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Minireview

Molecules and Cells

New Functional Aspects of Cathepsin D and Cathepsin E

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Cathepsin D (CD) and cathepsin E are representative lysosomal and nonlysosomal aspartic proteinases, respectively, and play an important role in the degradation of proteins, the generation of bioactive proteins, antigen processing, etc. Recenty, several lines of evidence have suggested the involvement of these two enzymes in the execution of neuronal death pathways induced by aging, transient forebrain ischemia, and excessive stimulation of glutamate receptors with excitotoxins. CD has also been shown to mediate apoptosis induced by various stimuli and p53-dependent tumor suppression. To gain more insight into *in vivo* functions of CD, mice deficient in this enzyme were generated. The mutant animals showed a progressive atrophy of the intestinal mucosa, a massive destruction of lymphoid organs, and a profound accumulation of ceroid lipofuscin, and developed a phenotype resembling neuronal ceroid lipofucinosis, suggesting that CD is essential for proteolysis of proteins regulating cell growth and tissue homeostasis. It has also been shown that CD molecules secreted from human prostate carcinoma cells are responsible for the generation of angiostatin, a potent endogenous inhibitor of angiogenesis, suggesting its contribution to the prevention of tumor growth and angiogenesis-dependent growth of metastases. Interestingly, pro-CD from human breast carcinoma cells showed a significantly lower angiostatin-generating activity than that from prostate carcinoma cells. Since deglycosylated CD molecules from both carcinoma cells showed a low angiostatin-generating activity, this discrepancy appeared to be attributed to the difference in the carbohydrate structures of CD molecules between the two cell types and to contribute to their potency to prevent tumor growth and metastases.

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Introduction

Cathepsin D (CD) and cathepsin E (CE) are analogous intracellular aspartic proteinases in mammalian cells. Besides their structural and immunological distinction (Azuma *et al*., 1989; Jupp *et al*., 1988; Kageyama *et al*., 1992; Okamoto *et al*., 1995; Yamamoto *et al*., 1980; 1987), they are different in tissue distribution and intracellular localization (Nishishita *et al*., 1996; Sakai *et al*., 1989; Saku *et al*., 1990; 1991; Sastradipura *et al*., 1998). CD represents a major portion of the proteolytic activity in the lysosomal compartment. This enzyme is usually localized in lysosomes of various tissues, although the level of expression varies with different cell types (Barrett, 1977; Yamamoto, 1999; Yamamoto *et al*., 1980). CD is also detected in endosomes of certain cell types, such as macrophages (Diment *et al*., 1988) and hepatocytes (Geuze *et al*., 1985). About 90% of the total CD activity in lysosomes is soluble, whereas about 20% of the activity is membrane-associated in endosomes. Lysosomal CD is considered to play a role in the proteolysis of intra- and extracellular proteins, whereas endosomal CD is postulated to be involved in proteolytic processing foreign antigens, invariant chain, and prohormons (Diment *et al*., 1988; Maric *et al*., 1994; Neefjes and Ploegh, 1992; Pillai and Zull, 1986; van Noort and Jacob, 1994). On the other hand, the overexpression and excretion of CD has been implicated in pathological processes such as inflammation (Barrett, 1977), tumor progression (Aaltonen *et al*., 1995; Leto *et al*., 1992; Mignatti and Rifkin, 1993; Sanchez *et al*., 1993; Tandon *et al*., 1990), and the release of β-amyloid peptides from amyloid precursor protein in Alzheimer's disease (Ladror *et al*., 1994; Siman *et al*., 1993).

In contrast to CD, CE has a limited distribution in cell types such as lymphoid tissues, gastrointestinal

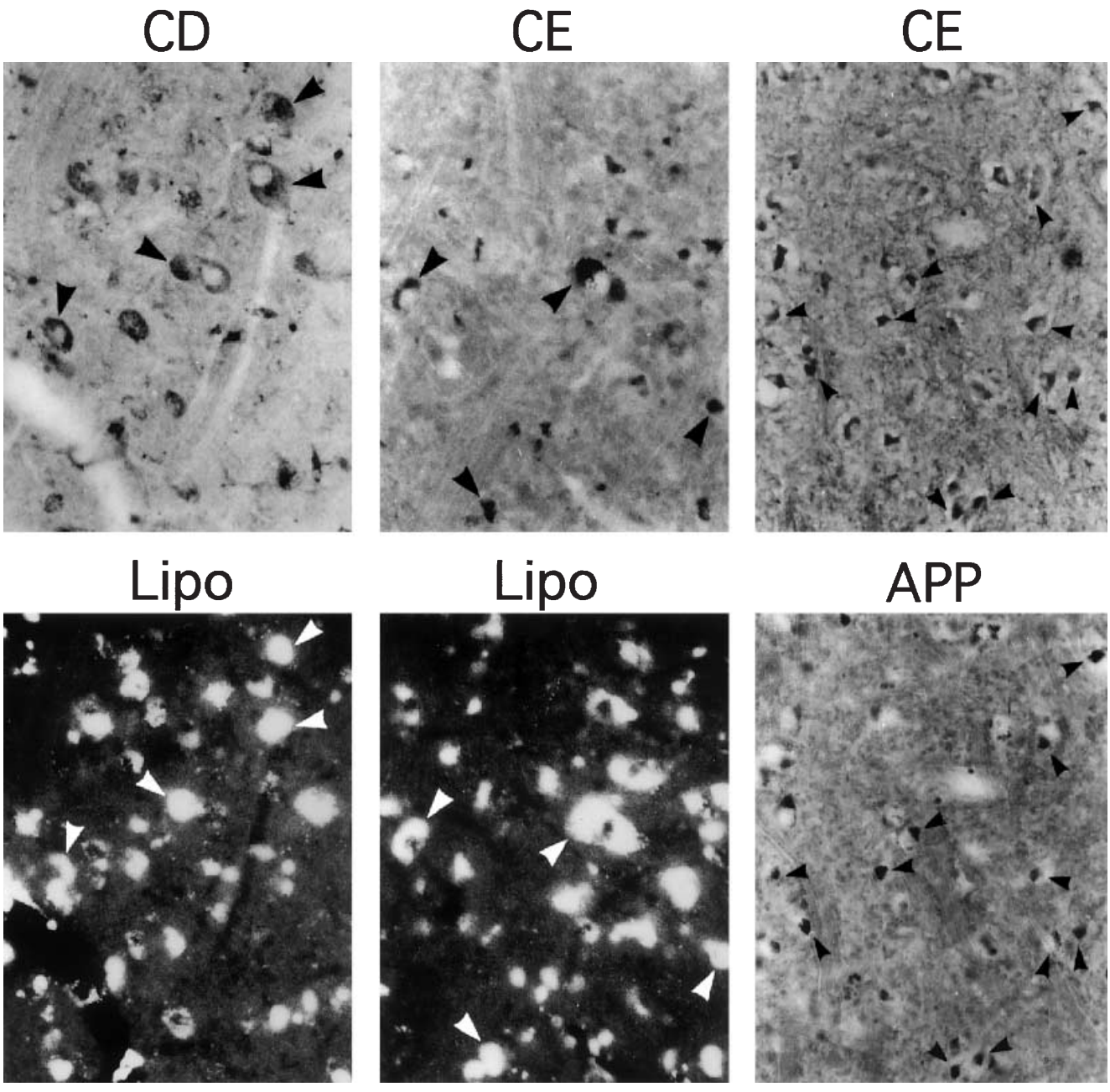

Fig. 1. Age-related increase in CD, CE, and $APP_{643–695}$ immunoreactivities and their colocalization with autofluorescent lipopigments in the brain stem neurons of 30-month-old rats. As shown by arrowheads, both CD and CE immunoreactivities in brain stem cells corresponded well with autofluorescent lipopigments observed under the ultraviolet light in the same section , as shown in the lower panels. The CE immunoreactivity in the brain stem cells also corresponded well with the $APP_{643–695}$ immunoreactivity in the same section.

tracts, urinary organs, blood cells, and microglia (Muto *et al.*, 1988; Sakai *et al.*, 1989, Sastradipula *et al.*, 1998). By immunoelectron microscopic studies CE has been shown to be different from CD in intracellular localization in various mammalian cells. Membrane association of CE is found in intracellular canaliculi of gastric parietal cells (Saku *et al.*, 1991), renal proximal tubule cells (Saku *et al.*, 1991), bile canaliculi of hepatic cells (Saku *et al.*, 1991), intestinal and tracheobranchial epithelial cells (Fiocca *et al.*, 1990; Saku *et al.*, 1989), osteoclasts (Yoshimine *et al.*, 1995), and erythrocytes (Yamamoto and Marchesi, 1984). The localization of CE in the endosome structures is observed in various cell types, such as gastric cells (Saku *et al.*, 1991), antigen-presenting B cell lymphoblasts (Bennett *et al.*, 1992), and microglia (Sastradipula *et al.*, 1998). The

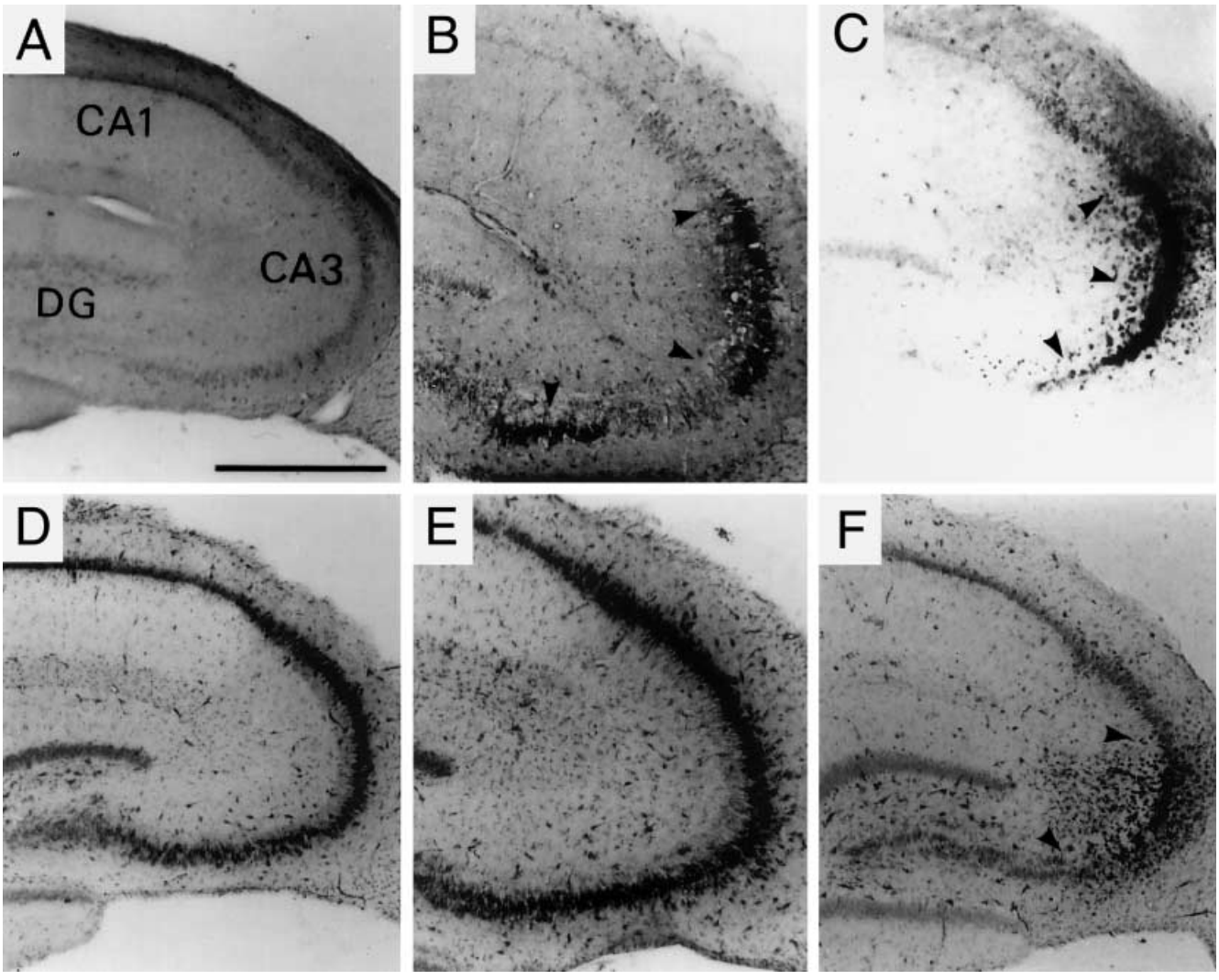

Fig. 2. Immunohistochemical localization of CE (**A–C**) and CD (**D–F**) in the hippocampi from control (**A, D**) and kainate-treated rats at day 1 (**B, E**) and day 3 (**C, F**) after intracerebroventricular postinjection. Bar = 500 μm. In the hippocampus of control rats, very little, if any, CE immunostaining was detected in a small number of neurons, whereas CD immunostaining was widely observed in the neurons. At day 1 after the kainate injection, CE immunostaining was dramatically enhanced in the CA3 hippocampal neurons. After day 3 postinjection, CE immunostaining in the CA3 region was further increased, whereas the numbers of CD-positive cells were also increased in the entire CA3 region.

enzyme is also found in the cisternae of the endoplasmic reticulum (Finley and Kornfeld, 1994; Sastradipula *et al.*, 1998; Tsukuba *et al.*, 1993). Despite its strategic localization, the detailed function of CE is currently unknown. In this minireview, we describe the current understanding of the biological significance of CD and CE.

Association of CD with apoptosis

Recent studies have emphasized a central role of the caspase cascade in apoptosis which is indispensable in developing and maintaining cellular homeostasis in multicellular organisms. Besides the caspase family of cysteine proteinases, CD has recently been shown to be involved in programmed cell death induced by interferon-γ, Fas, and TNF-α (Deiss *et al.*, 1996). The high CD antisense RNA levels were shown to protect the HeLa cells from cell death induced by these compounds. In addition, pepstatin A, a potent inhibitor of aspartic proteinases, was shown to suppress cell death in these systems and to interfere with TNF-α-induced programmed cell death of U937 cells. During these cell death processes, both CD mRNA and protein levels were shown to be significantly elevated. CD mRNA levels were also shown to increase in various p53-expressing human cancer cell lines undergoing adriamycin-induced apoptosis (Wu *et al.*, 1998); however, this elevation of CD levels was not observed in cells

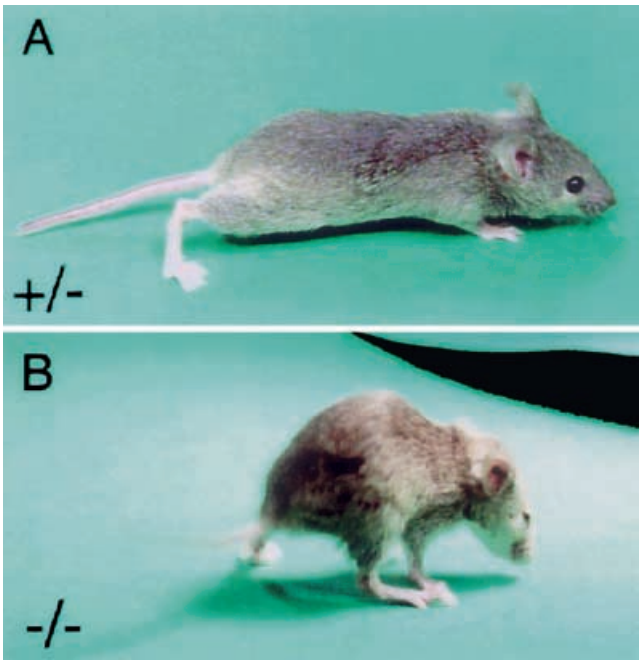

Fig. 3. Phenotype of CD−/− mice. During the first 2 weeks no phenotypic differences are detectable between CD−/− mice and wild-type litter-mates. Thereafter the weight of the CD−/− mice decreased. The CD−/− mice die between day 25 and 27 by massive intestinal necrosis, thromboembolia, and lymphopenia. During the last days of life, the CD−/− mice manifest seizures and become blind.

expressing a mutant p53 by adriamycin treatment. Moreover, pepstatin A could suppress p53-dependent apoptosis and the overexpression of CD inhibited cancer growth (Wu *et al.*, 1998). Since activation of the p53 pathway in mammalian cells can lead to cell cycle arrest or apoptosis (Levin, 1998), the abnormality of p53 was thought to contribute to tumor development and progression. It has thus been suggested that CD is an important mediator of p53-dependent apoptosis, like the bcl-2-family member bax that is the best-known mediator of p53-dependent apoptosis, by a transcriptional mechanism (Miyashita and Reed, 1995; Oltvai *et al.*, 1993).

Our recent experiments have provided evidence that CD and CE are involved in the execution of the neuronal death pathway induced by aging (Amano *et al.*, 1995; Nakanishi *et al.*, 1994; 1997), transient forebrain ischemia (Nakanishi *et al.*, 1993), and excessive stimulation of glutamate receptors with excitotoxins (Tominaga *et al.*, 1998). CD levels in all the brain tissues of aged rats (28–34 months) were about twice those of young rats (2 months). Although CE was barely detectable in all the brain tissues of young rats, its level markedly increased in the cerebral cortex and neostriatum of aged rats. The CD- and CE-immunopositive neurons of aged rats corresponded well with cells emitting autofluorescence for lipopigments and containing the carboxy-terminal fragments (634–695) of amyloid precursor protein (APP) (Fig. 1). CD and CE were also shown to be highly expressed in degenerating neurons and activated microglia in the hippocampal CA1 region for up to 7 d after transient forebrain ischemia. Taken together, the observation that the neuronal lesion and death induced by excitotoxins was consistent with the induction of the CD and CE gene response followed by the persistent expression of both enzymes in degenerating neurons and activated microglial cells (Fig. 2), suggesting that both enzymes are associated with the execution of neuronal death pathways.

Functions of CD revealed by analysis of CD-deficient (CD−/−) mice

To gain more insight into *in vivo* functions of CD, mice deficient in this enzyme were generated by gene targeting (Saftig *et al.*, 1995). The homozygous mutant (CD−/−) mice developed a progressive atrophy of the intestinal mucosa and a profound destruction of lymphoid tissues. Although the mutant mice could develop normally during the first 2 weeks, they stopped thriving in the third week, decreased in weight, and then died between 25 and 27 d (Fig. 3). At the cellular level, however, lysosomal bulk proteolysis was maintained in these mice. These results with CD−/− mice suggested that essential functions of CD were exerted by limited proteolysis of proteins regulating cell growth and/or tissue homeostasis. On the other hand, the important questions whether CD was involved in the generation of βA4-amyloid peptide from APP and the major histocompatibility complex (MHC) class-II-mediated antigen presentation were addressed directly using CD−/− mice (Deussing *et al.*, 1998; Saftig *et al.*, 1996). So far, several lines of evidence have suggested that CD participates in the amyloidogenic processing of APP as the β- and/or γ-secretases (Cataldo *et al.*, 1995; Dreyer *et al.*, 1994; Kohnken *et al.*, 1995; Siman *et al.*, 1993). Moreover, the upregulation of both CD mRNA and activity in the brains of patients with Alzheimer's disease and the existence of CD in patients with amyloid plaques have been demonstrated (Cataldo *et al.*, 1995; Diedrich *et al.*, 1991; Schwagerl *et al.*, 1995). It was thus considered to be of special interest to determine the role of CD in βA4-amyloid peptide generation. The primary cultures of hippocampal neurons derived from CD−/− mouse embryos expressing human APP showed a similar production of βA4-amyloid peptide as those from the wild-type mice, suggesting that CD was dispensable for βA4-amyloid peptide generation (Saftig *et al.*, 1996). On the other hand, recent studies have suggested that the generation of the antigenic determinants presented by MHC class II and the degradation of MHC class-II-associated invari-

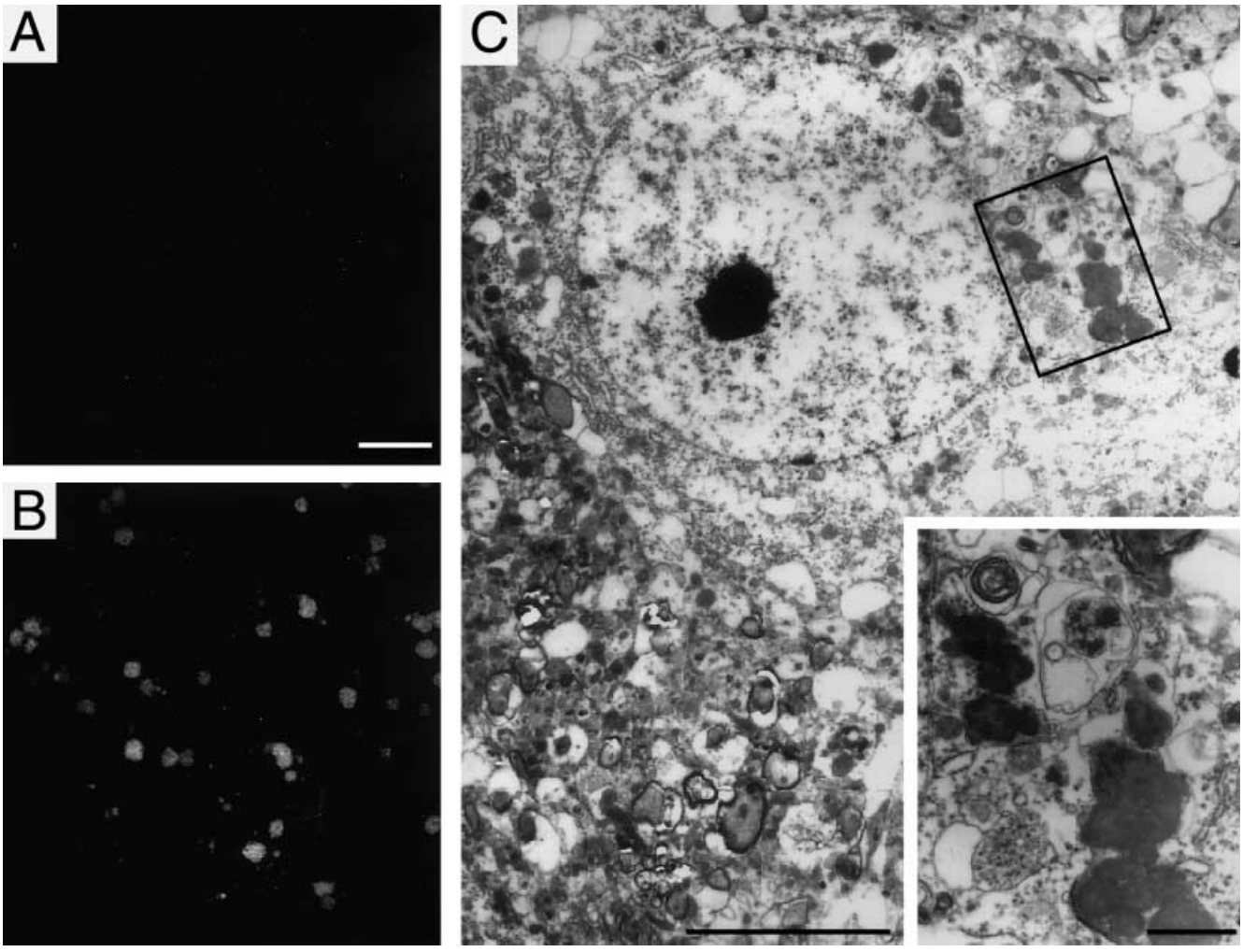

Fig. 4. Autofluorescence in neuronal cell bodies located in the CA1 region of the hippocumpi from the wild-type litter mates (**A**) and 23-day-old CD−/− mice (**B**). An electron micrograph of autophagosome/autolysosome-like bodies in the neuronal perikarya of a cerebral cortex neuron from a 23-day-old CD−/− mouse (**C**). A higher magnification of the perikaryon region indicated by a square is shown at the bottom right, where the dense granular bodies, fingerprint-like myelin figures, and granular osmiophilic deposits are seen.

ant chain (Ii) require the action of CD as well as cathepsins B (CB) and S (Adorini *et al.*, 1993; Manoury-Schwartz *et al.*, 1997; Maric *et al.*, 1994; Puri and Factorovich, 1988; Vidard *et al.*, 1991). However, biochemical analysis of MHC class II in antigen-presenting cells from CD−/− mice as well as CB−/− mice showed normal Ii degradation and antigen presentation, suggesting that CD was not essential for MHC class-II-mediated antigen presentation (Deussing *et al.*, 1998; Villadangos *et al.*, 1997).

During the course of our studies on morphological as well as functional changes in the brain tissues of CD−/− mice, we noticed that these animals showed neurological phenotypes with seizures near the terminal stage. The most striking feature found in the central nervous system (CNS) was a profound storage of autophagosome/autolysosome-like bodies with part of the cytoplasm, granular osmiophilic deposits, and fingerprint profiles (Fig. 3) (Koike *et al.*, 2000). Almost all neurons of these animals near the terminal stage autofluorescenced (Fig. 4), indicating the accumulation of ceroid lipofuscin in the lysosomal structures, as revealed by immunoreactivities for CB and subunit c of mitochondrial ATP synthase complex (F_1F_0-ATPase). Interestingly, however, the protein and activity levels of tripeptidyl peptidase I, whose deficiency causes one type of neuronal ceroid lipofucinose (NCL), were rather increased in the CD−/− mouse brain (Koike *et al.*, 2000). Considering that NCLs are a heterogeneous group of progressive neurodegenarative disorders with an autosomal recessive mode of inheritance and are characterized by serious symptoms including blindness, seizures, paralysis, dementia, and premature death and morphologically by massive lysosomal storage with an autofluorescent lipopigment in neurons (Goebel and Sharp, 1998) and that many types of NCLs, except for

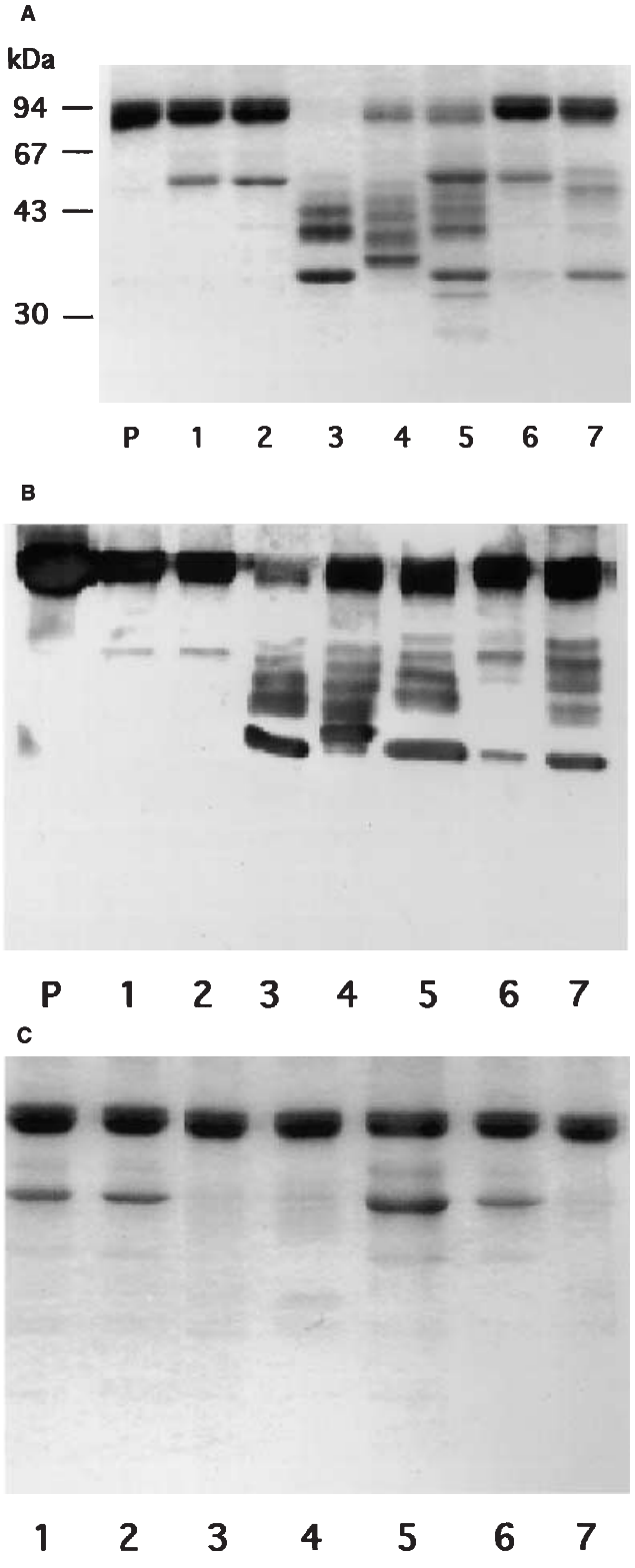

Fig. 5. Generation of angiostatic peptides from plasminogen by culture media from various carcinoma cells. Human plasminogen was incubated by the culture media from various carcinoma cells at 37°C and pH 4.0 overnight with (**A, B**) and without pepstatin A (**C**). The reaction products were subjected to SDS-PAGE and then stained by Coomassie brilliant blue (**A, C**) and analyzed by immunoblotting with antibodies against human plasminogen (**B**). P, plasminogen as a standard; 1, human fibroblast cell line (NKLF); 2, human umbilical vascular endothelial cells; 3, human prostate carcinoma cell line (PC-3); 4, human hepatic cancer cell line (HepG2); 5, human colon carcinoma cell line (colon-205); 6, human breast carcinoma cell line (MCF7); 7, mouse Lewis lung carcinoma cell line (LL/2). The 45- and 42-kDa angiostatic peptides are generated by PC-3, HepG2 and colon-205.

the infantile form of NCL, show a massive accumulation of subunit c of ATP synthase complex (Fearnley *et al.*, 1990; Hall *et al.*, 1991; Kominami et al; 1992; Palmer *et al.*, 1989), it is most likely that the CNS neurons in CD−/− mice represent a new form of lysosomal storage disease with a phenotype resembling NCL and that CD is essential for protecting the onset and development of this type of NCL.

More recently, the congenial ovine NCL was concurrently reported (Tyynelä *et al.*, 2000). This NCL was the first reported disease arising from a naturally occurring CD mutation. In this animal, a single nucleotide mutation in the CD gene resulted in substitution of a conserved active-site aspartate by asparagine at position 295. Since the active-site aspartate residue is essential for its catalytic activity, this mutation must produce a catalytically inactive protein. In fact, the cell extracts from the brain of this mutant animal did not express CD activity. Nevertheless, this protein appeared to be normally processed from the single-chain form to the double-chain form and was present in a stable form in the cells. Importantly, there were several differences between the CD−/− mouse and congenital ovine NCLs; a striking reduction of brain size was observed in the congenital ovine NCL compared to that of normal lambs, whereas no significant reduction in brain size was detected in CD−/− mice; the newborn congenital NCL lambs did not reveal any pathological changes in the lymphoid tissues, whereas CD−/− mice showed a massive loss of lymphoid cells in the spleen and thymus. These discrepancies may be due to the species difference in the maturity of the CNS and/or the gestation periods of 3 weeks for mice versus 21 weeks for sheep.

Generation of angiostatin by CD secreted by human carcinoma cells

More recently, we reported that CD secreted directly by human prostate carcinoma cells was responsible for the generation of angiostatic peptides comprising kringle domains 1–4 of plasminogen (Morikawa *et al.*, 2000). Angiogenesis is an important process of new microblood vessel formation under both physiological and pathological conditions. Although angiogenesis is crucial during

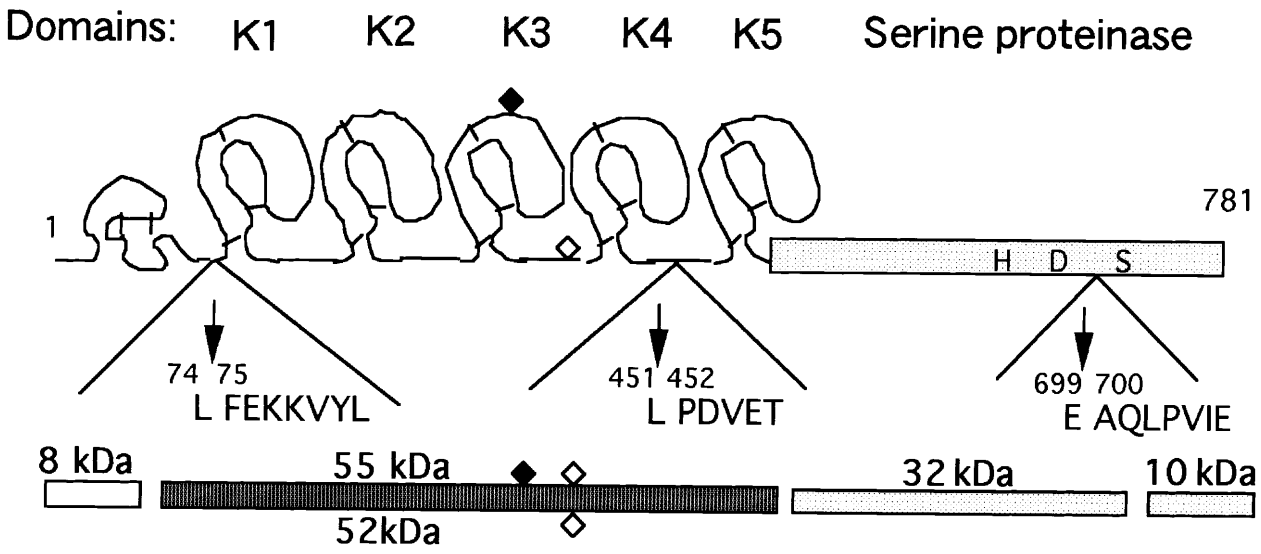

Fig. 6. Characterization of time-dependent generation of angiostatic peptides from plasminogen by pseudo-CD from the culture medium of PC-3 cells. A schematic model for the generation of angiostatic peptides from plasminogen by pseudo-CD. The cleavage of plasminogen by this enzyme, as indicated by arrows, first occurs at the Leu^{451}-Pro^{452} bond followed by the Leu^{74}-Phe^{75} bond. Then, an additional cleavage occurs at the Glu^{699}-Ala^{700} bond. ◆, an N-linked glycosylation site; ◇, an O-linked glycosylation site.

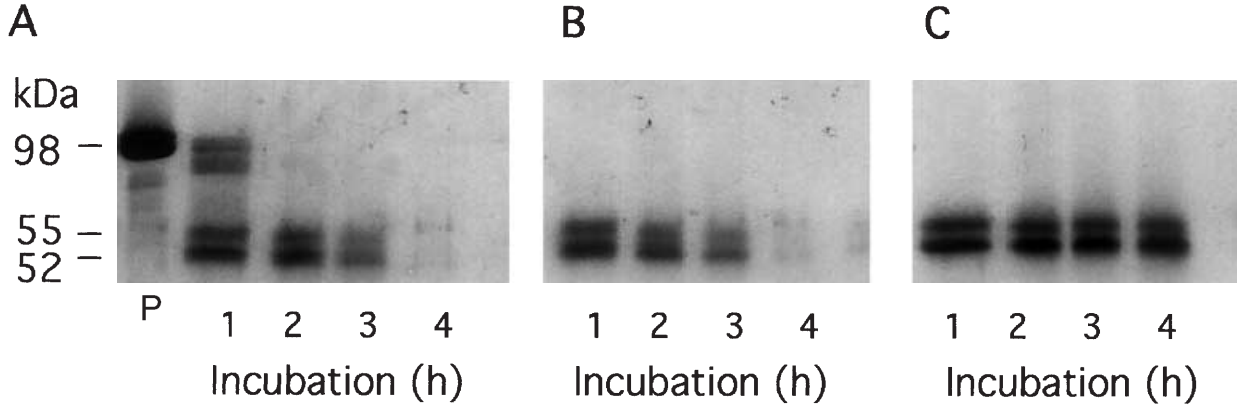

Fig. 7. Generation of angiostatic peptides from plasminogen by pepsin (**A**) and CE (**B**) compared with CD (**C**). Plasminogen was incubated at pH 4.0 and 37°C with human pepsin, CE, and CD. Aliquots were withdrawn from the reaction mixtures at the times indicated and analyzed by SDS-PAGE under reducing conditions. **P**, untreated human plasminogen as a standard.

embryonic development and all types of postnatal tissue growth and repair, it is also required for the metastatic growth of cartinoma (Folkman *et al.*, 1971). In addition to producing angiogenic growth factors such as a tumor-derived endothelial cell growth factor, certain types of tumors have also been known to generate angiogenic inhibitors (Folkman, 1995). Accordingly, the growth rate of a tumor may be the result of the balance between positive and negative effects. Angiostatin is a potent endogenious angiogenic inhibitor produced by proteolysis of plasminogen (Folkman *et al.*, 1971; O'Reilly *et al.*, 1997). Although the mechanism of angiostatin formation *in vivo*, as well as the molecular mechanism of angiostatin action, remains unknown, several lines of evidence *in vitro* have suggested that this protein is generated by multiple enzymic actions. These included matrix metalloproteinases (MMP-2, 3, 7, 9 and 12), plasmin, and tumor-cell-derived plasmin thiolreductase. When the conditioned media from various carcinoma cell lines were tested for angiostatin generating activity, prostate carcinoma cells PC-3, colon carcinoma 205 cells, and Lewis lung carcinoma LL/2 cells were found to secrete the enzyme(s) with the angiostatin generating activity (Fig. 5). To identify the proteinase(s) responsible for angiostatin generation, we isolated the enzyme with the proteolytic activity that is responsible for the generation of angiostatin from the culture medium of human prostate carcinoma cells and identified as pro-CD. The generation of angiostatic peptides with apparent molecular masses of 45 and 42 kDa from human

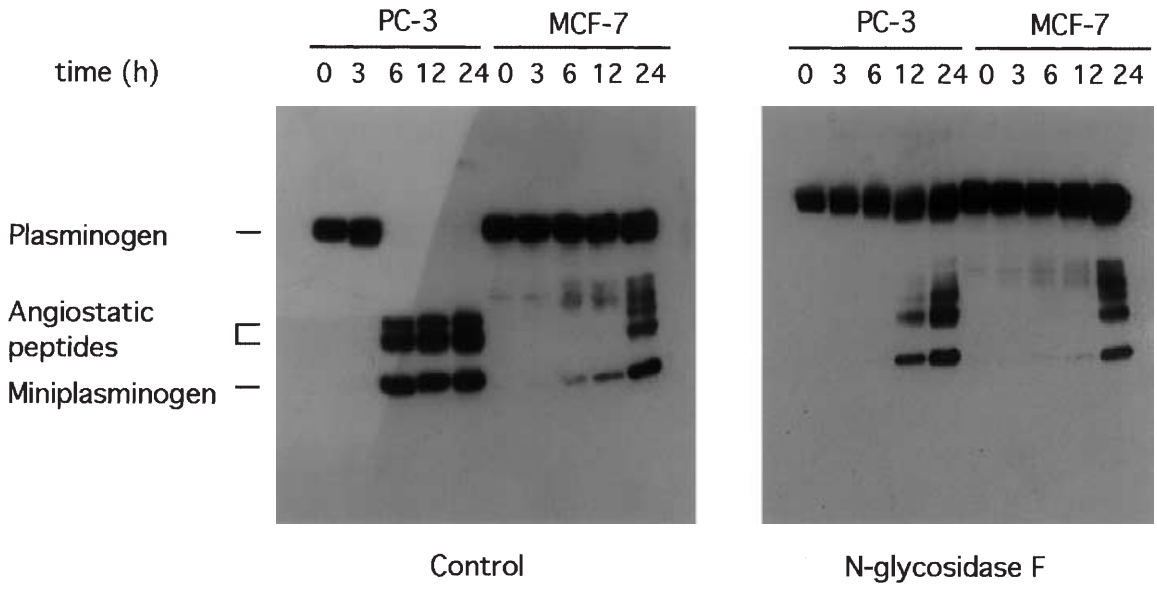

Fig. 8. Difference in the ability of angiostatin generation between procathepsin D molecules derived from PC-3 and MCF7 cells. Plasminogen was incubated at pH 4.0 and 37°C with pro-CD purified from the culture media of these carcinoma cells before and after N-glycosidase F treatment. Aliquots were withdrawn from the reaction mixtures at the times indicated and then analyzed by SDS-PAGE under nonreducing conditions.

plasminogen by pro-CD was observed in the pH range 3.0–6.8, most strongly at pH 4.0, *in vitro*. This reaction required the concomitant conversion of pro-CD to catalytically active pseudo-CD. Not only pro-CD but also mature CD could generate the angiostatic peptides from plasminogen together with the 35-kDa minoplasminogen. The generation of angiostatic peptides consisted of multiple steps (Fig. 6). The NH_2-terminal amino acid sequence analysis revealed that plasminogen was first cleaved by CD at the Leu^{451}-Pro^{452} bond followed by cleavage at the Leu^{74}-Phe^{75} bond and finally at the Glu^{699}-Ala^{700} bond. To determine whether other relevant aspartic proteinases have the ability to generate angiostatic peptides, plasminogen was treated with human CE and pepsin (Fig. 7). The results indicated that both CE and pepsin could efficiently generate angiostatic peptides. However, differing from CD, these proteinases further degraded the angiostatin peptides into smaller fragments by prolonged incubation.

Plasmin is known to play a key role in producing angiogenic factors such as MMPs and cytokines (Kwaan, 1992). Since cancer invasion is thought to be initiated by the activation of plasminogen, plasmin is important in cancer-mediated conversion of plasminogen to angiostatin. This suggests that inactivation of plasmin modulates angiogenesis in tumors. If the Glu^{699}-Ala^{700} bond in the serine proteinase domain of plasminogen is cleaved by CD, the plasminogen–plasmin converting activity must be lost. This possibility was substantiated by incubation of plasminogen with CD. The plasmin activity was lost time-dependently, indicating that angiostatin generation by CD resulted in the concomitant loss of plasminogen–plasmin converting activity.

It is well known that pro-CD is abundantly released by human breast carcinoma cells (Capony *et al.*, 1989; Cavailles *et al.*, 1993; Vignon *et al.*, 1986; Westley and May, 1987); however, as shown in Fig. 5, the conditioned medium from a human breast carcinoma cell line (MCF7) showed little or no angiostatin generating activity (Fig. 8). In contrast to the PC-3-derived CD, the MCF7-derived CD was found to scarcely generate angiostatic peptides from plasminogen. Interestingly, the N-glycosidase F-treated pro-CD from PC-3 cells showed a marked decrease in the angiostatin generating activity to a similar extent to that from MCF7 cells, suggesting that the structural difference of CD, mainly due to the different carbohydrate structures, is associated with their ability to generate angiostatic peptides. In conclusion, CD secreted by human prostate carcinoma cells is responsible for angiostatin gereration, thereby causing the prevention of tumor growth and angiogenesis-dependent growth of metastases.

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