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Participation of Cathepsins B and D in Apoptosis of PC12 Cells Following Serum Deprivation

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Cathepsin D, a lysosomal aspartic proteinase, has been shown to induce apoptosis of HeLa cells when overexpressed. To further understand regulatory mechanisms of cathepsin D-induced cell death, we examined whether lysosomal cysteine and aspartic proteinases are involved in apoptosis of PC12 cells following serum deprivation. In serum deprived culture, PC12 cells overexpressing cathepsin D died more rapidly than wild-type cells. When the active forms of cathepsins B and D were examined during the apoptotic process of wild-type cells, the amount of cathepsin B was drastically reduced 24 hr after the onset of culture, whereas that of cathepsin D considerably increased. The viability of PC12 cells overexpressing cathepsin B was significantly higher in serum-deprived culture than wild-type cells. In this situation, the amount of the cathepsin B protein did not decrease. The results suggest that there exists an apoptotic pathway regulated by lysosomal cathepsins B and D.

Cathepsins B and D are known to be representative cysteine and aspartic proteinases in lysosomes, respectively, which are ubiquitously present in mammalian cells and degrade unneeded intra- and extracellular materials into biological monomers for reutilization in the cells (28). Autophagy consisting of the sequestration of intracellular components and their degradation by lysosomal enzymes, usually occurs in normal cells to maintain cellular turnover (11). We have previously shown that the autophagy actively occurs in the apoptotic processes of CA1 pyramidal neurons in the gerbil hippocampus after brief ischemia and of effete epithelial cells at the villous tip of the small intestine (21, 26). The role of the autophagy frequently occurring in such dying cells is believed to protect the cells from death (7).

Apoptosis, characterized by morphological alterations consisting of shrinkage of cytoplasm, nuclear chromatin condensation, fragmentation of cells into apoptotic bodies and heterophagocytosis by neighboring cells, plays an important role in the elimination of surplus cells during embryogenesis and maintenance of tissue cell number (13, 27). It has been well established that the caspase family of proteinases play a central role in the execution of apoptosis (3, 19). Besides caspases, it is also interesting that cathepsin D is involved in apoptosis of HeLa cells and neurodegenerative processes (4, 8). Using antisense cDNA expression libraries, human cathepsin D is found to be one of positive mediators of apoptosis, and further, the enzyme is confirmed to induce apoptosis of the cells when overexpressed (8). Gene expression and cellular content of cathepsin D are up-regulated in pyramidal neurons of Alzheimer's disease brains (4). The caspase family of proteinases are required for induction of apoptosis, but little is known about the regulatory mechanism of cathepsin D in the cell death cascade or its relationship with the cascade using the caspase family of proteinases.

In the present study, we therefore examined whether or not, lysosomal cathepsins are involved in apoptosis of PC12 cells, a rat pheochromocytoma cell line, which are known to die by apoptosis, when cultured in the absence of serum and nerve growth factor (NGF). For this we prepared PC12 cells overexpressing cathepsin

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B or D, and examined their survival rates following serum deprivation. Here we show the presence of an apoptotic pathway regulated by lysosomal cathepsins; in the cascade cathepsin D acts as a death factor, but this death-inducing activity is usually suppressed by cathepsin B.

MATERIALS AND METHODS

Cells and culture. For the experiments, PC12 cells were used and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing a high level of glucose (4.5 g/L) supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). The PC12 cells transfected with or without cDNA of rat cathepsin B or D, which were harvested from the culture dishes, were washed several times with DMEM and plated at a density of $2.5 \times 10^3/cm^2$.

Isolation of cDNAs of rat cathepsins B and D and their transfection into PC12 cells. cDNA fragments covering the entire open reading frame of cathepsins B and D were directly amplified from the poly(A)$^+$ RNA of PC12 cells by the RT-PCR technique, using oligonucleotide primers (29). These cDNA fragments were subcloned into pcDNA3, and their sequences were determined and confirmed to be identical with the reported sequences of rat cathepsins B and D, respectively (2, 12).

The transfection of cDNAs of rat cathepsins B and D was carried out by using the calcium phosphate precipitation method(5), respectively, after open reading frames of cathepsins B and D were constructed into pcDNA3. Cells which expressed neomycin phosphotransferase were isolated by selection with G418 (450 μg/ml).

Antisera. Rabbit polyclonal antibodies to rat cathepsins B and D were purified by affinity chromatography, as previously reported (14, 15, 23).

Cell death assay. In each experiment, cells, cultured in the presence of serum, or in the absence of serum but in the presence of NGF (100 ng/ml), were used as controls. As experimental conditions, cells were cultured in the absence of serum or in the absence of serum but in the presence of 200 μM CA074 (N-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline), a specific inhibitor of cathepsin B and/or 100 μM pepstatin A. The survival assay of the cells were performed at 12 hr or 24 hr after the onset of culture by trypan blue exclusion test (17, 24). We also examined the survival rate of the cells by the TUNEL method as described below. These experiments were carried out in triplicate, and the data were statistically analyzed by the student's t-test.

Staining of TUNEL. PC12 cells, cultured under various experimental conditions were obtained 24 hr after the onset of culture and fixed with 4% paraformaldehyde buffered in 0.1 M phosphate buffer, pH 7.2 containing 4% sucrose at 4°C for 2 hr. The cells were thoroughly washed with PBS and treated with 0.3% H2O2 in methanol for 30 min. To detect nuclear DNA fragmentation, the TUNEL reaction was applied to the fixed cells according to the modified method of Gavrieli et al. (10, 21). Briefly, cells were incubated with 100 U/ml TdT and 10 nmol/ml biotinylated 16-2'-dUTP (Behringer-Manheim-Yamanouchi, Osaka, Japan) in TdT buffer (100 mM sodium cacodylate, pH 7.0, 1 mM cobalt chloride, 50 μg/ml gelatin) in a humid atmosphere at 37°C for 60 min. Further incubation with peroxidase-conjugated streptavidin or Texas red-conjugated avidin (Nichirei) was carried out for 30 min at RT. For cell death assay, staining for peroxidase was performed using 0.0125% diaminobenzidine and 0.002% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6) for 10 min.

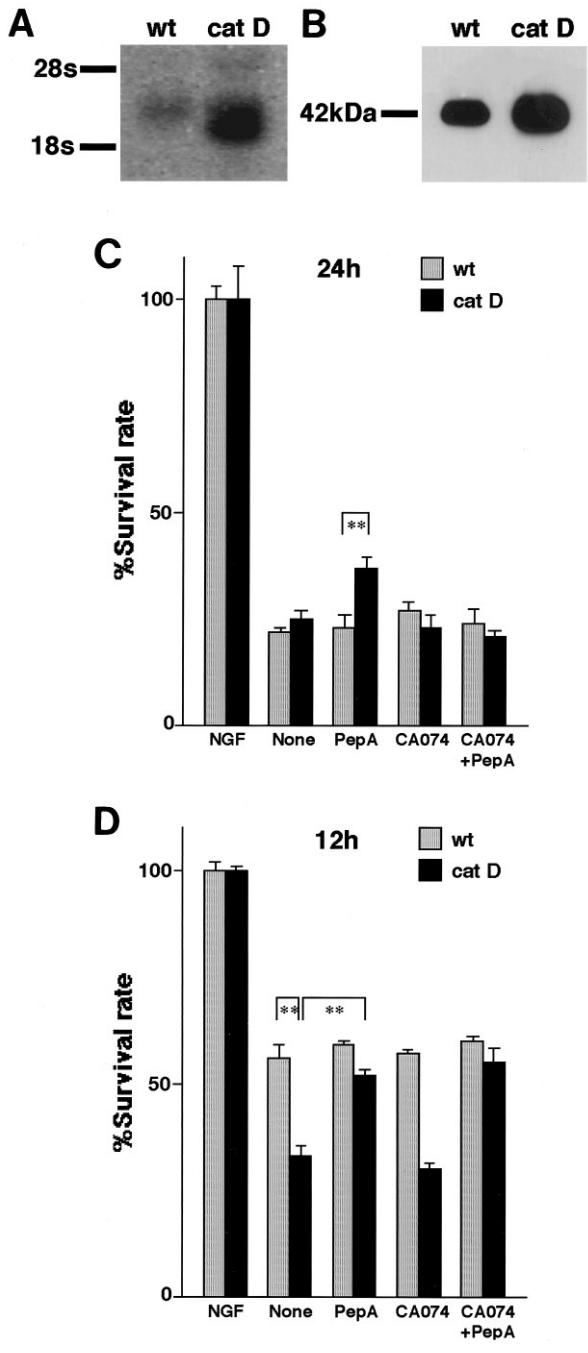

FIG. 1. Expression levels of the cathepsin D mRNA and protein in wild-type (wt) and cathepsin D-gene-transfected (cat D) PC12 cells and their survival rates. (A and B) Northern (A) and Western (B) blot analyses of cathepsin D in wt and cat D cells. Both mRNA and protein levels are highly augmented in cat D cells. (C and D) Survival rates of wt and cat D cells 24 hr (C) and 12 hr (D) after the onset of culture under serum deprivation with 100 ng/ml NGF, 100 μM pepstatin A (PepA), 200 μM CA074 or 200 μM CA074 and 100 μM pepstatin A. The numbers of surviving cells cultured in the absence of serum but in the presence of NGF were estimated as 100%. Vertical bars indicate $\pm$ standard deviation. ** indicates $P<0.02$; none indicates serum-free medium alone.

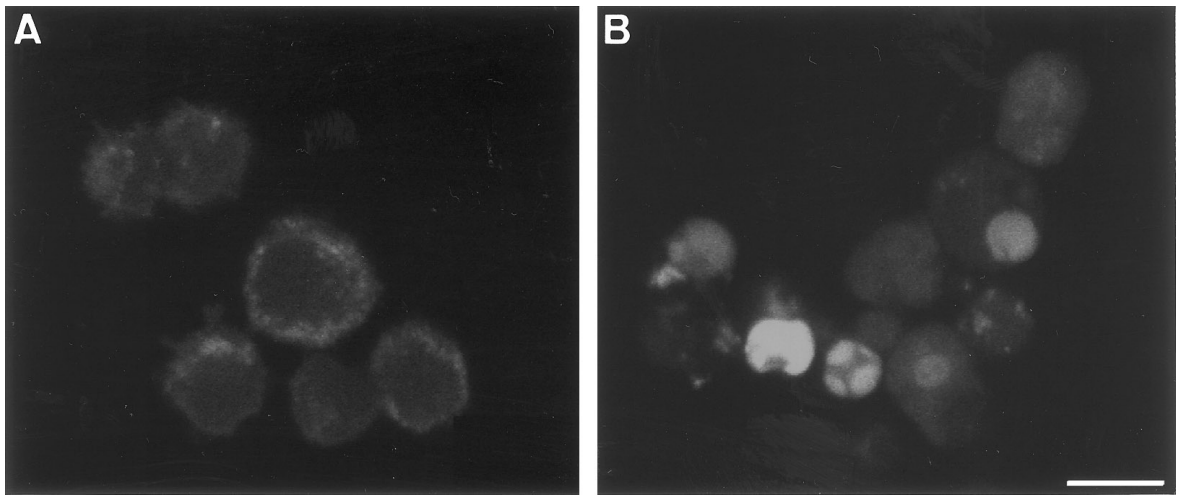

FIG. 2. Staining of TUNEL in wild-type (A) and cathepsin D-gene-transfected (B) PC12 cells cultured under serum deprivation for 24 hr. Bar indicates 10 μm.

Immunoblot analysis. Extracts from wt and cathepsin B or D-transfected PC12 cells before or after serum deprived culture were obtained by the treatment with cell lysis buffer containing 150 mM NaCl, 50 mM Tris, 1 % Triton X-100 including the protease inhibitor cocktail containing 0.06 mg/ml antipain-HCl, 0.01 mg/ml bestatin, 0.02 mg/ml chymostatin, 0.06 mg/ml E-64, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstain, 0.06 mg/ml phosphoramidon, 0.4 mg/ml Pefabloc SC, 0.2 mg/ml EGTA and 0.01 mg/ml aprotinin (Boehringer Mannheim). Ten μg of proteins from each sample was subjected to 12.5 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to PVDF membrane (Immobilon, Millipore). The blots were incubated with anti-cathepsin B or D and visualized with the ECL detection system (Amersham). Quantitative analysis of the immunosignals was carried out using a Scanning Imager (Molecular Dynamics).

Northern blot analysis. Total RNA was prepared from both wt cells and transfectant by the AGPC method (6). Each sample was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes. Hybridization was performed with a ^{32}P-labeled cDNA probe for rat cathepsin D. The membranes were subjected to autoradiography.

RESULTS AND DISCUSSION

Overexpression of cathepsin D induces apoptosis of HeLa cells (8). We tried to prepare PC12 cells which stably overexpressed cathepsin D. Most clones of the cells died during selection with G418, but we could establish one clone of the cells overexpressing cathepsin D. Expression levels of the cathepsin D mRNA and protein in the cells transfected with the cathepsin D gene were significantly higher than those in wt cells (Figure 1A,B). In serum-deprived culture, the cathepsin D gene-transfected cells and wt cells mostly underwent apoptosis 24 hr after the onset of culture and their survival rates were approximately 20% (Figure 1C). In this situation, pepstatin A increased the survival rate of the transfected cells, but CA074 (18), a specific inhibitor of cathepsin B, alone did not. When the viability of the transfected cells was examined 12 hr after the onset of culture, the survival rate was significantly lower than that of wt cells (Figure 1D). The decreased survival rate of the transfected cells was restored to the level of the survival rate of wt cells by the addition of pepstatin A to the culture medium, but the addition of CA074 alone did not change the survival rate of the transfected cells. We then examined the DNA fragmentation by TUNEL staining and found that nuclei of the transfected cells were positively stained by TUNEL in serum-deprived culture, whereas almost no TUNEL-positive cells was seen when NGF was present in the serum-free medium (Figure 2A,B). The results suggest that overexpression of cathepsin D facilitates apoptosis of PC12 cells, following serum deprivation.

We then examined changes in expression levels of cathepsin B and D proteins during the apoptotic process of wt PC12 cells by Western blotting. The amount of the active form of cathepsin B clearly decreased to approximately 20% of its initial amount 24 hr after the onset of culture, whereas that of cathepsin D increased to 300% (Figure 3A). We therefore prepared PC12 cells overexpressing cathepsin B. The survival rate of the cathepsin B gene-transfected PC12 cells elevated to about 68% 24 hr after the onset of culture under serum deprivation (Figure 3B). In this situation, no decrease in the amount of the 29 kDa cathepsin B protein was detected in the cells, although the amount of cathepsin D increased to about 250% of its initial amount (Figure 3C). When the activity of cathepsin B was inhibited by its specific inhibitor, CA074, the survival rate was decreased to 52% (Figure 3B). Moreover, this decreased survival rate of the cells overexpressing

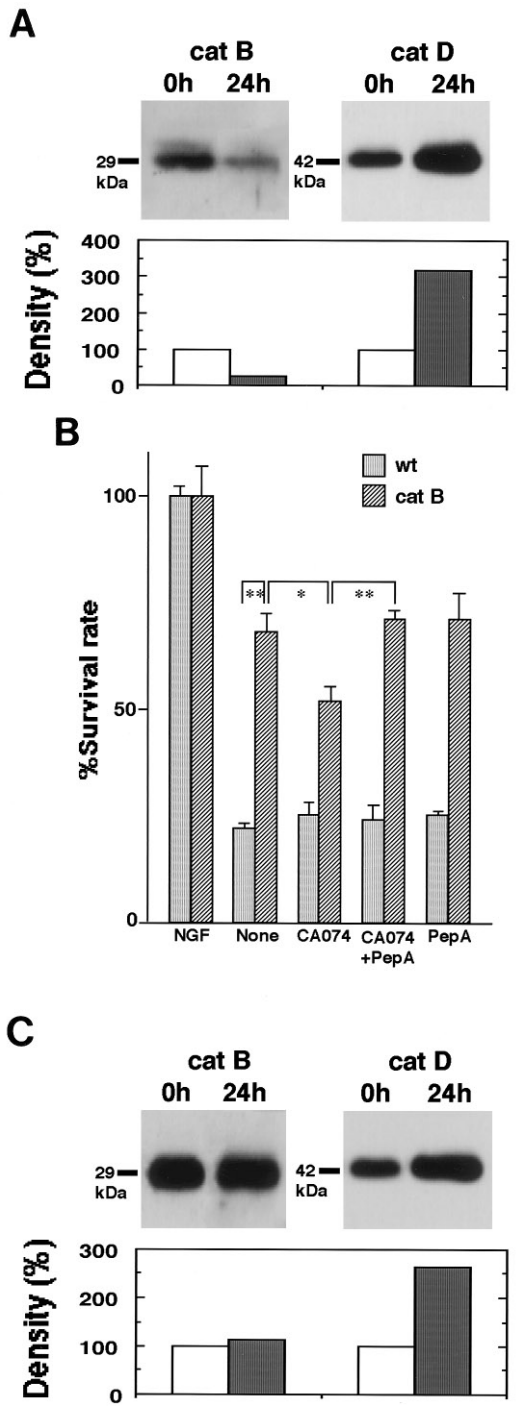

FIG. 3. Protein expression of cathepsins B (cat B) and D (cat D) in wild-type and cathepsin B-gene-transfected PC12 cells and their survival rates. (A) Western blot analyses (upper panel) of cathepsins B and D in wt cells and quantitative analyses (lower panel) of immunosignals of 29 kDa cathepsin B and 42 kDa cathepsin D proteins before (white column) and 24 hr (black column) after the onset of serum-deprived culture. (B) The survival rates of wild-type (wt) and cathepsin B-gene-transfected (cat B) PC12 cells 24 hr after the onset of culture under serum deprivation with 100 ng/ml NGF, 200 μM CA074, 200 μM CA074 and 100 μM pepstatin A (PepA), or 100 μM pepstatin A. The numbers of surviving cells cultured in the absence of serum but in the presence of NGF were estimated as 100%. Vertical bars indicate $\pm$ standard deviation. * indicates $P<0.05$ and ** $P<0.02$; none indicates serum-free medium alone. (C) Western blot analyses (upper panel) of cathepsins B and D in cathepsin B-gene-transfected cells and quantitative analyses (lower panel) of immunosignals of 29 kDa cathepsin B and 42 kDa cathepsin D proteins before (white column) and 24 hr (black column) after the onset of serum-deprived culture.

cathepsin B by CA074 was restored by the further addition of pepstatin A, but the addition of pepstatin A alone did not alter the survival rate (Figure 3C). These lines of evidence suggest that there exists an apoptotic pathway regulated by cathepsins B and D. In this cascade, cathepsin D acts as a death mediator, but this death-inducing activity of cathepsin D is prevented by cathepsin B.

Present results did not show the intracellular site where cathepsins B and D act as cell death regulators. However, the substrate which is specifically cleaved by cathepsin D and is associated with the cell death pathway normally undergoes degradation in lysosomal/endosomal compartments by cathepsin B, resulting in cell survival. Indeed, from the study of the homozygous cathepsin D-knockout mice, it has been suggested that essential functions of cathepsin D depend on limited proteolysis of biologically active proteins rather than bulk degradation of proteins in lysosomes (25). Until recently, subsets of the death machinery such as a complex of caspase-activated DNase (CAD) with inhibitor of CAD (ICAD) (DFF40 and 45) and the caspase family of proteinases have been clarified (9, 16, 20), although it remains unknown how these factors undergo degradation in cells. If these factors are sequestered into lysosomes, lysosomal cysteine proteinases, especially cathepsin B may be responsible for the degradation of them. Although no evidence has demonstrated nuclear or cytoplasmic factors which are activated in lysosomes, inactivation of cathepsin B may result in appropriate cleavage of death inducing factors by cathepsin D. Of course, further study is required to reveal these problems. Moreover, this lysosomal proteinase-dependent pathway for active cell death may function during metabolic imbalance, possibly associated with neurodegenerative diseases such as Alzheimer's disease (4, 22), between cathepsins B and D in lysosomal compartments.

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