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Altered expression of tissue-type plasminogen activator and type 1 inhibitor in astrocytes of mouse cortex following scratch injury in culture

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Abstract

The expression of plasminogen and plasminogen activators (PG/PAs) in reactive astrocytes was examined following scratch injury. In response to injury, casein-degrading activity could be observed around astrocytes. The protein expression of tissue-type plasminogen activator (tPA) was up-regulated, while the free form of urokinase-type plasminogen activator (uPA) was not detected. Consistent with these findings, results obtained with zymograph assay also revealed that tPA activity, but not uPA activity, was up-regulated. Moreover, the addition of 6-amino-caproitic acid (EACA) to casein-covered astrocytes significantly prevented the recovery of the injured astrocytes in a dose-dependent manner. Taken together, our data demonstrate that the expression of PG/PAs in cultured astrocytes is regulated following injury, suggesting that caseinolytic activity is an essential component during the process of astrocyte recovery.

Keywords: Astrocytes; Regeneration; Tissue-type plasminogen activator; Urokinase-type plasminogen activator; Type 1 plasminogen activator inhibitor; 6-Amino-caproitic acid

Astrocytes constitute a structural and supporting framework for nerve cells in the central nervous system (CNS), and serve an important role in neuromodulation as well as in transmitter uptake [14]. In response to insults such as brain injury, astrocytes are activated to proliferate and fill the damaged areas. The activation of astrocytes is especially important during the early stage of injury since it can rapidly and effectively restore the integrity of brain tissue. This kind of reactivity exemplifies the intriguing capacity of plasticity in the mature brain, i.e. repairing and remodeling the cell–cell communication following injury.

Plasminogen/plasminogen activators (PG/PAs) have been extensively studied in many cellular processes that are largely based on their ability to elicit limited proteolysis in the extracellular matrix [9]. Mammalian cells produce two molecular forms of PAs, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Astrocytes have been demonstrated to synthesize both of these two forms of activators, the expression of which can be developmentally regulated [3,7,10]. uPA is expressed solely in the immature astrocytes while tPA persists in the mature stages. Addition of uPA can stimulate the proliferation of astrocytes as well as outgrowth from cerebellar explants [10]. Recently, PG/PAs have been suggested to play functional roles in neural plastic events such as long term potentiation (LTP), nerve injury and regeneration [13,15,18,19].

The suggested role for PG/PAs in regeneration and neural plastic responses, together with the ability of cultured astrocytes to recover from injury, raise an interesting possibility that PG/PAs may play an potential role in the recovery of injured astrocytes. In the present study, the expression of tPA, uPA and PAI-1 was examined in cultured astrocytes after injury. We report here that the expression of components of the PG/PAs system could be regulated in cultured astrocytes following injury, suggesting that the caseinolytic activity is an essential component during the process of astrocyte recovery. Furthermore, our findings raise the interesting possibility that PG/PAs might play an important role during the process of injury and regeneration in the CNS.

Primary culture of astrocytes was prepared from the cerebral cortex of newborn ICR mice. The purity of the cultured

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astrocytes was estimated to be greater than 95% based on GFAP immunohistochemistry. Cultured astrocytes at 4 weeks were scratched with a sterile pipette tip according to a grid and the culture medium was immediately changed to remove the cell debris. The cell samples were collected at different time points (0 to 7 days) and total cellular protein was extracted by homogenizing the cells with PA extraction buffer (100 mM Tris–HCl, 10 mM EDTA, 0.4% Triton X-100, pH 7.5) on ice. Zymograph assay of the PA activity was performed as described previously [18]. For Western blot analysis, total cellular protein (40 μg) was run on a 10% PAGE gel, and then wet-transferred to Hybond-C membrane (Amersham, USA). The membrane was blocked by 5% milk and 0.05% tween-20 in PBS, followed by incubation with the primary antibodies [2] (1:500 dilution) and secondary antibody (biotinated goat anti-mouse IgG). The membrane was then stained by DAB.

After the astrocytes were scratched, the medium was changed immediately. The injured astrocytes were then covered with agarose-milk (0.2% agarose, 1.25% non-fat milk in DMEM medium). EACA medium was added, followed by incubation at 37°C for 3 days. Cells were fixed in 4% paraformaldehyde and stained with coommassie brilliant blue. To assay for pericellular caseinolytic activity, cultured astrocytes were overlayed with 1% non-fat milk in 0.01 M PBS supplemented with antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin), with or without additives (10 μg/ml plasminogen, BM, Germany; 50 mM EACA, Biological Inc, China). Cells were semi-dried under fume hood. Following incubation at 37°C for 5 h, cells were fixed in 4% paraformaldehyde, washed with 0.01 M PBS and stained by coommassie brilliant blue.

After scratch injury, astrocytes near the injury edge rapidly lost the polyglonal and flat properties, and became hypertrophy with processes that are characteristic of reactive astrocytes. An up-regulation of the total pericellular caseinolytic activity was observed around the reactive astrocytes using in situ caseinolytic assay (Fig. 1). When the astrocytes were at the quiescent stage (0 h), a weak and small zone appeared evenly around the flat astrocytes. At 1–3 days after injury, the caseinolytic zones substantially increased. The large caseinolytic zones initially appeared along the scratch line; fusion of some of the lytic zones was apparent, especially near the injury edge. The extent of caseinolytic activity subsequently declined to the basal level at 7 days following injury (Fig. 1).

The enzymatic activity results based on zymograph analysis showed a clear caseinolytic band of ~67 kDa which was dependent on PG; this band was not visible on the control gel in the absence of PG (Fig. 2). The enzymatic activities as revealed by these proteolytic bands indicated that tPA activity was up-regulated from day 0 to day 3, and then gradually declined from day 4 to day 7 concomitant with the recovery of astrocytes. The increase of tPA activity observed on day 3 may suggest the regulation of enzymatic activity at the post-translational level by proteases such as plasmin or trypsin [12] which are activated by scratch injury. In addition to the ~67 kDa band, another protein band of higher molecular weight was also detected by the tPA antibody (Fig. 3, top panel). It is possible that this high-molecular weight band is a complex of tPA and its inhibitors (such as PAI-1) as previously demonstrated [1]. Only one distinct band (~150 kDa) of uPA was detected by Western blot analysis and the free form of uPA (~50–55 kDa) could not be detected (Fig. 3, middle panel). It is possible that almost all the uPA has

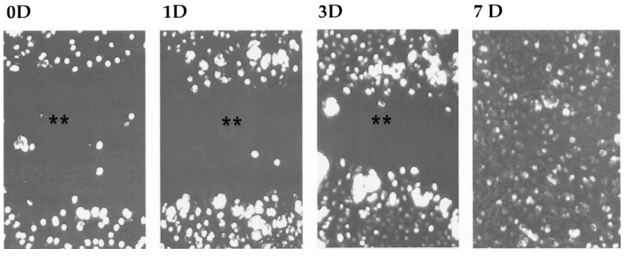

Fig. 1. The pericellular caseinolytic activity of the injured astrocytes. The enzymatic activity was depicted by the caseinolytic zone (bright zone) that revealed the degradation of the substrate by the pericellular enzymes. The undegraded substrate was stained by coommassie blue. An increased enzymatic activity around the astrocytes was observed at different time points after injury for up to 3 days (3D). When the cells recovered, the caseinolytic zone decreased accordingly. The double star indicated the scratched gap. Magnification, ×4.

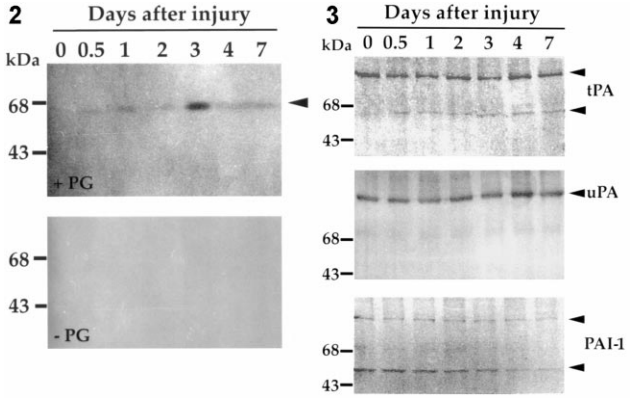

Fig. 2. The plasminogen-dependent caseinolytic activity in the astrocyte lysates. After a 24 h incubation of the overlayed indicating gel with PG, a caseinolytic band appeared at about 67 kDa (top panel). The caseinolytic band became more intense at day 3 (3D) following injury and then decreased to an undetectable level at day 7 (7D). The indicating control gel without PG did not show the caseinolytic band (bottom panel).

Fig. 3. Western blot analysis of the expression profiles of tPA, uPA and PAI-1 in astrocytes following injury. Top panel, two bands were detected by the anti-tPA antibody, with the ~67 kDa protein as the free form of tPA. Weak expression of the free-form tPA was detected at the quiescent stage (0D), but it increased and maintained at an elevated level in the reactive stages (0.5–7D). Middle panel, one band (~150 kDa) was detected by the anti-uPA antibody; free form of uPA (~50–55 kDa) was not detectable. Bottom panel, the expression of the free-form of PAI-1 (~50 kDa) was decreased during the recovery process. The size of the high molecular weight complex was ~120 kDa.

formed a complex of higher molecular weight and might account for the lack of detectable uPA activity on the zymograph. Similar to tPA, the expression of PAI-1 was also regulated in injured astrocytes during the recovery process. The free form of PAI-1 (~50 kDa) was down-regulated following injury (Fig. 3, bottom panel). The down-regulation of PAI-1 might act in concert with the up-regulation of tPA to result in an overall increase in tPA activity.

The addition of EACA onto the cultured astrocytes was found to significantly retard the recovery rate of the cultured astrocytes in a concentration-dependent manner. When astrocytes are covered with casein-agarose, the covered protein actually provided an artificial extracellular matrix barrier around the cells. The covered astrocytes need to degrade and penetrate the protein barrier in order to migrate towards the scratched gap. At 3 days after the addition of EACA, a significant difference in the extent of recovery was observed (Fig. 4). In control astrocytes, i.e. no EACA in the cultured medium, the gap at the injured site became smaller due to the migration of cells on both sides of the gap. Addition of EACA was found to retard the astrocyte recovery process by inhibiting the migration of the astrocytes in a concentration-dependent manner (5×10^{-2} to 5×10^{-4} M). In the presence of EACA at 1×10^{-1} M, only few astrocytes migrated towards the injured area (Fig. 4).

It has been reported that uPA is predominant in immature astrocytes with the loss of activity at the mature stage, while the activity of tPA persists in the mature stages [7]. This temporal expression profile suggests that each PA may play distinct role at different developmental stages. Furthermore, each PA not only participate in different growth factor-related process in astrocytes [8], but may also play a role in LTP in hippocampus and migration of cerebellar granule cells [4,13]. It is possible that tPA is involved in the structural modification and cell migration that accompany activity-dependent plasticity.

We report here the up-regulation of tPA activity in injured astrocytes. The transient elevation of tPA together with the decline of PAI-1 expression following injury comprises a differential regulatory pattern which would result in an increased tPA activity. We also found that EACA, which can inhibit PG and thus block the tPA mediated proteolytic cascade, can effectively retard the recovery of astrocytes at injured site. These findings suggest that tPA mediated caseinolytic activity may play an important role during astrocytes regeneration. Although the precise role of tPA in astrocytes remains to be elucidated, tPA may play an important role in the recovery process of injured astrocytes. It is possible that tPA may initiate the PG/PAs proteolytic pathway to modify some extracellular adhesive molecules and thus facilitate the formation of cell–cell connection and interaction. In addition, the activated tPA during the process of astrocyte recovery may not only play a functional role in the plastic response but may also initiate other signal cascades to coordinate the wide spectrum of effects in response to injury.

Unlike tPA, the expression of uPA in astrocytes appears to be in a high molecular weight complex with no apparent enzymatic activity. Increasing evidence indicates that uPA can act as a mitogen as well as a neuroglial signal during development. For example, uPA can induce proliferation of astrocytes, fibroblasts and certain tumors [8,10]. An up-regulation of uPA and uPA receptor in malignant astrocytoma was also observed [5]. More recently, an astrocyte cell line transfected with uPA showed enhanced ability to promote axon regeneration and this effect was contributed to uPA itself, independent of plasminogen [11]. Thus, uPA in mature astrocytes may mediate a PG-independent effect which is different from that of tPA.

EACA is usually used to treat brain trauma or stroke with hemorrhage. Because of the structural similarity between EACA and lysine-binding site of PG, EACA can competitively inhibit the activation of PG and block the extracellular proteolytic cascade (including thrombolysis) mediated by the PG/PAs system. EACA is therefore usually used as the PG inhibitor to evaluate the role of extracellular fibrinolysis in many studies. For example, it has been reported that EACA can reduce the degradation of basement

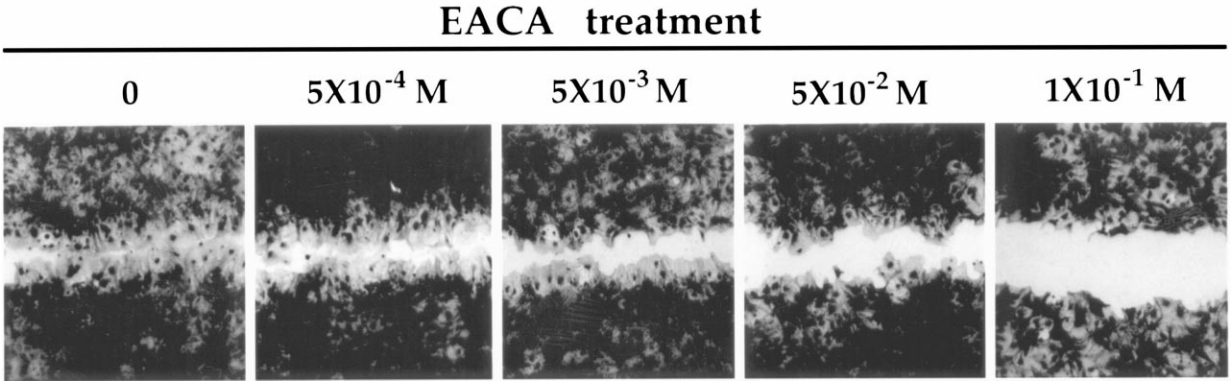

Fig. 4. The retardation effects of EACA on the recovery of injured astrocytes. After addition of EACA (5×10^{-4} to 1×10^{-1} M) onto the overlayed astrocytes, a concentration-dependent inhibitory effect on the recovery rate in filling up the gap was observed. In control, the injured gap was almost filled up by reactive astrocytes. Addition of EACA at various concentrations inhibited the migration of astrocytes to the gap.

membrane by human breast cancer cells in the presence of serum or PG [16] and completely inhibit the degradation of laminin by human colon carcinoma [17]. Furthermore, the neurite outgrowth from dorsal root ganglia within fibrin gel can be inhibited by EACA in a concentration-dependent manner [6]. In the present study, a physical barrier by casein was employed to explore the role of plasminogen activators during astrocyte recovery from injury. We report here that EACA can retard the process of astrocyte recovery after injury in a concentration-dependent manner. Our finding not only demonstrates the importance of the PG/PA pathway in the migration of astrocytes, but also suggests a potential implication of the role of EACA in neuronal regeneration. EACA, when used at an appropriate concentration to inhibit gliosis, may prevent the migration of the astrocytes to allow for the regeneration of the injured neurons.

Taken together, our findings demonstrate the regulation of expression of tPA and PAI-1 in injured astrocytes and suggest an important functional role of the PG/PAs system in the CNS following injury. While both neurons and microglial cells have also been identified to secrete these proteases and their inhibitor, the physiological implication of the balance of these factors, as well as the intricate co-ordination in the secretion of these factors in the CNS under physiological and pathological situations, remain to be elucidated.

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