An Ultrastructural and Immunohistochemical Study of PC12 Cells During Apoptosis Induced by Serum Deprivation with Special Reference to Autophagy and Lysosomal Cathepsins

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Received October 14, 1998

Summary. In addition to the caspase family of proteinases, cathepsin D, a lysosomal aspartic proteinase, has been suggested to act as a proapoptotic mediator in mammalian cells. To further understand the roles of cathepsins B and D in apoptosis of the cells, we examined the precise alteration processes of ultrastructures and immunoreactivity for these enzymes in PC12 cells cultured under serum deprivation.

Laser scanning microscopy showed immunoreactivity for cathepsins B and D to be finely distributed in the cytoplasm of PC12 cells at the onset of culture under serum deprivation. At 3h after the onset of culture, the immunoreactivity for cathepsin B slightly decreased in the cells, while immunodeposits for cathepsin D in the cells became more intense and larger in size than those at 0h. Positive staining for TUNEL in nuclei of the cells appeared at 6h, though fewer in number. Corresponding to the increase in the number of TUNEL-positive cells at 12h and 24h, the immunoreactivity for cathepsin B was drastically diminished in the cells, whereas that for cathepsin D was significantly augmented, especially in TUNEL-positive cells. Electron microscopically, autophagic vacuoles/autolysosomes appeared in the cytoplasm of the cells 3h after the onset of culture. A distinct nuclear change showing relatively condensed chromatin first appeared in the peripheral part of the nuclei at 6h. The number of PC12 cells having nuclei with chromatin condensation increased especially at 24h, while these cells showed shrinkage of both their cytoplasm and nuclei. Dense bodies and autophagic vacuoles with limiting membranes were seen in these cells.

These results showing the occurrence of autophagy and imbalance of protein amounts between cathepsins B and D during apoptosis may argue for our hypothesis that these enzymes are, in part, involved in the cell death cascade for PC12 cells following serum deprivation.

Lysosomes, as an acidic compartment with limiting membranes, are ubiquitous in all animal cells and play an important role in the maintenance of the cellular metabolic turnover by degrading unneeded intra- and extracellular materials into biological monomers which are then reutilized by the cells (UCHIYAMA et al., 1994). Cathepsins B and D are representative cysteine and aspartic proteinases in lysosomes of mammalian cells. They are involved in the Golgi-derived vesicles which fuse with autophagic/heterophagic vacuoles, forming auto/heterolysosomes. We have previously shown that 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 (NITATORI et al., 1995; SHIBAHARA et al., 1995). The role of autophagy, frequently occurring in such dying cells, is believed to protect the cells from death (CLARKE, 1990).

Apoptosis, a major type of active cell death, is accompanied by characteristic morphological alterations consisting of the shrinkage of cytoplasm, nuclear chromatin condensation, fragmentation of cells into small pieces, and heterophagocytosis by neighboring cells (KERR et al., 1972). It has been suggested that calpain, serine proteinases, granzymes and the ubiquitin-proteasome pathway of protein degradation play a role in the apoptotic process (SOLARY et al., 1998). Degradation of substrates by these proteinases may result in the release of apoptotic factors from mitochondria which activates the caspase family of proteinases, or the direct activation of the proteinases (SOLARY et al., 1998). In addition to these proteinases, we have recently demonstrated the existence of a cell death pathway...
regulated by lysosomal cathepsins B and D in which cathepsin D acts as a death mediator; this death-inducing ability of cathepsin D is normally inhibited by cathepsin B (KANAMORI et al., 1998; SHIBATA et al., 1998; ISAHARA et al., 1999). Thus, various types of proteinases are involved in the cell death cascade of mammalian cells except for the caspase family of proteinases. It is therefore important to understand the precise alteration processes of these proteinases in mammalian cells during apoptosis.

To understand further the relationship of structures and lysosomal proteinases during apoptosis, we examined the precise alteration-processes of subcellular structures and immunoreactivity for cathepsins B and D, using an in vitro system for apoptosis. For this we used PC12 cells, a rat pheochromocytoma cell line, which are known to die by apoptosis when cultured under conditions of serum and nerve growth factor (NGF) deprivation (BATISTATOU and GREENE, 1991, 1993). The present electron microscopic study revealed that autophagy actively occurs as an initial change in the structures of PC12 cells during apoptosis. Although autophagic vacuoles/autolysosomes appeared in the cells from the initial stage of culture, immunoreactivity for cathepsin B drastically decreased in the dying cells, and that for cathepsin D significantly increased. Considering cathepsin D to be a positive mediator of apoptosis, the present results showing the occurrence of autophagy and imbalance of protein amounts between cathepsins B and D in lysosomes during apoptosis may support our hypothesis that these enzymes regulate, in part, the cell death of PC12 cells following serum deprivation.

**MATERIALS AND METHODS**

**Cells and culture**

For the present experiments, PC12 cells were used and cultivated 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, penicillin (100 units/ml) and streptomycin (100 μg/ml). The PC12 cells were washed several times with DMEM and plated at a density of $1 \times 10^4$/cm$^2$.

**Antisera**

Rabbit antibodies to native (active) forms of rat cathepsin B and cathepsin D were purified by affinity chromatography, as previously reported (KOMINAMI et al., 1985; OHSAWA et al., 1993).

**Staining of TUNEL and lysosomal cathepsins**

PC12 cells cultured under serum deprivation were obtained at 0, 3, 6, 12, and 24 h 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 h. The cells were thoroughly washed with PBS and treated with 0.3% H$_2$O$_2$ in methanol for 30 min.

To detect nuclear DNA fragmentation, the TUNEL reaction was applied to the fixed cells according to the modified method by GAVRIELI et al. (1992) (NITATORI et al., 1995). 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 Texas red-conjugated avidin (Nichirei) was carried out for 30 min at room temperature.

To examine the immunoreactivity for cathepsins B and D, the cells were immunostained by respective antibodies against these enzymes. They were incubated with anti-cathepsin B (0.6 μg/ml) and cathepsin D (6.8 μg/ml) at 4°C overnight, respectively, and further incubated with FITC-conjugated anti-rabbit IgG for 30 min at room temperature. To analyze the correlation of TUNEL staining and immunostaining of cathepsin B or D in dying cells, double staining of each of these enzymes (FITC labeling) and nick end labeling of dUTP (Texas red labeling) was also carried out and viewed with a confocal laser scanning microscope (LSM-GB 200, Olympus, Tokyo, Japan).

**Electron microscopy**

PC12 cells were obtained at 0, 3, 6, 12, and 24 h after the onset of culture, fixed with 2% glutaraldehyde-
Fig. 1. Legend on the opposite page.
2% paraformaldehyde in PB for 2 h, and post-fixed with 2% OsO₄ for 1 h. After block-staining with a 2% aqueous solution of uranyl acetate, they were dehydrated with a graded series of ethanol and embedded in Epon 812. For light microscopy, semi-thin sections were cut 1 μm with an ultramicrotome (Reichert Ultracut N, Nissei, Japan) and stained with toluidin blue. After silver sections were cut with the ultramicrotome and stained with lead citrate and uranyl acetate, they were observed with a Hitachi H-7100 electron microscope.

RESULTS

Confocal laser scanning microscopy

Immunoreactivity for cathepsins B and D was intense and granular, and spread mainly in the peripheral region of PC12 cells prior to a serum-deprived culture (0 h) (Fig. 1a, b). Three h after the onset of culture under serum deprivation, cathepsin B-immunopositive granules either did not significantly change in number and intensity or became rather weak, compared with those at 0 h (Fig. 1c). Immunodeposits for cathepsin D in the cells, however, increased to become more intense and larger in size than that at 0 h (Fig. 1d). The distribution of these cathepsin D-immunopositive granules in the cytoplasm of the cells was similar to that at 0 h. At this stage TUNEL-positive cells were hardly observed. PC12 cells with TUNEL-positive nuclei appeared 6 h and 12 h after the onset of culture, but these cells were fewer in number until these time points (data not shown). Twenty-four h after the onset of culture, the cells with TUNEL-positive nuclei increased in number (over 50%) (Fig. 1e, f). In TUNEL-positive cells, the immunoreactivity for cathepsin D became highly intense (Fig. 1f), whereas that for cathepsin B was weak or faint (Fig. 1e).

Electron microscopy

To confirm alterations in immunoreactivity for cathepsin D in PC12 cells following serum deprivation, we examined the ultrastructures of the cells in the apoptotic process. PC12 cells prior to a serum-deprives culture possessed large nuclei with a homogenous electron-density and several small nucleoli, while small round mitochondria with distinct cristae, dense bodies, and the Golgi complex were scattered throughout the cytoplasm (Fig. 2a). Small secretory granules with dense cores were often seen in the periphery of the cytoplasm, and profiles of the rough endoplasmic reticulum were short in length and not prominent in the cytoplasm. Three h after the onset of culture under serum deprivation, dense bodies and membrane-bound vacuoles containing membranous structures and parts of the cytoplasm, which seemed to be autophagic vacuoles, appeared in the cytoplasm of the cells (Fig. 2b, c). Some of these vacuoles were encircled by double-membrane structures resembling the smooth endoplasmic reticulum (Fig. 2c); they were considered to be the initial stage of autophagy. Nuclei of these cells were similar to those at 0 h, although in some cases small amounts of heterochromatin appeared in the peripheral part of nuclei. In some PC12 cells obtained 6 h after the onset of culture, relatively condensed chromatin appeared in the peripheral part of nuclei (Fig. 2d). However, the margin of condensed chromatin was not sharply separated from the euchromatic region, so that its profile resembled that in cell division. Different from the nuclear figure of cell division, the nuclei of these PC12 cells possessed an intact nuclear envelope.

The number of PC12 cells undergoing apoptosis increased when examined 12 h and 24 h after the onset of culture, especially at 24 h. These dying cells had typical apoptotic nuclei with condensed chromatin which abutted on the nuclear envelope and was sharply separated from the other region of the nuclei, while their cytoplasmic and nuclear sizes were reduced, compared with those of the intact cells (Fig. 3a). In some dying cells, the nuclear envelope disappeared from the nuclei, and small and large masses of condensed chromatin were scattered in the cytoplasm (Fig. 3b). Moreover, there were other dying cells which possessed nuclei undergoing fragmentation into small pieces (Fig. 3c). The cytoplasm of these cells was also reduced in size, while the cytoplasmic organelles including dense bodies were often gathered in one place, although small and large vacuolar structures were scattered throughout the cytoplasm (Fig. 3b). Some cells contained numerous autophagic vacuoles in the cytoplasm (Fig. 3c). In our observations of the ultrastructures of PC2 cells during apoptosis, only one cell undergoing fragmentation and forming brebs could be seen, while apoptotic bodies having cytoplasmic organelles or small pieces of nuclei with condensed chromatin were hardly detected.

DISCUSSION

The present study demonstrated that an initial alteration in the ultrastructures of PC12 cells, a rat pheochromocytoma cell line, following serum deprivation
was the formation of autophagic vacuoles/autolysosomes in the cytoplasm. This morphological change in the cytoplasm of the cells at an early stage after the onset of serum deprived culture well corre-
sponded with an increase in the immunoreactivity for cathepsin D, a lysosomal aspartic proteinase.

**Fig. 2.** Electron micrographs of PC12 cells at 0 h (a), 3 h (b, c) and 6 h (d) after the onset of culture under serum deprivation. At 3 h (b, c), a PC12 cell, which possesses numerous membrane-bounded vacuoles containing membranous structures (thin arrow) and a part of the cytoplasm (thick arrows), is observed while some of these vacuolar structures are encircled by double membrane structures resembling the smooth endoplasmic reticulum (arrowheads, c). At 6 h (d), a PC12 cell having a nucleus with relatively condensed chromatin occasionally appears. Note that the chromatin figure in the cell is located in the periphery of the nucleus and its margin is not sharply separated from the other euchromatic region. a, c, d: ×6,000, b: ×30,000
The possible role of autophagic vacuoles/autolysosomes and cathepsins B and D in PC12 cells following serum deprivation

Autophagy consisting of the sequestration of intracellular components and their degradation by lysosomal enzymes usually occurs in normal cells to maintain cellular turnover, while it is known to greatly increase in cells under pathological conditions which cause cell dysfunction such as hypoxia, ischemia, endotoxin shock, and metabolic inhibition (GLAUMANN et al., 1981). In the process of autophagic alternations frequently occurring in dying cells, the role of the autophagy is believed to be an attempt by the cells to protect themselves from death (CLARKE, 1991). Our previous studies have shown that autophagy occurs in the CA1 pyramidal neurons of the gerbil hippocampus after brief ischemia and in effete epithelial cells at the villous tip of the human small intestine (NITATORI et al., 1995; SHIBAHARA et al., 1995; UCHIYAMA, 1995). Autophagic vacuoles/auto-

Fig. 3. Electron micrographs of PC12 cells 24 h after the onset of culture under serum deprivation. At 24 h (a-c), most PC12 cells have reduced in both cytoplasmic and nuclear sizes. a. A dying PC12 cell possesses a nucleus with condensed chromatin which abuts on the nuclear envelope. The cytoplasmic organelles are gathered in one place of the cytoplasm, although large and small vacuoles are scattered throughout the cytoplasm. b. In a PC12 cell, the nuclear envelope occasionally disappears and small and large masses of condensed chromatin (arrows) are scattered in the cytoplasm. c. The nucleus of a dying cell is undergoing fragmentation (FN) and masses of condensed chromatin (CM) are also seen in the cytoplasm. Numerous autophagic vacuoles and dense bodies are seen in the cytoplasm of the cell. a: ×8,300, b: ×6,600, c: ×8,000
lysosomes in these cells appear at the early stage of apoptotic processes. Moreover, it is interesting that the decrease in the uptake of amino acids in the CA1 pyramidal neurons of the gerbil hippocampus after brief ischemia occurs concomitantly with the increase in the immunoreactivity for lysosomal cysteine proteinases (Nitatori et al., 1995). That is, lysosomes in the CA1 pyramidal neurons may serve as a source of amino acids by actively degrading proteins in the early stage after ischemic insult, resulting in their protecting themselves from further alterations. In the present study, PC12 cells undergoing apoptosis were cultured in the absence of serum but in the presence of Dulbecco’s modified Eagle’s medium with a high level of glucose (4.5 g/L) which contains various sorts of amino acids, vitamins, and balanced salts. This indicates that the cells were not incubated in an environment with a deficiency of nutrients but rather with a lack of survival signals. Therefore, it seems likely that autophagy occurring in PC12 cells cultured under serum deprivation was not simply similar to that occurring in cells under starved conditions.

Our previous studies have shown that both the protein amount and proteolytic activity of cathepsin B in PC12 cells 24 h after the onset of culture under serum deprivation are drastically reduced and those of cathepsin D are significantly augmented, compared with their initial levels (Kanamori et al., 1998; Shibata et al., 1998; Isahara et al., 1999). These data are consistent with the present results showing that, in PC12 cells cultured under serum deprivation decreased drastically and that for cathepsin D significantly increased as the apoptotic process of the cells proceeded. It has been demonstrated that PC12 cells overexpressing cathepsin B become resistant to a serum-deprived culture, and that CA074, a specific inhibitor for cathepsin B decreases their viability (Shibata et al., 1998). Moreover, overexpression of cathepsin D promotes the apoptosis PC12 cells white pepstatin A, an aspartic proteinase inhibitor considerably rescues these transfected cells from apoptosis. From these lines of evidence, we hypothesize that the dissociation of the protein amounts between cathepsins B and D occurs, in part, responsible for the apoptosis of PC12 cells following serum deprivation; an apoptotic pathway appears to exist, which is regulated by the lysosomal proteinases wherein cathepsin D acts as a death factor and its death-inducing activity is usually suppressed by cathepsin B (Kanamori et al., 1998; Shibata et al., 1998; Isahara et al., 1999). In fact, an antisense cDNA fragment identical to human cathepsin D was isolated as one of the positive mediators of apoptosis by transfections with antisense cDNA expression libraries into HeLa cells exposed to death stimuli, and it has been confirmed that the enzyme induces apoptosis of the cells when overexpressed (Deiss et al., 1996).

In a serum-deprived culture, however, the apoptosis of PC12 cells is believed to be induced by the activation of the caspase family of proteinases. We have demonstrated that PC12 cells express caspase-3 mRNA, and when cultured under serum deprivation, they are positively double-stained for activated caspase-3 in the cytoplasm and for TUNEL in nuclei. Moreover, Sato et al. (1997) isolated cDNA clones of rat caspase-2 from a PC12 cell cDNA library and demonstrated its protein product by immunoblotting. Activation of caspase-2 or -3 has been shown to be required for apoptosis of PC12 cells after trophic factor withdrawal (Li et al., 1997; Takadera and Ohyashiki, 1997; Troy et al., 1997). Simultaneously, it is also true that, although the viability of PC12 cells in a serum-deprived culture is increased when the activation of caspase-3 is suppressed by acetyl-DEVD-cho, a specific inhibitor of caspase-3-like proteinases, this increased viability is significantly reduced when cultured in the presence of acetyl-DEVD-cho and CA074 (Isahara et al., 1999). Moreover, the further addition of pepstatin A to the culture medium restores the viability of the cells. These events indicate that the apoptotic cascade regulated by lysosomal cathepsins is usually blocked in PC12 cells by that regulated by the caspase family of proteinases. That is, inactivation of the caspase family of proteinases inhibits alterations in lysosomal cathepsins B and D. The present data showing that TUNEL-positive PC12 cells appearing 24 h after the onset of culture under serum deprivation are intensely positive for cathepsin D but only faintly so for cathepsin B suggest that the dissociation of the protein amounts between cathepsins B and D occurs in the dying PC12 cells. This may indicate that the cell death cascade regulated by lysosomal cathepsins is simultaneously ongoing when the cascade involving the caspase family of proteinases is activated; the two are able to function independently. From these results, it seems likely that the occurrence of autophagy and alterations in the amounts of lysosomal cathepsins in PC12 cells following serum deprivation are not attributed to a deficiency of nutrients but rather involved in the apoptotic cascade.

**Morphological changes in PC12 cells following serum deprivation**

It is well known that apoptosis processes rapidly proceed in cells. In particular, TNFR1 (tumor
necrosis factor receptor 1)/Fas-mediated apoptosis induces rapid alterations in morphology, since this type of death stimuli involves the activation of caspase-8 which further activates the caspase family of proteinases and CAD/DF40 (caspase-activating DNase/DNA fragmentation factor 40) (LIU et al., 1997; NICHOLSON and THORNBERY, 1997; ENARI et al., 1998; SOLARY et al., 1998; TAKAHASHI et al., 1998).

Cell death following trophic factor withdrawal is usually executed by a cascade different from the TNFR1/Fas-mediated pathway and proceeds more slowly than TNFR1/Fas-mediated apoptosis. The apoptosis of PC12 cells following serum deprivation proceeds slowly and is considered a typical model of cell death following trophic factor withdrawal.

As mentioned above, an initial alteration in the ultrastructures of PC12 cells, which was autophagic vacuoles/autolysosomes, occurred in the cytoplasm, but not in nuclei. Corresponding to the appearance of TUNEL-positive nuclei in the cells, an ultrastructural change in nuclei, in some cases, occurred 6 h after the onset of culture. Relatively condensed chromatin, which resembled the chromatin figure during cell division, first appeared in the peripheral part of nuclei, and the margin of condensed chromatin was not sharply separated from the euchromatic region. This type of chromatin figure, though observed less frequently, may correspond to the initial stage of DNA fragmentation into oligonucleosomes.

The apoptosis of thymocytes, lymphocytes and/or leukemia cells is often accompanied by a fragmentation of both cell bodies and nuclei into small pieces, resulting in the formation of apoptotic bodies (WYLLIE, 1981). In the present study we observed only one figure which showed a PC12 cell undergoing fragmentation into small pieces. Moreover, the dying PC12 cells showing a shrinkage of cell bodies and nuclei were seen 24 h after the onset of culture, but apoptotic bodies having cytoplasmic organelles or small nuclear pieces with condensed chromatin appeared less frequently. These results suggest that PC12 cells belong to a cell type in which cells are rarely/almost never fragmented into small pieces when cultured under trophic factor deprivation.

In the present study, the dying PC12 cells possessed many lysosomal structures in the cytoplasm. The appearance of autophagic vacuoles/autolysosomes in the cells having nuclei with condensed chromatin is consistent with the present immunohistochemical results showing that intense cathepsin D-immunodeposits co-exist with TUNEL-positive nuclei in PC12 cells. The limiting membrane of autophagic vacuoles/autolysosomes appeared intact in the dying PC12 cells, as has been suggested in apoptosis of hepatocytes by KERR (1971).

In conclusion, considering cathepsin D as a positive mediator of apoptosis, the present data showing the occurrence of autophagy and imbalance of protein amounts between cathepsins B and D in lysosomes during apoptosis strongly argues for our hypothesis that these enzymes are, in part, involved in the cell death cascade for PC12 cells following serum deprivation.

REFERENCES


Autophagy and Cathepsins B and D During Apoptosis


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