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Cystatin C induces apoptosis and tyrosine hydroxylase gene expression through JNK-dependent pathway in neuronal cells

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ABSTRACT

Cystatin C (CysC), an endogenous cysteine protease inhibitor, has been implicated in the apoptosis and differentiation processes of neuronal cells. In this study, we have investigated the pathway involved in the process. A human neuronal hybridoma cell line (A1 cell) was treated with CysC in both undifferentiated and retinoic acid (RA)-induced differentiated conditions, which decreased overall process length in both conditions. Also, CysC increased apoptotic cell number time-dependently, as revealed by TUNEL assay. Western blot analysis demonstrated that in differentiated A1 cells, CysC treatment decreased Bcl-2 and increased active caspase-9 protein level time-dependently. Immunocytochemistry results revealed that, CysC treatment significantly increased active form of Bax expressing cell number, which co-localized with mitochondria. Mitogen activated protein (MAP) kinase inhibition experiments showed that Bax mRNA induction and Bcl-2 mRNA inhibition by CysC treatment were c-Jun N-terminal kinase (JNK)-dependent. After RA-induced differentiation, choline acetyltransferase (ChAT) and neurofilament (NF) mRNA levels were increased in A1 cells. CysC treatment inhibited NF mRNA level in both undifferentiated and RA-differentiated, and increased TH mRNA in differentiated A1 neurons. Analysis of signal transduction pathway demonstrated that TH gene induction was also JNK-dependent. Thus, our results demonstrated the significance of JNK-dependent pathways on CysC-induced apoptosis and TH gene expression in neuronal cells, which might be an important target in the management of CysC dependent neurodegenerative processes.

Cystatin C (CysC) is an endogenous cysteine protease inhibitor, which is ubiquitously expressed in the nucleated cells and secreted in the body fluids. By inhibiting cysteine proteases such as cathepsins, it plays important roles in the regulation of diverse biological functions including inflammation, tumor invasion and neuronal cell differentiation. In the central nervous system (CNS), where CysC concentration is 5.5 times higher in cerebrospinal fluid (CSF) compared to serum [1], it might play an important role in CNS pathophysiology by balancing the protease activities. Indeed, its concentration is reported to be decreased in neuroinflammatory disease conditions [14], leptomeningeal metastasis [13] and cerebral amyloid angiopathy [19]. Conversely, enhanced CysC expression is observed in cell-stress conditions such as facial nerve axotomy [10], hypophysectomy [6], transient forebrain ischemia [17] and 6-OHDA-induced niagrostraiatal neuronal degeneration in vivo [22].

It has been shown that CysC possesses functions other than lysosomal cysteine protease inhibition. Such as, it could influence the fibrillation process of Alzheimer's amyloidβ peptide [11], or deposited intracellularly in endoplasmic reticulum (ER) [9]. Moreover, there are reports that have shown that CysC provides neuroprotection in serum deprived neuroblastoma cells [21], and increases nestin-positive neuronal progenitor cells and neurosphere [7]. On the other hand, we and others have found that CysC treatment increases the apoptosis of neuronal cells [2,11]. Interestingly, using CysC knockout mice, a report showed that CysC modulates both neurodegeneration and neurogenesis in a status

Abbreviations: CysC, cystatin C; RA, retinoic acid; ChAT, choline acetyltransferase; NF, neurofilament; TH, tyrosin hydroxylase; JNK, c-Jun N-terminal kinase; CNS, central nervous system; CSF, cerebrospinal fluid; ER, endoplasmic reticulum; DMEM, Dulbecco's modified eagle's medium; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; RT, room temperature; MAPK, mitogen-activated protein kinase.

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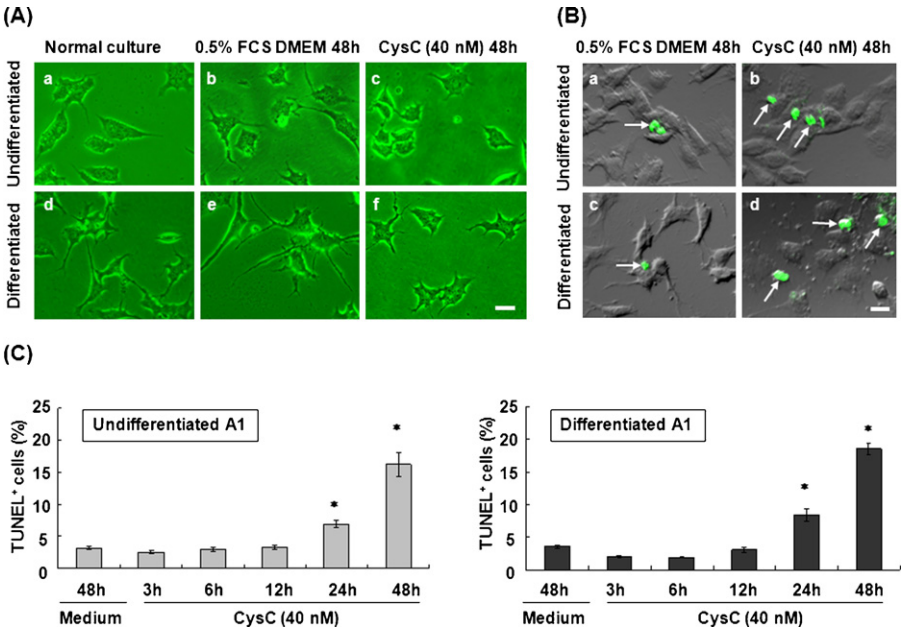

Fig. 1. Effects of CysC on the morphological changes and the apoptosis of A1 cells. The effects of CysC on the morphological changes are shown in (A); where photomicrographs of undifferentiated (a–c) and differentiated (d–f) A1 cells in normal culture condition (a and d), and cultured in DMEM medium containing 0.5% FCS alone (b and e) or 50 nM CysC (c and f) for 48 h are shown. (B) Shows the confocal microscope images of TUNEL$^+$ undifferentiated (a and b) and differentiated (c and d) A1 cells cultured in DMEM medium containing 0.5% FCS alone (a and c) or 50 nM CysC (b and d) for 48 h. Arrows indicate TUNEL positive cells. The quantification data of TUNEL$^+$ cells, counted in a microscopic field at 200× magnification, is presented in (C). TUNEL$^+$ cells were counted in 5 random microscopic fields of a slide, averaged and expressed as % of total cells in a field. The data presented in (C) are average ± SEM of 3 independent experiments. $^*p < 0.05$ vs medium-treated A1 cells for 48 h. Scale bar = 20 μm.

epilepticus model [18]. These results are implicating the diverse function of CysC, involving several signaling pathways in different cellular conditions. In this study, our aim was to investigate the signaling mechanisms of CysC-induce neuronal apoptosis and differentiation-specific gene expression in both undifferentiated and differentiated neuronal cells.

A1 neuronal hybrid cell line, generated by fusion of human cerebral neurons and human neuroblastoma cells [12], was cultured in DMEM supplemented with 10% fetal calf serum (Invitrogen, Camarillo, Canada). For differentiation, A1 neuron was treated with 1 μM retinoic acid (RA; Sigma, St. Louis, MO, USA), as described previously [12].

To analyze the apoptotic condition, we performed TUNEL assay using a kit (*In Situ* Cell Death Detection kit, POD, Roche, Mannheim, Germany), according to the manufacturer's instruction. After TUNEL reaction, cells were washed with PBS, mounted and examined under a laser-scanning confocal microscope (Olympus FV300).

Total RNA was isolated from A1 neurons using RNA Iso Plus (TaKaRa, Shigaken, Japan), according to the manufacturer's instructions. Then, to determine the mRNA level of target genes, real time PCR was performed with gene specific primers and SyBr green PCR master mix (power SyBr green, ABI systems, Foster, USA), using an ABI Prism 7000 Sequence Detector system (Applied Biosystems). GAPDH mRNA level was used as an internal control, and the target gene mRNA level in a sample was quantified by relative quantification method. The primer sequences used for PCR were, Bax: 5′-TGGAGCTGCAGAGGATGATTG-3′ (forward) and 5′-AGCTGCCACTCGGAAAAAGAC-3′ (reverse); Bcl-2: 5′-GCCCCCGTTGCTTTTCC-3′ (forward) and 5′-CCGGTTATCGTACCCTGTTCTC-3′ (reverse); tyrosine hydroxylase: 5′-TGTCCACGCTGTACTGGTTCAC-3′ (forward) and 5′-CGGCACCATAGGCCTTCA-3′ (reverse); low molecular weight-neurofilament (NFL): 5′-GATCTGCCTACGGCGGTTTA-3′ (forward) and 5′-TGGTGTAGTAGGACGGGAAGGA-3′ (reverse); choline acetyltransferase (ChAT): 5′-CGCTGGTGGCTAGAACA-3′ (forward) and 5′-TGATTGCAGCAGGCTACGAT-3′ (reverse); GAPDH: 5′-GCACCGTCAAGGCTGAGAA-3′ (forward) and 5′-TCTCGCTCCTGGAAGATGGT-3′ (reverse).

Total cellular protein was isolated as described previously [12]. Forty microgram of total protein was separated by SDS PAGE, using 10% polyacrylamide gel. The separated protein was transferred to a PVDF membrane, immunoblotted with primary antibody against Bax (rabbit, 1:1000, Abcam, Tokoyo, Japan), Bcl-2 (mouse, 1:1000, Upstate, NY, USA), caspase-8 (mouse, 1:1000, MBL, Nagoya, Japan), caspase-9 (mouse, 1:1000, MBL, Nagoya, Japan) and β-actin (mouse, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), followed by HRP-conjugated species-specific IgG, and immunoreactive proteins were visualized using an enhanced chemiluminescence kit (Amersham, Little Chalfont, Buckinghamshire, UK). Densitometric analysis of expressed protein was done using NIH image software.

For immunocytochemistry, cultured cells on the cover slip were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Cells were blocked with 5% normal goat serum for 30 min and then incubated with monoclonal mouse anti-Bax (6A7, 1:50, Santa Cruz) antibody overnight at 4 °C. The immunoreactive protein was detected with FITC-conjugated goat anti-mouse IgG (1: 100, Santa Cruz), and the fluorescence signals were visualized using a laser-scanning confocal microscope. Mitochondria were stained with MitoTracker Red (Lonza, Walkersville, USA) according to the manufacturer's protocol.

Data are expressed as means ± SEM (standard error mean). The statistical significance of the numerical data between groups was determined by one-way ANOVA or Student's *t* test. The significance level was defined as *p* values less than 0.05.

First, to analyze the effects of CysC on neuronal morphology and cellular apoptosis, both undifferentiated and RA-differentiated A1 neurons were treated with CysC (R&D Systems, Minneapolis, USA) up to 48 h. Undifferentiated A1 cells possess mostly a few short neuritis that were found extended after differentiation (Fig. 1A). CysC treatment for 48 h decreased the process length in both undifferentiated and differentiated conditions. TUNEL assay