REVIEW

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Autophagic Cell Death and Its Execution by Lysosomal Cathepsins

Yasuo UCHIYAMA

Department of Cell Biology and Neuroscience, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

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Summary. **In the last decade, the molecular mechanisms of apoptosis, a major type of active cell death (type I cell death) have largely been clarified in mammalian cells. Particularly, the caspase family of proteinases has been shown to play crucial roles in the execution of apoptosis. Differing from apoptosis, type II cell death is known to be associated with autophagosomes/autolysosomes and appear in the developing nervous system (CLARKE, 1990). We have previously shown that delayed neuronal death occurring in the CA1 pyramidal layer of the gerbil hippocampus after brief forebrain ischemia is apoptotic in nature and autophagosomes/autolysosomes abundantly appear in the neurons before DNA fragmentation. To further understand the roles of autophagosomes/autolysosomes in active cell death, we examined the apoptosis of PC12 cells using morphological and biochemical techniques. PC12 cells are known to undergo apoptosis when cultured in the absence of serum. In such an environment, the mitochondrial pathway of apoptosis is activated; cytochrome c is released from mitochondria, and caspase-9/caspase-3 are activated. We have first examined morphological features of PC12 cells during the apoptotic process following serum deprivation, and found that autophagy is induced from the early stage of the process in the cells before typical nuclear changes. When autophagy is inhibited in the cells by 3-methyladenine, an autophagy inhibitor, they are largely protected from apoptosis. In relation to the induction of autophagy in PC12 cells following serum deprivation, immunoreactivity, protein amounts, and the proteolytic activity of lysosomal proteinases, particularly cathepsins B and D, are all greatly altered; those of cathepsin B drastically decrease in the cells from the early stage of serum-deprived cultures, whereas those of cathepsin D increase. Moreover, PC12 cells overexpressing cathepsin D undergo apoptosis more rapidly in serum-deprived cultures than wild-type cells, whereas those overexpressing cathepsin B increase the viability. These lines of evidence suggest that autophagy is involved in PC12 cell death following serum deprivation, this type of cell death being regulated by lysosomal proteinases, cathepsins B and D, downstream autophagy.**

It has been well established that cells may die using their own death machinaries in response to appropriate death stimuli. This type of cell death is called active cell death and contrasts with necrosis, which is accidental cell death induced by changes in the living milieu. The main form of active cell death, namely apoptosis, occurs during morphogenesis including neurogenesis, corresponding to cell renewal in various mature tissues, and also under pathological conditions (KERR et al., 1972; UCHIYAMA, 1995). Molecular mechanisms of apoptosis have largely been clarified by recent progress in molecular cell biology. In particular, the caspase family of proteinases has been shown to play a major role in the execution of apoptosis. One other type of active cell death is known as autophagic or lysosomal cell death (or type II cell death), whose molecular mechanisms remain mostly unknown (CLARKE, 1990).

We have examined physiological and pathological active death in various cells and tissues using morphological and biochemical techniques and found that lysosomes and autophagy are activated in cells undergoing apoptosis (NITATORI et al., 1995; KANAMORI et al., 1998; OHSAWA et al., 1998; SHIBATA et al., 1998; ISAHARA et al., 1999; KOIKE et al., 2000). To analyze the precise apoptotic processes, we have 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; SATO et al., 1994). In these studies, we have noted that lysosomal cysteine and aspartic proteinases, cathepsins B and D, participate in the execution of active death. This review introduces cell death regulated by lysosomal proteinases in relation to the induction of autophagy.

I. PHYSIOLOGICAL ROLES OF LYSOSOMAL CATHEPSINS B AND D, AND AUTOPHAGY

For a precise understanding of autophagic cell death, we first explain the physiological aspects of various factors appearing in the death process. Lysosomes are ubiquitous in all animal cells as an acidic compartment with limiting membranes and contain various types of hydrolytic enzymes with acidic pH optima. They are able to degrade unneeded intra- and extracellular materials into biological monomers which are then reutilized by the cells. The lysosome is the centerpiece, consisting of acidic compartments known as the endosomal-lysosomal system, and includes Golgi-derived vesicles with newly synthesized enzymes as primary lysosomes, auto- and heterophagocytosed vacuoles fused with the primary lysosomes, and residual bodies with indigestible materials.

I-1. Characteristics of cathepsins B and D

As for lysosomal proteinases, cathepsins B and D are ubiquitously present in mammalian tissue cells (BARRETT, 1977; BARRETT and KIRSCHKE, 1981; WHITAKER et al., 1981; KOMINAMI et al., 1985; REID et al., 1986; UCHIYAMA et al., 1994). Cathepsin B (EC3.4.22.1), a cysteine proteinase, which has a cysteine residue in its active site, belongs to the papain superfamily. The molecular weight of cathepsin B is 39 kD as its proform and 29 kD and 26 kD as active single- and heavy-chain forms. PI values of the enzyme are present within 5.4–5.6 and z-Arg-Arg-MCA is most popularly used as a fluorogenic substrate. Cathepsin B deficient mice have been produced by PETERS and his colleagues, but their phenotype has not been reported yet except for two reports mentioning that cathepsin B is not involved in antigen processing (DEUSSIG et al., 1998) and plays a role in intrapancreatic trypsinogen activation and the onset of acute pancreatitis (HALANGK et al., 2000).

Cathepsin D (EC3.4.23.5), an aspartic proteinase which has two aspartate residues in its active center, belongs to the pepsin superfamily of proteinases. The molecular weight of procathepsin D is 56 kD, while active forms are 42 kD as a single chain form and approximately 30 kD as a heavy chain form. Cathepsin D is stable in the wide pH range and PI values of its major form are 5.7–6.5. We have recently produced fluorogenic substrates which are a valuable tool for routine assays (YASUDA et al., 1999). Through the generation of cathepsin D deficient mice, it has recently been shown that cathepsin D is involved in limited proteolysis rather than bulk proteolysis (SAFTIG et al., 1995); mice are born normally, but die at postnatal day 26 (P26) $\pm$ 1 day due to massive intestinal necrosis, thromboembolia, and lymphopenia. Moreover, neurons in the central nervous system (CNS) of cathepsin D deficient mice show a new form of lysosomal accumulation disease with a phenotype resembling neuronal ceroid lipofuscinosis, suggesting that cathepsin D plays an important role in protein degradation, particularly in CNS tissues (KOIKE et al., 2000).

I-2. Mannose 6-phosphate receptors needed for transport of cathepsins B and D from the trans Golgi network to prelysosomal compartments

Newly synthesized lysosomal enzymes — including cathepsins B and D — acquire mannose 6-phosphate (M6P) recognition signals which direct them from the trans-Golgi network (TGN) to the prelysosomal compartments such as nascent autophagosomes and early endosomes. Two receptors, cation dependent- and independent mannose 6-phosphate receptors (CD- and CIMPR), are involved in this sorting function in mammalian cells. CIMPR, a large transmembrane glycoprotein (Mr$\sim$300 kD), is also known as the insulin-like growth factor II (IGFII)/M6P receptor and contains two M6P binding sites, while CDMPR is a small receptor (Mr$\sim$46 kD) and has one M6P binding site. It is believed that CDMPR exists as a dimmer and/or tetramer on the membrane. Both receptors may appear on the cell surface where they are then internalized. CIMPR, but not CDMPR, binds extracellular ligands (VON FIGURA and HASILIK 1986; KORNFELD and MELLMAN 1989; LUDWIG et al., 1995; DAHMS, 1996; MUNIER-LEHMANN et al., 1996a, b). We have previously demonstrated the distribution of lysosomal cysteine proteinases, cathepsins B, H, and L, in various tissues and found that they show heterogeneous distribution patterns depending on the enzymes, cells, and tissues (UCHIYAMA et al., 1994). Moreover, the rat liver has shown different distribution of MPRs; CIMPR is localized in hepatocytes, whereas CDMPR is mainly distributed in non-hepatocytic cells including Kupffer cells and antigen-presenting cells (WAGURI et al., 2001). In fact, it has been suggested that MPRs preferentially bind different subgroups of lysosomal enzymes (LUDWIG et al., 1994; POHLMANN et al., 1995; MUNIER-LEHMANN et al., 1996a).

I-3. Recent understanding of autophagy

Autophagy consisting of the sequestration of intracellular components and their degradation by lysosomal enzymes is usually a major pathway for the bulk

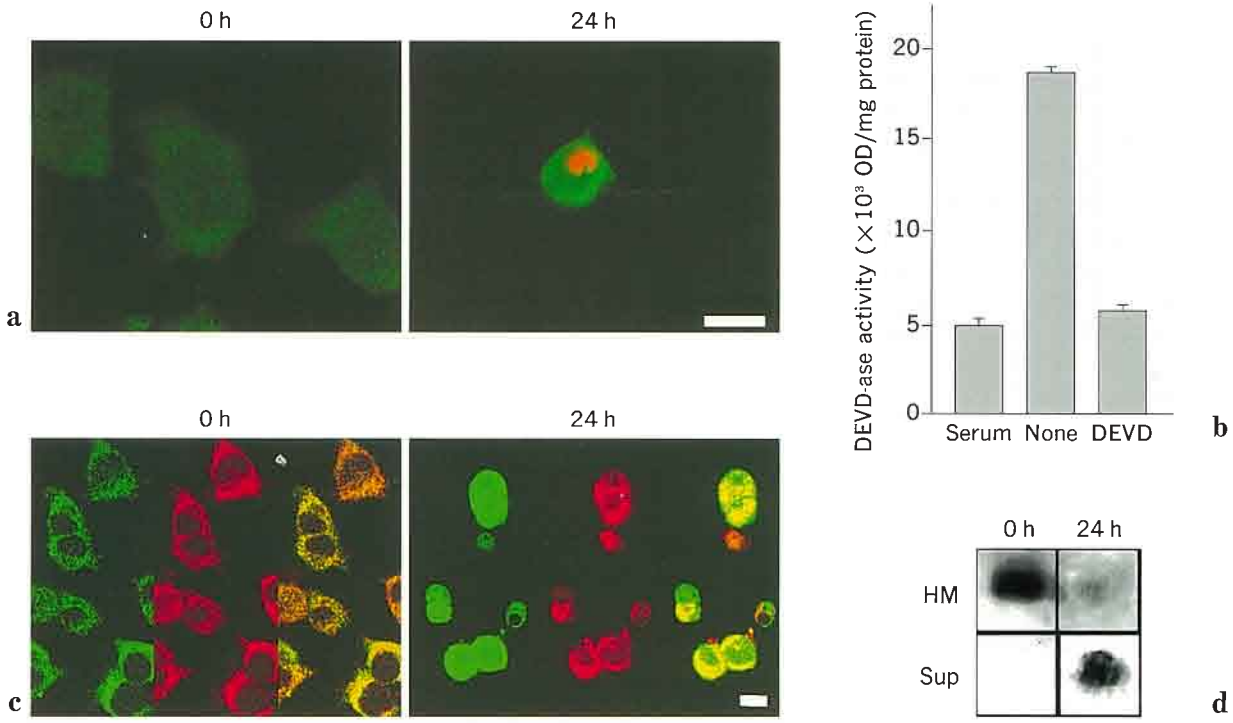


Fig. 1. Involvement of caspase-3 activation in apoptosis of PC12 cells following serum deprivation. **a.** Double staining images of activated caspase-3 and TUNEL in PC12 cells obtained at 0 h and 24 h after the beginning of serum-deprived culture. Positive staining of activated caspase-3 is localized in the cytoplasm of a cell whose nucleus is positive for TUNEL, whereas no staining is seen in the cells at 0 h. **b.** Proteolytic activity of caspase-3-like proteinases (DEVDase) in cytosolic extracts of PC12 cells obtained 24 h after the beginning of culture in the presence of serum (Serum), in the absence of serum (None) or in the absence of serum but in the presence of acetyl-DEVD-cho (DEVD). The activity is expressed as OD ($\times 10^3$)/mg protein. Vertical bars indicate $\pm$ standard deviation. **c.** Double immunostaining images of subunit β of mitochondrial ATP synthase (red color) and cytochrome c (green color) in PC12 cells obtained at 0 h and 24 h after the beginning of serum-deprived culture. Granular immunosignals for both cytochrome c and subunit β are seen in the cytoplasm of the cells at 0 h (right figure is merged), whereas diffuse staining of cytochrome c is clearly demonstrated in the cell at 24 h. **d.** Immunoblotting of cytochrome c in the heavy membrane fraction (HM) and the S-100 fraction (Sup) obtained from PC12 cells at 0 h and 24 h after the beginning of serum-deprived culture. Cytochrome c is translocated in the cytosolic fraction (S-100) at 24 h. Scale bars in a, c indicate 10 μm.

degradation of intracellular protein in lysosomes and occurs in normal cells to maintain cellular turnover (GLAUMANN et al., 1981; DUNN, 1990a, b). It is also known to greatly increase in cells under pathological conditions which cause cell dysfunction, such as hypoxia, ischemia, endotoxin shock, and metabolic inhibitors (GLAUMANN et al., 1981). The physiological roles of autophagy remain unknown, but it is induced when cells are cultured under starvation conditions. Therefore, its roles are believed to maintain an intracellular pool of amino acids which are required for protein synthesis and glyconeogenesis. Recent molecular studies using yeast *Saccharomyces cerevisiae* have contributed to the clarification of the molecular mechanisms of autophagy and found groups of genes (Apg/CVT genes) required for the formation of autophagosomes (see review by KLIONSKY and OHSUMI, 1999). The most important finding using yeast mutants defective autophagy is that a ubiquitination-like system is used for the complex formation of Apg5 and Apg12 and the modification of an Apg molecule (Apg8p) with phosphatidyl ethanolamine, which is speculated to recruit membrane sacs required for the sequestration of part of the cytoplasm (MIZUSHIMA et al., 1998, 2001; ICHIMURA et al., 2000). In these reactions, Apg7 and Apg10 act as E1 and E2, enzymes required for ubiquitination. Mammalian homologues of Apg8p are found to be LC3, a

microtubule associated protein, GATE, a regulator protein of transport within Golgi lamellae, and GABARAP, a GABA receptor associated protein (KABEYA et al., 2000). After cleavage by Apg4, a cysteine proteinase, the proform of LC3 is converted to LC3-I which works as a cytoskeleton protein, while LC3-I is further converted to LC3-II when autophagy is induced in the cells (KABEYA et al., 2000). In addition to Apg8p, beclin-1, a homologous protein of Apg6p, has been demonstrated to be deficient in MCF7 cells, a human breast cancer cell line, which show a low activity of the formation of autophagosomes (LIANG et al., 1999). Although our understanding of autophagy has been largely advanced, the molecular mechanisms of autophagosome-forming processes, especially of membrane dynamics to form autophagosomes, remain to be solved. That is, it presently remains unknown whether the membrane sac which encircles nascent autophagosomes originates newly synthetized membranes or the endoplasmic reticulum, as hitherto suggested by DUNN (1990a, b).

II. CASPASE: EXECUTIONERS OF MAJOR PATHWAYS IN APOPTOSIS

Apoptosis, which has primarily been defined by morphological observations, consists of cell shrinkage, nuclear chromatin condensation, and the fragmentation of cell bodies and nuclei into small pieces, i.e. apoptotic bodies, which are phagocytosed by adjacent cells, usually macrophages (KERR et al., 1972). The most important event in the apoptotic process is the formation of cell-fragmented pieces which are encircled by cell membranes and easily phagocytosed by macrophages without mediating inflammation. This differs from necrotic cell death which massively occurs in tissues following, for example, inflammation.

Apoptosis has been shown to occur in physiological and pathological situations following various death stimuli, but the final pathway of apoptosis is mediated by various machineries such as the caspase (cysteine-dependent aspartate-directed proteases) family of proteinases, Apaf-1, and DNA fragmentation factors (DFF40 and 45)/caspase activated DNase and its inhibitor (CAD and ICAD) (NICHOLSON and THORNBERRY, 1997; LIU et al., 1997, 1998; ENARI et al, 1998; SAKAHIRA et al. 1998). Two major pathways of apoptosis in mammalian cells have been noted (SALVESEN and DEXIT, 1999): 1) the ligation of death receptors, Fas/TNFR1 (TNF receptor 1) with their ligands which activates caspase-8; and 2) the release of death machinaries from mitochondria by cellular stress which activates caspase-9. In the first pathway, the molecule having a death domain (FADD, Fas associated death domain) associated with the death receptors recruits procaspase-8, which contains a death effector domain and is autoactivated by its oligomerizatin (BOLDIN et al., 1996). Activated caspase-8 activates caspase-3 or Bid. The latter attacks mitochondria, resulting in the activation of the mitochondrial pathway (YIN, 2000). Since execution factors of apoptosis are present in the cytoplasm of the cells, WANG and his colleagues have tried to search for these factors by the cell-free system (LIU et al. 1996, 1997, 1998; LI et al., 1997; ZOU et al., 1997). In this system, they have prepared a soluble fraction from the HeLa cell cytoplasmic fraction (S-100) and nuclei from the liver and found that the activation of caspase-3 and DNA fragmentation occur by mixing S-100 and nuclei in the presence of dATP. Then they have isolated apoptotic proteinase activating factors-1, 2 and 3, i.e., Apaf-1, which contains homologous domains of CED-3 and CED-4 of *Caenorhabdtis elegans* in the amino terminus portion and WD40 repeat in the carboxyl terminus portion, Apaf-2, which is cytochrome c, and Apaf-3, which is procaspase-9. These factors are involved in the second apoptotic pathway which is induced by cellular stress such as the deprivation of trophic factors and radiation which releases cytochrome c from mitochondria. The released cytochrome c together with dATP/ATP binds Apaf-1 by which procaspase-9 is recruited and autoactivated by the oligomerization. Activated caspase-9 activates caspase-3 which further activates CAD by cleaving ICAD (ENARI et al., 1998). Since CAD possesses a nuclear transport domain, it translocates into nuclei and cleaves DNA into oligonucleosomes.

Death machinaries thus mediate apoptosis. In neurodegenerative diseases and senile neuronal changes, pathological situations progress chronically. At present, it remains unknown whether cell death occurring in these disease situations is mediated by apoptotic machinaries or other novel factors. We have paid attention to autophagy and lysosomal cathepsins as death mediators for a novel cell death pathway.

III. INVOLVEMENT OF AUTOPHAGY IN ACTIVE CELL DEATH AND LYSOSOMAL PROTEINASES AS DEATH MEDIATORS DOWNSTREAM AUTOPHAGY

We have previously shown that autophagy actively occurs in both the apoptotic processes of CA1 pyramidal neurons in the gerbil hippocampus after brief forebrain ischemia and of effete epithelial cells at the

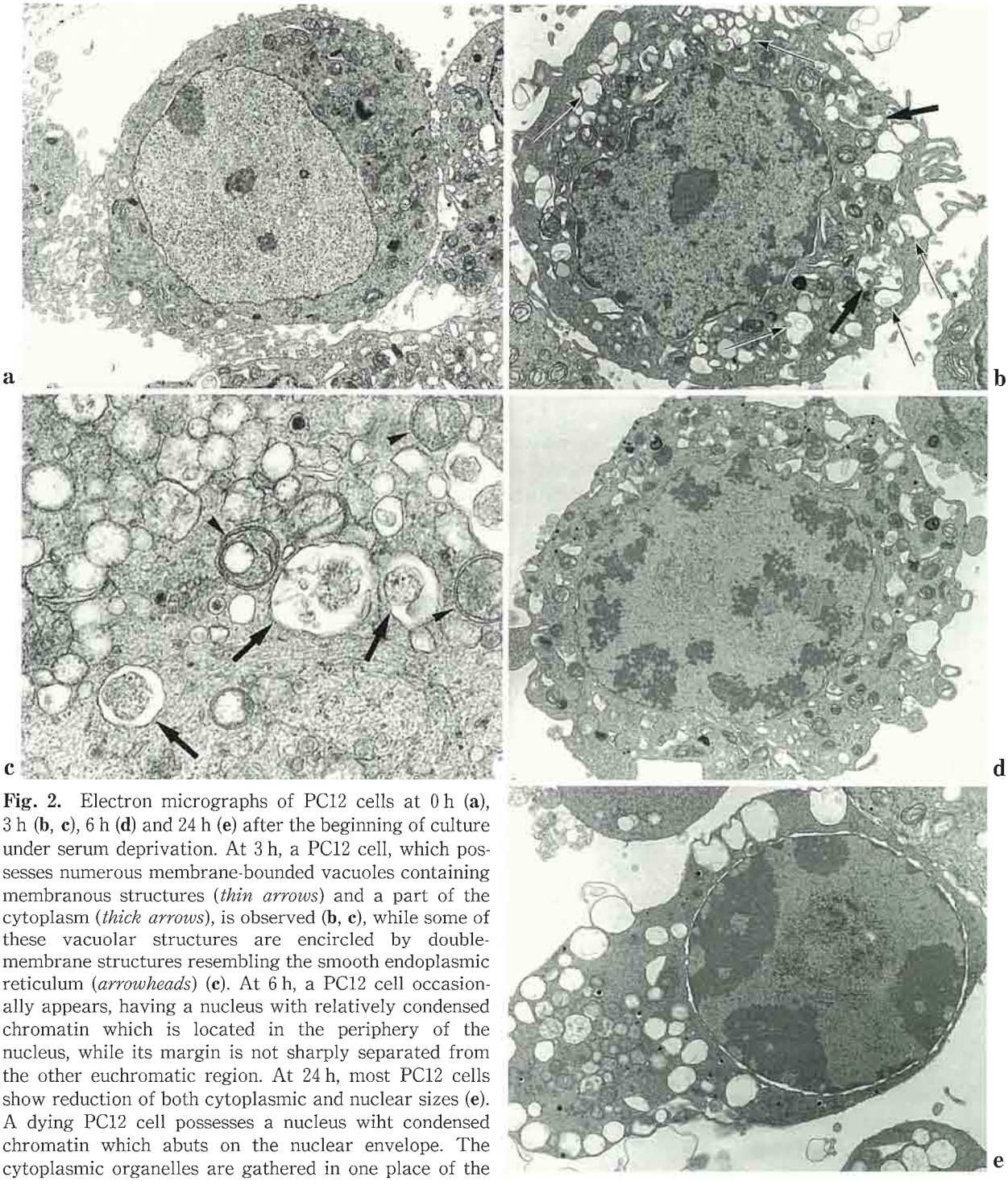


Fig. 2. Electron micrographs of PC12 cells at 0 h (**a**), 3 h (**b**, **c**), 6 h (**d**) and 24 h (**e**) after the beginning of culture under serum deprivation. At 3 h, a PC12 cell, which possesses numerous membrane-bounded vacuoles containing membranous structures (*thin arrows*) and a part of the cytoplasm (*thick arrows*), is observed (**b**, **c**), while some of these vacuolar structures are encircled by double-membrane structures resembling the smooth endoplasmic reticulum (*arrowheads*) (**c**). At 6 h, a PC12 cell occasionally appears, having a nucleus with relatively condensed chromatin which is located in the periphery of the nucleus, while its margin is not sharply separated from the other euchromatic region. At 24 h, most PC12 cells show reduction of both cytoplasmic and nuclear sizes (**e**). A dying PC12 cell possesses a nucleus wiht 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. a: ×4,800, b, d: ×6,000, c: ×30,000, e: ×8,300

villous tip of the human small intestine (NITATORI et al., 1995; SHIBAHARA et al., 1995). The role of autophagy, which frequently occurs in such dying cells, is believed to protect the cells from death (CLARKE, 1990), However, BURSCH et al. (1996) have shown that anti-estrogen drugs such as tamoxifen induce autophagic vacuoles in MCF-7 cells, a human mammary cancer cell line, resulting in their death, which is prevented by 3-methyladenine, an inhibitor of autophagy. Moreover, a lysosomal aspartic proteinase, cathepsin D, has been shown to be involved in apoptosis of cultured cells and neurodegenerative processes (CATALDO et al., 1995; DEISS et al., 1996). An antisense cDNA fragment identical to human cathepsin D is isolated as one of 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). It is interesting that gene expression and cellular content of cathepsin D are upregulated in pyramidal neurons of brains with Alzheimer's disease (CATALDO et al., 1995). Thus, lysosomal proteinases and autophagy have been suggested to be involved in cell death.

We therefore examined the relationship between active cell death and autophagy or lysosomal proteinases using cultured neuronal cells.

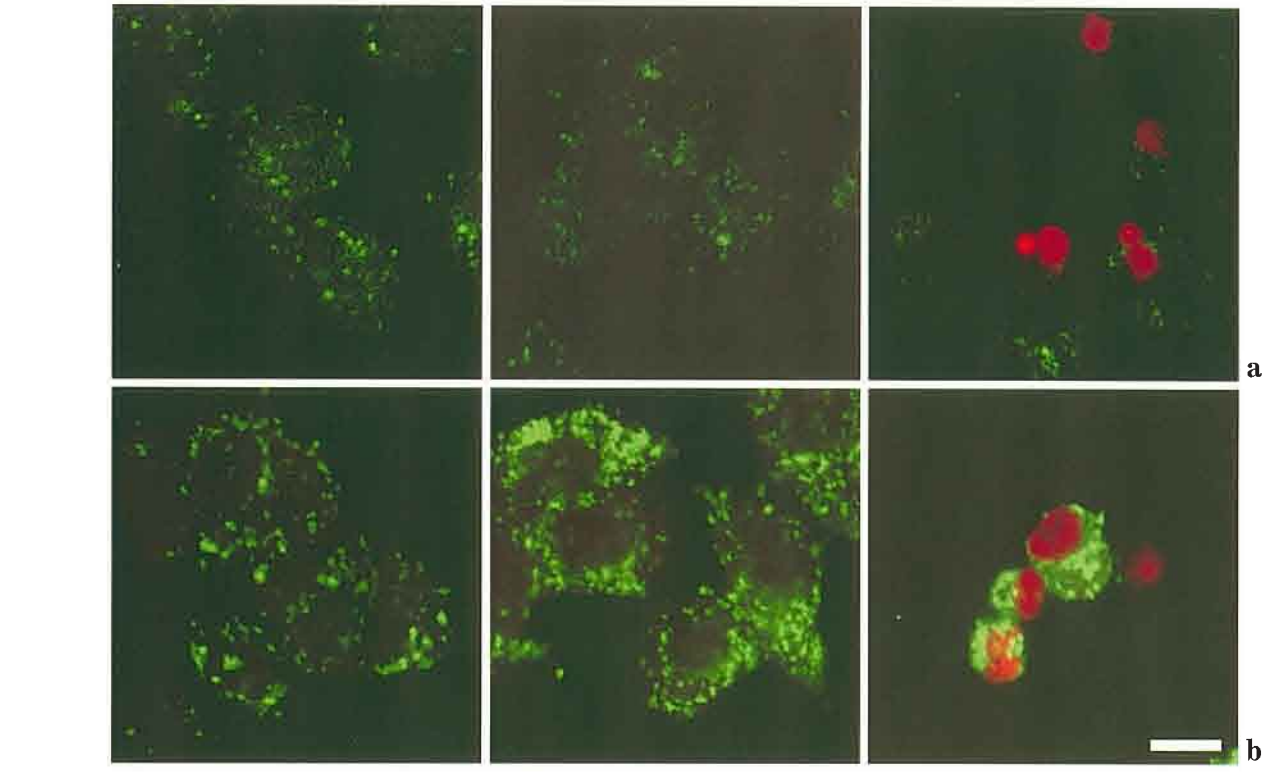
III-1. Trophic factor deprivation activates the lysosomal system in apoptosis of neuronal cells

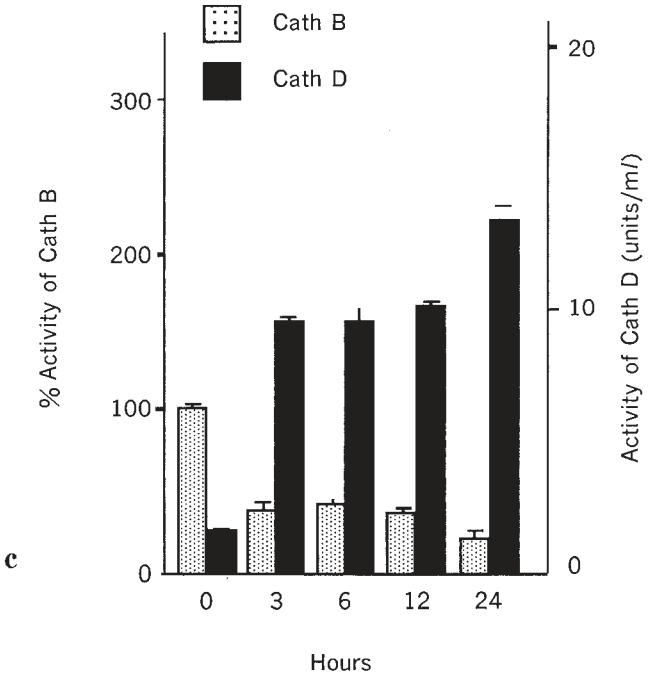
As stated above, PC12 cells die by apoptosis when cultured in the absence of serum (BATISTATOU and GREENE, 1991, 1993; SATO et al., 1994). In fact, caspase-3-like proteinase activity increases and activated caspase-3 is co-stained with TUNEL in cells obtained 24 h after the beginning of serum-deprived cultures (Fig. 1) (ISAHARA et al., 1999). Moreover, immunoreactivity for cytochrome c co-localizes well with subunit β of mitochondrial ATP synthase in intact PC12 cells, whereas its immunosignals become diffuse in the cytoplasm of the cells 24 h after the beginning of serum-deprived cultures (Fig. 1). This tendency of cytochrome c in PC12 cells undergoing apoptosis has also been confirmed by immunoblot analysis; cytochrome c is distributed only in the heavy membrane (mitochondrial) fraction when intact PC12 cells are cell-fractionated, whereas it is translocated to the soluble fraction in the cells undergoing apoptosis, suggesting that apoptosis of PC12 cells following serum deprivation is executed by the caspase-9/caspase-3 cascade.

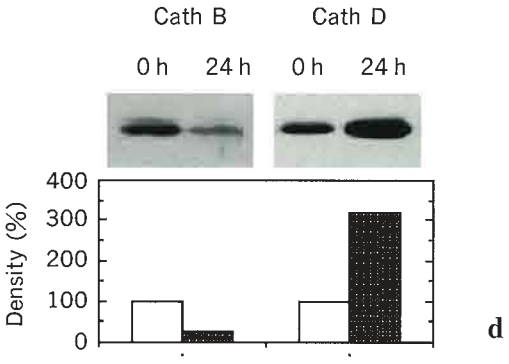
As for ultrastructural changes in PC12 cells, autophagosomes/autolysosomes which contain part of the cytoplasm appear in the cytoplasm of the cells 3 h after the beginning of serum-deprived cultures (Fig. 2). A distinct nuclear change showing relatively condensed chromatin first appears in the peripheral part of the nuclei at 6 h. The number of PC12 cells having nuclei with chromatin condensation increases especially at 24 h, while these cells show shrinkage of both the cytoplasm and nuclei. Dense bodies and autophagic vacuoles with a limiting membrane are seen in these cells. Moreover, immunoreactivity for cathepsins B and D in lysosomes of the cells undergoing apoptosis greatly changes. As shown in Figure 3, positive signals for cathepsins B and D are finely distributed in intact PC12 cells. Immunoreactivity for cathepsin B decreases even at 3 h and drastically at 24 h after the beginning of serum-free cultures, whereas that for cathepsin D distinctly increases during serum-free culturing. These changes in immunoreactivity for cathepsins B and D have also been confirmed in PC12 cells by measuring proteolytic activity and protein amounts of these enzymes (Fig. 3) (SHIBATA et al., 1998; ISAHARA et al., 1999).

Since autophagy is induced and lysosomal proteinases greatly alter in the PC12 cells during the apoptotic process, the next question is the issue of

Fig. 3. Changes in lysosomal cathepsins B and D during the apoptotic process of PC12 cells following serum deprivation. **a** and **b.** Double immunostaining of TUNEL (red color) and cathepsin B (green color) (**a**) or D (green color) (**b**) in PC12 cells at 0 h (left), 3 h (middle) and 24 h (right) after the onset of culture under serum deprivation. Immunoreactivity for cathepsin B becomes weaker in PC12 cells at 3 h (**a**, middle) than that at 0 h (**a**, left), while only faint immunoreactivity is detected in TUNEL-positive cells at 24 h (**a**, right). On the other hand, cathepsin D-immunopositive granules become more intense and larger in size in the cells at 3 h (**b**, middle) than those at 0 h (**b**, left). At 24 h, TUNEL-positive cells possess highly intense immunoreactivity for cathepsin D (**b**, right). Confocal laser scanning microscopic images. Bar indicates 10 μm. **c.** Proteolytic activity of cathepsins B (Cath B) and D (Cath D) in PC12 cells during the apoptotic process. Cathepsin B activity drastically decreases during the process, whereas cathepsin D activity increases. **d.** Immunoblotting of cathepsins B (Cath B) and D (Cath D) in extracts of PC12 cells obtained at 0 h and 24 h after the beginning of serum deprived culture (upper panel). Protein amounts of both enzymes change in parallel to their immunoreactivity and proteolytic activity during the apoptotic process. Lower panel shows bar graphs of the density in the upper panel.







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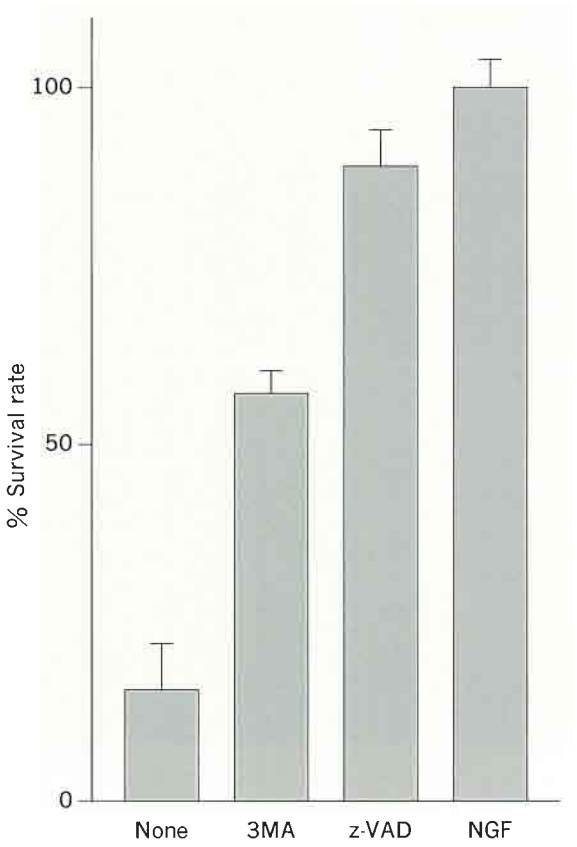

Fig. 4. Survival rates of PC12 cells cultured in the absence of serum (None), and in the absence of serum but in the presence of 3 methyladenine (3MA), z-VAD-fmk (z-VAD) or nerve growth factor (NGF). The 3MA, an autophagy inhibitor, significantly increases the viability of the cells, although its effect on the survival rate is weaker than those of z-VAD, a pan-caspase inhibitor. The survival rates were determined by lactate dehydrogenase activity of living cells at 24 h after the beginning of serum-free culture and the survival rate of the cells cultured in the presence of NGF was estimated as 100%.

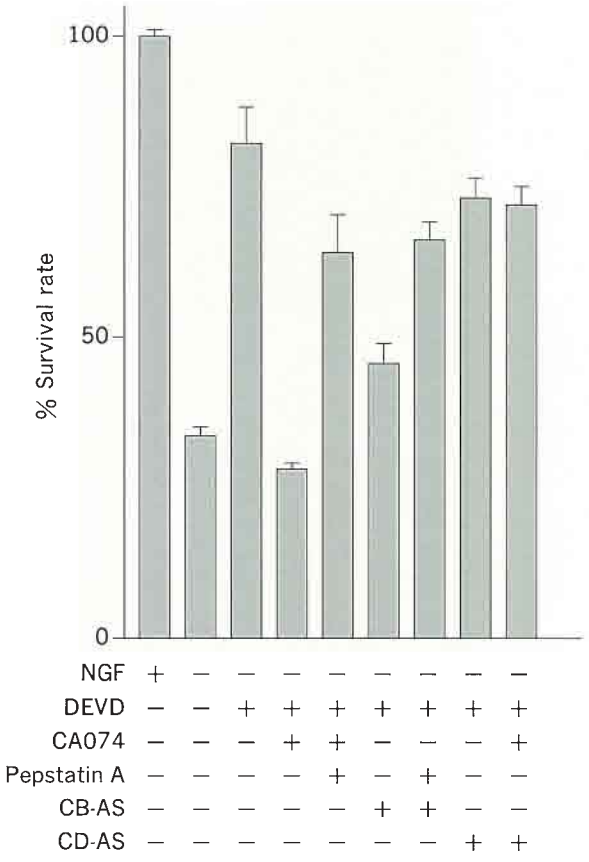

Fig. 5. Effects of specific inhibitors of cathepsins B and D or their antisense oligonucleotides on the survival rates of PC12 cells cultured under various conditions. The survival rate was determined by the trypan blue exclusion method at 15 h after the beginning of cultures and the survival rate of the cells cultured in the presence of NGF was estimated as 100%. DEVD: acetyl-DEVD-cho (an inhibitor of caspase-3-like proteinases), CA074: a specific inhibitor of cathepsin B, pepstatin A: an inhibitor of cathepsin D, CB-AS: antisense oligonucleotide of cathepsin B, CD-AS: antisense oligonucleotide of cathepsin D.

whether autophagy is involved in PC12 cell death following serum deprivation. When PC12 cells are cultured, for 24 h, in the absence of serum but in the presence of 10 mM 3-methyladenine, an inhibitor of autophagy, the survival rate of the cells is greatly increased, compared with that of the intact cells (Fig. 4). These results suggest that there exists a cell death pathway through autophagy in PC12 cells following serum deprivation. The induction of autophagy has also been demonstrated in superior cervical ganglion (SCG) neurons and autophagosomes appearing before DNA fragmentation when the neurons were cultured in the absence of NGF or in the presence of cytosine arabinoside (XUE et al., 1999). Moreover, XUE et al. (1999) have shown that 3-methyladenine delayed the apoptosis of SCG neurons and this neuroprotection occurs concomitantly with the inhibition of cytochrome c release from mitochondria and prevention of caspase activation, suggesting that sutophagy may mediate caspase-independent neuronal death.

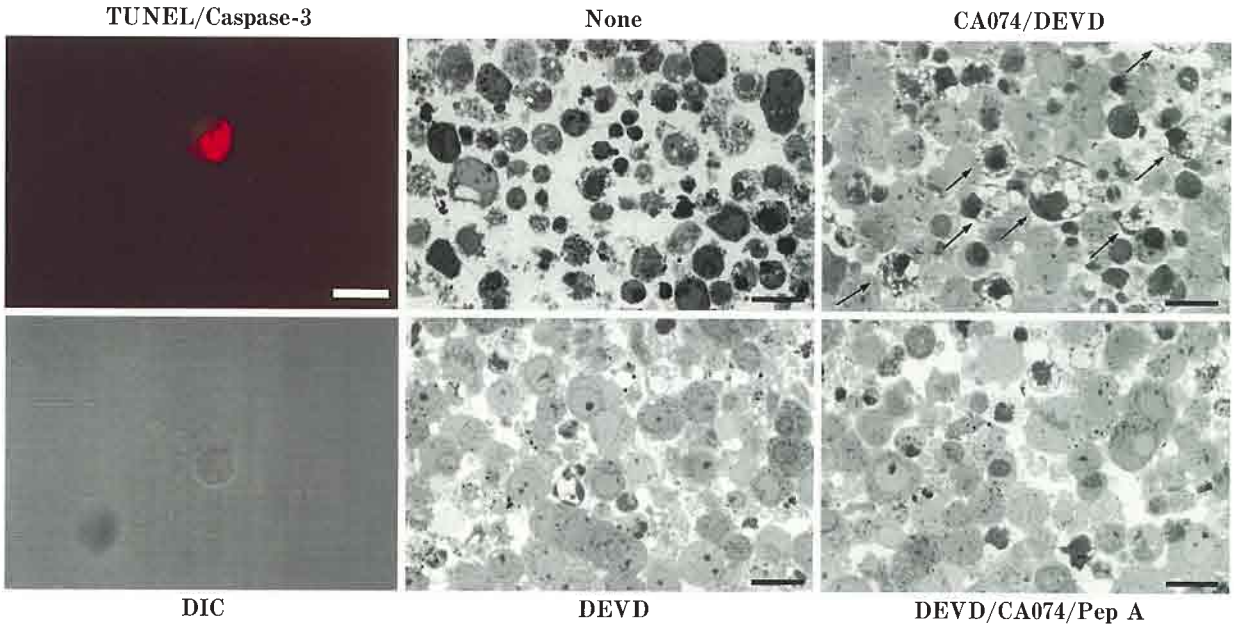

Fig. 6. Effects of specific inhibitors of cathepsins B and D on PC12 cells cultured in the absence of serum. Double staining of activated caspase-3 and TUNEL in PC12 cells cultured in the absence of serum but in the presence of CA074 and acetyl-DEVD-cho shows that nuclear staining of TUNEL is positive in a cell where activated caspase-3 is immunonegative (left upper panel, confocal laser microscopic image). Left lower panel indicates a differential interference image (DIC) of the left upper panel. In semithin sections, most PC12 cells undergo shrinkage and nuclear chromatin condensation when cultured in the absence of serum (None), whereas they are largely rescued from apoptosis when acetyl-DEVD-cho, an inhibitor of caspase-3 is present in the serum-deprived medium (DEVD). The addition of CA047, an inhibitor of cathepsin B, induces the active death of PC12 cells with large cell bodies (*arrows*) when cultured in the absence of serum but in the presence of acetyl-DEVD-cho (CA074/DEVD). This situation of the cells is largely restored by the further addition of pepstatin A, an inhibitor of cathepsin D (DEVD/CA074/Pep A). Bars indicate 10 μm.

III-2. Involvement of cathepsins B and D in neuronal death

Since autophagy is involved in the active death of PC12 cells and intracellular concentrations of lysosomal proteinases are greatly changed during the apoptotic process, the issue of whether lysosomal proteinases, particularly cathepsins B and D, play roles in the execution of PC12 cell death also becomes important. As stated above, the activity of caspase-3-like proteinases (DEVDase) is elevated in PC12 cells following serum deprivation. The survival rate of the cells may be maintained by treatment with acetyl-DEVD-cho, a specific inhibitor of caspase-3 (Fig. 5). To examine the participation of cathepsins B and D in the cell death of PC12 cells, specific inhibitors and antisense oligonucleotides of these enzymes have been applied to cultures of the cells treated with acetyl-DEVD-cho (ISAHARA et al., 1999). CA074, a specific inhibitor of cathepsin B or its antisense oligonucleotides, induces the active death of the cells, which is suppressed by the addition of pepstatin A, an inhibitor of cathepsin D or its antisense oligonucleotides (Fig. 5). By double staining with TUNEL and activated caspase-3, the dying cells treated with CA074 are positive for TUNEL staining but negative for activated caspase-3 (Fig. 6). Since caspase-3 is inhibited by acetyl-DEVD-cho, cytoskeletons of the cells are not degraded, their ultrastructures show relatively large cell profiles with chromatin condensation of the nuclei (Fig. 6). Moreover, dorsal root ganglion (DRG) neurons obtained from rat embryos on the 15 th gestational day, a time when they require NGF for survival and differentiation in cultures, have also been analyzed to confirm the presence of active cell death associated with autophagy and lysosomal proteinases in neurons other than PC12 cells. When cultured in the absence of NGF, the DRG neurons survive in the presence of acetyl-DEVD-cho or acetyl-YVAD-cho. Under these conditions, CA074 reduces the survival rate of the neurons, which is subsequently restored by the further addition of pepstatin A.

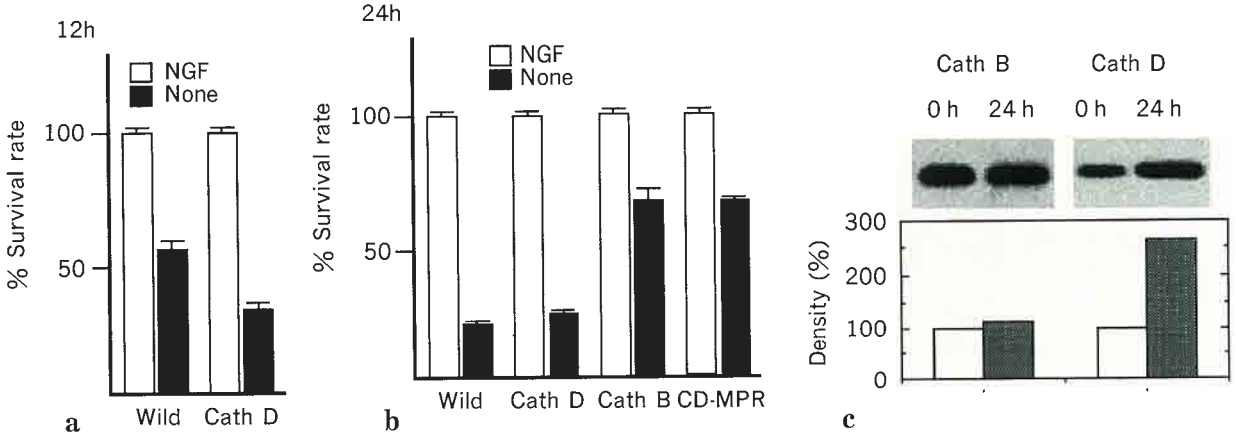

Fig. 7. Overexpression of cathepsin B or D, or CD-MPR changes the viability of PC12 cells following serum deprivation. **a.** The viability of PC12 cells overexpressing cathepsin D is significantly lower than that of wild-type PC12 cells when examined 12 h after the beginning of serum-deprived culture. **b.** The survival rates of PC12 cells overexpressing cathepsin D, cathepsin B, or CD-MPR cultured for 24 h in serum-free conditions. The survival rate of the cells overexpressing cathepsin D is low, similar to that of wild-type cells, whereas that of the cells overexpressing cathepsin B or CD-MPR is significantly increased. The viability of the cells was determined by the trypan blue exclusion method and the survival rate of the cell cultured in the absence of serum but in the presence of NGF was defined as 100% (**a**, **b**). **c.** Changes in protein amounts of cathepsins B and D in PC12 cells overexpressing cathepsin B at 0 h and 24 h after the beginning of serum-deprived culture. No change in the protein amount of cathepsin B (Cath B) is detected by immunoblotting, although the protein amount of cathepsin D (Cath D) increases to 2.5 times its original amount after 24 h culturing. Upper panel indicates Western blots and lower panel shows bar graphs of densities of each protein bands.

These results suggest that a pathway for initiating cell death exists, and which is regulated by lysosomal cathepsins, in which cathepsin D acts as a death factor. We speculate that this death-inducing activity is normally suppressed by cathepsin B. Since this cell death pathway is inhibited by 3-methyladenine, an autophagy suppressor, its regulation by cathepsins B and D is located downstream autophagy.

III-3. Overexpression of lysosomal proteinases alter the viability of PC12 cells

To further confirm the participation of lysosomal proteinases in active cell death which is initated by the autophagosome formation, PC12 cells overexpressing cathepsin B or D have been prepared (KANAMORI et al., 1998; SHIBATA et al., 1998). Establishment of PC12 cells which stably overexpress cathepsin D is difficult, and most clones of the cells are easily die during selection with G418. We have established one clone of the cells overexpressing cathepsin D. In serum-deprived cultures, the cathepsin D gene-transfected cells and wild-type cells largely undergo apoptosis 24 h after the beginning of cultures, and their survival rates are low (Fig. 7). When the viability of the transfected cells is examined 12 h after the beginning of cultures, the survival rate is significantly lower than that of wild-type cells (Fig. 7), indicating that overexpression of cathepsin D facilitates apoptosis of PC12 cells, following serum deprivation. By TUNEL staining, nuclei of the transfected cells are positively stained in serum-deprived cultures,whereas almost no TUNEL-positive cells are seen when NGF is present in the serum-free medium.

As stated before, in correspondence to decreases in immunoreactivity and proteolytic activity of cathepsin B during the apoptotic process of wild-type PC12 cells, its expression level clearly decreases to approximately 20% of its initial amount 24 h after the beginning of culture, although that of cathepsin D increases to 300% (Fig. 3). This drastic decrease in the protein amount of cathepsin B indicates its importance in the maintenance of cell viability. In fact, the survival rate of the cathepsin B gene-transfected PC12 cells is elevated to about 70% 24 h after the beginning of serum-deprived culture (Fig. 7). In this situation, no decrease in the amount of the 29 kD

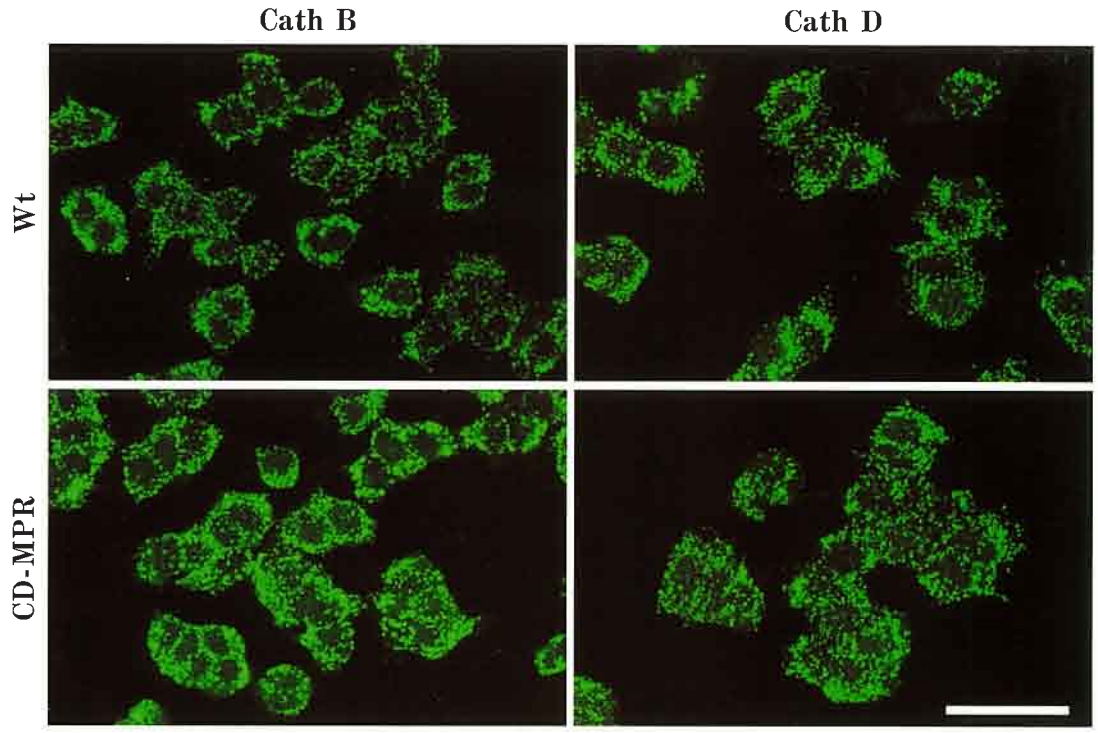

Fig. 8. Confocal laser microscopic images of cathepsins B (Cath B) and D (Cath D) in wild-type (Wt) and CD-MPR gene-transfected PC12 cells. Immunodeposits for cathepsin B become more intense and coarser in the transfected cells than in the wt cells, whereas those for cathepsin D is similar between the wt and transfected cells. Bar indicates 10 μm.

cathepsin B protein is detected in the cells, although the amount of cathepsin D increases to about 250% of its initial amount (Fig. 7).

We have also examined the expression levels of MPRs in wild-type PC12 cells which are involved in the transport of lysosomal enzymes from the trans Golgi network to autophagosomes/heterophagosomes/endosomes. Immunoreactivity for CIMPR is intensely detected in the cells, but that of CDMPR is faint or low. We have therefore cloned cDNA of the rat CDMPR and transfected the gene into PC12 cells (KANAMORI et al., 1998). Interestingly, the transfected cells show coarser cathepsin B-immunodeposits in the cytoplasm than the wild-type cells, although no clear-cut alteration is detected in the immunoreactivity for cathepsin D in the transfected cells (Fig. 8). Corresponding to the elevation of the immunoreactivity for cathepsin B, the survival rate of the transfected cells is significantly augmented in serum-deprived cultures (Fig. 7). In this situation, the protein amount of cathepsin B is similar to the level of intact PC12 cells. These results strongly argue for the presence of a cell death pathway regulated by lysosomal cathepsins B and D. In this pathway, cathepsin D acts as a death factor, while the death inducing activity of cathepsin D is suppressed by cathepsin B. Moreover, transport of cathepsin B from the trans Golgi network to prelysosomal compartments in PC12 cells may be largely dependent on CDMPR.

In our cell death model where cathepsin D acts as a death-mediator, cathepsin B is suggested to suppress the action of cathepsin D. Therefore, it is reasonable to hypothesize that both actions occur within lysosomal compartments, although the precise mechansims of this cell death pathway after lysosomes remain unknown. Recent studies using tumor cell lines or primary cultured hepatocytes have shown that cathepsin B is released from lysosomes after treatment with tumor necrosis factor-α (TNF-α) and induces apoptosis (GUICCIARDI et al., 2000; FOGHSGAARD et al., 2001). Isolated hepatocytes treated with TNF-α in the presence of actinomycin D accumulate cathepsin D in the cytosol, while caspase-8 causes the release of active cathepsin B from purified lysosomes in cell-free systems where released cathepsin B further releases cytochrome c from

mitochondria (GUICCIARDI et al., 2000). This apoptotic process is largely reduced when hepatocytes from the liver of cathepsin B deficient mice are used, suggesting that a caspase-mediated release of cathepsin B from lysosomes enhances mitochondrial release of cytochrome c and subsequent caspase activation in TNF-α-treated hepatocytes (GUICCIARDI et al., 2000). On the other hand, when tumor cells are treated with TNF-α in the presence of low doses of z-VAD-fmk, a pan-caspase inhibitor, cathepsin B is released into the cytosol and induces cell death with apoptotic features (FOGHSGAARD et al., 2001). In the latter case, cathepsin B deficient fibroblasts are not resistant to TNF-α treatment, suggesting that tumor cell death mediated by TNF-α occurs in a caspase-independent manner (FOGHSHAARD et al., 2001). In such cases where TNF-α initiates cell death cascades, the issues of why only cathepsin B is released from lysosomes after treatment with TNF-α and also of whether the cathepsin B-mediated cell death pathway is caspase-dependent or independent remain to be solved. In contrast with these two studies, KAGEDAL et al. (2001) have shown that cathepsin D is implicated in the onset of apoptosis in fibroblasts exposed to oxidative stress generated by redox cycling of naphthazarin. In this model, cathepsins D, B and L are released from lysosomes into the cytosol and only cathepsin D is responsible for the activation of the caspase cascade, since released cathepsins B and L may be inhibited by the presence of the endogenous inhibitor of cysteine proteinases. Indeed, the proteolytic activity of cathepsin B is rapidly decreased after the beginning of cultures. Moreover, in comparison with our results, the role of cathepsin B in the cell death processes seems to largely differ depending on cell type. However, these lines of evidence suggest that there certainly exist cell death pathways mediated by lysosomal proteinases.

IV. CONCLUSIONS

Our study on "the roles of autophagy/lysosomal proteinases in active cell death" has been based on the evidence that autophagy and lysosomal proteinases are activated during the process of CA1 pyramidal cell death in the gerbil hippocampus after brief froebrain ischemia (NITATORI et al., 1995). Using PC12 cells and DRG neurons, we have shown that autophagy is involved in active death of these cells, while cathepsin D acts as a death mediator downstream autophagy and cathepsin B inhibits this death-inducing ability of cathepsin D. Our next aim on neuronal cell death mediated by autophagy is to clarify the issue of whether delayed neuronal death in the CA1 pyramidal region of the hippocampus after ischemic insult is regulated by lysosomal proteinases in relation to autophagy. According to our unpublished data, immunoreactivity for cathepsin B is drastically decreased in TUNEL-positive CA1 pyramidal neurons of the gerbil hippocampus after brief ischemia, whereas that for cathepsin D is increased in the neurons. The behavior of these proteinases in the dying CA1 pyramidal neurons after ischemic insult is similar to that in PC12 cells and DRG neurons following serum/trophic factor deprivation. Further studies are required to obtain a definitive answer to these question.

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Prof. Yasuo UCHIYAMA
Department of Cell Biology and Neuroscience
Osaka University Graduate School of Medicine
2-2 Yamadaoka, Suita, Osaka
565-0871 Japan
Tel: +81-6-6879-3120
Fax: +81-6-6879-3129
E-mail: uchiyama@anatl.med.osaka-u.ac.jp

内山 安男
565-0871 吹田市山田丘2-2
大阪大学大学院医学系研究科
医学部第一解剖学教室