Expression of heparanase in nestin-positive reactive astrocytes in ischemic lesions of rat brain after transient middle cerebral artery occlusion

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Abstract

Heparanase is an enzyme that cleaves heparan sulfate proteoglycans, an important component of the extracellular matrix to generate heparan sulfate fragments, leading to the remodeling of the extracellular matrix and the basement membrane particularly during cancer metastasis. A growing body of evidence suggests that heparanase serves multiple functions in normal tissues including the central nervous system. In this study, we showed that heparanase is expressed in reactive astrocytes in the peri-infarct lesion of a rat brain whose middle cerebral artery was transiently occluded for 90 min. RT-PCR and Western blot analyses revealed that heparanase expression was markedly upregulated during the subacute phase of ischemia (from 3 to 7 days post-reperfusion (dpr)). As revealed by immunohistochemical study, heparanase was localized in astrocytes located in the peri-infarct region. Heparanase+ astrocytes expressed nestin that is known as a marker of reactive astrocytes. Infiltrated neutrophils were weakly heparanase+. After 7 dpr, the expression level of heparanase+ astrocytes considerably decreased. Therefore, the maximum expression of heparanase by astrocytes may correlate with the time of migration of reactive astrocytes toward the ischemic core, which may result in astrogliosis. These findings suggest a novel role of heparanase in the pathophysiology of brain ischemia.

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Heparanase is an endo-β-d-glucuronidase involved in the cleavage of the heparan sulfate (HS) chain of HS proteoglycans (HSPGs) and hence participates in degradation and remodeling of the extracellular matrix (ECM) and basement membrane (BM) [6,22,15]. Heparanase activity is well correlated with the metastatic ability of various tumor-derived cells [21,5,7]. In addition to tumor-derived cells, heparanase is also expressed in normal cells, including leukocytes such as neutrophils, macrophages, endothelial cells and astrocytes [15,16,3,10]. Heparanase expressed by these cells has been considered to facilitate cellular migration and invasion that are associated with autoimmunity, inflammation and angiogenesis. A recent study demonstrated that heparanase is also expressed in the neuronal cells and glial cells, suggesting that it plays some roles in the normal function of the central nervous system (CNS) [24]. However, the types of cells that normally express heparanase in the CNS or express heparanase in the pathologic CNS have not yet been determined. The present study was conducted to determine the cell types that express heparanase in the ischemic brain using a rat brain ischemia model, in which the right middle cerebral artery (MCA) was transiently occluded.

Male Wistar rats (8–10 weeks old; body weight, 230–280 g) were used in this study. The animals were handled in accordance with the Guidelines for Animal Experimentation of Ehime University School of Medicine. The transient right MCA occlusion was carried out as described elsewhere [11]. Briefly, the rats were anesthetized with 1% halothane in 30% oxygen and 70% nitrogen, and their body temperature was maintained at 37 °C using a heating lamp during the operation. Focal cerebral ischemia was induced by 90 min MCA occlusion using a 4.0 siliconized filament in rats. The rats were evaluated for evidence of their neurologic deficits at 1 h after the operation, as described elsewhere [1]. At 3 and 7 days post-reperfusion (dpr), brains from the rats were fixed by transcardial perfusion with phosphate-
buffered saline (PBS) containing 4% paraformaldehyde and 2 mM MgCl₂. After the dissected brains were cryoprotected in PBS containing 15% sucrose at 4 °C overnight, they were rapidly frozen in powdered dry ice and sliced at the caudoputamen level into 4-μm coronal sections using a cryostat. To identify the ischemic region, some sections were stained with hematoxylin. For double-immunofluorescent staining, the sections were incubated overnight at 4 °C with a mixture of FITC- and Cy3-labeled secondary antibodies (Chemicon) for 2 h at room temperature. Hoechst 33258 (Sigma) was used for nuclear staining. The immunostained sections were visualized using alkaline phosphatase-conjugated secondary antibodies (1:1000; Promega, WI) followed by nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) stainings [19].

We first examined the temporal expression of heparanase mRNA and protein after MCA occlusion using RT-PCR and Western blot analyses (Fig. 2). Although the heparanase mRNA expression was almost undetectable in the control brain following the 30 PCR cycles, it was significantly detected at 1 dpr and reached its maximum level at 3 dpr. Thereafter, the mRNA expression level decreased by 14 dpr (Fig. 2A). The change in protein expression level was monitored by Western blot analysis on protein from the same ischemic parietal cortex (Fig. 2B). Similarly to the RT-PCR results, the Western blot analysis showed the presence of a latent-form (65 kDa) of heparanase that significantly increased from 3 dpr to 7 dpr. The active-form (50 kDa) bands were much less significant than the latent-form bands. The active-form is converted from the latent-form through cleavage by lysosomal proteases such as cathepsin D and L [17]. The latent-form of heparanase can also act as an adhesion molecule by lysosomal proteases such as cathepsin D and L [17]. The active-form is converted from the latent-form through cleavage by lysosomal proteases such as cathepsin D and L [17]. The latent-form, the 50-kDa band, was much less significant than the latent-form bands. The active-form is converted from the latent-form through cleavage by lysosomal proteases such as cathepsin D and L [17]. The latent-form of heparanase can also act as an adhesion molecule by lysosomal proteases such as cathepsin D and L [17].

Fig. 2. Increased expression levels of heparanase mRNA and protein in ischemic cortex during acute and subacute phases of brain ischemia. (A) Representative data of RT-PCR analysis on temporal profile of heparanase mRNA expression. Note that the expression level of heparanase mRNA reached its maximum around 3 dpr. (B) The expression level of heparanase protein as detected by Western blot analysis increased by 3 dpr, and the expression profiles of heparanase mRNA and protein correlated well. β-Actin mRNA (A) and immunoreactivity (B) were used as internal controls.
Fig. 3. Representative immunohistochemical data on heparanase localization in ischemic region denoted in Fig. 1. The immunostained brain sections were prepared from ischemic brains fixed at 3 dpr, except for that shown in (D), which is a 7 dpr-brain section. (A) Astrocytes in the contralateral region faintly express GFAP that are not heparanase+. No significant heparanase staining was found in the contralateral hemisphere. (B) Strongly GFAP+ astrocytes in the peri-infarct region bearing thick processes were heparanase+. (C) Heparanase+ astrocytes expressed nestin. (D) At 7 dpr, heparanase+ reactive astrocytes (pink arrowheads) were rare. (E) There are many CD11b+ cells in the peri-infarct region at 3 dpr, many of which are heparanase− macrophage-like cells. There are also smaller faintly heparanase+ round cells with segmented nuclei (yellow arrowheads), which may be neutrophils. (F–H) Double-immunostaining of heparanase with β-Tubulin III, MBP and NG2 in the peri-infarct cortex at 3 dpr. β-Tubulin III+ neurons, MBP+ oligodendrocytes and NG2 glia were heparanase−. Blue arrowheads in H denote NG2+ macrophage-like cells, which are heparanase−. Scale bars = 25 μm (E) and 50 μm (others).
independent of its enzymatic activity [4,18], suggesting that both form of heparanase may function in the pathophysiology of brain ischemia.

To determine the cellular source of heparanase in ischemic lesions, ischemic brain sections were fixed at 3 dpr and subjected to immunohistochemical staining, since the heparanase protein expression level was at a maximum at 3 dpr (Fig. 3). No significant heparanase immunofluorescence was observable in the contralateral hemisphere (Fig. 3A). In contrast, many heparanase-immunoreactive cells were present particularly in the peri-infarct region. Heparanase+ cells located in the boxed region in Fig. 1 are shown in Fig. 3B–H. The immunoreactivities of heparanase and GFAP were primarily colocalized, indicating that astrocytes were the source of heparanase in ischemic lesions (Fig. 3B). The heparanase+ astrocytes displayed hypertrophied cell bodies and processes with a GFAP-immunoreactivity stronger than that of normal astrocytes (compare cells in Fig. 3B with those in Fig. 3Aa), which are typical morphologic characteristics of reactive astrocytes. Furthermore, such astrocytes expressed nestin, which has been recognized as a marker of reactive astrocytes [9] (Fig. 3C). Although reactive astrocytes were still present in the peri-infarct region at 7 dpr, heparanase-immunoreactivity was hardly detectable in most astrocytes at this time point (Fig. 3D). CD11b+ macrophage-like cells and neutrophils densely accumulate in ischemic lesions during acute and subacute phases of brain ischemia. Such blood–borne cells have been shown to express heparanase [8]. However, by double-immunofluorescence staining using antibodies to CD11b and heparanase, most CD11b+ cells, which were presumably infiltrated macrophages, were found to be heparanase−. Faint staining with the anti-heparanase antibody was detected only in CD11b+ neutrophils with segmented nuclei (Fig. 3E). β-Tubulin III (neurons), MBP (oligodendrocytes) and NG2 (NG2 glia)-positive cells were all heparanase− (Fig. 3F, G and H, respectively).

In response to various types of CNS damage, astrocytes become activated and accumulate in the damaged area, causing glial scarring or astrogliosis. It is likely that 3 dpr is the time point when reactive astrocytes migrate from the peri-infarct region toward the ischemic core in the present stroke model [23]. Thus, this was the time point when the maximum heparanase expression level was observed. As revealed by immunohistochemical study, heparanase expression was observed mostly in reactive astrocytes, but markedly abated by 14 dpr, when astrogliosis is almost completed. Heparanase mRNA and protein were hardly detectable in the control brain. It has been suggested that the emergence and migration of reactive astrocytes may contribute to the repair of injured tissues and the restoration of CNS functions [14,13]. Furthermore, HSPGs have been implicated in migration of astrocytes after CNS damage [2]. The present results support the notion that heparanase is involved in the migration of reactive astrocytes and astrogliosis in the ischemic brain through HSPG degradation in ECM in and around CNS lesion. Note that the environment in the ischemic region is acidic, which is optimal for heparanase activity [15,20].

Direct evidence supporting this notion, however, should be obtained.

In CNS lesions, ECM and BM networks may be remodeled by various matrix-degrading enzymes, such as matrix metalloproteases, endoglycosidases, and aspartic/cystein/serine proteases, to facilitate the release of growth factors and cytokines, the angiogenesis, the infiltration of immune cells, and the migration of resident glial cells. As a part of these processes, the present results suggest the involvement of heparanase in the healing process following CNS damage through the enhancement of the migration of reactive astrocytes.

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References


