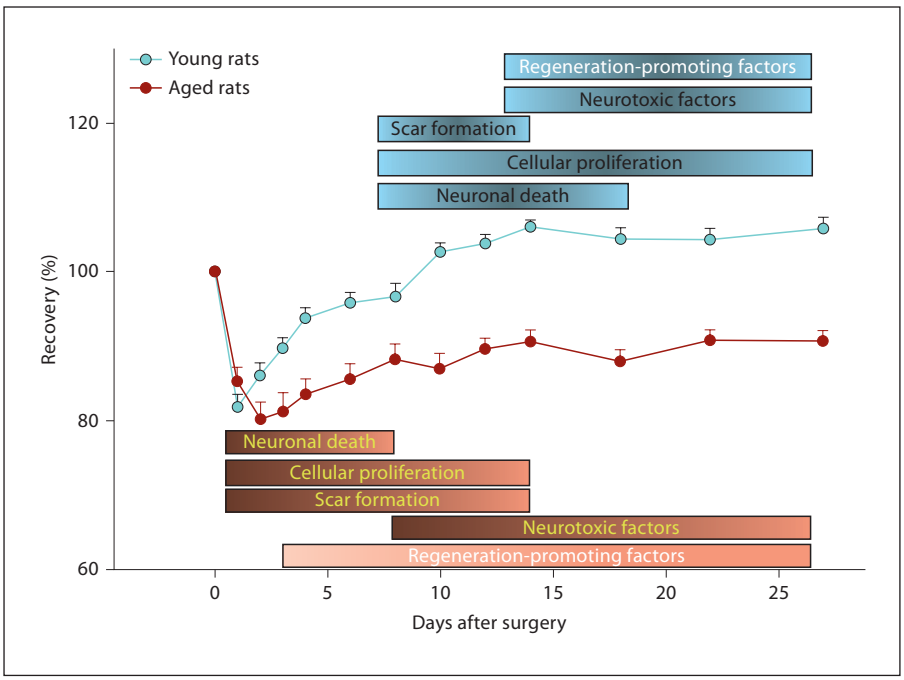

Fig. 1. General time course of functional recovery after stroke in young and aged rats, along with the duration and intensity of underlying major cellular and molecular events such as neuronal death, phagocytosis, scar formation, neurotoxic factors and regeneration-promoting factors.

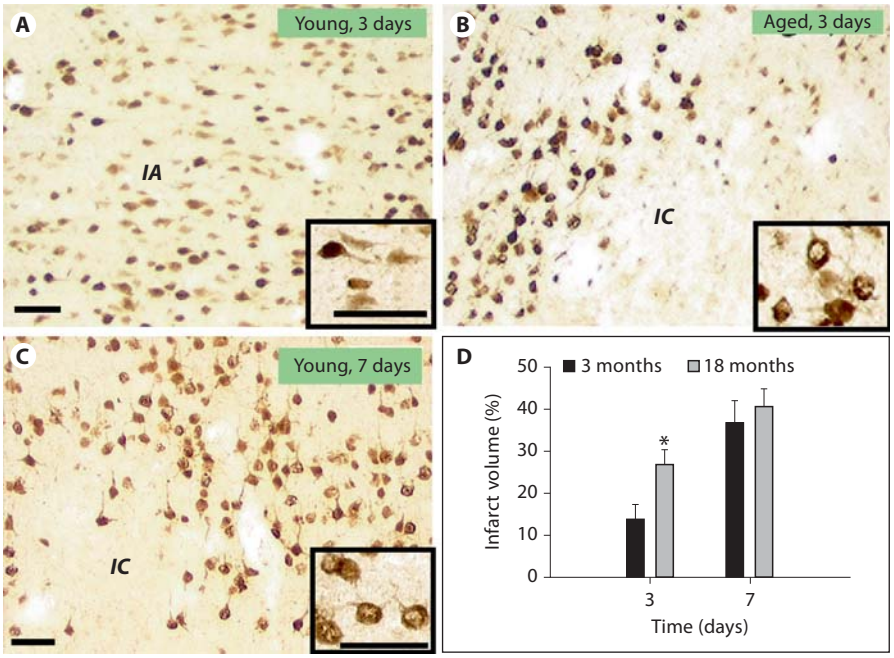

Fig. 2. Infarct development is accelerated in aged animals. NeuN immunohistochemistry showed a mild episode of cerebral ischemia caused moderate neuronal degeneration on post-ischemia day 3 (**A**) compared to the high degree of degeneration seen in aged animals (**B**). On day 3, the infarcted area comprised about 7% of the cortical volume in young animals and 28% in aged rats (* $p < 0.02$). By day 7, the volumes of cortical infarcts were nearly equal in both age groups (**C**, **D**). IC = Infarct core; IA = infarcted area (i.e. the ischemic region in a mild or incipient stage of degeneration); PI = periinfarct. **A**, **C** Bars = 50 μm.

ing the performance level of aged rats is the infarct size, such that functional impairments in the group with the largest infarcts (20% tissue loss) were more severe than the functional impairments in the rats with 4% tissue loss [15]. Figure 1 summarizes all of these differences between young and aged rats in the timing and completeness of recovery following MCAO. The behavioral tests used to assess the recuperation after stroke are given, along with the biological significance of each test, in table 1.

Neurobiology of Tissue Recuperation after Stroke in Aged Animals

Poor recovery may reflect the combination of the more aggressive activation of factors leading to infarct progression (neuronal degeneration, apoptosis, phagocytosis), factors impeding tissue repair (astroglial scar, neurite inhibitory proteins) and neurotoxic factors.

At the same time, factors promoting brain plasticity and growth may be less responsive. Growth-promoting factors include growth-associated proteins GAP43 and CAP23, the growth-promoting transcription factor c-jun, the growth-promoting cell guidance molecule L1, the CDK5-inhibitor p21, microtubule-associated proteins MAP1B and MAP2, immature neurons marker doublecortin, and stem cell marker nestin [26, 29–31]. Pathogenesis of tissue damage is mainly due to inflammatory interactions involving cytokines, chemokines and leukocytes and neurotoxic factors like the C-terminal fragment of β-amyloid (Aβ) [11, 23, 26, 32–34]. One of the main findings is that both timing and magnitude of these factors is dysregulated in the postischemic aged rat brain (fig. 1).

Infarct Development Is Accelerated in Aged Animals

Functional imaging studies after stroke have shown that the reorganization in peri-infarct cortex or connected cortical regions correlates closely with functional recovery [35–37]. Therefore, these regions are mostly studied at cellular and molecular levels.

There are a number of studies on the evolution of infarct volume in aged rats. We recently found that aged rats usually develop an infarct within the first few days after ischemia [27].

In contrast to young animals where the infarct area represented 7% of the ipsilateral hemisphere (fig. 2A), on day 3, the necrotic zone of aged rats lacked NeuN immunopositivity in 28% of the ipsilateral cortical volume (fig. 2B). The infarcted area continued to expand, and by day 7 reached 35–41% of the ipsilateral cortical volume in both in young (fig. 2C) and aged rats (fig. 2D). This suggests that the timing of neuronal loss in aged rats is accelerated, but the ultimate extent of brain cell loss is not significantly different from that in young rats. It should be noted, however, that the greater number of degenerating neurons in aged rats are seen only if the infarct area is relatively large; for small infarcts there is no age difference in the number of surviving neurons in the ischemic border zones [15, 17].

Table 1. Behavioral tests

Behavioral test	Description
Neurological status	rat is pulled gently by the tail and the presence or absence of circling is observed
Limb-placement symmetry	rat is held gently by the tail at the edge of a table; symmetry or asymmetry of forelimb placement is observed
Body proprioception	rat is touched lightly on each side of the body with a blunt probe; tests sensorimotor responsiveness
Response to vibrissae touch	a blunt stick is brushed against the vibrissae on each side, and presence or absence of response is noted; tests sensorimotor responsiveness
Beam-walking test (rotarod)	rat is tested for its ability to maintain balance while walking on a rotating rod; assesses fine vestibulomotor function
Inclined plane	the ability of each animal to maintain its position at a given angle on an inclined plane is determined
Spontaneous activity	rat is placed in a large cage and the number of crossings of a bisecting line is determined; assesses interest in exploration of a novel environment
T-mazes	rat is placed in a t-maze in which one of the arms of the maze is baited with a reward; tests working and reference memory
Radial-arm maze	rat is placed in an 8-arm radial maze, elevated 60 cm above the floor; tests spatial working memory

Neuronal Degeneration and Loss through Postischemic Apoptosis Are Accelerated in Aged Rats

Fluoro-Jade B staining showed that aged rats had an unusually high number of degenerating neurons in the infarct core as early as day 3 – 3.5-fold vs. young rats (fig. 3A–C). Interestingly, the number of degenerating neurons did not rise further in aged animals, even though 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. 3C) [23, 29].

A major cellular event that contributes to early infarct development in aged rats is augmented apoptosis [38]. Aging increases the susceptibility of the central nervous system to apoptotic events [39]. One possible mechanism

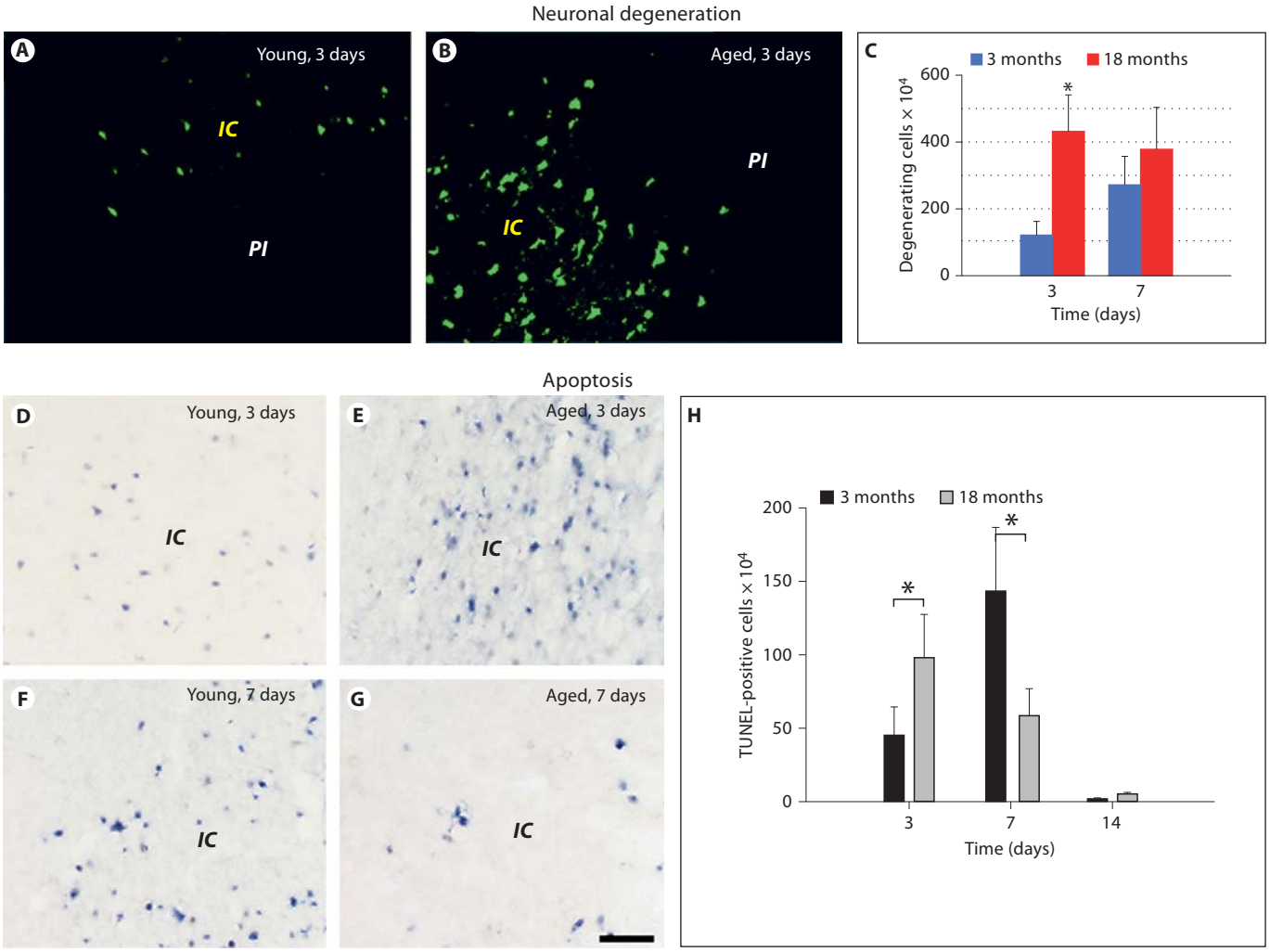

Fig. 3. A–C The number of degenerating cells was greatly increased in aged rats shortly after stroke. Young rats: Fluoro-Jade B staining showed only a few degenerating neurons in the infarct core on day 3 (**A**). Their number then increased rapidly and reached a maximum at days 7–14 (4-fold vs. day 3; $p < 0.001$) (**C**). In contrast, aged animals had a large number of degenerating neurons in the infarct core already on day 3 (**B**) (3.5 times higher than young rats, * $p < 0.001$) (**C**). The number of degenerating neurons was roughly equal in both age groups at day 7 (**C**). **D–H** Through immunohistochemistry, apoptosis in young rats became detectable in the infarct core at day 3 (**D**) and was fully developed by day 7 (**F**). Apoptosis in aged rats was fully developed by day 3 (**E**) and began to decline by day 7 (**G**). Quantitatively, at day 3 the number of apoptotic cells in the infarct core of aged rats outnumbered that of young rats 2-fold ($p < 0.02$). **H** On day 7, however, the ratio was reversed, i.e. apoptotic cells in young rats outnumbered those in old rats 1.7-fold (* $p < 0.05$). IC = Infarct core; PI = periinfarct. **D–G** Bars = 50 μm.

of increased expression of pro-apoptotic proteins in aged animals is via increased NO production by constitutive NO synthase isoforms in a model of transient global ischemia [40]. The particular vulnerability of the aged brain to apoptosis is confirmed by our finding that aged rats had considerably more apoptotic cells 3 days after ischemia (fig. 3E) than young rats (fig. 3D) (2-fold increase over young rats, $p < 0.02$) (fig. 3H) [27]. On day 7, the ratio was unexpectedly reversed such that aged rats (fig. 3G) now had a *smaller* number of apoptotic cells than young rats (fig. 3F) (1.7-fold difference, $p < 0.05$, fig. 3H). However, if the damage to the cerebral cortex is extensive, there is no difference in infarct size or the number of cells undergoing apoptosis between aged and young adults [17].

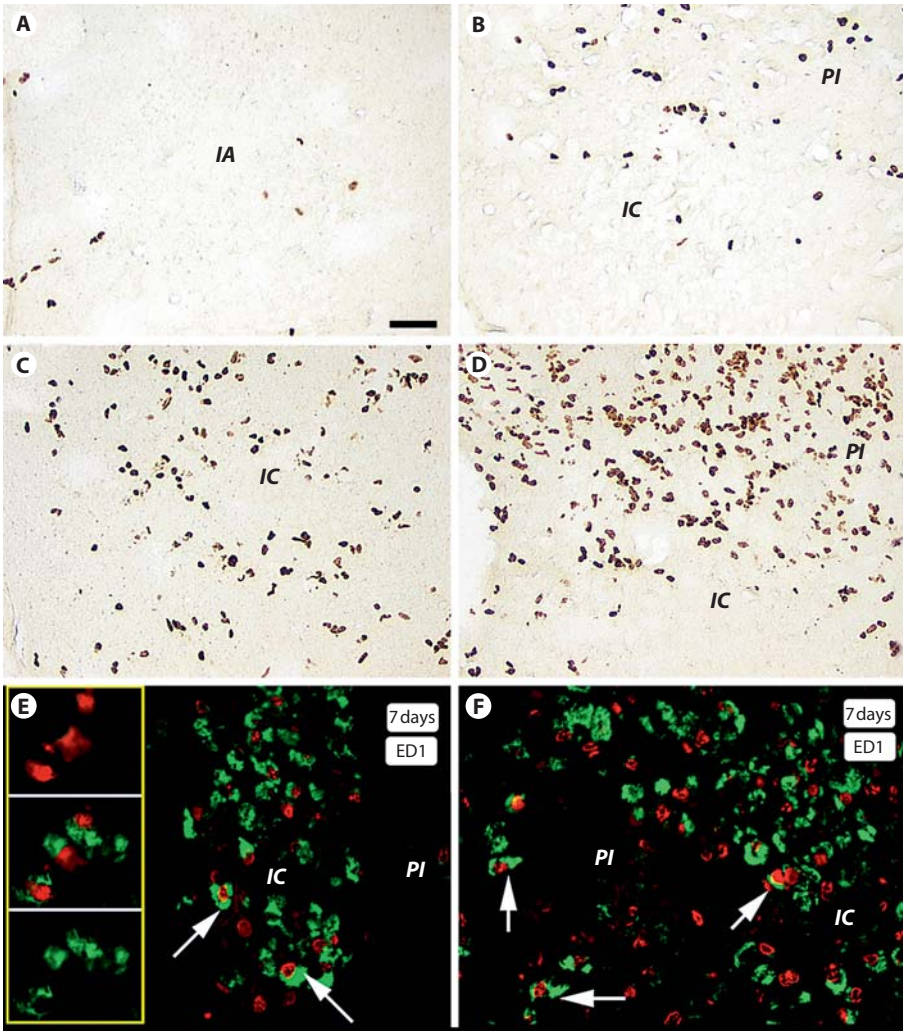

Fig. 4. The number of BrdU-positive (proliferating) cells increased early in aged rats after stroke. Young rats: BrdU-positive cells increased by 5.7-fold vs. sham-operated rats ($p < 0.01$) on day 3 (**A**), reached a maximum (8-fold vs. sham-operated rats; $p < 0.01$) on day 7, and then decreased to control levels by days 14–28 (not shown). Day 0 represents the value for sham-operated rats. Aged rats: On day 3, the infarcted hemisphere of aged rats showed significantly more BrdU-positive cells than in young rats (4-fold increase; $p < 0.001$, **B**). The number of these cells peaked on day 7 post-stroke (4.8-fold higher than in young rats; $p < 0.001$, **D**) and then abruptly declined by day 14 (not shown). Colocalization of BrdU-positive cells (red) with microglial cells (green) was maximal on day 7 in young (**E** inset, arrows) and aged (**F**, arrows) rats. IC = Infarct core; IA = infarcted area (i.e. the ischemic region in a mild or incipient stage of degeneration); PI = periinfarct. **A–D** Bars = 50 μm.

Postischemic Cellular Proliferation Is Prematurely Increased in Aged Rats and Contributes to an Early Scar Buildup

Our data not only show greater cell death in the infarct zone of aged rats on day 3 poststroke, but also that there are more newly generated cells at this time.

Pulse labeling with BrdU shortly before sacrifice revealed a dramatic increase in proliferating cells in the infarcted area of aged rats on day 3 (fig. 4B), which significantly exceeded the number in young rats at the same time point (fig. 4A). By day 7, the number of BrdU-positive cells had increased substantially in the infarcted area of young rats, too (fig. 4C). Nevertheless, the difference in the number of BrdU-positive cells remained at day 7 poststroke (fig. 4D), at which point the number of BrdU-positive cells peaked in both age groups before abruptly declining to control levels by days 14–28 (not shown).

After multiple BrdU treatments of post-stroke rats, the colocalization of monocytic (ED1) and proliferation (BrdU) markers was maximal in the infarct core on day 7 post-stroke for both young (fig. 4E) and aged (fig. 4F) rats. Although the young rats had a slightly higher cumulative rate of co-expression, i.e. about 45% of the BrdU-positive cells also were ED1 positive, compared to a 37% co-expression rate in aged rats, although this difference is not statistically significant.

The reason for the early accumulation of BrdU-positive cells in the lesioned hemisphere of aged rats remains unknown. We speculate that 2 age-associated factors could be important: (1) a decreased plasticity of the cerebral vascular wall [41] and (2) an early, precipitous inflammatory reaction to injury [23]. The increased fragil-

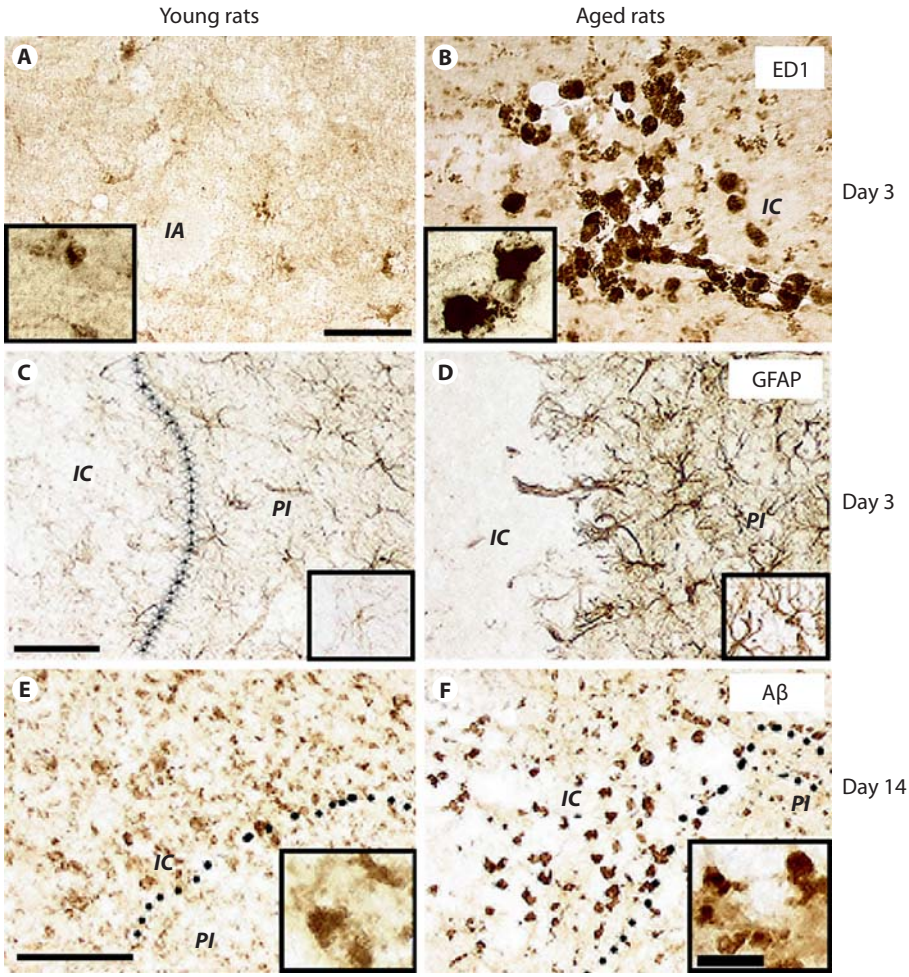

Fig. 5. Comparative analysis of major cellular events in poststroke young and aged rats. **A**, **B** Inflammatory reaction. Early (day 3) appearance of phagocytic cells in the periinfarcted area of aged (**B**) young (**A**) rats. **C**, **D** Scar formation. Astrocytes become activated early after stroke in aged rats (**D**) and contribute to a premature formation of the glial scar. In young rats the astrocytes are much less activated (**C**). **E**, **F** Neurotoxic factors. Immunohistochemical staining of the neurotoxic C-terminal β-APP. The C-terminal β-APP-positive cells increased both in number and staining intensity, a process that was augmented in aged rats on day 14 (**F**) compared to young rats (**E**). IA = Infarct area; IC = infarct core; PI = periinfarct. **A–F** Bar = 50 μm.

ity of aged blood vessels due to decreases in distensible components of the microvessels such as elastin [42] may lead, upon ischemic stress, to the fragmentation of cerebral capillaries that would promote the leakage of hematogenous cells into the infarct area [43, 44].

With double-labeling techniques, the proliferating cells in the aged rats brain after stroke were identified either as reactive microglia (45%), oligodendrocyte progenitors (17%), astrocytes (23%), CD8+ lymphocytes (4%), or apoptotic cells (<1%) [27].

Early, Fulminant Phagocytic Activity of Brain Macrophages in the Postischemic Aged Rat Brain

Pathogenesis of tissue damage is mainly due to inflammatory interactions involving cytokines, chemokines and leukocytes, and to accumulation of neurotoxic factors like the C-terminal fragment of Aβ [11, 23, 26, 32–34]. Unfortunately, there are very few studies done on such factors in post-stroke aged animals.

Upon examining the phenotype, we found that many proliferating BrdU-positive cells also express markers of brain macrophages, such as ED1. The phagocytic activity of brain macrophages may contribute to the early, rapid development of the infarct in aged animals, and is part of the more general inflammatory reaction occurring after stroke [11, 23, 45, 46]. Markedly increased activity of activated microglia/monocytes has also been reported in senescence-accelerated mice following intracerebral hemorrhage [28].

Activated macrophages generate free radicals, the production of which is augmented in aged subjects following cerebral ischemia [47, 48]. A related consideration is that the vulnerability of brain tissue to traumatic injury [49], especially to DNA damage and oxidative stress, also in-

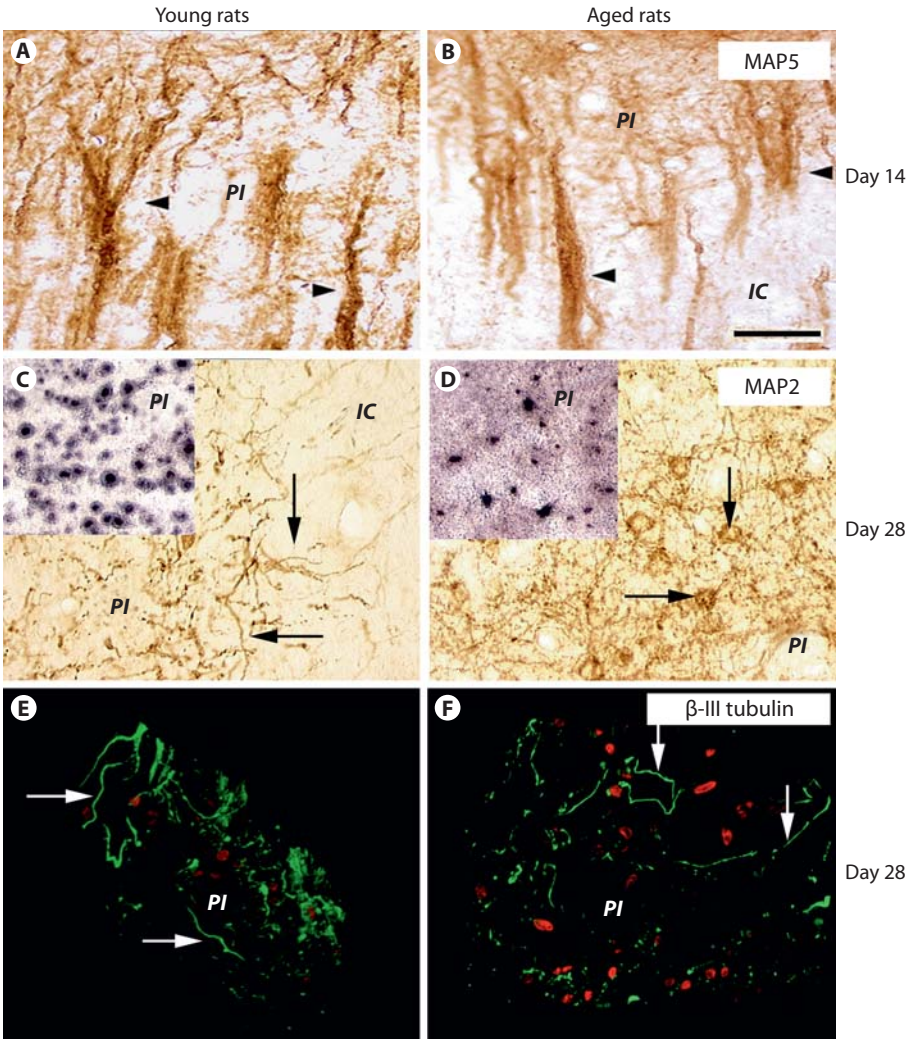

Fig. 6. The regenerative potential of the aged brain is not exhausted. **A**, **B** Note the vigorous expression of MAP1B in the periinfarct of 3-month-old (**A**) and, to a lesser extent, 20-month-old rats (**B**) on day 14 following the stroke. **C**, **D** Similarly, MAP2 protein and mRNAs were upregulated in the periinfarcted area at almost the same levels both in young (**C**) and aged (**D**). The insets show in situ hybridization for MAP2 mRNA in the periinfarcted cortex of post-stroke rats. Note that in young rats (**C**) the transcript levels are higher than in aged rats (**D**). **E**, **F** The axonal growth marker, β-III-tubulin, had lower levels of expression in the periinfarcted area of aged rats (**F**) as compared to young rats (**E**). Morphologically, the axonal branches were thinner in aged rats. Cellular nuclei are in red. IC = Infarct core; PI = periinfarct. **A–D** Bars = 50 μm.

creases with age [17, 50]. Consistent with such observations is our finding of pronounced microglia activation 3 days poststroke in aged (fig. 5B) but not young (fig. 5A) rats [23].

Rapid Delimitation of the Infarct Area by Scar-Forming Nestin- and GFAP-Positive Cells

In aged animals the infarcted area was already visible at day 3 and was circumscribed by a rim of activated astrocytes (fig. 5D). At this time point there was no accumulation of activated astrocytes in the periinfarcted area of young rats (fig. 5C).

The proliferating astrocytes lead to a premature formation of the scar in aged rats, a phenomenon that limits the recovery of function in aged animals. It should be noted that there are at least 3 cell types contributing the formation of the astroglial scar: nestin-positive cells that are the first to delineate the scar in the brains of aged rats (3 days), followed by GFAP-positive astrocytes (7 days) and finally by cells expressing the N-terminal fragment of β-APP (14 days) [23, 26, 51].

Precipitous and Persistent Expression of the Neurotoxic C-Terminal Fragment of β-APP in the Infarcted Area of Aged Rats

Cerebral ischemia promotes conditions that are favorable to the focal accumulation of neurotoxic factors such as Aβ, especially in the aged brain [11]. In aged rats, the

neurotoxic C-terminal fragment of β-APP steadily accumulated over time and reached a maximum on day 14 in aged rats (fig. 5F) compared to young rats (fig. 5E).

Evidence derived from mice expressing the 100-aminoacid carboxy-terminal fragment of β-APP indicates that this fragment may promote synaptic degeneration and neuronal death [51] and impair learning [52]. Notably, the neurodegeneration is accelerated with increasing age [51]. It seems that, in general, an overexpression of β-APP695 in postmitotic neurons results in neuronal degeneration due to intracellular accumulation of this isoform [53].

Regenerative Potential of the Brain Appears to Be Competent up to 20 Months of Age

To explore the potential of older animals to initiate regenerative processes following cerebral ischemia, we studied the expression of the juvenile-specific cytoskeletal protein, microtubule-associated protein 1B (MAP1B); the adult-specific protein, microtubule-associated protein 2 (MAP2); the axonal growth marker, βIII-tubulin, in male Sprague-Dawley rats at 3 months and 20 months of age.

Focal cerebral ischemia, produced by reversible MCAO, resulted in vigorous expression of both MAP1B penumbra of 3-month-old (fig. 6A) and, to a lesser extent, 20-month-old rats (fig. 6B) at 14 days following the stroke [26, 29]. Similarly, MAP2 protein and mRNA were upregulated in the periinfarcted area at almost the same levels both in young (fig. 6C) and aged (fig. 6D). Somewhat lower levels of expression were noted for the axonal growth marker, βIII-tubulin, in the periinfarcted area of aged rats (fig. 6F) compared to young rats (fig. 6E). Collectively, these results suggest that the regenerative potential of the brain at the structural level is competent up to 20 months of age.

Recent studies confirm that mechanisms for self-repair in the young brain also operate in the aged brain. For example, stroke causes increased numbers of new striatal neurons despite lower basal cell proliferation in the subventricular zone in the aged brain [54, 55]. However, despite conserved proliferative activity in the subventricular zone, the number of neurons that reach the injury site is quite modest, as was shown recently for doublecortin-positive neurons in the infarcted area of aged rats [27]. One possible explanation is that lateral ventricle-derived nestin-positive cells do not pass the corpus callosum barrier, and therefore cannot contribute to generation of neurons in the neocortex. Indeed, current evidence indicates that the great majority of newly formed cells in the adult brain are non-neuronal [56–58].

Recent studies also indicate that the molecular profile of growth-promoting genes is very different between aged and young adult groups during the sprouting response to lesions in the CNS. Aged individuals activate most growth-promoting genes at later time-points following stroke than young adults. This includes a delayed induction of GAP43, CAP23 and the growth-promoting transcription factor c-jun. The growth-promoting cell guidance molecule L1 and the CDK5 inhibitor p21 are actually downregulated during the axonal sprouting process in aged individuals compared with a robust and early upregulation of these 2 molecules in young adults [30, 31].

Few neuroprotectants are effective in aged rodents. A major goal of clinical research is to limit the infarct size and one major line of investigation has involved the hypothesis that infarct size is determined by the degree of excitotoxicity. This line of reasoning is based on the observation that excessive concentrations of glutamate can lead to neuronal death.

The failure of multiple clinical trials to demonstrate any neuroprotective efficacy of several glutamate or N-methyl-D-aspartate receptor antagonists has led investigators to search for other potential causative mechanisms. Good candidates are antagonists to the N-methyl-D-aspartate receptor antagonist, e.g. MK-801, and the AMPA receptor antagonist, e.g. NBQX. However, both MK-801 and NBQX were found to be less-effective neuroprotectants in aged than in young rats [59]. Nevertheless, a more recent study showed that treatment of aged rats with sildenafil, a phosphodiesterase type 5 inhibitor used to enhance cGMP-mediated relaxation of pulmonary vasculature, improves functional recovery following stroke in both young and aged rats. This treatment may exert its effects by promoting brain plasticity through enhancement of angiogenesis and synaptogenesis [18].

A more general method of neuroprotection that is efficacious in young rats is ischemic preconditioning. However, the degree of protection was reduced in aged compared to young rats [60]. A likely explanation is that the brains of aged animals showed a reduced stress response that is likely to act neuroprotectively to stroke [17].

Neurosteroids have been recently shown to be effective as neuroprotective agents for ischemic stroke. Treatment with physiological concentrations of estradiol decreases ischemic injury by almost 50% compared to sham-operated controls, in both young and aging rats

[61, 62]. It is possible that the protective function of estradiol in this model is the suppression of apoptosis in the infarct area, resulting in enhanced neuronal survival in the penumbral region of the infarct [61, 62].

The use of stem cells to replace neurons lost after stroke potentially offers a novel approach to treatments aimed at improving recovery of tissue and function [63, 64]. Such a treatment might utilize the endogenous reserves of stem cells located in the subventricular zone or the subgranular zone of the hippocampus. One major concern, however, with any therapy designed to boost neurogenesis following stroke is that the capacity to produce new neurons may be diminished in the hippocampus and olfactory bulb of aged animals [65–71]. Despite this, a cause for optimism is that a variety of treatments, such as environment enrichment [72], administration of growth factors [66, 73] and induction of epileptic seizures [74] can increase the production of new neurons in aged animals, although at a lower level than in young animals. Even more encouraging is a recent study demonstrating the same degree of neurogenesis in the striatum of old and young animals [55]. Even though this study reported lower levels of neuron production by aged animals in the subgranular and subventricular zones, the report of equivalent levels in the striatum indicates that the potential for self-repair following stroke persists in the aged brain. While the use of the organism's own stem cells has many advantages, this technique is in its infancy, and the field still awaits an unambiguous proof of principle.

Another experimental approach that has received considerably more attention is the use of external sources of stem cells. One important question is the type of cells that should be used. Both fetal [75] and murine stem cell lines [76, 77] have been used successfully as grafts to improve functional deficits after experimental stroke in rats. Adult stem cells, such as those derived from human umbilical cord blood, have also proven efficacious [78–81].

The appropriate route of stem cell administration must also be determined. One approach is transplantation either into the lesioned hemisphere, the contralateral hemisphere or both. Other possible targets for stem cell administration are the striatum [55, 77], the cortical parenchyma or the cerebral ventricles [76]. Following unilateral stroke, the grafted stem cells appear to be attracted both to the site of damage and to the corresponding contralateral region, suggesting the existence of both local repair processes and those involved in plastic changes in contralateral motor pathways [76].

An additional second route of administration of stem cells is via the circulation, either intravenously [78, 82–84] or by injection into the carotid artery [85]. The field of stroke therapy using stem cells is a new but promising area, and it is hoped that studies to be carried out in the near future may validate a general therapeutic approach.

Conclusions

These results show that: (1) compared to young rats, 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; (2) in both aged 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 (3) 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.

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Mild systemic inflammation has a neuroprotective effect after stroke in rats

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Abstract

Stroke is accompanied by a strong inflammatory reaction in the brain. Periodontal disease is a chronic local infection which causes a systemic low grade inflammation. We hypothesized that a mild systemic inflammatory reaction as caused by periodontal disease prior to stroke onset, may exert a neuroprotective effect in a rat model of focal ischemia.

To test this hypothesis, marginal periodontitis was induced by ligatures on the second maxillary molars in BB/LL Wistar rats for 3 weeks. Two weeks after periodontitis initiation, focal cerebral ischemia was produced by reversible occlusion of the right middle cerebral artery. After a survival time of 7 days after ischemia, rats were killed and bone loss was determined on the buccal and palatinal surfaces of the defleshed jaw. In addition, markers of systemic inflammation were determined in a different group of laboratory animals at 14 days after the onset of periodontitis. The infarct size and markers of the inflammatory reaction in the brain were determined by immunohistochemistry.

We found: (i) rats with ligatures exhibited significantly more periodontal bone loss than the control rats; (ii) the development of periodontitis was associated with an elevated gene expression for several markers of systemic inflammation (interleukin-10, transforming growth factor beta 1, tumor necrosis factor alpha, interleukin-1beta and interferon gamma; (iii) rats with periodontitis and a mild systemic inflammation had a significantly reduced infarct volume and a significant reduction in the number of brain macrophages in the infarcted area. In conclusion we found that that mild systemic inflammation elicited prior to stroke onset may have a neuroprotective effect in rats by reducing the infarct volume and tissue destruction by brain macrophages.

Key words: animal model, stroke, neuroprotection, experimental periodontal disease, inflammation.

INTRODUCTION

Periodontal disease is an inflammatory disorder caused by a gram negative anaerobic microflora in the dental plaque. Matured dental plaque is associated with an immune-inflammatory response, which causes loss of supporting connective tissue and of alveolar bone. Cytokines may be considered as markers of the progression and severity of periodontitis. In the inflamed gingiva the levels of the cytokines, transforming growth factor beta 1 (TGFbeta1), tumor necrosis factor alpha (TNF alpha), interleukin-1beta (IL-1beta) and interferon gamma (INF gamma) are upregulated and can be detected in the crevicular fluid and antiinflammatory cytokines such as interleukin-10 (Il-10) are downregulated. Associations between periodontal disease and markers of low grade chronic systemic inflammation have been found for C-reactive protein [1-3], serum white blood cells [2], plasma fibrinogen [4], and across different study types including population-based human studies [2-4] and experimental animal studies [1] .

Cerebral ischemia produces a necrotic core in which the blood brain barrier is disrupted. At cellular level, focal cerebral ischemia elicits a strong inflammatory reaction initiated by the activation of astrocytes, resident microglia and perivascular macrophages. After transient ischemia, activated microglia express numerous antigens, including CD4 and major histocompatibility complex (MHC) class I and II antigens, as well as phagolysosomes recognized by the monoclonal antibody ED1. In young animals, microglia in the periinfarct area evolves after 3-7 days into a phagocytosing phenotype [5-7]. It is widely believed that the microglia contributes to the pathophysiology of cerebral ischemia and neuronal cell death [8] and is a major factor that limits functional post-stroke recovery [9].

Ischemic preconditioning is a procedure whereby brief episodes of ischemia are protective against a subsequent, more severe insult [10]. One factor that may mediate the neuroprotective effect of ischemic pre-conditioning is inflammation [11, 12]. Preconditioning with low doses of the proinflammatory agent lipopolysaccharide (LPS) in the rat provides a delayed tolerance and neuroprotection against subsequent challenge via focal ischemia in the brain [13, 14]. Two mechanisms have been put forward to explain the neuroprotective effect of proinflammation, one that depends on inducible NO synthase and peroxynitrite [14, 15], and a second one hypothesizing that LPS preconditioning suppresses neutrophil infiltration into the brain and microglia/macrophage activation in the ischemic hemisphere, which is paralleled by suppressed monocyte activation in the peripheral blood [16]. Therefore, we reasoned that low grade, chronic systemic inflammation, such as that occurring in periodontal

disease, may have a pre-conditioning effect and may be neuroprotective in a rat model of cerebral ischemia.

To test this hypothesis we subjected a rat strain that developed periodontitis and mild systemic inflammation, to cerebral ischemia and measured the infarct volume and several markers of systemic inflammation as well as markers of brain inflammatory reaction after 7 days survival period.

MATERIALS AND METHODS

Animals

Seventy two male BB/LL Wistar rats, 4 months old, were bred and maintained in our own animal facility under strict hygienic conditions and maintained at a 12-h light and dark cycle (5 AM/ 5 PM). The reason for choosing this rat strain is given under Results. All rats had free access to food (Ssniff ®; Soest, Germany) and acidulated water.

Thirty-five rats (one rat died during surgery) received dental ligatures and 37 received the sham procedure for dental ligature (2 died during surgery). Seven animals in each group received sham surgery for middle cerebral artery occlusion (MCAO), in which the artery is surgically exposed but not ligated.

To measure the systemic effect of experimental periodontitis 7 rats received dental ligatures and 7 received the sham procedure for dental ligatures. These rats were sacrified 14 days after the onset of experimental periodontitis to measure systemic inflammation. The experiment was approved by the local animal care committee.

Experimental periodontitis model

To evoke periodontal disease in the maxilla, under general anesthesia silk ligatures (strength 5/0, *RESORBA,* Nürnberg, Germany) were tied around the neck of the second maxillary molar teeth as previously described [17]. Sham controls were rats in which the ligature was put in place around the neck of the second maxillary molar teeth and withdrawn after 1 minute.

The ligatures were left in place until the animals were killed. After decapitation, the maxillae were biologically and chemically defleshed and stained with methylene blue. Using a light microscope with an video camera (OLYMPUS Optical Co., Hamburg, Germany) and an image analysis program (analySIS 3.0, Soft Imaging System, Münster, Germany), an image of the oral and buccal surfaces of the posterior maxillary dentition were captured with a 30X magnification. Standardization of the recordings was assured by fixed reference points on the rat jaws. Ten oral and 10 buccal distances were measured from the cemento – enamel

junction to the alveolar bone crest. Each of 5 oral and buccal distances lay outside or within the placement zone of the ligature. Repeated measurements for this method yielded a relative coefficient of variation of 1.03%.

Surgery

On day 15 after induction of periodontal disease, blood flow through the middle cerebral artery was transiently interrupted as previously described [18].

Throughout surgery, anesthesia was maintained by spontaneous inhalation of 1-1.5% halothane in a mixture of 75% nitrous oxide and 25% oxygen. Body temperature was controlled at 37°C by a Homeothermic Blanket System (Harvard Apparatus). The middle cerebral artery was slowly lifted with a tungsten hook attached to a micromanipulator (Maerzhaeuser Precision Micro-manipulator Systems, Fine Science Tools) until blood flow through the artery was completely interrupted.

The common carotid arteries were then occluded by tightening the pre-positioned thread loops. The surgical field was kept warm and moist with artificial cerebrospinal fluid (Liquicheck^{TM}, Spinal Fluid Control, BioRad) for the duration of the procedure.

The local changes in blood flow were monitored using a laser doppler device placed on the parietal bone (Perimed, Stockholm, Sweden). The occlusion was considered to be successful if the blood flow dropped below 20% of the pre-surgery value. Sham operations were conducted by removing the hook within 60 seconds.

After 90 min the middle cerebral artery and the common carotid arteries were re-opened, allowing full reperfusion of the brain. After survival time of 7 days, the rats were deeply anesthetized with 2.5% halothane in 75% nitrous oxide and 25% oxygen, and perfused with buffered saline followed by buffered, 4% freshly depolymerized paraformaldehyde. The brain was removed, post-fixed in 4% buffered paraformaldehyde for 24 hr, cryoprotected in 20% sucrose prepared in 10 mmol/l phosphate buffered saline, flash-frozen in isopentane and stored at -70°C until sectioning.

Gene expression analysis

Two weeks after ligature, seven test and seven control rats were used for relative gene expression analysis in blood which was obtained by orbital puncture under light anesthesia (Sevofluran, Abbott, Wiesbaden Germany). The total RNA of blood was isolated, transcribed in cDNA and used for real-time PCR as detailed before [19].

Each quantitative PCR was performed in triplicate. Target cDNA was amplified by

primer sets of *Il-10 (*Acc. no. NM_012854, F: 5' CAGAGAACCATGGCCC AGAA 3' R: 5' AGCGTCGCAGCTGTATCCA 3'), *TGF beta1* (Acc. no. NM_021578, F: 5' TGGGCACCATCCATGACAT 3', R: 5' GCAGTTCT TCTCTGTGGAGCTG 3'), *TNF alpha* (Acc. no. NM_012675; F: 5'GACAAGGCTGCCCCGACTA 3'R: 5'AAGGGCTCTTGATGGCAGAGA 3'), *IL-1beta* (Acc. no. NM_031512, F: 5' CACCTCTCAAGCAGAGAGCACAGA 3' R: 5' GGGTTCCATGGTGAAGTCAACT 3'), and *INF gamma* (Acc. no. NM_138880, F: 5' TCGCACCTGATCACTAACTTCTTC 3' R: 5'GTGGGTTGTTCACCTCGAACTT 3').

The rat 18srRNA gene served as the endogenous reference gene. The melting curve was done to ensure specific amplification. The standard curve method was used for relative quantification. For each experimental sample, the amounts of targets and endogenous reference, 18srRNA, were determined from the calibration curve. The target amount was then divided by the endogenous reference amount to obtain a normalized target value.

Immunohistochemistry

Sections (25 µm) were cut on a freezing microtome and processed for immunohistochemistry as free-floating material as previously described [18, 20, 21].

To obtain uniform staining of all tissue sections (about 360 per experiment), we constructed a semi-automated device (PCT, Device assembly for preparing and analyzing tissue for microscopic examinations, DE 199 45 621 A1 and WO 01/22052 A1, 2001) making it possible to process and stain all sections simultaneously under identical conditions. In short, after blocking in 3% donkey serum, 10 mmol/l PBS, 0.3% Tween 20, sections were incubated overnight at 4°C with monoclonal antibodies recognizing, (i) a cytoplasmic determinant of brain macrophages (clone ED1, Serotec, UK); (ii) the neuronal viability marker, mouse anti-NeuN (1:1000, Upstate/Chemicon, CA, USA); (iii) brain edema marker, rabbit anti-aquaporin-4 (AQP4)(1:500, Upstate/Chemicon, CA, USA) diluted in PBS containing 3% normal donkey serum and 0.3% Tween 20. The primary antibody was detected using the ABC system (Vectastain Elite Kit, Vector, Burlingame, CA). Then sections were incubated in a chromogen solution consisting either of nitro-blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 250 µg/ml levamisole in alkaline phosphatase buffer (AQP4 staining) or 0.025% 3',3' diaminobenzidine and 0.005% hydrogen peroxide in 100 mmol/l Tris buffer (pH 7.5)(ED1 and NeuN staining). Finally, the sections were mounted on slides, air-dried, and coverslipped with a xylene-based mounting medium.

Determination of Infarct Volume

To assess the size of the infarct induced by transient focal ischemia, every twentieth section was stained with an immunological marker of neuronal viability (NeuN).

Low power images of the stained sections were taken with a video camera and the images were printed. Then the infarcted area was demarcated using a pencil and the images were scanned. Finally, the area and the partial volume of every twentieth section of the ipsilateral cerebral hemisphere was obtained using the NIH image analysis program. Integration of the resulting partial volumes gave the volume of the ipsilateral hemisphere along with the volume of the cortical infarct [21].

Phenotype quantitation by 3D-reconstruction

Because cell quantitation by stereological methods is not bias-free, the ratio between fully activated macrophages and an activated microglia-like phenotype was determined by counting the cells in volume units employing a 'random-systematic' protocol (random start point for a systematic series of every 10^{th} section through the infarcted volume) using a 3D-reconstruction approach.

To this end, the macrophages were fluorescently labelled using a mouse anti-rat ED1-FITC (1:100, Serotec, UK) primary antibody followed by goat anti-mouse-FITC secondary antibodies as previously described [21]. A sequence of confocal images of 161x242x25 µm, spaced 0.1 µm apart across a 25µm-thick section and covering 30% of the infarcted area, was taken for fluorescently labeled cells [21]. The resulting images were loaded into the 3-D analysis software "Volocity" (IMPROVISION, Coventry, UK) and computed using a Macintosh computer. By rotating the 3-D image, we were able to determine precisely the number of cells per volume unit.

Activated microglial cells and macrophages have distinct morphologies, but the cross-sectional areas of the nuclei are nearly equal; therefore, the distinction between activated microglial cells and phagocytic macrophages had to be undertaken manually by microscopic evaluation. Finally, a mean ratio between the number of cells displaying a macrophage-like and activated microglia-like phenotype was calculated.

Light microscopy

For light microscopy, a Nikon microscope was used. Images (768 x 1024 pixels) were captured electronically using a CCD camera (Nikon, Duesseldorf, Germany). For a group of micrographs, the camera settings for exposure, gain, and contrast enhancement were similar.

Confocal analysis of sections was done using a Nikon Eclipse microscope equipped with a laser device from Visitech (Munich, Germany). Fluorescence signals were detected using a Nikon E800 microscope,

Statistical analysis

The Mann-Whitney U test for unpaired and the Wilcoxon test for paired comparisons were used to compare bone loss from the test and control group. The main effects of treatment (ligature placement) and microglia phenotype were evaluated by one-way analysis of variance analyses using SPSS software (SPSS Inc., Chicago, IL). The level of significance (two-tailed threshold) was set at $p \leq 0.05$. Data are given as means ± standard deviations.

RESULTS

Bone loss

In preliminary experiments we compared ligature-induced bone loss in several rat strains and choose for further experiments the BB/LL Wistar rat strain [22] that showed the greatest bone loss from all the rat strains tested (Fig. 1A)

Bone loss within the ligature effect zone was then compared to bone loss in areas outside the ligature zone in the test (ligature-induced periodontitis) (Fig. 1B) and control rats (Fig. 1C).

In rats killed at 14 days after the onset of experimental periodontitis to determine the systemic level of biomarkers of inflammation, bone loss was significantly greater in the region of "within ligature" than the bone loss in the adjacent tissue, without ligature, or the corresponding region in the control rats (Fig. 1D). After 3 weeks of ligature placement, bone loss around the 2nd molar was even more pronounced and significantly exceeded that from surrounding tissue without ligature as well as that from the control rats (Fig. 1D). In the control group, the bone loss was not significantly different at any time point or region (Fig. 1C, D).

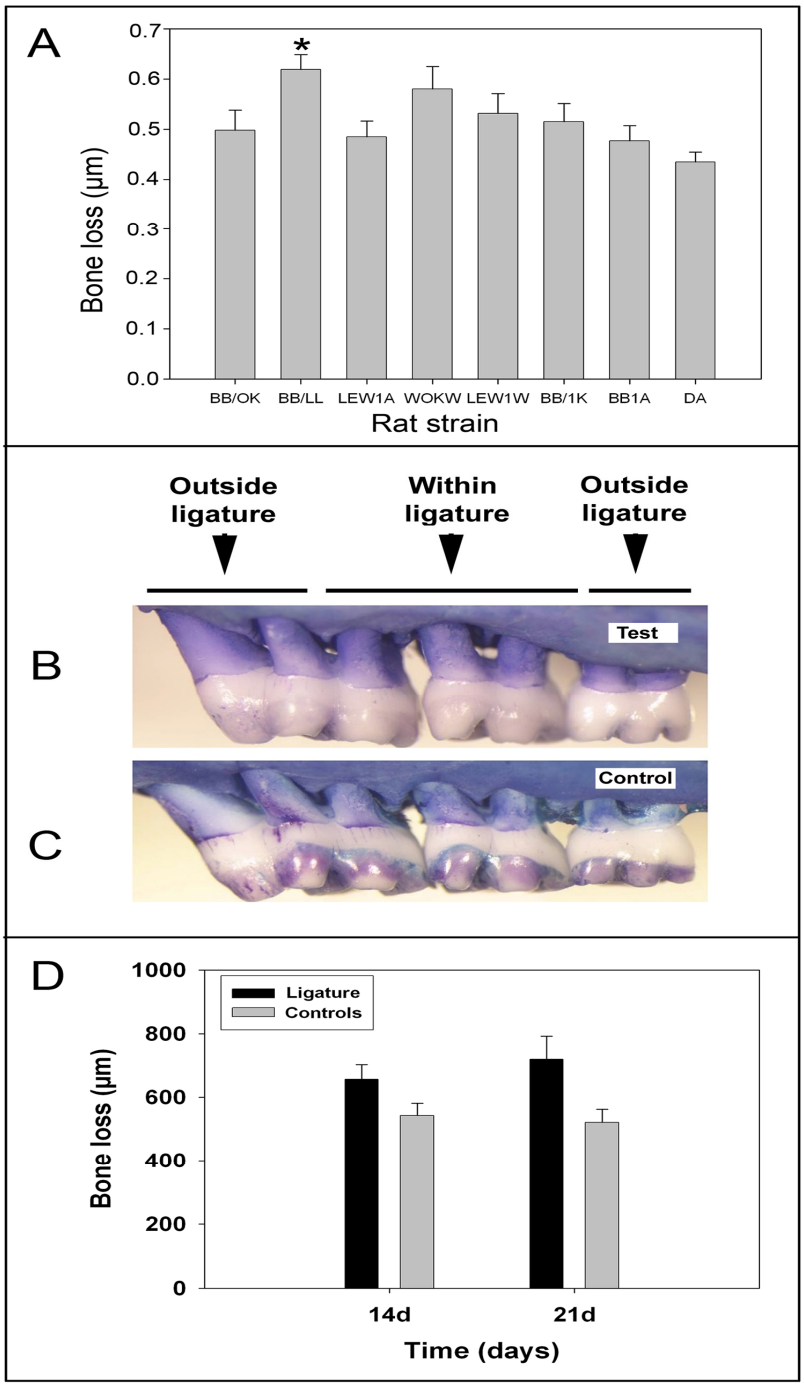

Figure 1

(A) Ligature-induced bone loss in several rat strains**.** Note that bone loss was greatest in the BB/LL Wistar strain, 618 µm ($p < 0.05$). Data are given as mean ± SD.

(B, C): The arrows point either to the "within ligature" region showing bone loss around the 1st and 3rd molar as compared to the "outside ligature" region in test (**B**) and control (**C**) rats.

(**D**): Mean (± SD) bone loss in test (ligature-induced periodontitis) and control rats (without ligature). In rats subjected to periodontitis bone loss after two weeks was significantly more pronounced than in the control group (657 vs. 543µm; $p < 0.019$). After 3 weeks bone loss in the periodontitis group was further increased as compared to control rats (720 vs. 521 µm; $p < 0.01$).

Body weight

Following stroke, the animals ate less and body weight declined progressively. To mitigate problems of feeding in aged animals during the first three days post-stroke, we fed them moistened, soft food pellets. Despite these precautions, some weight loss occurred in the first week following stroke, especially in the control group. There was, however, no significant weight difference between control and ligated rats. Sham-operated rats also lost some weight after surgery, most likely due to surgical stress (Fig. 2A).

Systemic inflammatory cytokines levels

Measurement of inflammatory cytokines expression by RT-PCR revealed that by day 14 the mRNA level of all cytokines was higher in the parodontitis than in the control group. Interleukin-10 (*IL-10*), *TGFbeta1*, and *INF gamma* were statistically significant higher in the test rats than in the control rats. Interleukin 1b (*IL-1beta*) and *TNF alpha* were also higher but did not attain statistical significance (Fig. 2B).

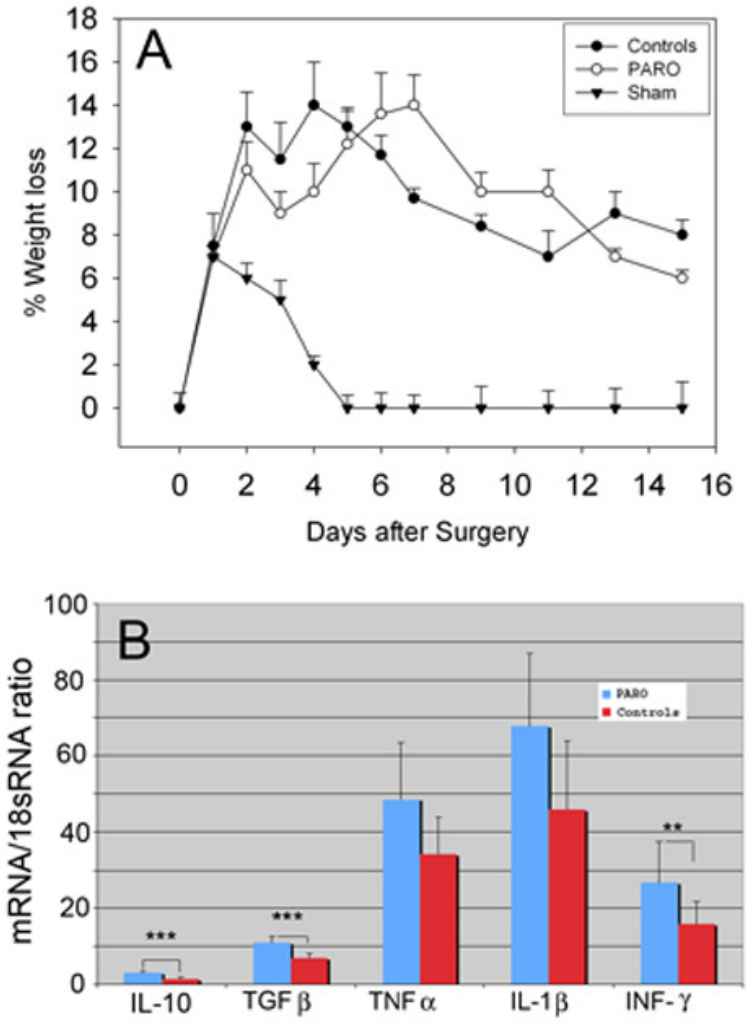

Figure 2

(**A**): Body weight regulation after stroke. Some weight loss occurs in the first week following stroke, however, there was no significant weight difference between control (filled circles) and ligated rats (open circles). Except days 1-3 post-stroke, the body weight of sham-operated rats did not significantly vary after surgery (filled triangles).

(**B**) Measurement of systemic inflammatory cytokines levels by RT-PCR in rats with (PARO) and without ligature (Controls). Note that the mRNA levels for interleukin-10 (*Il-10)*, transforming growth factor beta1 (*TGFbeta1)*, and interferon gamma (*INF gamma)* were statistically significant higher in the periodontitis than in the control rats. Interleukin 1b (*IL-1beta*) and tumor necrosis factor alpha (*TNF alpha)* were also higher but did not attain statistical significance (N =7 control animals; N=7 animals with ligature).

Infarct volume

The infarct core was visualized by immunohistochemistry for NeuN, a sensitive marker of neuronal degeneration [9].

Based on this stain, the areas most affected by stroke were the parietal and, to a lesser extent, posterior frontal cortex. The morphology of viable neurons that stained positively for NeuN in the periinfarct region (PI) was not essentially different between the controls (Fig. 3A, inset) and treated group (Fig. 3B, inset). However, measurement of the infarct volume using NeuN immunohistochemistry revealed a significant 30% decrease in the volume of cortical infarcts in the periodontitis group (Fig. 3C).

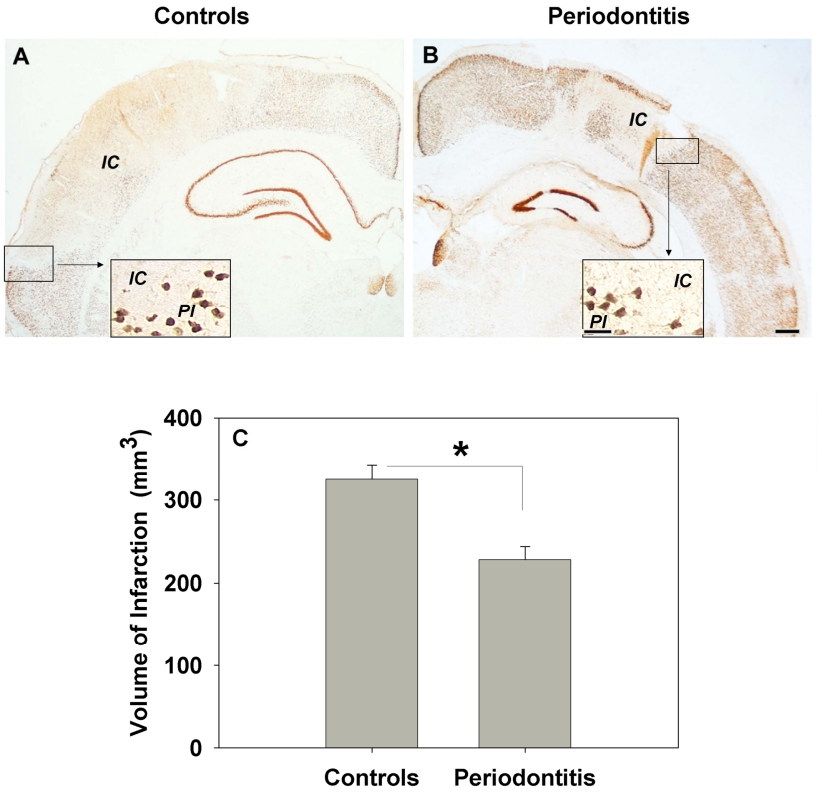

Figure 3. Comparison of infarction volume between the ligation- and control groups.

(**A,B**): Infarct visualization by anti-NeuN immunohistochemistry. The periinfarct region (PI) stained positively for NeuN while the infarct core (IC) was devoid of NeuN immunoreactivity There was not difference in the morphology of neurons in the periinfarcted region between controls (**A**, inset) and treatment group (**B**, inset). However, measurement of the infarct volume using NeuN immunohistochemistry revealed a significant 30% decrease ($p < 0.03$) in the volume of cortical infarcts in the ligation group (**C**). Bars: 400 µm (**A**, **B**); 10 µm (insets). The insets represent an enlarged view of the enclosed area.

Brain edema

Following stroke, water may enter in the periinfarcted area via aquaporin-4 (AQP4) water channels that are localized in astrocyte end-feet membrane domains adjacent to brain capillaries [23] and brain swelling (brain edema) does occur.

Brain edema may influence the volume of the infarcted hemisphere and thus may affect the infarct volume calculation. Since aquaporin-4 expression is related to brain edema, we made an indirect estimation of brain edema by measuring aquaporin-4 expression by semi-quantitative immunohistochemistry at days 1, 3 and 7 post-stroke. At day 1 post-stroke, AQP4 was robustly upregulated in blood vessels in the infarcted area (Fig. 4A). After 7 days, however, AQP4 expression in the periinfarcted area of animals with periodontitis (Fig. 4C) was only slightly above baseline levels of sham-operated animals (Fig. 4B).

By quantitative immuhistochemistry, there was, as expected, a significant increase in AQP protein expression at day 1 as compared to sham controls (Fig. 4D). Thereafter the AQP4 levels in blood vessels of test animals remained slightly above the sham-control levels (1.4-fold)(Fig. 4D). There was no significant difference between the peridontitis and the ligature-control group (Fig. 4D).

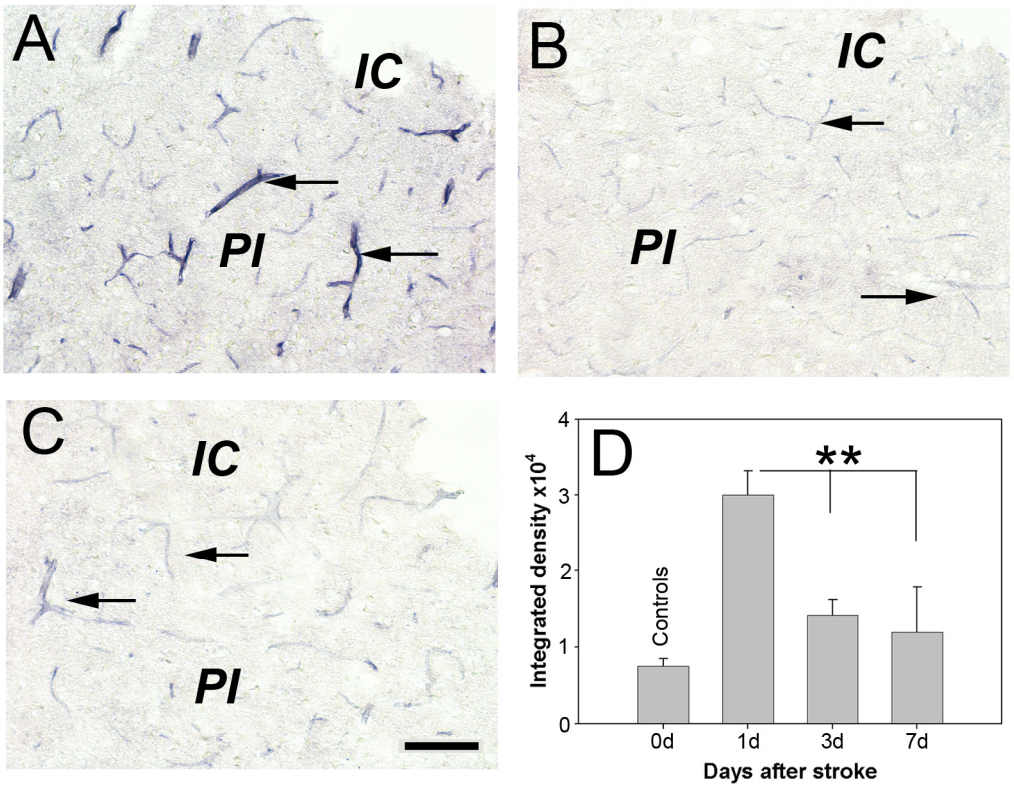

Figure 4. **Time course of aquaporin-4 expression in the infarcted area**.

(**A**): At day 1 post-stroke, AQP4 was robustly upregulated in blood vessels (arrows) in the infarcted area. (**C, D**): After 7 days AQP4 expression in blood vessels in the periinfarcted area of animals with peridontitis (**C,** arrows) was slightly above baseline levels of ligature controls (**B**). By quantitative immunohistochemistry there was a highly significant 4.5-fold ($p < 0.02$) increase in AQP4 protein expression in blood vessels (arrows) at day 1 as compared to sham controls (**D**). There was no significant difference between the peridontitis and the ligature-control group (**D**). Bar, 100 µm. Abbreviations: *IC*, infarct core; *PI*, periinfarct.

Phenotype of microglial cells expressing ED1

Using a monoclonal antibody raised against the cytoplasmic determinant of brain macrophages (clone ED1), we found 2 distinct populations of ED1-positive cells in the infarct core. In the control group (Fig. 5A), the macrophage-like phenotype predominated (Fig. 5C, E), while in the periodontitis group (Fig.5B), the activated microglia-like phenotype predominated (Fig. 5D). We then calculated the ratio between phenotyped cells and found that, in the control group, the macrophage-like cells predominated over the activated microglia-like cells (Fig. 5E). In the periodontitis group, on the contrary, the ratio between the macrophage-like and activated microglia-like phenotypes was reversed (Fig. 5E).

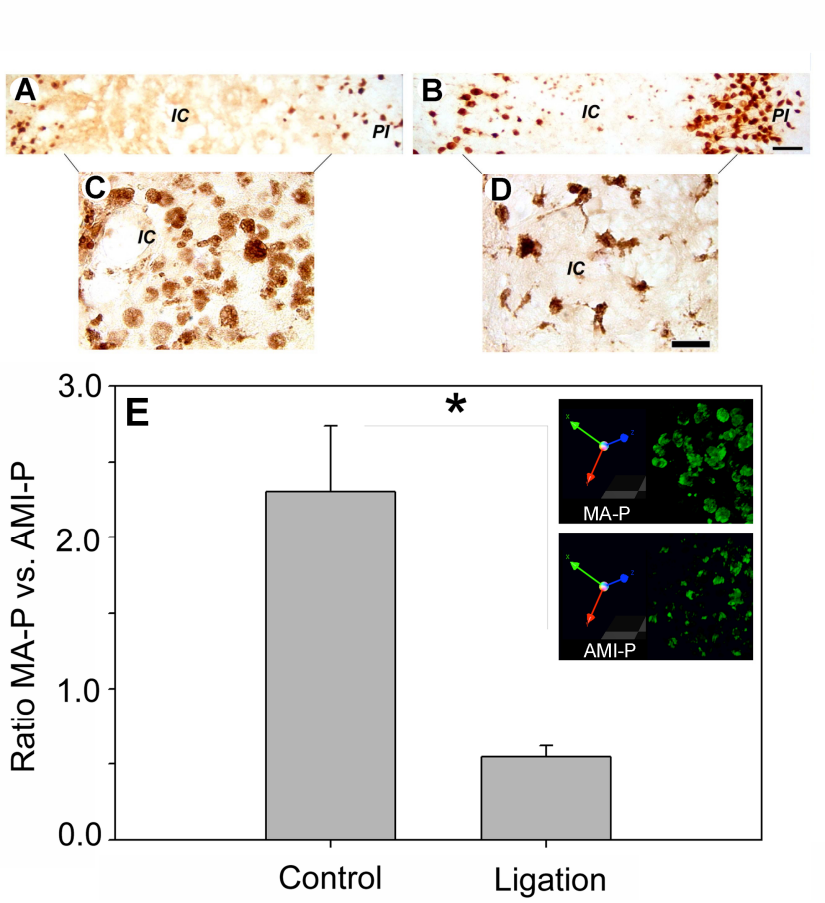

Figure 5. Phenotyping of microglial cells in the infarct core after stroke.

In the control group (**A**), the macrophage-like phenotype predominated (**C**)**,** while in the periodontitis group (**B**) the activated microglia-like phenotype predominated (**D**). In the control group the ratio of macrophage-like to activated microglial cells was 2.2 ($p < 0.01$)(**E**). In the periodontitis group, on the contrary, the ratio between the macrophage-like and activated microglial cells was 0.66 ($p < 0.01$) (**E**). **C** and **D** represent details of **A** and **B**, respectively. Insets illustrate the macrophage- and the activated microglial-like phenotypes.

Abbreviations: ***MA-P***, macrophage-like phenotype; ***AMI-P***, activated microglia-like phenotype. Bars: 50 µm (**A**, **B**); 20 µm (**C**, **D**).

DISCUSSION

Post-stroke inflammation contributes traditionally to worsening of neurological outcome. We found, surprisingly, that a *mild* systemic inflammation elicited prior to stroke in a rat model for periodontitis, has a neuroprotective effect by reducing the infarct volume in a rat model for cerebral ischemia. We hypothesized that the reduction in the infarct volume was due to a reduction in the number of macrophage-like cells that when present cause an enlargement of the infarcted area.

Although animals lost weight after stroke, there was no statistical difference between controls and animals suffering from parodontitis, suggesting that short-term bone loss due to parodontitis does not affect eating.

One possible pitfall of our study is that entry of water into the brain because of compromised blood-brain barrier may have influenced our calculations of the infarcted area. It is well recognized that the aquaporin family of water channel proteins is the major pathway by which water rapidly crosses cell membranes [23]. It was recently reported that AQP4 expression is associated with brain edema [24], a finding confirmed by other studies indicating that AQP4 contributes to the development of brain edema [25, 26]. Transgenic mice expressing reduced AQP4 levels display delayed onset of brain edema following water intoxication [24, 27], further confirming the role of AQP4 in brain edema.

However, we have shown that brain edema, measured indirectly as aquaporin-4 expression, was at its maximum between days 1-3 after stroke, a finding that is in accordance with data in the literature, suggesting that any influence of edema on estimation of infarct volume would be minimal. Even if a small influence of edema remained at the time of our measurements, it would have had no influence on our overall conclusions, since aquaporin-4 levels were similar in ligated and unligated animals.

The tissue damage that ensues following stroke is, to a considerable extent, due to inflammatory reaction involving cytokines, chemokines and leukocytes [9, 18, 28, 29].The phagocytic activity of brain macrophages may contribute to the early, rapid development of the infarct in aged animals, and is part of a more general inflammatory reaction occurring after stroke [9, 18, 30]. In this work using an induced periodontitis model in rats, we showed that the development of periodontitis was associated with a mild systemic inflammation, reflected in elevated levels of *Il-10*, *TGF beta*, *TNF alpha*, *IL-1beta* and *INF gamma* mRNAs.

We hypothesize that a proinflammatory milieu may act protectively by reducing the infarct size. One possible mechanism for this is that preconditioning by systemic inflammation may suppress neutrophil infiltration into the brain and microglia/macrophage

activation in the ischemic hemisphere, which is paralleled by suppressed monocyte activation in the peripheral blood [16].

The phagocytic activity of microglia in the presence of a proinflammatory milieu is likely to be regulated by the balance between pro- and anti-inflammatory cytokines. For example, pretreatment of microglia with interferon-gamma raised the proportion of microglia capable of phagocytosing apoptotic cells to 75% above the untreated controls. In contrast, preincubation of microglia with TNF alpha, TGF alpha and IL-10 did not alter phagocytosis [31] or even inhibited microglial activity [32]. Further, chronic stimulation with lipopolysaccharide (LPS), that mimics a proinflammatory milieu, induced microglial cells to release immunoregulatory and neuroprotective agents (prostaglandin E2, TGF beta, and nerve growth factor), whereas the synthesis of pro-inflammatory molecules (TNF alpha and nitric oxide) was inhibited [33]. Collectively, these data suggest that in our model the proinflammatory milieu before stroke had a protective effect by attenuating the strong inflammatory reaction that occur normally in response to stroke.

Our hypothesis is supported by a recent publication showing that selective ablation of proliferating resident microglia was associated with a marked alteration in the temporal dynamics of proinflammatory cytokine expression, and a significant increase in the size of infarction [34].

Numerous studies have confirmed the neuroprotective efficacy of ischemic preconditioning in animal models [12, 35, 36]. Although it was known that inflammation elicited prior stroke could be a factor mediating the protective effect of ischemic pre-conditioning [11] it was only recently that a neurotoxin like LPS, was successfully used to mimic the ischemic pre-conditioning by modulating the inflammatory response [16].

The association of periodontitis with cerebrovascular events and stroke, has been studied with different designs such as prospective studies [2, 37] and in case–control studies [38, 39]. Our findings seem contradictory to epidemiological studies, in which periodontitis has been linked to coronary heart disease as a potential risk factor. However, it should be kept in mind that long-term (years) chronic periodontitis in humans may contribute, via systemic inflammation, to the occlusion of coronary arteries. Our rat model differed from the human situation in at least two aspects: the occlusion was not due to atherosclerosis-induced thrombosis and the vessel did not undergo any atherosclerotic longterm change. Thus, periodontitis-induced inflammation may not meet the biological conditions necessary to aggrevate the biological consequences of a stroke. Furthermore, in our model the short-term (14 days) systemic inflammation was too short to allow accumulation of arteriosclerotic

plaques that are at the origin of most embolic cortical infarcts.

*In conclusion***,** we have shown that mild systemic inflammation as a result of periodontitis, is an efficacious method to achieve neuroprotection in a rat model of stroke. Further investigations are necessary to understand and dissect the exact mechanisms underlying this effect.

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Eidesstattliche Erklärung

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbständig verfaßt und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät vorgelegt worden.

Ich erkläre, daß ich bisher kein Promotionsverfahren erfolglos beendet habe und daß eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Datum
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DEGREES AND CERTIFICATES

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June 1993: The Medical Council of New Zealand, Wellington, New Zealand: eligible for medical registration after passing the NZREX (New Zealand Medical Registration Examinations)

March 1995- February 1999: The University of Auckland, New Zealand: Master of Health Sciences in Pharmacology with upper class honors

June 1997: Step 1 USMLE (United States Medical Licensing Examination)

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July 1999-June 2003: Resident and Teaching Fellow in Pathology, Boston

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Publikationsliste

POSTERS AND ORAL PRESENTATIONS

1. Freeman L , Lam A, Petcu E , Smith R, Khalil D , Vari F , Catley L, Hart D, Vuckovic S. Development of human multiple myeloma in NOD/SCID mice with multiple bone involvement. Australasian Society for Immunology 37th Annual Scientific Meeting, Sydney NSW, 2-6 December 2, 2007

2. Petcu EB, Sascau M, Sfredel V, Walker LC, Grisk O, Kessler C, Popa-Wagner A. Development of seizure susceptibility is coincident with a tight time window that is required for accumulation of neural progenitors in the dorsal hippocampus. Joint meeting of the German Society of Neuropathology and Neuroanatomy (DGNN) and the Polish Association of Neuropathologists with international participation in conjunction with the 52nd Annual Meeting of the German Society of Neuropathology and Neuroanatomy (DGNN). Greifswald, 5-8 September 2007, Germany. Acta Neuropathologica, Vol 114 (3): 321-322, 2007

3. E Petcu, S Weinstein, N Johnson, A Lam. Oral cancer in the Republic of Kiribati: a case series. First World Congress of the International Academy of Oral Oncology. Amsterdam, The Netherlands, May 17-20, 2007.

4. E B Petcu, MW Assaad, M Hornish, R Lorenzana, RA Goulart. Endometrial biopsy results in patients with endometrial cells on Pap Test utilizing Bethesda System 2001. USCAP 93rd Annual Meeting, Vancouver, BC, Canada, March 6-12, 2004.

5. E Petcu, L Berman, H Farber, J Hayes, F Predescu. Severe Pulmonary Hypertension in a patient with Systemic Lupus Erythematosus complicated by Fenfluramine treatment. The XXIV Congress of The International Academy of Pathology, Amsterdam 5-10 Oct, 2002, Netherlands.

6. E B Petcu, A De Las Morenas. Cytological evaluation of the thyroidian carcinomas using CK 19, galectin and morphometric measurements. "United States and Canadian Academy of Pathology, 91 st Annual Meeting, Feb 23-March 2, 2002, Chicago, USA"

7. EB Petcu, A Gonzalez-Serva. Metastatic prostatic adenocarcinoma with tertiary extramammary Paget's Disease. Journal of Cutaneous Pathology, Vol 28, Oct 2002.

8. Petcu EB, Schug SA. A double blind evaluation of the analgesic effect of a single dose of pamidronate infusion on cancer pain due to bone involvement. "The Australian-NZ

Pain Society Annual Meeting: The Culture of Pain in New Zealand, 21-23 May 1999, Rotorua, New Zealand"

9. Petcu EB, Schug SA. A clinical evaluation of the early analgesic effect of pamidronate in metastatic bone pain. "The II Congress of the European Federation of IASP Chapters: Pain in Europe. 23-27 September 1997, Barcelona, Spain".

10. Petcu EB, Doggrell SA. Atrial hyper-responsiveness to isoprenaline in prehypertensive but not hypertensive rats. J. of Mol. and Cell. Pharmacol. Sept 1996, A27

11. Doggrell SA, Barnett CW, Petcu EB. The effects of BAAM on cardiac responses in normo- and hypertensive rats. "ASCEPT, 29th Annual Meeting. The University of Adelaide, 3-6 December 1995, Adelaide, Australia". Proc. Aust. Soc. Clin. Exp. Pharmacol. Toxicol. 1995; 2:57.

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BOOKS

Short T, Petcu EB. The inhalational anaesthetics. The 10th chapter in Side Effects of Drugs Annual 21, Editor Aronson, Elsevier Science, 1998.

PUBLICATIONS

1. Salajegheh A, Petcu EB, Smith RA, Lam AK. Follicular variant of papillary thyroid carcinoma: a diagnostic challenge for clinicians and pathologists. Postgrad Med J. 2008 Feb; 84(988):78-82

2. Petcu EB, Sfredel V, Platt D, Herndon JG, Kessler C, Popa-Wagner A. Cellular and Molecular Events Underlying the Dysregulated Response of the Aged Brain to Stroke. Gerontology. 2007 Dec 21;

3. Petcu EB, Dunoiu C, Ioana M, Pirici D, Mogoantă L, Popa-Wagner A. Modified variant of the Rieske iron-sulfur protein in the hippocampus of kindled rats and human epileptic patients. Rom J Morphol Embryol. 2007;48(1):17-24

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