Increased Expression of Cathepsins E and D in Neurons of the Aged Rat Brain and Their Colocalization with Lipofuscin and Carboxy-Terminal Fragments of Alzheimer Amyloid Precursor Protein

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Abstract: Age-related changes in the expression and localization of two distinct intracellular aspartic proteinases, cathepsin E (CE) and cathepsin D (CD), were investigated in the rat cerebral cortex and the brainstem by immunocytochemical and quantitative methods using discriminative antibodies specific for each enzyme. Nonlysosomal CE was barely detectable in these two brain tissues in the embryonic stages, whereas relatively high expression of lysosomal CD was observed in embryonic tissues. After birth, CE was increasingly expressed in these tissues with aging to attain maximal levels at 30 months of age. Western blot analyses revealed that CE existed predominantly as the mature enzyme at 2 and 17 months of age, whereas it was present as not only the mature enzyme but also the proenzyme at 30 months of age. On the other hand, CD was mainly present in the mature form throughout development, although its level in these tissues was also significantly increased with aging. The CE-positive cortical and brainstem neurons of the aged rat corresponded well with cells emitting autofluorescence for lipopigments. By the double-staining technique, most of the CE-positive cortical and brainstem neurons of the aged rat were also positive for antibody to the carboxy-terminal fragments of amyloid precursor protein (APP53-695), intracellular accumulation of which is thought to be associated with age-related changes in the endosome/lysosome system. It is important that electron microscopy revealed that CE in brainstem neurons of the aged rat colocalized with CD in the lipofuscin-containing lysosomes. These results indicate that aging results in the increased expression and lysosomal localization of CE in cortical and brainstem neurons and changes in the endosomal/lysosomal proteolytic system, which may be related to lipofuscinosesis and altered intracellular APP metabolism. Key Words: Cathepsin E—Cathepsin D—Aspartic proteinase—Lipofuscin—Amyloid precursor protein—Aging—Neurodegeneration.


In past years attention has been paid to the altered intracellular proteolytic system in relation to different neuropathological conditions, as irregular cellular proteolysis of physiologically important neuronal proteins has severe consequences for the integrity of neuronal structure and function. The disorder or breakdown of the endosomal/lysosomal proteolytic system has also been suggested to be of pathological significance in neurodegenerative diseases such as Alzheimer’s disease (Nixon and Cataldo, 1993). Therefore, it has been believed that alterations in proteolytic systems in neurons play a crucial role in cellular events that leads to neuropathological conditions.

Lipofuscin is a major type of residual body observed in various types of neurons during the aging process and is considered to be formed initially by autophagocytosis of cytoplasmic components and ultimately defined as a membrane-bound lysosomal organelle containing lipoidal moieties. Although the chemical composition of lipofuscin is very heterogeneous, the formation of lipofuscin is thought to be related to a decrease in lysosomal proteolytic activity (Sohal and Wolfe, 1986). On the other hand, several lines of evidence indicate that levels of amyloidogenic C-terminal fragments generated from amyloid precursor protein (APP) increase in neurons of the aged brain (Benowitz

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Abbreviations used: AEC, aminoethylcarbazole; APP, amyloid precursor protein; CD, cathepsin D; CE, cathepsin E; LDH, lactate dehydrogenase; P fraction, particulate fraction; PBS, phosphate-buffered saline; S fraction, soluble fraction; SDS, sodium dodecyl sulfate.
et al., 1989; Takemura et al., 1993; Beeson et al., 1994). It is generally accepted that the formation and deposition of the amyloidogenic APP fragments are closely associated with specific proteolytic events, especially the altered endosomal/lysosomal system (Bennowitz et al., 1989; Cole et al., 1989; Golde et al., 1992; Haass et al., 1992), and that the amyloidogenic APP fragments are routinely produced in an acidic compartment by noncysteine proteases and that they are eliminated by lysosomal cysteine proteinases such as cathepsins B and L (Siman et al., 1993). The importance of lysosomal cysteine proteinases in elimination of the potentially amyloidogenic APP has also been suggested by accumulation of potentially amyloidogenic C-terminal APP fragments after treatment with the general cysteine proteinase inhibitors E-64 and leupeptin (Hayashi et al., 1992; Bahr et al., 1994; Hajimohammadreza et al., 1994) and by its possible association with the decreased activities of cathepsins B and L in aged rat brain tissues (Nakanishi et al., 1994; Amano et al., 1995b). Taken together, it is of special interest to note that the injection of leupeptin or chloroquine, both inhibitors of endosomal/lysosomal proteolysis, into young rat brains causes the formation of lysosomal granular aggregates that closely resemble the ceroid-lipofuscin (Ivy et al., 1984). Therefore, age-related accumulation of lipofuscin and the C-terminal APP fragment is thought to be associated with an alteration of the endosomal/lysosomal system.

Recently, the activity of cathepsin D (CD; EC 3.4.23.5), a typical lysosomal aspartic proteinase, has been shown to increase in various brain regions of both the aged rat and human (Matus and Green, 1987; Kenedy et al., 1989; Nakamura et al., 1989a; Banay-Schwartz et al., 1992), especially in neurons from aged rat brain (Nakanishi et al., 1994), suggesting the age-dependent enhancement of the endosomal/lysosomal system in these cells. It has been shown that the enzymatically active CD, as well as the lysosomal cysteine proteinase cathepsins B and L, is present extracellularly at high levels in the senile plaques (Bernstein et al., 1989; Cataldo and Nixon, 1990; Cataldo et al., 1991). Furthermore, secondary lysosomes and residual bodies are markedly increased in affected neurons of Alzheimer's disease brain (Nixon and Cataldo, 1993; Cataldo et al., 1994). On the other hand, cathepsin E (CE; EC 3.4.23.34) is another intracellular aspartic proteinase, whose biochemical and catalytic features such as substrate specificity, susceptibility to various inhibitors, and pH optimum, are similar to those of CD (Yamamoto et al., 1978, 1979, 1985; Yonezawa et al., 1987). However, CE is apparently distinct from CD in tissue distribution and cellular localization. CE is found to be localized mainly in the endosome-like structure, the endoplasmic reticulum, and the plasma membrane but not in a typical lysosomal compartment in normal tissues (Iehimaru et al., 1990; Saku et al., 1991; Solcia et al., 1993). Taken together, the limited tissue distribution of CE suggests its regulated gene expression and involvement in different proteolytic events from CD. Increased expression and abnormal localization of CD have been well described in the brain during aging, but less attention has been given to age-dependent changes in CE of the brain. Recently, CE was shown to be highly expressed in degenerating neurons and to be localized in the core of the senile plaques of Alzheimer's disease brain (Bernstein and Wiederanders, 1994). In a previous study (Nakanishi et al., 1994), we found that CE was greatly expressed in both neurons and microglia from the aged rat brain, although it was barely detectable in the normal young rat brain. We also showed the alteration in immunostaining pattern of CE in neurons from a diffuse type to a granular type during the aging process. However, it is not clear whether the age-related increase in activity of CE and CD is associated with the maturity or senescence processes. Also, there is little understanding of the molecular forms of these two enzymes and their accumulation sites in aged neurons.

In view of the evidence linking the endosomal/lysosomal proteolytic system to the pathogenesis of lipofuscin and amyloid deposits, it is important to determine the association of the proteolytic events relating to CE and CD with the age-related neuronal degeneration process. In this study, we report their expression, molecular forms, and cellular localization in aged neurons. Furthermore, to define the altered endosomal/lysosomal system in aged neurons, the cellular localization of CE and CD was compared with that of lipofuscin and C-terminal APP fragments.

MATERIALS AND METHODS

Materials

Fischer 344 rats were divided into four different age groups: embryonic (embryonic day 18), young (2 months of age), middle-aged (17 months of age), and aged (30 months of age) rats. Antibodies against rat spleen CE (Yamamoto et al., 1978) and CD (Yamamoto et al., 1979) were raised in rabbits and purified by affinity chromatography as described previously (Yamamoto et al., 1985). Antibody specific for the synthetic peptide Ser-Glu-Leu-Ser-Glu-Phenylalanine-Lys-Ser-His-Asn-Leu-Asp-Met, which corresponds to the prosequence comprising residues Ser"Met" of human pro-CE cDNA (Azuma et al., 1989), was raised in rabbits and purified on the peptide-Sephrose 4B affinity column. Monoclonal antibodies against the N-terminus of APP (22C11 clone; its epitope lies between residues 60 and 100 of APP) and the C-terminal portions (anti-APP21–68, its epitope lies between residues 643 and 695) were purchased from Boehringer Mannheim Biochemica.

Sodium dodecyl sulfate (SDS)-gel electrophoresis and immunoblotting

Four rats each were used for immunoblotting analysis at 2, 17, and 30 months of age. Fourteen embryonic rats were used for immunoblotting analyses. The detailed immunoblotting procedure has been described previously (Nakanishi et al., 1994).
al., 1994; Amano et al., 1995b). In brief, the cerebral cortex and the brainstem were obtained from rats in each age group that were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and killed by intracardiac perfusion with isotonic saline. The soluble and particulate fractions obtained from the cerebral cortex and brainstem homogenates by differential centrifugation as described below were electrophoresed in SDS-polyacrylamide gels. For immunoblotting, proteins on SDS gels were transferred electrophoretically at 100 V for 12–15 h from the gels to nitrocellulose membranes according to the method of Towbin et al. (1979) and then incubated at 4°C overnight under gentle agitation with one of the following primary antibodies: anti-CE IgG (0.50 μg/ml), anti-CD IgG (1.55 μg/ml), 22C11 (5 μg/ml), or anti-APP663–695 IgG (10 μg/ml). After washes, the membranes were incubated with 0.5% horseradish peroxidase-labeled donkey anti-rabbit IgG (Amersham). Subsequently, membrane-bound horseradish peroxidase-labeled antibodies were detected by the enhanced chemiluminescence detection system (ECL Kit; Amersham) on x-ray film (X-Omat; Kodak) 30–60 s after exposure. As a control, the primary antibody was replaced by nonimmune rabbit IgG. The protein bands of CE and CD on x-ray film were scanned and densitometrically analyzed by a densitometer (Personal Scanning Imager PD110; Molecular Dynamics).

Subcellular fractionation

Three rats each were used for subcellular fractionation at 2 and 30 months of age. The cerebral cortex and the brainstem obtained from rats in each age group were homogenized in 3 volumes of 0.25 M sucrose/0.2 M KCl (pH 7.4) with a Teflon–glass homogenizer (20 strokes). Each homogenate was centrifuged at 650 g for 10 min at 4°C. The supernatant was further centrifuged at 10,000 g for 20 min at 4°C. The resulting precipitate was resuspended in the same buffer and designated as the particulate fraction (P fraction), which is rich in lysosomes and mitochondria. The supernatant was centrifuged at 105,000 g for 1 h at 4°C. The final supernatant was designated as the soluble fraction (S fraction).

Determinations

Acid proteinase activity was determined at pH 5.5 using 1.5% acid-denatured hemoglobin as a substrate as described (Sakai et al., 1989; Nakanishi et al., 1993). β-Glucuronidase was assayed with 4-methylumbelliferyl-β-D-glucuronide as a substrate by the method of Robins et al. (1968). Lactate dehydrogenase (LDH) was assayed by the method of Wacker et al. (1956). Protein content was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Preparation of cell cultures

Primary cultures of cortical neurons were prepared from embryonic day 18 rats. The cerebral cortices were dissected and transferred into ice-cold oxygen-bubbled L-15 medium (GIBCO, Gaithersburg, MD, U.S.A.). Then, the tissues were enzymatically dissociated by incubation twice for 15 min at 37°C in Ca2+– and Mg2+-free phosphate-buffered saline (PBS) containing papain (Worthington, Freehold, NJ, U.S.A.; 90 units), DNase I (Worthington; 2,000 units), d,l-cysteine-HCl (Sigma, St. Louis, MO, U.S.A.; 2.23 mg), bovine serum albumin (Nacalai, Kyoto, Japan; 2 mg), and glucose (50 mg). After digestion, the tissue fragments were resuspended in a culture medium consisting of 90% of a 1:1 mixture of Dulbecco’s modified Eagle’s (GIBCO) and Ham’s F12 (GIBCO) media, 5% heat-inactivated horse serum (GIBCO), and 5% heat-inactivated fetal bovine serum (GIBCO) containing 15 mM HEPES buffer (pH 7.2), 30 nM selenium, 1.9 mg/ml sodium bicarbonate, 100 units/ml penicillin G, and 100 mg/ml streptomycin sulfate. The tissue fragments were then dissociated by gentle passage through plastic tips with three different diameters. Dissociated cells were filtered through a nylon cell strainer with a pore size of 70 μm (Falcon, Franklin Lakes, NJ, U.S.A.) and centrifuged; then the cells were resuspended in the culture medium and plated in dishes (105 cells per dish) precoated with polylysine (Sigma) and maintained in a humidified CO2 incubator (95% air/5% CO2) at 37°C. After 2 days, nonneuronal cell division was halted by 24 h of exposure to 10−5 M cytosine arabinoside (Sigma). Subsequent medium replacement was carried out every 2 days. After 7 days in vitro, cultures were subjected to immunoblot analyses.

Immunolight microscopy

Four rats each were used for immunohistochemical analyses at 2 and 30 months of age. The detailed immunohistochemical procedure has been described previously (Nakanishi et al., 1994; Amano et al., 1995b). In brief, specimens were obtained from rats in each age group, which were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and killed by intracardiac perfusion with isotonic saline followed by a chilled fixative consisting of 4% paraformaldehyde in 0.2 M PBS (pH 7.4). After perfusion, the brain was removed and further fixed by immersion in the same fixative for 24 h at 4°C and then immersed in 20% sucrose (pH 7.4) for 24 h at 4°C. Floating parasagittal sections (30 μm thick) of the brainstem and the cerebral cortex were prepared by a cryostat and stained by anti-CD IgG (1.55 μg/ml) or anti-CE IgG (0.50 μg/ml) with the avidin–biotin–peroxidase complex method. After PBS washes, sections were reacted with aminomethylcarbazole (AEC; Dako Corp., Carpinteria, CA, U.S.A.) for 5–20 min. For double immunostaining, AEC was used as a chromogen yielding a xylene-soluble orange precipitate. After immunostaining for CE or CD, AEC precipitates for CE or CD labeling were removed by incubating the sections in 50% alcohol for 5 min and then in xylene for 30 min at room temperature. After washing with PBS, the sections were stained with 22C11 (10 μg/ml) or anti-APP663–695 IgG (5 μg/ml) with the avidin–biotin–peroxidase method followed by reaction with 0.025% 3,3'-diaminobenzidine/0.4% (NH4)2Si(SO4)3/0.09% H2O2/0.1 M Tris-buffered saline solution for 5–10 min. All sections were thoroughly rinsed with PBS, mounted, and covered-slipped with glycerol gelatin (Sigma). As an immunohistochemical control, the sections were incubated with nonimmune rabbit IgG and treated as described above.

Immunoelectron microscopy

For immunoelectron microscopy, a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde containing 0.05% CaCl2 and 0.1% tannic acid in 0.1 M cacodylate buffer (pH 7.4) was used for a fixative. After perfusion, the brain was removed and further fixed by immersion in the same fixative for 1 h. After washing in 50 mM glycine in cacodylate buffer, the tissue pieces were rinsed at 4°C overnight in cacodylate buffer containing 7% sucrose. The specimens were subsequently postfixed in ferrocyanide-reduced 1% OsO4 at 4°C for 30 min, dehydrated, and embedded in LR
White. They were then polymerized by ultraviolet irradiation at −25°C for 72 h. Ultrathin sections were mounted on Formvar-coated 150-mesh nickel grids. Nonspecific staining was blocked by treatment in 1% bovine serum albumin in PBS containing 0.1 M lysine hydrochloride for 30 min. The grids were reacted for 3 h by floating them on drops of anti-CE IgG (10 μg/ml) or anti-CD IgG (37 μg/ml). After rinsing with PBS, the grids were incubated for 1 h on 10-nm colloidal gold-conjugated anti-rabbit IgG diluted to 1:40 in 1% bovine serum albumin in PBS. After rinsing with PBS, the immunolabeled sections were stained with uranyl acetate for 5 min and observed using an H-7000 transmission microscope (Hitachi, Tokyo, Japan) operated at 75 kV.

RESULTS

Immunoblot analyses of CE and CD

The activity levels of aspartic proteinase (CE plus CD) were analyzed in the brain extracts prepared from the cerebral cortex and the brainstem of embryonic and 2-, 17-, and 30-month-old rats. Increased age was found to be associated with elevated levels of the activity in both the cerebral cortex and the brainstem. The mean ± SEM specific activity in the cerebral cortex of embryonic and 2-, 17-, and 30-month-old rats was 2.5 ± 0.1 (n = 14), 5.1 ± 0.7 (n = 4), 5.0 ± 0.1 (n = 4), and 9.6 ± 1.8 (n = 4) units/mg of protein, respectively. On the other hand, the mean ± SEM specific activity in the brainstem of embryonic and 2- and 30-month-old rats was 2.5 ± 0.1 (n = 14), 15.3 ± 4.6 (n = 4), and 29.0 ± 4.2 (n = 4) units/mg of protein, respectively. On the other hand, the mean ± SEM specific activity in the cerebral cortex and brainstem from rats of four age groups. Immunoblot analyses using the polyclonal antibody for CE, which can recognize both the pro- and mature forms of CE, revealed that a definitive immunoreactive band was barely detectable in the tissue extract of embryonic brain. However, the extracts of both brain tissues at 2, 17, and 30 months of age revealed the increased expression of the 42-kDa band, and an additional band with a molecular mass of 46 kDa was observed especially at 30 months of age (Fig. 1a and b). Densitometric scanning showed that the CE levels in the cerebral cortex and brainstem at 30 months of age were increased ~1.4 and 1.7 times as much as those at 2 months of age, respectively. On the other hand, immunoblot analyses using the antibody specific for CD showed an intense band with a molecular mass of 44 kDa, which corresponded to the mature form, throughout the entire age (Fig. 1c and d). The CD levels were also increased with advancing age by up to 1.5-fold in the cerebral cortex and 1.7-fold in the cerebral cortex at 30 months of age as compared with those at the embryonic stage.

The cortex extracts from 2-month-old rats and the extracts of primary cultured cortical neurons from the embryonic rat showed a 42-kDa protein band, whereas two bands with apparent molecular sizes of 46 (major) and 42 (minor) kDa were clearly observed in the spleen extracts of 2-month-old rats (Fig. 2a). The 46-kDa form in the spleen extract was sensitive to conversion to the 42-kDa form by a brief acid treatment at pH 3.5 at 37°C for 10 min, whereas the 42-kDa form was resistant to this treatment (Fig. 2a). Furthermore, the antibody specific for the synthetic peptide Ser-Gln-Leu-Ser-Glu-Trp-Lys-Ser-His-Asn-Leu-Asp-Met reacted with the 46-kDa band in the spleen extracts but not with the 42-kDa bands in either tissue (Fig. 2b). Therefore, the 46- and 42-kDa forms were found to be the mature and proform of CE, respectively.

FIG. 1. Immunoblot analyses of whole extract of cerebral cortex (CX) and brainstem (BS) from rats of four different ages by anti-CE (a and b) and anti-CD (c and d) antibodies. Each column represents the mean relative immunoreactivity of four experiments. The relative immunoreactivity of CE and CD was determined as compared with 2 months of age and embryonic, respectively. E, embryonic day 18; 2M, 2 months old; 17M, 17 months old; 30M, 30 months old.
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FIG. 2. Immunoblot analyses of whole extracts from various rat tissues by (a) anti-CE and (b) anti-pro-CE antibodies. Extracts without (−) and with (+) a brief acid treatment were analyzed in a. CX, cerebral cortex; BS, brainstem; SP, spleen; CL, cortical cell cultures.

Subcellular fractionation
To examine the age-related alteration in intracellular distribution of CE and CD, their levels in the subcellular fractions prepared from tissue extracts of the cerebral cortex and the brainstem of 2- and 30-month-old rats were measured. At 2 months of age, the P and S fractions were found to be rich in the lysosome and the cytoplasmic matrix, respectively, as judged by the distribution of β-glucuronidase (a marker for the lysosome) and LDH (a marker for the cytosol). A small amount of β-glucuronidase was detected in S fractions of the cerebral cortex (6%) and the brainstem (7%). However, its level in the S fractions was markedly increased in the brain from 30-month-old rats (16% for the cerebral cortex and 21% for the brainstem). On the other hand, the LDH content recovered in the P and S fractions was independent of age, suggesting that the age-dependent increase of autophagy has no significant effect on distribution of the cytosolic components. As shown in Fig. 3a and b, CE was exclusively confined to P fractions of both the cerebral cortex and the brainstem from 2-month-old rats. However, in tissues from 30-month-old rats, CE was found in the S fractions to a similar or even higher extent than shown in the P fractions. The CE levels in the P fraction between 2- and 30-month-old rats were not significantly changed in the cerebral cortex but increased 1.7-fold in the aged brainstem. On the other hand, CD was also mainly found in the P fraction and partially in the S fraction of 2-month-old rat brain (Fig. 3c and d). The CD levels in both brain tissues were significantly increased in 30-month-old rat brain as compared with 2-month-old rats. The marked increase of CD level in the S fractions of the aged rat tissues may represent the increased fragility of the lysosomal membrane of aged cells.

Localization of CE and CD at light and electron microscopic levels
At 2 months of age, CE was barely detectable in both the frontal portion of the cerebral cortex (Fig. 4a) and the pontine reticular formation of the brainstem (Fig. 5a), whereas CD was widely but unevenly distributed as coarse intraplasmic granules in these tissues (Figs. 4c and 5c). At 30 months of age, marked increase in CE immunoreactivity was observed in both cortical (Fig. 4b) and brainstem (Fig. 5b) neurons. It was noted that CE was greatly accumulated as large granular immunoreactive products in large neurons of

FIG. 3. Immunoblot analyses of the extracts of P and S fractions of brain tissues from young and aged rats by (a and b) anti-CE and (c and d) anti-CD antibodies. Each column represents the mean relative immunoreactivity of three experiments. The relative immunoreactivity of both CE and CD was determined as compared with the S fraction of 2-month-old rats. CX, cerebral cortex; BS, brainstem; 2M, 2 months old; 30M, 30 months old.
FIG. 4. Age-related increase in CE and CD immunoreactivities (upper panels) and their colocalization with autofluorescent lipopigments (lower panels) in cortical neurons. a and b: Immunostaining of CE in (a) 2- and (b) 30-month-old rats. c and d: Immunostaining of CD in (c) 2- and (d) 30-month-old rats. e–h: Autofluorescent lipopigments observed under the ultraviolet light in the same section shown in a–d before immunostaining, respectively. Colocalization of CE immunoreactivities and autofluorescent lipopigments in cortical neurons of the aged rat was indicated by arrowheads in b and f. Colocalization of CD immunoreactivities and autofluorescent lipopigments in cortical neurons of the aged rat was indicated by arrowheads in d and h. Bar = 50 μm.

these brain regions. Microglia in the white matter were also intensely immunostained with anti-CE antibody (data not shown). Immunoreactivity of CD was increased in both neurons and glial cells in these brain regions of 30-month-old rats (Figs. 4d and 5d).

The lower panels in Figs. 4 and 5 show age-dependent accumulation of lipofuscin granules in the same sections of the upper panels. At 30 months of age, intense autofluorescent granules were found to accumulate in the focal cytoplasmic site of the majority of cortical (Fig. 4f and h) and brainstem (Fig. 5f and h) neurons. These autofluorescent pigments were considered to be lipofuscins because they exhibited yellow-brown fluorescence under ultraviolet light. The localization of the autofluorescent pigments corresponded well with that of CE (Figs. 4f and f and 5b and f). CD was also partially colocalized with autofluorescent pigments (Figs. 4d and h and 5d and h). In contrast, the cortical and brainstem neurons from 2-month-old rats showed no such autofluorescence (Figs. 4e and g and 5e and g). The immunoreaction products with both antibodies had to be weakly demonstrated, because the intense immunostainings interfered with clear demonstration of the autofluorescence of lipofuscin. The discrepancy in immunoreactivity between CE and CD was mainly due to the practical difficulty in demonstrating low concentrations of the cellular CE. However, it was confirmed that CE was present in most of the lipofuscin-positive neurons, if not all (data not shown).

Ultrastructurally, lipofuscin is known to accumulate in lysosomes as electron dense granules and lipid-like granules. As shown in Fig. 6b, immunogold particles indicating antigenic sites for CD were mainly associated within such lipofuscin-containing organelles in the pontine reticular formation neurons of the brainstem of 30-month-old rats. A small number of lysosomes without lipofuscin-like materials was also labeled by immunogold particles for CD. Because CD, as well as cathepsins B and L, is known as a representative lysosomal hydrolase, the lipofuscin-like materials were found to accumulate in secondary lysosomes. It is interesting that immunogold particles indicating antigenic sites for CE were predominantly found in lipofuscin-containing lysosomes accumulated near the nuclear membrane (Fig. 6a). In serial thin sections, the same granules containing lipofuscin-like materials were labeled by immunogold particles for both CD and CE (data not shown). These results indicate the
colocalization of CE and CD in lipofuscin-loaded lysosomes of neurons from 30-month-old rats.

**Colocalization of CE and CD with APP**

Figures 7 and 8 show a marked difference between 2- and 30-month-old rat brain tissues in immunostaining with the antibody to the C-terminal fragment of APP (APP<sub>643-695</sub>). At 2 months of age, the immunoreactivity was scarcely detectable in either the frontal portion of the cerebral cortex or the pontine reticular formation of the brainstem (Figs. 7b and 8b). At 30 months of age, however, the immunoreaction with this antibody was evident in the majority of large frontal cortical pyramidal cells in the layer V (Fig. 7d) and relatively large pontine reticular neurons (Fig. 8d). The number of the positive frontal cortical pyramidal cells in the layer V and pontine reticular neurons counted through a drawing tube attached to the microscope was 176 ± 61 and 66 ± 24 cells/mm<sup>2</sup>, respectively. Double immunostaining revealed that the appearance of cells positive for the C-terminal fragment was highly consistent with the appearance of CE-positive cells in the cortical (Fig. 7c and d) and brainstem (Fig. 8c and d) neurons. On the other hand, anti-APP<sub>60-100</sub> antibody intensely stained the cortical and brainstem neurons of 2- and 30-month-old rats, where there was no obvious difference in staining patterns between both groups (data not shown).

**Immunoblot analyses of APP**

To confirm the age-related neuronal accumulation of the C-terminal fragment of APP, the tissue extract...
of brainstems from embryonic and 2- and 30-month-old rats was subjected to SDS-polyacrylamide linear gradient gel electrophoresis from 5 to 12% (wt/vol) to separate full-length APP and APP fragments. In the brainstem extracts from 2- and 30-month-old rats, two immunoreactive bands of ~19 and 22 kDa corresponding to the C-terminal fragment of APP were clearly detected with anti-APP_{643-695} antibody, and their levels greatly increased at 30 months of age (Fig. 9). However, these protein bands were barely detectable at the embryonic stage. The discrepancy between immunohistochemical (Figs. 7 and 8) and immunoblotting data (Fig. 9) on the young brainstem with regard to immunoreaction with the antibody APP_{643-695} appeared to be due to the difference in immunoreactivity between the natural and denatured proteins. Namely, it is likely that APP in the young neurons exists mostly as the native and normally processed forms that are unable to react with the antibody, probably owing to the steric

**FIG. 7.** Double-staining immunohistochemistry shows the colocalization of CE and APP in cortical neurons of a 30-month-old rat. a and b: Double-staining with anti-CE antibody and anti-APP antibody in cerebral cortex of a 2-month-old rat. c and d: Double-staining with anti-CE antibody and anti-APP antibody in cerebral cortex of a 30-month-old rat. As shown by arrowheads in c and d, most CE-positive cortical cells were contained with APP-immunoreactivity. Bar = 100 μm.

**FIG. 8.** Double-staining immunohistochemistry shows the colocalization of CE and APP in brainstem neurons of a 30-month-old rat. a and b: Double-staining with anti-CE antibody and anti-APP antibody in brainstem of a 2-month-old rat. c and d: Double-staining with anti-CE antibody and anti-APP antibody in brainstem of a 30-month-old rat. As shown by arrowheads in c and d, most CE-positive brainstem cells were contained with APP-immunoreactivity. Bar = 100 μm.
The present study clearly showed that CE and CD were increasingly expressed in both the cerebral cortex and the brainstem during the normal aging process and that these changes were associated with senescence of the brain. CD was exclusively found in the mature form in both young and aged rat brain tissues. Although CE was mainly present in the mature form in the young rat brain tissues, a significant amount of pro-CE as well as the predominant mature form was detected in the aged brain tissues. These results suggest that CE is increasingly expressed in these rat brain tissues with aging and greatly processed to the mature CE. On the other hand, CE in most rat tissues such as spleen and stomach has been shown to be predominantly present as the enzymatically inactive proform (Yonezawa et al., 1993; Okamoto et al., 1995). This protein is also known to exist in normal human erythrocyte membranes in the proform, which can be easily activated by cell aging or oxidant challenge (Yamamoto et al., 1989). Furthermore, the recombinant human CE expressed in Chinese hamster ovary cells (Tsu Kubu et al., 1993) and mouse L cell fibroblasts and monkey Cos1 kidney cells (Finley and Kornfeld, 1994) was primarily present as the proenzyme in the endoplasmic reticulum. Finley and Kornfeld (1994) have suggested that CE in the endoplasmic reticulum served as a reservoir that could be mobilized in response to certain stimuli. More recently, Nishishita et al. (1996) reported that CE in rat thymus was exclusively confined to thymocytes, in which it was present in the proform.

They have also shown that the thymocyte pro-CE was converted into the mature form by in vivo dexamethasone administration, suggesting that the proenzyme is transported from the primary storage sites, such as the endoplasmic reticulum and the cis- or trans-Golgi, to the functional sites, where it is processed to the catalytically active enzyme. Although the functional sites of CE are currently unknown, the proteolytic maturation of pro-CE appears to proceed within an acidic environment, because in the pulse-chase experiment in cultured rat microglia the proteolytic processing has been shown to be blocked by an increase in the intracellular pH with bafilomycin A1 and NH4Cl (D. F. Sastradipura et al., unpublished data).

In the present study, we provide the first evidence for the colocalization of CE with CD in the lipofuscin-containing lysosomes in the brainstem neurons of aged rats. Because CE predominantly existed as the intact form in the brain tissues and cortical cell cultures, it seems likely that the newly synthesized pro-CE in these tissues is transported to the intracellular processing sites connected with its activation. Previous immunoelectron microscopic studies have consistently demonstrated that intracellular localization of CE in nonneuronal cells was different from that of CD (Ichimaru et al., 1990; Saku et al., 1991; Solcia et al., 1993), where the enzyme appeared to be present mostly in the mature form (Finley and Kornfeld, 1994). In gastric and lymphoid cells, CD was exclusively confined to lysosomes, whereas CE was distributed diffusely in the cisternae of the rough endoplasmic reticulum and of the nuclear envelope and often in the cytoplasmic matrix. Therefore, CE in nonneuronal cells, except for macrophages and microglia, is assumed to be exclusively concentrated in the storage sites, but the nature of the storage sites of CE remains to be determined. In contrast, CE in neurons is likely to be transported to the final processing acidic sites.

It is widely accepted that the initial step in the formation of lipofuscin is autophagocytosis of cytoplasmic components. These autophagosomes fuse with primary or secondary lysosomes to form autolysosomes. In the next step, lipofuscin undergoes maturation to convert into the characteristic fluorescent material. Therefore, colocalization of CE with CD or lipofuscin suggests the age-related alteration of the endosomal/lysosomal system in the neurons, together with the association with enhanced autophagy in the neuronal aging process. In addition to the immunocytochemical observation, the fusion of CE-containing organelles with lysosomes and the enhanced endosomal/lysosomal system in the aged brain tissues are supported by the fact that the amount of CE in the S fraction of the aged rat brain tissues was very large, although the enzyme was barely detectable in the S fraction of the young brain tissues. This may also be related to the age-dependent increase in the fragility of the endosomal/lysosomal system. It has also been proposed that the increase in
the cytosolic CD level in the aged brain is probably due to disintegration of the lysosomal membrane (Matus and Green, 1987; Nakamura et al., 1989b). The leakage of CE as well as CD into the cytoplasm of neurons may be associated with age-related pathologi-
ical changes because CE and CD have been shown to be capable of degrading cytoskeletal proteins and bioactive peptides (Matus and Green, 1987; Kageyama, 1993).

The present results also demonstrate that CE was colocalized with the C-terminal APP fragments that reacted with anti-APP643–695 antibody in the aged neu-
rons. Immunoblot analysis revealed the age-related in-
crease in content of two smaller proteins of APP, with apparent molecular masses of 19 and 22 kDa, which appeared to represent proteolytic C-terminal APP frag-
ments. The size of these proteins strongly suggested that they might contain an intact βA4 domain and therefore be amyloidogenic. As APP has been shown to accumulate in lipofuscin-containing lysosomes in neurons from normal and Alzheimer’s disease subjects (Benowitz et al., 1989) and as CE was predominantly found in lipofuscin-containing lysosomes of aged rat neurons (Fig. 6), CE may be involved in the altered APP catabolism to produce amyloidogenic C-terminal APP fragments. Recently it has been suggested that CD as well as CE efficiently cleaves the wild-type APP, as well as a Swedish mutant, to yield amyloidogenic-
genic fragments (Ladror et al., 1994). Further study will be needed to determine whether CE can process full-length APP into small amyloidogenic fragments.

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