Increased Severity of Hemorrhage in Transgenic Mice Expressing Cerebral Protease Nexin-2/Amyloid β-Protein Precursor

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Background and Purpose—Secreted isoforms of amyloid β-protein precursor (AβPP) that contain the Kunitz proteinase inhibitor domain, also known as protease nexin-2 (PN2), are enriched in brain. Although little is known of its physiological function, the potent inhibition of certain prothrombotic proteinases by PN2/AβPP suggests that it may function to regulate cerebral thrombosis during vascular injury events.

Methods—To examine the antithrombotic function of cerebral PN2/AβPP in vivo, we performed measurements of carotid artery thrombosis and experimental intracerebral hemorrhage in transgenic mice with specific and modest overexpression of PN2/AβPP in brain. Comparisons were made with wild-type mice and Tg-rPF4/APP mice, a model that possesses specific and modest overexpression of PN2/AβPP in platelets and exhibits reduced thrombosis in vivo.

Results—Modest overexpression of PN2/AβPP in transgenic mouse brain had no effect on intraluminal carotid arterial thrombosis but resulted in larger hematoma volumes and hemoglobin levels (23.1±2.7 mm³ [n=6; P<0.01] and 1411±202 µg/hemisphere [n=12; P<0.01], respectively), compared with wild-type mice (15.9±2.2 mm³ [n=6] and 935±418 µg/hemisphere [n=12], respectively).

Conclusions—These findings indicate that cerebral PN2/AβPP plays a significant role in regulating thrombosis in brain and that modest age-related increases in the cerebral levels of this protein could markedly enhance the extent of cerebral hemorrhage. (Stroke. 2007;38:2598-2601.)

Key Words: β-amyloid precursor protein ♦ intracerebral hemorrhage ♦ proteinase inhibitor ♦ thrombosis ♦ transgenic

The amyloid β-protein precursor (AβPP) is most often recognized as the parent molecule to the amyloid β-protein (Aβ) that accumulates in the brains of patients with Alzheimer disease and related disorders. Although much has been elucidated regarding the proteolytic processing of AβPP involved with liberating Aβ, little remains known of AβPP function. AβPP is expressed predominantly as 3 isoforms of increasing length (695, 751, and 770 amino acids) with the 2 larger containing a Kunitz proteinase inhibitor (KPI) domain. Secreted KPI-containing 751/770 isoforms of AβPP are analogous to the cell-secreted proteinase-inhibitor known as protease nexin-2 (PN2). PN2/AβPP is abundant in brain and its expression increases with age in the cerebral compartment.

PN2/AβPP is a potent inhibitor of several key prothrombotic proteinases. In addition to brain, PN2/AβPP is abundantly expressed in circulating blood platelets. Recently, we demonstrated that modest overexpression of PN2/AβPP in the platelets of transgenic mice results in decreased thrombosis in vivo. Although these results support the function of platelet PN2/AβPP in regulating thrombosis in vivo the potential role of brain PN2/AβPP in limiting cerebral thrombosis is unclear.

To determine whether elevated cerebral PN2/AβPP levels in mice are associated with an increased severity in hemorrhage, transgenic mice that specifically and modestly overexpress PN2/AβPP in brain were quantitatively analyzed in experimental models of carotid artery thrombosis and intracerebral hemorrhage and were compared with transgenic mice that modestly overexpress PN2/AβPP in platelets and wild-type mice.

Materials and Methods

Transgenic Mice
All work with animals followed National Institutes of Health guidelines and was approved by the Stony Brook University Institutional Animal Care and Use Committee. The generation and characterization of transgenic mice specifically expressing PN2/AβPP either in platelets under control of the rat platelet factor 4 promoter (Tg-rPF4/APP) or in neurons in brain under control of the mouse Thy1 promoter (Tg-Thy1/APP) were recently described.

In all experiments transgenic and wild-type mice were 3 months of age and all on a pure C57Bl/6 background.
Quantitative Immunoblot Analysis for PN2/AbPP
The levels of human PN2/AbPP and total PN2/AbPP (mouse+human) in isolated mouse platelet or forebrain homogenates were determined by quantitative immunoblotting using monoclonal antibody (mAb) P2-1 that is specific for human AbPP. PN2/AbPP is expressed only in platelets of Tg-rPF4/APP mice or brain of Tg-Thy1/APP mice. Immunoblot analysis of total (mouse+human) PN2/AbPP in platelet (B) or brain (E) homogenates prepared from different mice using mAb22C11 that recognizes both human and mouse AbPP as described.9,10

Carotid Artery Thrombosis
This technique was performed according to the protocol of Eitzman et al11 as recently described.9

Experimental Intracerebral Hemorrhage
This procedure was performed according to the protocol of Clark et al.12 Briefly, 500 nL of bacterial collagenase/saline (150 U/mL) was stereotaxically delivered to the caudate/putamen at a depth of 4.0 mm unilaterally to promote collagen degradation leading to vessel rupture. Eighteen hours after initiation of hemorrhage the mice were perfused with PBS, the brains were harvested and then analyzed for hematoma volumes as described.9 Alternatively, harvested perfused brains were divided midline sagitally and the hemoglobin levels were determined in the hemorrhage and contralateral hemispheres using a spectrophotometric assay as a measure of the extent of hemorrhage in the lesioned hemispheres of the mice.13

Statistical Analysis
Comparison of results obtained from transgenic mice and wild-type mice were analyzed by 1-way ANOVA between the groups followed by post-hoc pair-wise Tukey-Kramer tests at the 0.05 significance level using GB-Stat 6.5 software.

Results
Analysis of transgene-encoded human PN2/AbPP expression in the different transgenic lines confirmed that the protein is specifically expressed in platelets in the Tg-rPF4/APP mice (Figure 1A) and in brain in the Tg-Thy1/APP mice (Figure 1D). Quantitation of PN2/AbPP expression in each transgenic line showed a comparable modest level of overexpression of the protein that only approximately doubled the amount of PN2/AbPP in platelets in the Tg-rPF4/APP mice (Figure 1B and 1C) or in brain in the Tg-Thy1/APP mice (Figure 1E and 1F).

To determine the effect of modest brain parenchymal increases in PN2/AbPP on intraluminal arterial thrombosis we performed a quantitative carotid artery thrombosis procedure in Tg-Thy1/APP mice. In all experiments, Tg-rPF4/APP...
mice were also included because we have recently shown that they exhibit reduced thrombosis in vivo. In contrast to Tg-rPF4/APP mice, the Tg-Thy1/APP mice exhibited no effect and were indistinguishable from wild-type mice in the time to vessel occlusion and cessation of blood flow (Figure 2). This indicates that modestly elevated PN2/αβPP levels in the brain parenchyma do not influence thrombosis occurring within the lumen of an intact vessel of Tg-Thy1/APP mice.

We next determined the effect of increased brain parenchymal PN2/αβPP levels on the severity of experimental intracerebral hemorrhage. In this case, the hematoma volumes were significantly larger (P<0.01) in both Tg-Thy1/APP mice (23.1±2.7 mm³) and Tg-rPF4/APP mice (24.4±4.7 mm³) compared with wild-type mice (15.9±2.2 mm³) (Figure 3A through 3D). Similarly, the hemoglobin content of the hemorrhagic brain hemispheres was significantly higher in Tg-Thy1/APP mice (1411±202 μg/hemisphere, P<0.01) and Tg-rPF4/APP mice (1830±306 μg/hemisphere, P<0.01) compared with wild-type mice (946±418 μg/hemisphere) as an independent measure of hemorrhage severity (Figure 3E). This finding indicates that when cerebral vessel rupture occurs modest increases in brain parenchymal PN2/αβPP can significantly reduce thrombosis and increase the severity of hemorrhage.

Discussion
In the present study, we show that the Tg-Thy1/APP mouse is a useful model to investigate the effects of modestly increased brain PN2/αβPP levels in regulating thrombosis during cerebral vascular injury. Cerebral PN2/αβPP levels are reported to increase with age and after brain injury. Increased brain parenchymal PN2/αβPP had no effect on carotid artery thrombosis because it is inaccessible to prothrombotic mediators that are activated in the intact vessel lumen. On the other hand, with vessel rupture during experimental intracerebral hemorrhage extravasated prothrombotic proteinases are now exposed to both cellular and secreted PN2/αβPP in the brain parenchyma resulting in inhibition and more extensive bleeding. Aβ peptide, the amyloidogenic fragment of AβPP, can affect cerebral blood flow. Although we have not been able to detect Aβ in Tg-rPF4/APP mice, Tg-Thy1/APP mice show very small amounts of soluble Aβ in brain parenchyma. Whether this would have an effect on cerebral blood flow during experimental hemorrhage is presently unknown. The findings in this study suggest that small increases in brain PN2/αβPP levels can have a significant impact on cerebral thrombosis and are consistent with age- and disease-related increases of this protein in brain that may contribute to the severity of hemorrhage.

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Disclosures
None.

References
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