Short communication

Roles of urokinase type plasminogen activator in a brain stab wound

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Abstract

Urokinase type plasminogen activator (uPA) may influence brain pathophysiology after injury. We studied disruption of the blood–brain barrier (BBB) and changes in the vasculature after a brain stab wound in uPA-deficient, uPA receptor-deficient, and PA inhibitor-1 (PAI-1) deficient mice. The extravasation of immunoglobulin was greater in PAI-1 deficient mice; less pronounced in uPA-deficient mice; similar to controls in uPA receptor-deficient mice. Vasculatures in the wound proliferated in PAI-1 deficient mice. Our study shows that uPA affects BBB disruption. PA enhances angiogenesis after brain injury.

Theme: Disorders of the nervous system

Topic: Trauma

Keywords: Blood–brain barrier; Brain edema; Knock out mouse; Microglia; Plasminogen activator; Urokinase

Extracellular matrix (ECM) proteins provide structure, regulate the micro-environment in the brain, and maintain the blood–brain barrier (BBB). For the healing process to start after injury, degradation of the ECM by proteases is necessary [15]. Therefore, after sustaining an injury, brain tissues express increased levels of proteases to break down the ECM [11]. Collagen type IV, one of the ECM proteins, maintains the basement membrane of blood vessels in brain tissue. The breakdown of the basement membrane by proteases such as type IV collagenases may result in disruption of the BBB leading to brain edema, hemorrhage. Plasmin is converted from plasminogen by plasminogen activators (PA). Because PA and plasmin contribute to the conversion of inactive type IV collagenases [8], the expression of PA may trigger a cascade of extracellular proteolysis. There are two kinds of PA, tissue-type PA (tPA) and urokinase-type PA (uPA). Usually, tPA has a high affinity for fibrin and contributes to intravascular fibrinolysis. On the other hand, uPA binds to uPA receptors on the surface of various types of cells and contributes to cell-mediated fibrinolysis. However, the role of uPA in brain injuries remains unclear. In this study we investigated the role of uPA, uPA receptor (uPAR), and PA inhibitor-1 (PAI-1), a major inhibitor of uPA, in experimental brain stab wounds.

Mice deficient in uPA, uPAR, and PAI-1 were produced by standard gene targeting in embryonic stem cells. Details have been published elsewhere [3]. Ten-week-old mice of both sexes were used. Age-matched male or female C57/BL6 mice were the controls. The animals were anesthetized with an intraperitoneal injection of 400 mg/kg chloral hydrate. The head of each mouse was fixed in a stereotaxic instrument. A small burr hole was then drilled in the skull at a site on the bregma and 2.0 mm lateral to the sagittal suture on the right side. A stainless needle (0.5 mm diameter with a sharpened tip) was gently inserted through the hole into the cortex and the striatum to a depth of 2.5 mm from the cortical surface. A stereotaxic brain atlas of the mouse was consulted for precise positioning of the experimental brain stab wound [9]. At 3, 8 and 15 days...
after placing the stab wound, the mice were deeply anesthetized with sodium pentobarbital, and their brains were fixed by transcardiac perfusion with heparinized physiological saline and ethanol acetic acid solution (ethanol:glacial acetic acid mixture 6:1, v/v). The brains were removed and processed for paraffin embedding using standard histological techniques. All brains were sectioned coronally at 6 μm thickness. Sections including the site of the stab wound were submitted for immunohistochemistry. We used monoclonal rat anti-F4/80 as a marker of macrophage/microglia (Serotec, Oxford, UK), polyclonal rabbit anti-mouse collagen type IV (LSL, Tokyo, Japan), polyclonal rabbit anti-human von-Willebrand factor (Factor VIII-related antigen) (DAKO, Carpinteria, CA, USA) and biotinylated polyclonal swine anti-mouse immunoglobulin (DAKO). For visualization of the primary antibody, we used the LSAB® 2 Kit (DAKO), alkaline phosphatase–streptavidin complex. Then the brain sections were incubated for 10 min at room temperature with non-specific binding-blocking solution (DAKO). Sections mounted on glass slides were incubated with these primary antibody solutions or a control solution containing non-specific rat or rabbit immunoglobulin for 60 min at room temperature. After washing with buffer solution, all sections except those treated with biotinylated polyclonal swine anti-mouse immunoglobulin were incubated with biotinylated anti-rabbit or anti-rat immunoglobulin solution for 30 min at room temperature.

The specimens were then incubated with the alkaline phosphatase–streptavidin complex solution for 10 min at room temperature. For visualization of alkaline phosphatase, we used the New Fuchsin System® (DAKO). Hematoxylin was used for counterstaining. The areas stained with anti-mouse immunoglobulin were quantitatively evaluated using a computer-assisted image-analyzing system. The degree of von Willebrand factor immunoreactive tubular structures was scored; 0, nearly similar to the contralateral cortex (<10 immunoreactive tubular structures/0.3 mm²); 1, moderately increased in number of von Willebrand Factor-positive tubular structures compared to the contralateral cortex (10–25 immunoreactive tubular structures/0.3 mm²); 2, remarkably increased in number of von Willebrand Factor-positive tubular structures (>25 immunoreactive tubular structures/0.3 mm²).

In control mice, marked extravasation of immunoglobulin was observed at 3 days after lesioning; it declined at 8 and 15 days after lesioning. In uPA-deficient mice, the extravasation of immunoglobulin was significantly smaller compared to the controls at 3 days. In PAI-1-deficient mice, there was marked immunoglobulin extravasation at 3 and 8 days. In uPAR-deficient mice at 3 days post-injury, immunoglobulin staining was similar to that seen in control animals, however it was remarkably reduced at 8 days (Table 1). Immunohistochemistry against collagen type IV showed characteristic irregular staining and the proliferation of microvasculature surrounding the lesion in 80% of PAI-1 deficient mice and in about 14% of the control mice at 8 days post-injury (Fig. 1 and Table 2).

<table>
<thead>
<tr>
<th>Areas of immunoglobulin extravasation (mm²)</th>
<th>3 days</th>
<th>8 days after lesioning</th>
<th>15 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.5±1.4 (n=6)</td>
<td>1.0±0.6 (n=7)</td>
<td>0.4±0.7 (n=7)</td>
</tr>
<tr>
<td>PAI-1 deficient</td>
<td>4.2±0.8 (n=5)</td>
<td>3.2±1.3* (n=5)</td>
<td>0.7±0.8 (n=6)</td>
</tr>
<tr>
<td>uPAR deficient</td>
<td>3.0±1.6 (n=5)</td>
<td>0.6±0.9 (n=5)</td>
<td>0.2±0.5 (n=5)</td>
</tr>
<tr>
<td>uPA deficient</td>
<td>1.0±0.8* (n=4)</td>
<td>1.0±1.0 (n=5)</td>
<td>0.0±0.0 (n=4)</td>
</tr>
</tbody>
</table>

Table 1

*P<0.05 (Mann–Whitney U-test) vs. controls. Values are mean±S.D.

Several inhibitors of PA are known such as PAI-1, PAI-2, and PAI-3. Among them, PAI-1 most effectively inhibits the proteolytic activity of PA. Lipopolysaccharide induces an increase in the production of PAI-1 by macrophages [6]. mRNAs encoding uPA and PAI-1 are elevated in the mouse brain following seizure after intraperitoneal excitotoxin injection [7]. Injury may provoke an increase in the production of proteases in brain tissue [12]. We observed that the degree of immunoglobulin staining in uPA-deficient mice was reduced compared to control mice. We also noted remarkable extravasation of immunoglobulin surrounding the stab wound in PAI-1 deficient mice. These findings suggest strongly that PA and tissue fibrinolysis are involved in the traumatic BBB disruption and that PAI-1 inhibits its spread. Similarly, excess matrix metalloproteinases lead to cerebral hemorrhage and brain edema, and their inhibitors block the development of secondary brain injury [13].

There are two kinds of PA: tPA and uPA. Tsirka et al. [16] demonstrated that tPA was necessary for excitotoxin-induced hippocampal neuronal death. Upon excitotoxin injection, tPA converts plasminogen to plasmin which degrades neuronal laminin [4]. The destruction of neuronal laminin leads to neuronal cell death. In ischemic brain tissues, tPA increases brain damage in relation to excess glutamate [17]. Striato-pallidal destruction results in excess glutamatergic inputs to the substantia nigra pars reticulata.
Fig. 1. Left, immunostaining against collagen type IV of PAI-1 deficient mouse 8 days after the stab wound. Note irregular and proliferating collagen type IV positive microvasculature (arrows). Right, immunostaining against F4/80 antigen of PAI-1 deficient mouse 8 days after the stab wound. Microglia cells and/or macrophages accumulated in the same area where the irregular and proliferating microvasculature is observed. Bar=50 μm.

Table 2
Number of mice with irregular collagen type IV (+) microvascular structures

<table>
<thead>
<tr>
<th></th>
<th>3 days</th>
<th>8 days after lesioning</th>
<th>15 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 (n=6)</td>
<td>1 (n=7)</td>
<td>0 (n=7)</td>
</tr>
<tr>
<td>PAI-1 deficient</td>
<td>0 (n=5)</td>
<td>4* (n=5)</td>
<td>2 (n=6)</td>
</tr>
<tr>
<td>uPAR deficient</td>
<td>0 (n=5)</td>
<td>0 (n=5)</td>
<td>0 (n=5)</td>
</tr>
<tr>
<td>uPA deficient</td>
<td>0 (n=4)</td>
<td>0 (n=5)</td>
<td>0 (n=4)</td>
</tr>
</tbody>
</table>

* P<0.05 (Fisher’s exact probability test) vs. controls.

(SNR). This excess glutamate is closely related to the transneuronal degeneration of the SNR [14]. However, we recently reported that tPA did not contribute to the development of transneuronal degeneration in the SNR following a striato-pallidal lesion [5]. Brain tissue tPA was expressed only in young mice, its expression rapidly declined as the animals grew older [1]. In damaged brain tissues, the exact role of tPA remains unclear. Unlike tPA, uPA was consistently detected in homogenized adult brain tissue [1]. uPA and fibrinolytic activities are regulated and accelerated in the cell surface level via uPA receptor. However, our study indicated that in uPA receptor-deficient mice and in the controls, the same degree of BBB disruption was observed shortly after lesioning; it was remarkably reduced by the 8th day post-injury. By comparison, in uPA-deficient mice, the degree of BBB disruption was less pronounced at 3 days after the stab wound. This shows that uPA enhances the pathophysiology of brain injury in the acute stage, and uPA works without binding to its receptors in uPA receptor-deficient mice. Similarly, it is reported that uPA supplies sufficient proteolytic activities to clear fibrin deposits from the tissues in the absence of uPA receptor [2].

In PAI-1-deficient mice 8 days after lesioning, the number of von Willebrand factor-positive vasculatures significantly increased, and strong irregular staining against collagen type IV was observed. The irregular staining against collagen type IV may reflect the proliferation of microvasculature. If this structure reflects the breakdown of the microvasculature, it should be observed at 3 days after lesion placement. These findings suggest that absence of PAI-1 facilitates angiogenesis. Microglia and/or macrophages accumulated in the irregular, proliferating microvasculature at 8 days after brain injury. Previous in vivo studies showed that microglia secreted uPA and expressed uPAR [10,18]. Roles of microglia on the angiogenesis after brain injury should be elucidated in relation to PA.

The present study clearly shows that uPA contributes to the development of secondary brain damage due to the breakdown of the microvascular ECM, and that uPA may work without binding to its receptor in the acute stage.
PAI-1 inhibits traumatic brain edema. On the other hand, plasminogen activator may enhance the angiogenesis necessary for the healing process after brain injury.

References


