Age-related changes in cellular localization and enzymatic activities of cathepsins B, L and D in the rat trigeminal ganglion neuron

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Abstract

Altered localization and cellular level of three distinct lysosomal proteinases, cathepsins B (CB), L (CL), and D (CD), with aging were investigated in the rat trigeminal ganglion (TG) by immunohistochemical and quantitative analyses. At the light microscopic level, the intracytoplasmic distribution of these three enzymes was found to change with aging: These lysosomal proteinases in the TG of young rats (2–3 months of age) were widely and evenly distributed throughout the cytoplasm as coarse intracytoplasmic granules, whereas they were localized at focal cytoplasmic sites of the TG neurons of aged rats (28–31 months of age) as coarse aggregates. A similar distribution was observed with a major lysosomal membrane sialoglycoprotein having an apparent molecular mass of 107 kDa (LGP107). The cellular distribution of the three cathepsins as well as LGP107 in the TG neurons of aged rats corresponded well with that of autofluorescent lipofuscin. At the electron microscopic level, the age-related redistribution of these cathepsins in the TG neurons was found to be due to their great accumulation in autolysosomes localized at the focal perinuclear sites. The cellular levels of CB and CL determined by activity measurement in the TG of the young rats were 1.8 and 1.7 times as much as those of the aged rats respectively. In contrast, no significant difference was observed between the CD activities in the two age groups. These results

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strongly suggest that age-related changes in localization and cellular level of CB, CL, and CD in TG neurons are closely linked with the increased formation of autolysosomes and lipofuscin, which is the most ubiquitous age-related cytological alteration.

**Keywords:** Aging; Trigeminal ganglion; Cathepsins; Lysosome; Phagocytosis

1. **Introduction**

Cathepsins B (CB), L (CL), and D (CD) are typical and well-characterized lysosomal proteinases that are widely but unevenly distributed in a variety of mammalian cell types including neuronal cells [3,4,25]. Besides their intralysosomal proteolysis for intracellular protein turnover, these enzymes have been suggested to be involved in a variety of pathological conditions such as inflammation [12,20] and neoplastic transformation [5,21]. More recently, there is increasing evidence that the disorder or breakdown of lysosomal systems has pathological significance in neurodegenerative diseases such as Alzheimer's disease. In Alzheimer's disease, enzymatically active CB and CD have been found extracellularly at high levels in the senile plaques [7] and there was a marked accumulation of secondary lysosomes and residual bodies in affected neurons [6,18]. In rat brain, it has been reported that enzymatic activities of CD [10,14,15,17] and cathepsin E (CE), a non-lysosomal aspartic proteinase, were significantly increased with advancing age [17]. Lysosomal leakage in the aged rat brain has also been demonstrated to result in increased cytosolic CD activity [14, 15]. Furthermore, these cathepsins are known to exhibit the ability to degrade physiologically significant neuronal constituents such as neurofilaments, microtubule-associated proteins and myelin basic proteins [2, 14, 19, 24]. The abnormality of lysosomal systems is considered to have pathological significance in the ageing process of the central nervous system.

In contrast to the central nervous system, little or no information is available about age-related alterations of the lysosomal system in the peripheral nervous system. The present study was thus undertaken to elucidate age-related changes in localization and cellular level of CB, CL, and CD as well as CE in the rat trigeminal ganglion (TG) neurons.

2. **Materials and methods**

2.1. **Materials**

Antibodies against rat spleen CB, CD, and CE were raised in rabbits and purified by affinity chromatography as described previously [16,17]. Antibodies specific for CL and the 107 kDa lysosomal membrane sialoglycoprotein (LGP107) were provided from Drs. Y. Nishimura and Y. Tanaka (Department of Physiological Chemistry, Kyushu University Faculty of Pharmaceutical Sciences, Japan), respectively. \( N-(L-3\text{-trans-}\text{propyl}-\text{carbamoyloxirane-2-carbonyl})L\text{-isoleucyl-L-proline} \) (CA-074) was kindly supplied by Taisho Pharmaceutical Co. Ltd (Tokyo, Japan). Two age groups of Fischer 344 male rats (young: 2–3 months of age; aged: 28–31 months of age) were used in the present study.
2.2. Immunocytochemical procedures

Detailed immunohistochemical procedures were described previously [16,17]. Briefly, young (2–3 months, n = 5) and aged (28–31 months, n = 5) rats anaesthetized with sodium pentobarbital were perfused with chilled isotonic saline and then with chilled fixative consisting of 4% paraformaldehyde in 0.2 M phosphate buffered saline (PBS, pH 7.4). After perfusion, the TG was removed, fixed in the same fixative for 24 h at 4°C, and then immersed in 20% sucrose (pH 7.4). Floating parasagittal sections (30–40 μm thick) were prepared by a cryostat and stained by anti-CB (6.5–8.0 mg/ml), CL (0.5–0.8 mg/ml), CD (10–20 μg/ml), LGP107 (50 μg/ml) IgG at dilution of 1: 800–2400 in PBS with the avidin-biotin-peroxidase complex method using a Vectastain Kit (Vector Laboratories, Burlingame, CA, USA) followed by the diaminobenzidine reaction. For double immunostaining of CB and LGP107, aminoethylcarbazole (AEC) was used as a chromogen yielding a xylene-soluble orange precipitate. After immunostaining for CB, AEC precipitates for CEI labeling, were removed by incubating the sections with xylene and then the sections were further stained by anti-LGP107 IgG. For immunoelectron microscopic examination, aged rats (31 months, n = 3) were perfused with chilled isotonic saline and then with chilled fixative (4% paraformaldehyde and 0.5% glutaraldehyde in 0.2 M PBS). After perfusion, the TG was removed, fixed in the same fixative for 3 h at 4°C. Sections (20–25 μm thick) were cut by a Microslicer (DTK1000, Dosaka EM, Kyoto, Japan) and processed similarly to those for light microscopy, except that all procedures were performed at 4°C and they were not treated with Triton X-100. After immunostaining as described above, the sections were further postfixed with 0.5% osmium tetroxide, dehydrated through graded ethanol and embedded in Epon 812. Ultrathin sections were examined with a JEOL200CX electron microscope. Control sections were treated in the same way except that the primary antibodies were removed or replaced by a non-immune rabbit IgG.

2.3. Determination of cathepsins activities

Detailed methods for determination of enzymatic activities were also described previously [16,17]. Shortly, the TG extracts from young (2–3 months, n = 6–7) and aged rats (28–31 months, n = 4–5) were prepared by ultrasonication of the homogenate in the presence of 0.1% Triton X-100 followed by centrifugation. CB and CL activities in the extract were measured with benzyloxycarbonyl-phenylalanyl-arginine-4-methyl-7-coumarylamide (Z Phe-Arg-MCA) at pH 6.0. Knowing the total activity against Z-Phe-Arg-MCA, the levels of CB and CL were determined by measuring the activity sensitive and insensitive to the CB specific inhibitor CA-074 [9], respectively. One unit for CB and CL was defined as the amount of each enzyme required to release 1 nmol of 7-amino-4-methylcoumarin liberated from the substrate per min. CD and CE activities were measured with acid-denatured hemoglobin (Hb) as a substrate at pH 3.5. The levels of CD and CE were determined from the total activity by immunoprecipitation utilizing discriminative antibodies specific for each enzyme. One unit for CD and CE was defined as the amount of each enzyme required to release 1 μg of tyrosine per min. Protein was
3. Results

3.1. Age-related changes in cellular localization of cathepsins B, L and D

At the light microscopic level, three distinct lysosomal proteinases CB, CL, and CD were abundantly and evenly distributed through all the TG neurons of young rats (Fig. 1A–C). These enzymes were found in the cells as coarse intracytoplasmic granules. The neuronal nuclei were devoid of immunoreactive products for each enzyme. No significant difference in staining pattern of the respective cathepsins was observed among different sized TG neurons with a diameter ranging 10–60 μm. In aged rats, the relative amount of the medium-sized TG neurons was increased and the mean diameter was shifted from 27 to 32 μm (n = 100). More than 60% of the TG neurons of aged rats showed intense and local immunostaining for CB, CL, and CD at the focal cytoplasmic sites as large coarse aggregates (Fig. 1D, E, F). When changes in the cellular localization of CB, CL and CD were also examined in the middle age group, a similar focal distribution of these three enzymes was found in TG neurons of 20-month-old rats, but barely detectable in those of 12-month-old rats (data not shown), suggesting that changes in their localization are closely associated with such senescent cytological alterations as increased formation of autolysosomes and lipofuscins. On the other hand, CE was not detectable in TG neurons of either young or aged rat groups. Only macrophage-like cells in this tissue from both age groups gave diffuse staining for CE in the cytoplasm (data not shown).

In the great majority of TG neurons from the aged rat group, intense autofluorescent granules were found to accumulate in the focal cytoplasmic site (Fig. 2A). These autofluorescent pigments were considered to be lipofuscins since they exhibited yellow-brown fluorescence under ultraviolet light. The TG neurons from the young rat group showed faint or no such autofluorescence (data not shown). Granular immunoreactive products of both CB (Fig. 2B) and LGP107 (Fig. 2C) were also found in the focal cytoplasmic site of the TG neurons and their distribution corresponded well with that of autofluorescent pigments. All the CB-positive cells contained both lipofuscin and LGP107, but LGP-positive and lipofuscin-containing cells were not always positive for CB. Similar observations were obtained for CL and CD, although there were slight regional differences in immunostaining patterns among these cathepsins (data not shown).

At the electron microscopic level, the TG neurons from the aged rat group were characterized by the presence of a number of autolysosomes, which engulfed swollen cytoplasmic organelles such as mitochondria, vacuoles and lipofuscin pigments (Fig. 2D,E). The immunoreactive materials for CB appeared in the form of fine electron-dense granules were observed in lysosomes (Fig. 2D, single arrow heads) and autolysosomes which contained degrading cytoplasmic organelles such as mitochondria and electron-dense lipid vacuoles (Fig. 2D, double arrow heads). CB-positive autolysosomes often consisted of two membranes, indicating
Fig. 1. Immunohistochemical staining of CB, CL and CD in TG neurons of the young and aged rat. The left and right columns are TG neurons from the young and aged rat, respectively. The top panels (A,D): staining of CB; the middle panels: staining of CL; the bottom panels: staining of CD. Intense granular immunoreactive materials of each enzyme are homogeneously distributed through the cytoplasm of the young TG neuron. In the aged TG neuron, however, focal accumulations of immunoreactivities of these enzymes are noted. Bar = 10 μm.
Fig. 2A–C. Localization of lipopigment autofluorescent (A), CB (B) and LGP107 (C) in the same section of the TG from the aged rat. D: immunocytochemical staining of CB in the TG neurons of the aged rat. Immunoreactive products of CB are observed as fine electron-dense granules in both lysosomes (marked by arrow heads) and autolysosomes (marked by double arrow heads). E: control staining. Both lysosomes (marked by arrow heads) and autolysosomes (marked by double arrow heads) are devoid of immunoreactive materials. Bar = 40 μm (A), 1 μm (D).

Table 1
Enzymatic activities of CB, CL and CD in the trigeminal ganglion from young and aged rats

<table>
<thead>
<tr>
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<th>Cathepsin B</th>
<th>Cathepsin L</th>
<th>Cathepsin D</th>
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<tbody>
<tr>
<td>Young</td>
<td>18.4 ± 5.6 (n = 7)</td>
<td>9.8 ± 3.6 (n = 7)</td>
<td>13.5 ± 1.8 (n = 6)</td>
</tr>
<tr>
<td>Aged</td>
<td>10.4 ± 0.5 (n = 4)**</td>
<td>5.8 ± 0.6 (n = 6)*</td>
<td>14.2 ± 2.7 (n = 5)</td>
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Values are presented as mean ± S.D. in units/mg protein. *p is calculated according to Student’s t-test for the differences between young and aged groups.

*P < 0.05.

**P < 0.01.
that they are in the early stage of autophagocytosis. No fine electron-dense granule representing immunoreactivity was observed in any lysosomal structures when tissues were incubated without anti-CB antibody or when anti-CB antibody was replaced by non-immune IgG (Fig. 2-E).

3.2. **Age-related changes in enzymatic activities of cathepsins B, L and D**

Table 1 shows the activity levels of CB, CL, and CD in the Triton X-100 soluble extract of the TG from both young and aged rat groups. In either group, the Z-Phe-Arg-MCA- and Hb-hydrolyzing activities in the TG extract were totally inhibited by E-64 and pepstatin, respectively. The results strongly suggest that the Z-Phe-Arg-MCA- and Hb-hydrolyzing activities were composed of the cysteine proteinases CB and CL and the aspartic proteinases CD and CE, respectively. The CB and CL activities were determined by measuring the activity sensitive and insensitive to CA-074, respectively. In both groups, about 65% of the total Z-Phe-Arg-MCA-hydrolyzing activity was attributed to CB and about 35% to cathepsin L. Thus, the ratio of the CB to CL contents in the tissue was likely to be independent of aging. On the other hand, the Hb-hydrolyzing activities in the TG extracts from both groups were totally immunoprecipitated by increasing amounts of the anti-CD IgG but not the anti-CE IgG, indicating that this tissue contained only CD activity. As shown in Table 1, the levels of CB and CL in the young rat group were significantly higher than those in the aged rat group by 55% ($P < 0.01$) and 60%, ($P < 0.05$), respectively. In contrast, the CD level in the tissue was not significantly changed with aging.

4. **Discussion**

The present study provides evidence that the lysosomal system in the TG neurons is clearly altered with aging. The TG neuron from the young rat group exhibited wide and homogeneous distribution of cathepsins as well as the lysosomal membrane protein LGP107 through the cytoplasm, whereas most of the TG neurons from the aged rat group showed their limited and focal distributions in the perinuclear regions. In order to examine whether changes in the cellular localization of lysosomal cathepsins occurred between young age and maturity, we examined their expression in TG neurons of the middle aged rats. A similar limited and focal distribution of these enzymes in TG neurons was found in rats of 20 months of age, but barely detectable in rats of 12 months of age. Therefore, it is more likely that changes in the expression of these enzymes are associated with senescence but not maturity. By immunoelectron microscopic study, strong antigenecities for these enzymes in the TG neurons from the aged rat group were mainly shown in autolysosomes which were focally formed in the perinuclear cytoplasmic sites, indicating enhanced autophagocytosis. These autolysosomes contained engulfed cytoplasmic organelles such as mitochondria and lipofuscin pigments. A similar prominent accumulation of autolysosomes and lipofuscins has been reported in some pathological states. In Alzheimer’s disease, CB and CD positive lysosomes containing lipofuscin pigments were markedly increased in cortical pyramidal
neurons in the absence of atrophic, chromatolytic or neurofibrillary changes [6,18]. CD-positive lipofuscin granules have been shown to dramatically increase in affected spinal cord neurons of the motor neuron degeneration mouse, a mutant strain of which motor neurons degenerate only in late adulthood [18]. In the sciatic nerve of the rat, axonal degeneration also corresponded to sites of the accumulation of CD and secondary lysosomes [22]. Taken together, the age-related redistribution of cathepsins in the TG neurons is closely associated with the increased formation of autolysosomes and lipofuscins which is the most ubiquitous age-related cytological alteration [23]. Furthermore, resultant accumulation of such autolysosomes is likely to sterically interfere with cell functions such as intracellular vesicular transport.

The reason for the age-related decrease in the activity levels of CB and CL but not CD in the TG remains unknown. However, endogenous cysteine proteinase inhibitors such as cystatins B and C may be also increased and accumulated in the TG neurons with advancing age, since the cellular level of calpastatin, an endogenous inhibitor of calpain, is known to increase in the aged rat brain [11]. Interestingly, injection of leupeptin, a cysteine proteinase inhibitor, or chloroquine, a general lysosomal inhibitor, into young rat brains was found to induce the formation of lysosome-associated granular aggregates which closely resemble the ceroid-lipofuscin [8]. Furthermore, age-related change in CB activity was not found in the rat brain, while CL activity was significantly decreased with advancing age [17]. Therefore, reduced activities of cysteine proteinases are considered to be closely related with the formation of lipofuscin. On the other hand, CD activity in the TG showed no age-related change, whereas CD activities in both human and rat brains have been reported to increase with aging by two-fold [1, 10, 14, 15, 17]. The major factor responsible for this discrepancy between the TG and the brain may be due to difference in cellular constituents between the two different tissues, since the increased activity of CD with aging in the brain is considered to be mainly associated with glial proliferation [17].

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References


