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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.

Keywords: Heparanase; Reactive astrocytes; Ischemia

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 neurological 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-

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buffered saline (PBS) containing 4% paraformaldehyde and 2 mM $MgCl_2$. 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 combination of the following rabbit polyclonal and mouse monoclonal primary antibodies: rabbit anti-mouse heparanase (1:2000; [8]), mouse anti-glial fibrillary acidic protein (GFAP, a marker of astrocytes, 1:500; Chemicon, Temecula, CA), mouse anti-nestin (a marker of reactive astrocytes and neural stem cells, 1:500; Chemicon), mouse anti-β-tubulin III (a marker of neurons, clone Tuj1, 1:500; BAbCO, Berkeley, CA), mouse anti-NG2 chondroitin sulfate proteoglycan (NG2, a marker of NG2 glia, 1:500; Chemicon), mouse anti-CD11b (a marker of microglia/macrophage lineage, clone OX42, 1:250; Serotec, Oxford, UK), and mouse anti-myelin basic protein (MBP, a marker of oligodendrocytes, 1:250; Chemicon). Anti-mouse heparanase antibody reacts with both the latent-form (65 kDa) and the active-form (50 kDa), as described previously [8]. After rinsing in Tris-buffered saline containing 0.1% Tween 20, the sections were incubated 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 specimens were observed under a BX-52 Olympus (Tokyo, Japan) conventional microscope equipped with a CCD camera.

For RT-PCR and Western blot analyses, we dissected the parietal cortex containing the ischemic core and peri-infarct regions of rat brains that were transcardially perfused with chilled PBS. Ischemic regions in the parietal cortex were identified on the basis of the 2% 2,3,5-triphenyltetrazolium hydrochloride (TTC; Wako, Osaka, Japan) staining or hematoxylin staining (Fig. 1 [11]). For RT-PCR analysis, we isolated total RNA from the ischemic regions using ISOGEN (Nippon gene, Tokyo, Japan) and obtained cDNA by reverse transcriptase reaction using an oligo (dT) primer [12]. The DNA fragments were amplified by GoTaq DNA polymerase (Promega) using the following primer sets: heparanase [sense, TTT GAT CCC AAC AAG GAA CCC AC; antisense, GTA GTG ATG CCA GGT GAG AGA GTC; product size, 584 bp, Gene Bank accession number: NM_022605], and β-actin as an internal standard [sense, AGA AGA GCT ATG AGC TGC CTG ACG; antisense, TAC TTG CGC TCA GGA GGA GCA ATG; product size, 351 bp] [12]. The PCR conditions were as follows: 94 °C for 1 min, followed by 30 (heparanase) or 22 (β-actin) cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. For Western blot analysis, tissue lysates were prepared with Laemmli's sample solution. Equal amounts of total protein were separated on 12.5% SDS-PAGE and transferred onto nitrocellulose membranes. The blots were probed with antibodies to heparanase (1:500) and β-actin (clone AC15, 1:1000; Sigma), and 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

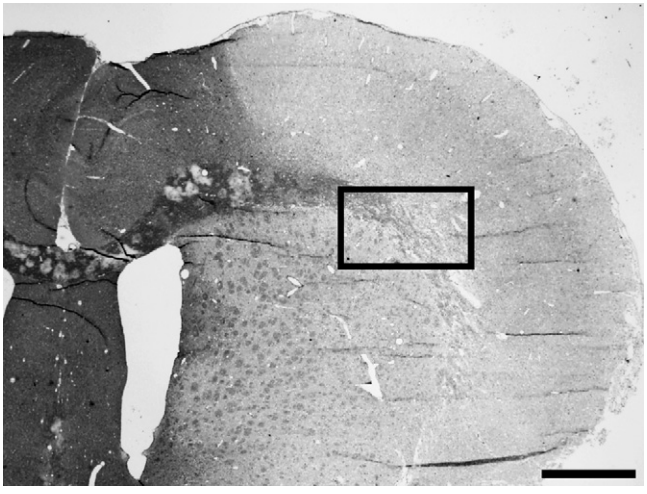

Fig. 1. Ischemic region in right hemisphere at caudoputamen level at 3 dpr, as revealed by hematoxylin staining. The ischemic region was weakly stained with hematoxylin and appears white in the image. The boxed peri-infarct region around the corpus callosum denotes the area where immunohistochemical study was conducted (see Fig. 3). Scale bar = 1 mm.

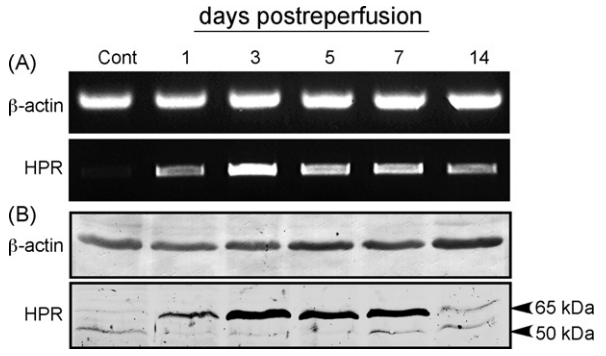

Fig. 2. Increased expression levels of heparanase mRNA and protein in ischemic cortex during acute and subacute phases of brain ischemia. (A) Representative data of RT-PCR analysis on temporal profile of heparanase mRNA expression. Note that the expression level of heparanase mRNA reached its maximum 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.