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Expression of cystatin C prevents oxidative stress-induced death in PC12 cells

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Abstract

Cystatin C, an inhibitor of cysteine proteinases, is suggested to be involved in oxidative stress-induced apoptosis of cultured CNS neurons and various neuronal diseases in vivo; however, little is known about its mechanism of action. To address the role cystatin C plays in oxidative stress-induced neuronal cell death, we established PC12 cell lines that stably expressed rat cystatin C. These cystatin C-expressing PC12 cells showed remarkable resistance to high (50%) oxygen atmosphere. This resistance correlate with expression levels of cystatin C, demonstrating that cystatin C has a protective effect on high oxygen-induced cell death. In contrast, in a normal (20%) oxygen atmosphere neither control nor cystatin C-expressing PC12 cells showed a significant change in the number of living cells, indicating that cystatin C does not play an important role in the regulation of cellular proliferation. Furthermore, the cystatin C-expressing cell line also resisted other oxidative stresses, including glutamate- and 13-L-hydroperoxylinoleic acid (LOOH)-induced cell death. These results demonstrate that cystatin C has protective effects against various oxidative stresses that induce cell death.

Keywords: ROS; High oxygen; Glutamate; LOOH; Neuronal cell death; Neurodegenerative disorders

1. Introduction

When neural cells are under oxidative stress, excessive reactive oxygen species (ROS) are produced that induce neuronal death. This neuronal death is related to a variety of neuronal degenerative disorders that are caused by aging, cerebrovascular injury and neuropathology [1,8]. Most neurodegenerative disorders, including Alzheimer's disease (AD), familial amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) are closely associated with oxidative damage, as suggested by many studies [6,13,30,32]. Additionally, naturally occurring cell death is related to ROS [10].

Cystatin C, a member of family 2 of the cystatin superfamily, is an alkaline protein with an apparent molecular mass of 13.5 kDa that inhibits the activity of the lysosomal cysteine proteinases, cathepsin B, H and L [2,16]. The cystatin C protein is widely expressed in almost all tissues, including the brain, and is secreted into various biological fluids, including urine, semen, saliva and cerebrospinal fluid [16]. Immunohistochemical studies have revealed that in the brains of rats, monkeys and humans, cystatin C is expressed mainly in astrocytes, in addition to weak and variable staining in some neurons [35]. High levels of cystatin C mRNA have also been detected in the cerebral cortex, hippocampus, hypothalamus and cerebellum of rodent brains [35]. We have previously revealed that cystatin C mRNA and protein are remarkably up-regulated in rat cerebral cortical neurons cultured in a 50% oxygen atmosphere, which induces apoptosis, demonstrating that cystatin C is implicated in oxidative stress-induced cell

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death of CNS neurons [26]. Studies on oxidative stress in vivo support that cystatin C is implicated in some neuronal diseases. Cystatin C-positive neurons are abundant in the cerebral cortex of some elderly people and AD patients, whereas in the normal brain cystatin C-immunoreactivity is found mainly in astrocytes, rather than in neurons [12,34]. In AD brains, cystatin C-immunoreactivity is primarily limited to pyramidal neurons in cortical layers III and V, where neurons are most susceptible to cell death in AD [12]. Cystatin C also accumulates within beta-amyloid deposits in AD brains [34]. Furthermore, polymorphisms in the cystatin C gene are associated with late-onset AD [11,15]. Additionally, a mutation in the cystatin C gene is related to hereditary cerebral hemorrhage with amyloidosis-icelandic type [23]. In ALS, cystatin C-immunoreactivity is intense in small eosinophilic intraneuronal inclusions within the remaining lower motor neurons, the Bunina bodies, which are a specific pathological hallmark of ALS [28]. In transient ischemia, the delayed death of CA1 pyramidal neurons shows apoptotic features [27]. In this case, immunoreactivity for cystatin C, as well as immunoreactivity for cathepsins B, H and L, whose activities are inhibited by cystatin C, is increased in the CA1 pyramidal neurons [18,29]. Cathepsins B and D leak into cytosolic regions of the cell because of a variety of oxidative stresses that induce destabilization of the lysosomal membrane; this cathepsin leakage leads to the release of cytochrome *c* from mitochondria, and consequently induces cell death [7,19,25,36]. Considering that cystatin C inhibits the cathepsins, these studies strongly suggest the possibility that cystatin C prevents the neuronal cell death by inhibiting the cathepsins penetrated into cytosolic region, but it remains unclear what role cystatin C plays in these diseases. In the present study, to clarify the role cystatin C plays in neuronal death caused by oxidative stress, we established the rat pheochromocytoma PC12 cell line that stably expressed rat cystatin C and evaluated the resistance of these cells to various oxidative stresses.

2. Materials and methods

2.1. Transfection and selection of PC12 cell clones

Rat PC12 cells were grown in monolayer cultures as described below and incubated for 2 days with a mixture of Lipofectoamine 2000 reagents (Life Technologies, USA) and the pcDNA3.1/Myc-His vector containing cDNA for rat cystatin C or the pcDNA3 vector. Following transfection, cells were incubated in medium containing 1 mg/mL G418. One week after transfection, G418-resistant cells were seeded at a low density to form colonies. Colonies were screened for Myc-His-tagged cystatin C expression by Western blot analysis, as described below.

2.2. Cell culture

PC12 cell lines were plated at a final density of 6.25×10^3 cells/cm^2 on polyethylenimine-coated 12-well culture plates (Coaster, USA) using Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 5% precolostrum newborn calf serum (PNCS, Mitsubishi Chemical, Japan), 5% heat-inactivated horse serum (HS) in 5% (v/v) CO_2, and 20 or 50% (v/v) O_2 atmosphere in a N_2–O_2–CO_2 gas incubator (Tabai BNP-110M, Tabai, Japan) at 37 °C. Zero, 2, 4 or 6 days after incubation, cell viability was detected as described below. Primary cultures of dissociated cerebral cortical neurons were prepared from embryonic day 20 rats (Wistar ST, both sexes) as described previously [26]. Briefly, the cells were cultured in a medium consisting of 5% PNCS, 5% HS and 90% (v/v) DF medium consisting of a 1:1 mixture of DMEM and Ham's F12 media (Gibco, USA) containing 15 mM HEPES buffer, pH7.4, 30 nM selenium and 1.9 mg/mL sodium bicarbonate. The each cell were plated at final cell density of 5×10^5 cells/cm^2 (cortical neurons) or 3×10^5 cells/cm^2 (hippocampal neurons) on a polyethylenemine-coated culture plates. The cells were incubated in 5% (v/v) CO_2 and 20% (v/v) O_2 atmosphere in a N_2–O_2–CO_2 gas incubator at 37 °C for 24 h. The medium was changed to serum-free DF medium containing transferrin (5 μg/mL), insulin (5 μg/mL) and progestrone (20 nM), and then the culture plates were transferred to a 50% (v/v) O_2 and a constant 5% (v/v) CO_2 atmosphere in a N_2–O_2–CO_2 gas incubator. Zero and 72 h after incubation under the high oxygen atmosphere, cell lysates were collected, as described below. The use of live animals was performed according to the Guideline of Experimental Animal Care issued by the Prime Minister's Office of Japan.

2.3. Western blot analysis

Cultured cells were washed three times with ice-cold PBS, and lysed in a buffer containing 1% (w/v) SDS, 10 mM Tris–HCl (pH 7.4), 2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Lysates were boiled for 3 min at 100 °C, and then centrifuged at 100,000 × *g* for 30 min. The concentration of the clarified lysates was determined by BCA protein assay (Pierce, USA) and lysates were dissolved in sample loading buffer containing 100 mM Tris–HCl (pH 6.8), 4% (w/v) SDS, 25% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.005% (w/v) bromophenol blue. The lysates were resolved by electrophoresis on 15% SDS-polyacrylamide gels. Proteins were then transferred onto polyvinylidene difluoride membranes (Millipore, USA) in transfer buffer consisting of 25 mM Tris–HCl (pH 7.4), 192 mM glycine and 20% (v/v) methanol using a semi-dry electrophoretic transfer system (Bio-rad, USA). The membranes were blocked with 0.1% (w/v) Tween 20/TBS (T-TBS) containing 5% (w/v) nonfat dried milk (Morinaga, Japan) at room temperature for 1 h, then incubated with anti-cystatin C polyclonal antibody (DAKO, USA) diluted to 1:1000 with T-TBS containing 5% (w/v) nonfat dried milk at room temperature for 1 h. After three washes with T-TBS, membranes were incubated with peroxidase-coupled goat anti-rabbit IgG antibody (Jackson Immuno Research, USA) at room temperature for 1 h. The