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Cystatin C prevents degeneration of rat nigral dopaminergic neurons: in vitro and in vivo studies

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Destruction of nigrostriatal dopaminergic (DA) pathway triggers various persistent responses, such as inflammation and increased synthesis of neural growth factors, both in striatum and in substantia nigra. The pathological processes involved in such responses are poorly characterized and could contribute to secondary damage and/or regeneration in the central nervous system (CNS). Cystatin C was previously implicated in the process of neurodegeneration. However, its biological role during neurodegeneration is not understood and remains controversial. The present study identified an increased cystatin C mRNA level in the DA-depleted rat striatum, starting from the second week following a 6-OHDA-induced lesion. Immunohistochemical analysis confirmed the increase in cystatin C protein level in the striatum following DA depletion. Double-labeled fluorescence immunohistochemistry revealed that nigrostriatal neurons, astrocytes, and microglia contributed to the elevated level of cystatin C. Exposure to 6-hydroxydopamine, a DA-specific neurotoxin, resulted in DA neurons loss in the fetal mesencephalic cultures, an effect which could be partially reversed by treatment with cystatin C. Moreover, in vivo DA neurons survival study showed that administration of cystatin C in rats with 6-OHDA-induced lesion partially rescued the nigral DA neurons. The results indicate that the 6-OHDA lesioning induced a relatively slow but sustained up-regulation of cystatin C expression and suggest that the inhibitor may exert a neuroprotective action on DA neurons. The findings raise the possibility that cysteine proteinase inhibitors may be new candidates for neuroprotective treatment of Parkinson's disease. Cystatin C may be useful therapeutically in limiting neuropathy in Parkinson's disease.
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Introduction

Parkinson's disease, a common neurodegenerative disease, is characterized by degeneration of dopaminergic (DA) neurons in the substantia nigra (SN) (Dawson and Dawson, 2003). The characteristic features of the disease can be reproduced to some extent in animals through the administration of various neurotoxic agents disturbing the DA neurotransmission. For example, intraparenchymal injections of the neurotoxin 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle in rats destroy catecholamine-containing neuronal cell bodies and nerve terminals (Ungerstedt and Arbuthnott, 1970). The 6-OHDA animal model is widely used to examine the function of neural transplants and the alterations in the expression of receptors and their sensitivity to agonist drugs (Betarbet et al., 2002). The lesion causes robust biochemical and structural changes in the SN and its target striatum leading to apparent reorganization of the nigrostriatal pathway. Specifically, the lesion differentially affects mRNA expression levels of subtypes of DA receptor, cannabinoid CB1 receptor, and nicotinic acetylcholine receptor subunit (Elliott et al., 1998; Romero et al., 2000). The lesion also initiates inflammation by elevating the tumor necrosis factor-α in the DA-denervated striatum, which can be suppressed by the immunosuppressant FK506 (Mogi et al., 2000). These processes appear to be accompanied by neural growth-associated activity as evidenced by increased expression of a number of factors that are known to

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stimulate the growth of central neurons in vitro, such as the neurotrophins brain-derived neurotrophic factor (including its specific receptor trkB) and members of glial cell line-derived neurotrophic factor family (Hida et al., 2003; Numan and Seroogy, 1997; Zhou et al., 1996, 2000). Consistent with these changes, it was shown that the striatal extracts from patients with Parkinson's disease promotes growth of DA neurons in fetal mesencephalic cultures (Carvey et al., 1993), suggesting a trophic activity of the striatum on DA neurons. Thus, the striatum appears to play a role in self-neuronal protection when neurodegeneration of midbrain DA neurons occurs. However, the molecular mechanisms underlying the neural plasticity of the DA-denervated striatum are far from being elucidated.

Cystatin C, a cysteine protease inhibitor, was recently identified by an approach based on suppression subtractive hybridization (SSH) as being differentially expressed in DA-depleted striatum of the 6-OHDA-lesioned rats (Xu et al., 2000). Cystatin C is a member of family 2 of the cystatin superfamily (for reviews, see Akopyan, 1991; Barrett and Kirschke, 1981; Barrett et al., 1984), widely expressed by various tissues (Abrahamson et al., 1990; Lofberg et al., 1982, 1983; Paraoan et al., 2001) and present in various biological fluids including urine, blood, and cerebrospinal fluid (Abrahamson et al., 1986; Lofberg et al., 1980; Sickmann et al., 2000). Involvement of cystatin C in various degenerative processes in the central nervous system (CNS) was only relatively recently described. Delayed expression of cystatin C by CA1 pyramidal cells and reactive astrocytes of rat hippocampus was reported in transient forebrain ischemia (Ishimaru et al., 1996; Palm et al., 1995). However, cystatin C was persistently up-regulated in neurons and glia in a rat model for medial temporal lobe epilepsy (Aronica et al., 2001). In addition, cystatin C protein level was found increased in neurons cultured in the high oxygen atmosphere, suggesting that oxidative stress stimulates the expression of cystatin C in cultured neurons and that cystatin C might therefore have a role in regulation of apoptosis elicited by oxidative stress (Nishio et al., 2000). Recent studies showed that, in patients with Alzheimer's disease (AD), cystatin C is colocalized with amyloid beta-protein (Abeta) in parenchymal and vascular amyloid deposits (Levy et al., 2001).

The role of cystatin C in the neurodegeneration associated with PD is unknown. The present study demonstrates that the expression of cystatin C is up-regulated, both at mRNA and protein level, in the DA-depleted striatum following 6-OHDA treatment. Moreover, the evidence provided by in vitro and in vivo studies suggests a role of cystatin C in the prevention of degeneration of mesencephalic DA neurons, thus implying that cystatin C might be a potential neuroprotectant of nigral DA neurons.

Materials and methods

Animals

All animal experiments were carried out in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female Sprague–Dawley rats (180–220 g) provided by the Animal House, Shanghai Institutes for Biological Sciences, were caged in groups of three with food and water given ad libitum. The animals were kept in a temperature controlled environment at 21°C on a 12:12-h light–dark cycle. Timed pregnant rats were used as the cell culture source.

Induction of unilateral lesion in medial forebrain bundle (MFB) by 6-OHDA and animal behavioral tests

All surgery was performed under Equithesin anesthesia (0.3 ml/100 g) and adequate measures were taken to minimize pain or discomfort. Unilateral DA degeneration of the striatum was achieved by stereotaxic injections of 6-OHDA (Sigma, St. Louis, MO, USA) into the ascending medial forebrain bundle as described previously (Ungerstedt and Arbuthnott, 1970). Briefly, 4 μl of 6-OHDA (2.5 μg/μl in 0.2 mg/ml ascorbate–saline) were injected at 4.4 mm caudal to bregma, 1.2 mm lateral to midline, 7.8 mm below dura. Sham-lesioned animals received administration of 4 μl of ascorbate vehicle. The lesion parameters used in the present study result in the selective destruction of virtually all dopaminergic neurons in the SN (>95%) and the ventral tegmental area (>80%), as indicated by reduction in mRNA level of tyrosine hydroxylase (TH) (Zhu et al., 1993), DA contents, and TH immunoreactivity (Zhou et al., 1996). One week after lesioning, rotational behavior was assessed with apomorphine (0.05 mg/kg). Animals that exhibited adequate turning (i.e., at least six full body turns per min for rats and three full body turns per min for mice contralateral to the lesion side) were used in the study.

Isolation of total RNA and Northern blot analysis

The animals were sacrificed by decapitation at various time points after the lesion was induced and the brains were removed within 5 min postmortem. For each time point, four to five 6-OHDA-lesioned animals were used. The lesion-side striata were dissected and stored at −80°C until used. The tissues were homogenized and total RNA was isolated using a Totally RNA isolation kit (Ambion, Austin, TX). Polyadenylated RNA was further purified with Oligotex mRNA Mini Kit (Qiagen, Germany).

Northern blot analysis followed standard procedures (Sambrock et al., 1989) with a few modifications. Thirty micrograms of total RNA isolated from DA-depleted and sham-lesioned striata of rats at 1, 2, and 5 weeks postlesion were size fractionated by 1.0% formalin-denatured agarose gel electrophoresis and transferred to a nylon membrane (Amersham). A ^{32}P-labeled cystatin C-specific cDNA probe was produced using random primer DNA Labeling Kit (TaKaRa Biotechnology, Dalian, China) followed by *Eco*RI digestion. The specific activity of the probe was approximately 10^9 dpm/μg DNA. After hybridization with the labeled probes at 42°C, the filters were sequentially washed with 2× SSC, 0.1% SDS at room temperature followed by 0.2× SSC, 0.1% SDS at 42°C, and 0.1× SSC, 0.1% SDS at 65°C. Autoradiography was performed using an intensifying screen at −80°C and the exposure time was varied so that the band intensity was kept within the linear range. Densitometric scanning of blots allowed the calculation of the ratio of signal obtained with cystatin C transcript and 28S ribosome in both denervated striatum and control. The results were expressed as percentage of the control (100%).

Semiquantitative reverse transcriptase (RT)-PCR

Single strand (ss) cDNA was synthesized from 1 μg total RNA in a volume of 20 μl containing 50 pmol random hexamers (Gibco BRL, UK), 0.2 mM each dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI, USA), 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 50 mM DTT, 0.75 U RNasin (Promega), and either 200 U

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