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Proteinase 3 induces oxidative stress-mediated neuronal death in rat primary cortical neuron

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HIGHLIGHTS

- PR3 induces neuronal cell death in vitro and in vivo.
- PR3 increases intracellular ROS level in vitro and in vivo.
- PR3 activates procaspase-3 and alters expression level of Bcl-2, Bax, and Bcl-xL.

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ABSTRACT

The recruitment of neutrophils into the cerebral microcirculation occurs, especially, in acute brain diseases like a focal cerebral ischemia and plays important role in pathological processes. Proteinase 3 is one of the three major proteinases expressed in neutrophils but no reports are available whether proteinase 3 can modulate neuronal survival. In this study, treatment of cultured rat primary cortical neuron with proteinase 3 induced overt reactive oxygen species production and decreased total glutathione contents as well as disruption of mitochondrial transmembrane potential. Proteinase 3 induced neuronal cell death as evidenced by MTT analysis as well as propidium iodide staining, which was prevented by pretreatment with an antioxidant, N-acetyl cysteine. Proteinase 3 increased activation of procaspase-3 and altered expression level of apoptotic regulator proteins, such as Bcl-2, Bax, and Bcl-xL. Similar to in vitro data, a direct microinjection of proteinase 3 into striatum of rat brain induced neuronal death, which was mediated by reactive oxygen species. These results suggest that proteinase 3 is new essential regulator of neuronal cell death pathway in a condition of excess neutrophil encounter in neuroinflammatory conditions.

1. Introduction

During acute brain injuries, such as cerebral ischemia and trauma, plasma leukocytes extensively interact with endothelial cells within a few hours leading to the infiltrating neutrophils and other inflammatory cells into brain parenchyma [8]. Neutrophils have drawn intense interests in that they are among the first infiltrating cells into ischemic brain, possibly regulating physiological and pathological processes, such as delayed inflammation [6].

The infiltrated neutrophils aggravate brain damages by amplifying cerebral inflammatory responses, resulting in blood brain barrier (BBB) breakdown, brain edema, neuronal death, and hemorrhage transformation [10].

In ischemic brain the importance of neutrophil infiltration on the modulation of neuroinflammatory responses and neuronal cell death was increasingly implicated in recent studies. Migration of activated neutrophil into the brain injury contributed to neuronal death with extracellular proteases such as MMP-9 and a loss of

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intracellular de-condensed DNA [1]. A clinical study in patients with acute cerebral infarction showed that neutrophil to lymphocyte ratio was significantly increased with mortality [19]. Deficiency of leukocyte adhesion molecules (ICAM-1) reduced infarct size and mortality in ischemic stroke model [9].

In neutrophils, three types of serine proteases, i.e. neutrophil elastase, proteinase 3 (PR3), and cathepsin G, are stored in large quantities in cytoplasmic azurophilic granules [10]. Among these, PR3 is implicated in a variety of biological processes, such as degradation of extracellular matrix (ECM) components, cleavage of inflammatory mediators, and induction of endothelial cell apoptosis [15]. PR3 has been characterized as the major autoantigen for autoantibodies in granulomatosis with polyangiitis (GPA), a systemic vasculitic disease [5]. In addition to cytoplasmic granules, PR3 is also localized in secretory vesicles as well as cell surface of quiescent or activated neutrophils [18].

Surprisingly enough, while some experimental evidences are available in terms of possible roles of neutrophil elastase and cathepsin G in the regulation of BBB breakdown, neuronal cell death, and activation of CNS resident immune cells, such as microglia and astrocytes [13,17], no reports are available regarding the possible role of PR3 in the regulation of neuronal cell death.

In this study, for the first time, we investigated the role of PR3 in ROS generation and neuronal cell death in cultured rat primary neuron. In addition, the effects of PR3 microinjected into rat striatum were also examined.

2. Materials and methods

2.1. Materials

Male or pregnant female Sprague-Dawley (SD) rats (250–300 g, 8 weeks) were purchased from the Orient Co., Ltd, a branch of Charles River Laboratories (Seoul, Korea). Animals were housed four or five per cage and allowed access to water and food ad libitum. The cages were maintained at a constant temperature ($23 \pm 1\,^{\circ}C$) and relative humidity ($60 \pm 10\%$) under a 12-h light/dark cycle (lights on from 07:30 to 19:30). All experimental procedures were carried out using protocols approved by the Institutional Animal Care and Use Committee of the Konkuk University. PR3 (NM_002777) and elastase was purchased from Elastin Products Company (Ownsville, MO, USA). The cytosine-β-arabinofuranoside (Ara-C), monochlorobimane (mBCl), N-acetyl-cysteine (NAC) and D-glucose were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Primary neuronal cell culture

Primary cortical neuronal cultures were obtained from dissociated embryonic day-18 (E18) cortex of SD rats. Briefly, the cortices were mechanically dissociated and gently triturated three times in the MEM culture medium. The cells were seeded onto 50 μg/ml poly-D-lysine coated plate in the culture medium supplemented with 5% FBS, 5% HS, and 2 mM glutamine. The cultures were maintained at 37 °C in a humidified, 5% CO_2 incubator. For pure neuron cultures, 2 μM Ara-C was added after 2 days. Cultured neuronal cells were used at DIV 8.

2.3. Determination of ROS, GSH content and mitochondrial membrane potential

Intracellular ROS formation in cultures was measured using indicator, H_2DCF-DA. To measure the ROS in the brain tissue, rats were injected with dihydroethidium (DHE, 1 mg/kg, i.p.) at 30 min prior to be sacrificed. Brain sections were used for fluorescence imaging of oxidized DHE by confocal microscopy.

Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were estimated by the uptake of a cell-permeant fluorescent dye, rhodamine 123. Treated cells were incubated with rhodamine 123 (10 μM) at 37 °C for 20 min. To measure the total GSH content, the cultured cells were added mBCl (20 μM) and incubated at 37 °C for 20 min.

2.4. Measurement of neuronal cell death

Cell viability was also assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. MTT (5 mg/ml) was added to the cell-culture medium at 37 °C for 2 h. The absorbance was read at 570 nm. Cell viability was also assessed by propidium iodide (2 μg/ml) staining. Fluorescence image was observed under the fluorescence microscope (LSM10; Carl Zeiss, Dublin, CA).

2.5. Microinjection of PR3

SD rats were anesthetized with Rompun/Zoletil 50® (1:2, 200 μl/kg, i.p.) and were placed in a stereotaxic frame (Stoelting, Wood Dale, IL). PR3 (3 μl; 1.5 μg) was microinjected (0.5 μl/min) at the following coordinates (in millimeters relative to bregma and the surface of the dura mater): anterio-posterior (AP), +0.7; mediolateral (ML), ±2.1; dorso-ventral (DV), −5.0. Injections of PR3 in the right striatum were done in 5 rats; 5 rats injected the vehicle (PBS). Rats were injected with NAC (300 mg/kg, i.p.) 1 day before PR3 injection.

2.6. Histological study

After 24 h of PR3 microinjection, animals were sacrificed and perfused with ice-cold 4% PFA in PBS (pH 7.4) for 20 min. Sections were immersed with blocking buffer (10% HS, 0.3% triton X-100 in PBS) for 1 h at room temperature and incubated overnight at

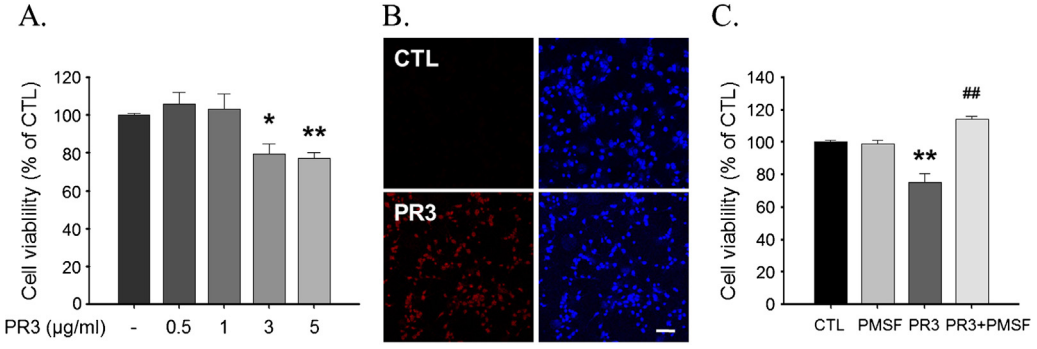

Fig. 1. PR3 induces cell death in rat primary cortical neurons. Cells were treated with PR3 (0.5, 1, 3 and 5 μg/ml) for 24 h. Cell viability was measured by MTT assay (A) and PI staining (B). Bar size = 50 μm. (C) Effects of PMSF on PR3-induced cell death. Values are expressed as the mean ± SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control, $^{\#}p < 0.05$ vs. PR3.