Overexpression of Human S100B Exacerbates Brain Damage and Periinfarct Gliosis After Permanent Focal Ischemia

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Background and Purpose—We have previously demonstrated that augmented and prolonged activation of astrocytes detrimentally influences both the subacute and chronic phases of cerebral ischemia. Furthermore, we have suggested that the astrocyte-derived protein S100B may be important in these pathogenic events. However, the causal relationship between S100B and exacerbation of brain damage in vivo remains to be elucidated.

Methods—Using transgenic mice overexpressing human S100B (Tg huS100B mice), we examined whether S100B plays a cardinal role in aggravation of brain damage after permanent middle cerebral artery occlusion (pMCAO).

Results—Tg huS100B mice had significantly larger infarct volumes and worse neurological deficits at any time point examined after pMCAO as compared with CD-1 background strain-matched control mice. Infarct volumes in Tg huS100B mice were significantly increased from 1 to 3 and 5 days after pMCAO (delayed infarct expansion), whereas those in control mice were not significantly altered. S100, glial fibrillary acidic protein, and Iba1 burdens in the periinfarct area were significantly increased through to 7 days after pMCAO in Tg huS100B mice, whereas those in control mice reached a plateau at 3 days after pMCAO.

Conclusions—These results provide genetic evidence that overexpression of human S100B acts to exacerbate brain damage and periinfarct reactive gliosis (astrocytosis and microgliosis) during the subacute phase of pMCAO. (Stroke. 2008;39:2114-2121.)

Key Words: astrocyte ■ delayed infarct expansion ■ focal cerebral ischemia ■ microgliia ■ S100B

Recent evidence suggests that a number of biologically active molecules produced by activated astrocytes may play a dichotomous role in various central nervous system pathologies, including stroke.1,2 Among these molecules, S100B (the ββ form of S100 protein) is an astrocyte-derived protein involved in multiple intracellular processes that functions as a neurotrophic factor.3 S100B is a well-known biomarker for severity of brain damage and has been shown to predict prognosis after ischemic or traumatic brain injury.4,5 In addition, detrimental effects of S100B have been reported, including that S100B (1) at micromolar concentrations produces neuronal damage by causing overexpression of inducible nitric oxide synthase and subsequent release of nitric oxide; (2) can stimulate activation of microglia and astrocytes through a cytokine cycle; and (3) activates nuclear factor-κB, a key transcription factor in inflammatory responses.6

We have proposed that reactive astrocytes play a detrimental role in ischemic brain damage, given the tight association between apoptotic cells and reactive astrocytes in the periinfarct area after cerebral ischemia. Furthermore, astrocyte-derived S100B may be a candidate molecule in this effect because of the following findings: (1) the occurrence of delayed infarct expansion after cerebral ischemia is associated with increased S100B production by activated astrocytes; (2) pharmacological inhibition of astrocytic S100B synthesis by arundic acid leads to significant amelioration of both astrocytic activation and delayed infarct expansion; (3) apolipoprotein E4 knock-in mice exhibit significant aggravation of astrocytic activation and delayed infarct expansion; and (4) this detrimental effect is abolished by pharmacological blockade of S100B biosynthesis by arundic acid.7 Despite these findings, it is important to note more generally that proinflammatory glial activation may promote or oppose brain cell demise depending on the type of brain injury.8

Therefore, we sought to determine the causal relationship between S100B and exacerbation of brain damage. Thus, we...
investigated whether overexpression of S100B in astrocytes exacerbates brain damage and delayed infarct expansion after permanent middle cerebral artery occlusion (pMCAO) using transgenic mice overexpressing human S100B (Tg huS100B mice). Furthermore, we aimed to evaluate whether there is a correlation between the severity of delayed infarct expansion and perifocal gliosis.

**Materials and Methods**

**Animals**

We maintained Tg huS100B mice (The Jackson Laboratory, Bar Harbor, Maine; carrying approximately 10 copies of the human S100B gene under endogenous regulatory control13) as homozygotes on an outbred CD-1 genetic background (Charles River Laboratories, Wilmington, Mass). These mice overexpress S100B in cortical astrocytes by 400% to 600% over CD-1 controls.13

**Surgical Procedures and Neurological Evaluation**

Surgical procedures were in accordance with the guidelines of the Institutional Animal Use Ethics Committee. Anesthesia was induced and maintained with halothane (1.5% to 2% and 0.5%, respectively) in N2O/O2 with spontaneous ventilation. Focal cerebral ischemia was induced by tandem occlusion of the left common carotid artery and distal segment of the middle cerebral artery with normothermic rectal temperature.10 Evaluation of neurological deficits was performed at 24-hour intervals after pMCAO (score 0, no neurological deficit; score 1, forelimb flexion; score 2, decreased resistance to lateral push and forelimb flexion without circling; score 3, same behavior as grade 2, with circling; and score 4, inability to walk spontaneously).14

**Determination of Brain Damage**

A total of 48 mice (Tg huS100B mice, n=24; and CD-1 mice, n=24; 10-week-old males; n=6 at each time point for each mouse line) were randomly allocated for death at 1, 3, 5, and 7 days after pMCAO. Serial parafin sections (5 μm) of the cerebrum were separated by 1-mm intervals were sequentially labeled as sections 1 to 6 (1, bregma +1.78 mm; 2, +0.78 mm; 3, −0.22 mm; 4, −1.22 mm; 5, −2.22 mm; 6, −3.22 mm)15 and stained with hematoxylin & eosin or cresyl violet. The infarct area was measured using Scion Image software (Scion Corp) followed by calculating the number of pixels in regions of interest. In preliminary experiments, we also determined brain damage in sham-operated Tg huS100B and CD-1 control mice. We did not observe any infarct area in these mice, even at 7 days after pMCAO (data not shown).

**Evaluation of Cerebral Vasculature, Physiological Parameters, and Regional Cerebral Blood Flow**

We examined the cerebral vasculature on the brain surface and microvessels in cortical regions of interest that we studied in a separate set of animals (n=10 each) after perfusion with India black ink. Samples for physiological data (PaO2, PaCO2, pH, mean arterial blood pressure, blood glucose, body temperature, and regional cerebral blood flow) were taken from a separate set of animals (n=6 each). After baseline measurement, regional cerebral blood flow was continuously monitored every 5 minutes for 60 minutes with 2 laser-Doppler flow probes (OmegaVate) at 2 points (the core and the periphery of the infarct area) of the dorsolateral cerebral cortex of the ischemic hemisphere.10

**Immunohistochemistry and Image Analysis**

For S100, glial fibrillary acidic protein (GFAP), and Iba1 burden analyses in the perifarct area, additional paraffin sections adjacent to the coronal brain slice at the level of bregma −1.22 mm were used. Rabbit anti-S100, -GFAP, and -Iba1 polyclonal primary antibodies (undiluted; DAKO, 1:500; DAKO, and 1:1,000; WAKO, respectively) and a Vectastain Elite ABC kit (Vector Laboratories) were used. For quantitative analyses, regions of interest within the cortex abutting the outer border of the infarct were subdivided into 5 different subfields, from which images were captured with a threshold optical density that discriminated staining from background and analyzed using SimplePCI software (Compix, Inc Imaging Systems). S100, GFAP, or Iba1 burden in the periinfarct area is presented as a percent (positive pixels/total pixels) calculated for regions of interest. In a separate set of adult (10-week-old) male mice (n=6 for each genotype), we quantified constitutive levels of S100 and GFAP in cortical regions of interest corresponding to those that we studied by average pixel intensity or burden analyses, respectively.

**Statistical Analysis**

Data are presented as the mean±SD. If data were not normally distributed and/or were ordinal level, Kruskal-Wallis H test was performed followed by the Mann-Whitney U test. If data were normally distributed, statistical analysis was performed using analysis of variance followed by Bonferroni or Dunnett T3 methods. In instances of single mean comparisons, t test for independent samples or Mann-Whitney U test was performed. Repeated-measures analysis of variance was used for regional cerebral blood flow data. Bivariate correlations were performed using Pearson’s R. Alpha levels were set at 0.05 (SPSS; SPSS Inc).

**Results**

**Enhanced Brain Damage and Worsened Neurological Deficits in Tg huS100B Mice**

The absence of subarachnoid hemorrhage or intracerebral hematoma was visually confirmed in all animals subjected to pMCAO. Mortality was not encountered before the end of the experiment. Notably, delayed infarct expansion occurred in Tg huS100B mice, as evidenced by significantly increased values when comparing 3 or 5 days to 1 day after pMCAO (#P<0.01 for each comparison). By contrast, infarct volumes of CD-1 mice did not significantly increase with time after pMCAO. Furthermore, at each time point, infarct volumes in Tg huS100B mice were significantly larger than those in control mice (***P<0.01 at 1 day, ***P<0.001 at 3, 5, and 7 days; Figure 1).

In ipsilateral hemispheres of Tg huS100B mice, extensive pallor (the ischemic area), highlighted in hematoxylin & eosin- and cresyl violet-stained specimens, was restricted to the cortices at 1 day after injury and extended partially to the white matter adjacent to the ischemic area.
of the cortex at 3, 5, and 7 days after pMCAO, whereas the ischemic area in control mice was restricted to the cortices at any time point. At every time point, neurological scores were significantly higher in Tg huS100B mice than in control mice (†P<0.01 at 1 to 5 days; *P<0.05 at 6 and 7 days after pMCAO; Table).

**Tg huS100B Mice Do Not Have Altered Cerebral Vasculature, Physiological Parameters, or Regional Cerebral Blood Flow After Ischemia**

To determine if expression of the huS100B transgene resulted in cerebral vasculature developmental abnormality, we injected India black ink into Tg huS100B or CD-1 mice and examined cerebral vessels. In the middle cerebral artery and its territory, no apparent between-groups differences in the formation of the M1 segment, in the branching pattern of the more distal segments, or in distribution of microvessels were observed, except for a minor variation of the branching angle. Moreover, both of the posterior communicating arteries are detected in 2 lines of mice (inset). Arrowhead, posterior communicating artery; PCA, posterior cerebral artery; BA, basilar artery. Scale bar=50 μm. **B, Regional cerebral blood flow after pMCAO in Tg huS100B and CD-1 mice.** Statistical analysis revealed a within-each line of mice main effect of time after pMCAO (**P<0.001 for the core, ***P<0.01 for the periphery). CCAO indicates common carotid artery occlusion; CBF, cerebral blood flow.
each mouse group when considering either the core (**P<0.01) or the periphery (***P<0.001) of the ischemic area, revealing a significant time-dependent decrease in regional cerebral blood flow. However, there was no significant interaction between time after pMCAO and the huS100B transgene when considering either the core or the periphery of the ischemic area, showing no significant difference in regional cerebral blood flow between Tg huS100B mice and controls (Figure 2B).

Increased Periinfarct S100 Burden in Tg huS100B Mice

As expected owing to overexpression of the S100B transgene, constitutive S100 levels in Tg huS100B mice were significantly higher versus CD-1 mice (135±0.6 versus 113±0.5, P<0.001) in cortical regions of interest that we studied. In both the nonischemic areas of the ipsilateral hemispheres and the contralateral hemispheres, S100 expression appeared to be enhanced in Tg huS100B mice compared with controls at any time point examined after pMCAO. S100-expressing astrocytes were observed in both hemispheres (excluding the infarct site) in Tg huS100B and control mice. Numerous minute S100-positive granules in astrocytic processes were dispersed between neurons. At 1 day after pMCAO, astrocytic nuclei in the periinfarct area were moderately positive and their processes were faintly stained for S100 in both lines of mice, probably reflecting the early stage of activation. At 3, 5, and 7 days after pMCAO, both the astrocytic nuclei and swollen processes in the periinfarct area were strongly positive for S100 in both lines of mice, indicating typical reactive astrogliosis. Of note, S100-expressing astrocytes in the periinfarct area were more prominent in Tg huS100B mice than in CD-1 mice at any time point (Figure 3A).

Image analysis revealed that S100 burden in the periinfarct area was significantly enhanced at 3, 5, and 7 days versus 1 day after pMCAO (†††P<0.001 for each mouse line). Moreover, S100 burden in control mice demonstrated a plateau at 3 days after pMCAO, whereas S100 burden in Tg huS100B mice continued to increase until 7 days after pMCAO (###P<0.001 at 3 days versus 5 or 7 days after pMCAO). Importantly, at any time point examined, S100 burden in Tg huS100B mice was significantly larger than in control mice (**P<0.01 for each time point; Figure 3B). Bivariate correlation analysis between infarct area (as determined in section 4) and S100 burden (as determined in a section serially adjacent to section 4) across all days did not reach statistical significance in control mice, whereas it was significant in Tg huS100B mice (r=0.73, P<0.001).

Figure 3. A, Expression of S100 in the periinfarct areas. In both lines of mice, S100 immunoreactivity appears to be enhanced at 3, 5, and 7 days versus 1 day after pMCAO. Most importantly, S100 immunoreactivity appears to be more pronounced in Tg huS100B mice versus CD-1 mice at all time points after pMCAO. Scale bar=50 μm. B, Five open rectangles in the inset of the coronal brain section diagram designate the regions of interest. ***P<0.001 (versus CD-1 mice); †††P=0.001 (versus 1 day); ###P<0.001 (versus 3 days).
Increased Periinfarct Glial Fibrillary Acidic Protein and Iba1 Burdens in Tg huS100B Mice

Constitutive GFAP levels did not show significant differences between Tg huS100B and CD-1 mice (0.84±0.06% versus 0.81±0.05%), showing no greater basal astroglial activation in adult Tg huS100B mouse cortical regions of interest studied. In both the nonischemic areas of the ipsilateral hemispheres and the contralateral hemispheres of Tg huS100B and control mice subjected to pMCAO, GFAP-expressing astrocytes were observed near the pia mater and the small penetrating vessels in the cortex as well as in the white matter, whereas Iba1-expressing microglia (morphologically characterized by small cell bodies and ramified thin processes) were diffusely distributed throughout the entire cortex, white matter, and striatum. Astrocytic GFAP or microglial Iba1 expression was particularly confined to swollen astrocytic or microglial processes and was slightly enhanced at 1 day after pMCAO in both lines of mice. At 3 days after pMCAO, astrocytes and microglia were more pronounced in the periinfarct area and were strongly GFAP-positive or Iba1-positive, respectively. At 5 and 7 days after pMCAO, reactive astrocytes, earmarked by enhanced GFAP expression, hyperplasia, and gemistocytic changes, formed a glial barrier in the periinfarct area. Furthermore, numerous reactive microglia, characterized by enhanced Iba1 expression, hyperplasia, and highly branched processes, were also observed in the periinfarct area, whereas numerous infiltrating macrophages could be found in the outer boundary of the infarct area. GFAP-expressing astrocytes and Iba1-expressing microglia in the periinfarct area were more prominent in Tg huS100B mice than in control mice at 3 to 7 days after pMCAO (Figures 4A and 5A).

Image analysis revealed that GFAP and Iba1 burdens in the periinfarct area were significantly enhanced at 3, 5, and 7 days versus 1 day after pMCAO (†††P<0.001 versus CD-1 mice); †††P<0.001 (versus 1 day); ###P<0.001 (versus 3 days).

![Figure 4. A, Expression of GFAP in the periinfarct areas. GFAP immunoreactivity appears to be enhanced at 3, 5, and 7 days versus 1 day after pMCAO in both lines of mice. Most importantly, GFAP immunoreactivity appears to be more prominent in Tg huS100B mice versus CD-1 mice at 3, 5, and 7 days after pMCAO. Scale bar=50 μm. B, Five open rectangles in the inset of the coronal brain section diagram designate the regions of interest. ***P<0.001 (versus CD-1 mice); †††P<0.001 (versus 1 day); ###P<0.001 (versus 3 days).]
Discussion

The principle finding of our study is that infarct volume, neurological deficits, and periinfarct-reactive gliosis (astrocytosis and microgliosis) during the time interval between 1 and 7 days after pMCAO were significantly aggravated in Tg huS100B mice versus control mice, providing evidence for increased susceptibility of Tg huS100B mice to ischemia.

Regarding increased gliosis in Tg huS100B mice, we noted significant increases in S100, GFAP, and Iba1 burdens in the periinfarct area through to 7 days after pMCAO, whereas control mice reached a plateau at 3 days after pMCAO, suggesting that increased levels of S100B promote periinfarct glial activation after pMCAO. Our interpretation of these findings is that proinflammatory events associated with accelerated glial activation in the periinfarct area may lead to exacerbation of brain damage. In further support of this hypothesis, we found a statistically significant correlation between reactive astrocytosis

Table. Neurological Scores After pMCAO in Tg huS100B and CD-1 Mice

<table>
<thead>
<tr>
<th>Days After pMCAO</th>
<th>Neurological score</th>
<th>Tg huS100B</th>
<th>CD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (n=24)</td>
<td>2 (n=18)</td>
<td>3 (n=18)</td>
</tr>
<tr>
<td>Neurological</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>score</td>
<td></td>
<td>0 22 2 0 0</td>
<td>0 0 14 4 0</td>
</tr>
<tr>
<td>Tg huS100B</td>
<td></td>
<td>0 6 13 0 0</td>
<td>0 5 13 0 0</td>
</tr>
<tr>
<td>CD-1</td>
<td></td>
<td>0 6 8 0 0</td>
<td>0 2 4 0 0</td>
</tr>
</tbody>
</table>

*P<0.05; †P<0.01 (versus CD-1 mice).
and microgliosis along the infarct border and the occurrence of delayed infarct expansion in Tg huS100B mice, but not in control mice. These results provide further support of the idea that enhanced and prolonged activation of glia induces a detrimental influence in the rodent brain during the subacute phase (1 to 7 days) of pMCAO.8,10,11 Our results also lend further support to the hypothesis that the astrocyte-derived protein, S100B, is an important mediator of these deleterious effects.8,11,16 It should be noted that we focused on measuring gliosis in the peri-infarct area because activated glia are most prominent in this region, being much less evident within the infarct itself due to progressive pan necrosis. In addition to our finding of increased S100 burden in Tg huS100B mice in the peri-infarct area after pMCAO, we also noted higher constitutive levels of S100 in these mice versus CD-1 mice. This distinction between constitutive and ischemic brain injury-induced S100 is important, because increased S100 burden at baseline may predispose to worse initial brain damage insult, whereas further inducible S100 burden would likely promote time-dependent exacerbation of brain injury.

Given their classic role in maintaining ionic homeostasis in the brain, astrocytes have long been regarded as “the silent partners of the working brain”. Accumulating evidence, however, has demonstrated that astrocytes may be more active participants in processes, including transport of substances between blood and neurons, cerebral blood flow metabolism control, modulation of synaptic transmission, synaptogenesis, and neurogenesis.2,17 However, astrocytes may also exert detrimental influences in certain central nervous system pathologies. In fact, subacute and chronic central nervous system disorders often have a component of glial activation, as manifested by the infiltration of activated microglia and astrocytes into the region of damaged tissue.12 Reactive astrocytes likely exert their effects in collaboration with activated microglia, which have a harmful influence on the disease process by producing a number of toxic substances, including cytokines, nitric oxide, toxic prostanoids, and reactive oxygen species.18 Like microglia, activated astrocytes produce a myriad of neurotoxic substances in addition to neurotrophic substances.1 Activation of microglia triggers and promotes astrocytic activation through the release of cytokines such as tumor necrosis factor-α and interleukin-1, demonstrating a synergistic relationship between these glial cells through the formation of a vicious positive feedback loop, namely “the cytokine cycle”.19

S100B is a well-known biomarker for severity of brain damage and has been shown to predict prognosis after acute ischemic or traumatic central nervous system injury.5,5 Based on these reports, S100B has been hypothesized to be a candidate molecule in exacerbation of brain damage. The S100 protein family has primarily been implicated in Ca2+-dependent regulation of a variety of intracellular functions, including protein phosphorylation, enzyme activities, cell proliferation/differentiation, dynamics of cytoskeleton constituents, structural organization of membranes, intracellular Ca2+ homeostasis, and protection from oxidative cell damage.3 In addition to these roles, there is evidence that S100B exerts a detrimental influence by overproducing inducible nitric oxide synthase and subsequent release of nitric oxide at high levels.6 By this and other mechanisms, it has been demonstrated that S100B is critically involved in central nervous system inflammatory responses. For example, S100B activates nuclear factor-κB, a key transcription factor in inflammatory responses.7 Furthermore, receptor for advanced glycation end products has been shown to be a receptor for S100/calgranulin-like molecules, and it triggers cellular activation, culminating in the generation of key proinflammatory mediators.20 The fact that the promoter of receptor for advanced glycation end products has functional nuclear factor-κB sites reinforces the likelihood that this might contribute to the pathogenesis of inflammation.21

Somewhat paradoxically, astrocytes and astrocyte-derived S100B have been found to play dichotomous roles in the progression of central nervous system pathologies, although it is still controversial whether such detrimental effects outweigh neuroprotective effects or vice versa. In this regard, there is evidence showing the therapeutic potential of S100B for traumatic brain injury. Specifically, intraventricular S100B infusion induces neurogenesis within the hippocampus, which is associated with enhanced cognitive function after experimental traumatic brain injury.22 Thus, a dual role of activated glia in pro- or anti-inflammatory processes and in mediating repair or disease progression depending on the timing and intensity of the insult appears to exist. In the present study, we provide genetic evidence that overexpression of human S100B significantly enhances ischemic brain damage and exacerbates neurologic deficits after pMCAO in transgenic mice, supporting a detrimental effect of human S100B in ischemic brain damage. In concert with our data, it has been reported that S100B transgenic or knockout mice exhibited worsening and attenuation, respectively, of brain damage induced by perinatal hypoxia-ischemia.23 Notably, the present results showing a significant correlation between peri-infarct reactive gliosis and delayed infarct expansion in Tg huS100B mice are congruent with our previous report using a pharmacological approach to blocking S100B biogenesis,11 lending further support to the notion that enhanced and prolonged peri-infarct gliosis acts to exacerbate ischemic brain damage. Because S100B has been shown to be trophic to glia,24 this may be related to the mechanism underlying hyperplasia of peri-infarct reactive glia after pMCAO in Tg huS100B mice brains. Interestingly, it has been reported that aged rats have accelerated glial reactivity in response to cerebral ischemia, which coincides with stagnation of functional recovery.25 Thus, the worsening of neurological deficits in Tg huS100B mice after pMCAO may represent an event caused by inappropriately accelerated glial responses.

Given that S100B and GFAP are both expressed by activated astrocytes, a tenable hypothesis would be that, similar to overexpression of S100B, GFAP might exert a deleterious effect on ischemic brain damage. In this regard, it has been reported that GFAP-null astrocytes are better substrates for neuronal survival and neurite outgrowth versus wild-type astrocytes26 and that GFAP-null mice exhibit high susceptibility to cerebral ischemia,27 suggesting a protective role of GFAP against cerebral ischemia. Nonetheless, human GFAP overexpressing transgenic mice manifest fatal enceph-
aloapathy, whereas GFAP-null mice show relatively subtle effects on neural development. Thus, although the increase in astrocytic GFAP expression is most likely in response to tissue repair and inflammation, its relationship to S100B expression and brain damage warrants further investigation. In addition, although GFAP plays a major role in glial scar formation around a lesion site as a dominant event, there is evidence that a glial scar–astrocyte phenotype produces inhibitory extracellular matrix molecules such as chondroitin and keratin sulfate proteoglycans, which inhibit axonal and tissue regeneration. Thus, one potential therapeutic strategy might be to minimize the extent of glial scar formation after cerebral ischemia. In support of this idea, cotreatment of antibodies to transforming growth factor-β1 and -β2 resulted in reduction of glial scar formation.

In conclusion, we have provided genetic evidence that overexpression of human S100B aggravates brain damage after pMCAO, which is associated with enhanced reactive gliosis (astrocytosis and microgliosis) in the periinfarct area. Such an association between enhanced brain damage and greater perinifarct reactive gliosis may allow for exploring the putative etiologic contribution of reactive glia to exacerbation of ischemic brain damage as well as the mechanism underlying the detrimental influence of S100B.

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**Disclosures**

None.

**References**

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