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Cathepsin L coexists with Cytotoxic T-lymphocyte Antigen-2 alpha in distinct regions of the mouse brain

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ABSTRACT

Cathepsins B and L are two prominent members of cystein proteases with broad substrate specificity and are known to be involved in the process of intra- and extra-cellular protein degradation and turnover. The propeptide region of cathepsin L is identical to Cytotoxic T-lymphocyte antigen-2α (CTLA-2α) discovered in mouse activated T-cells and mast cells. CTLA-2α exhibits selective inhibitory activities against papain and cathepsin L. We previously demonstrated the distribution pattern of the CTLA-2α protein in mouse brain by immunohistochemistry, describing that it is preferentially localized within nerve fibre bundles than neuronal cell bodies. In the present study we report colocalization of cathepsin L and CTLA-2α by double labeling immunofluorescence analysis in the mouse brain. In the telencephalon, immunoreactivity was identified in cerebral cortex and subcortical structures, hippocampus and amygdala. Within the diencephalon intense colocalization was detected in stria medullaris of thalamus, mammillothalamic tract, medial habenular nucleus and choroid plexus. Colocalization signals in the mesencephalon were strong in the hypothalamus within supramammillary nucleus and lateroanterior hypothalamic nucleus while in the cerebellum was in the deep white matter, granule cell layer and Purkinje neurons but moderately in stellate, and basket cells of cerebellar cortex. The distribution pattern indicates that the fine equilibrium between synthesis and secretion of cathespin L and CTLA-2α is part of the brain processes to maintain normal growth and development. The functional implication of cathespin L coexistence with CTLA-2α in relation to learning, memory and disease mechanisms is discussed.

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1. Introduction

Several kinds of proteolytic enzymes of mammalian proteases have been identified including aspartic, cysteine, metallo, serine and threonine (Rawlings et al., 2014). Cathepsins are cysteine proteases belonging to the papain subfamily. They are predominantly endopeptidases located intracellularly in endolysosomal vesicles. Various types of cathepsins have been discovered including cathepsin B, D, H, L, S and P (Barrett et al., 1981; Maubach et al., 1997). Cathepsins B, L, and H are found in most cell types and body tissues where they regulate diverse normal biological processes such as cell death, proliferation, migration, invasion and protein turnover (Barrett et al., 1981; Reddy et al., 1995; Maubach et al., 1997; Deussing et al., 2002; Cowan et al., 2005). Cathepsin L in secretory vesicle has been demonstrated for production of active peptides required for cell to cell communication in the nervous and endocrine systems (Funkelstein et al., 2010).

The expression of some cathepsins is high and regulated in specific cell types. Cathepsin B and L are expressed constitutively and thought to participate in protein turnover and diseases. Studies in mice deficient in cathepsin B or L have indicated a role for the cathepsins in normal brain development. Mice deficient in both cathepsin L and B show brain atrophy due to massive apoptosis of cerebral and cerebellar neurons (Felbor et al., 2002). However, prolonged activation of cathepsin B is associated with neuronal degeneration in Alzheimer's disease (Callahan et al., 1998; Nixon, 2000) while inhibition results in reduction of brain β-amyloid peptides and significant improvement in memory in a mouse model of Alzheimer's disease (Hook et al., 2009). Similarly, gene-expression profiling by cDNA microarrays also show that CTLA-2α is highly expressed in mice brain tissues susceptible to cerebral malaria (Delahaye et al., 2006). Regulation of cathepsin activity appears to have a significant role in health and disease.

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Structural information indicates that cathepsins consist of a signal peptide, a propeptide, and a catalytic domain which is a mature proteolytically active enzyme (Mach et al., 1994; Ménard et al., 1998; Turk et al., 2012). The propeptides of some proteases are reported to be potent inhibitors of the proteases from which they were derived. The structure of Cytotoxic T-lymphocyte antigen-2 alpha (CTLA-2α) is homologous to the proregion of cathepsin L (Denizot et al., 1989; Yamamoto et al., 2002) and that CTLA-2α is a potent inhibitor of cathepsin L – like cystine proteases (Delaria et al., 1994; Carmona et al., 1996; Deshapriya et al., 2010; Kurata et al., 2003). Other propeptide-like cysteine proteinase inhibitor proteins homologous to CTLA-2α have been identified in other organisms including the Bombyx cysteine protease inhibitor (BCPI) identified in Bombyx mori (Yamamoto et al., 1999a, 1999b; Kurata et al., 2001) and the crammer peptide (CG10460 gene product) found in *Drosophila melanogaster* (Comas et al., 2004).

The Drosophila crammer gene (CG10460) which is homologous to mouse CTLA-2α gene, was found to be expressed in Drosophila glial cells and mushroom bodies, the Drosophila olfactory memory centre, that form a prominent bilateral structure of the insect brain. The concentration of expressed crammer is shown to be critical for the establishment of long-term memory, suggesting a role of this inhibitor in memory formation through regulation of cathepsin activity (Comas et al., 2004). In the hippocampus concurrent inhibition of multiple cysteine proteases induces a decrease in long-term formation but not short-term spatial memory in mouse (Dash et al., 2000).

In this context, information on the colocalization of cathepsin L (a family of cysteine proteinases) with CTLA-2α in the central nervous system is pertinent to several aspects of learning, memory establishment and diseases. This study was therefore aimed at examining simultaneous localization of cathepsin L and CTLA-2α in the mouse brain by double labeling immunofluorescence microscopy.

2. Materials and methods

2.1. Animals and tissue preparation

A total of 10 mice were kept in a room at 19–21 °C temperature, with free access to food and water. All experimental procedures were performed according to the guide for protection and control of animal experimentation in Japan. Permission to use animals in experiments was approved by the Animal Protection and Control committee of Yamaguchi University. Ten adult male and female mice five in each, aged 12 months were studied, sagittal and coronal cutting planes were prepared. The mice were anesthetized with sodium pentobarbital (70 mg/kg) by intraperitoneal injection and transcardiac perfusion with 0.01 M phosphate buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO) in 0.1 phosphate buffer (PB; pH 7.4). Brain tissues were dissected and postfixed in 4% PFA for 2 h at 4 °C. The tissues were then processed through graded ethanol series to paraffin wax and sectioned at a thickness of 4 μm using a microtome, then used for immunofluorescence analysis.

2.2. Generation of antibodies

Recombinant cathepsin L and CTLA-2α were purified using methods described previously with minor modifications. Affinity-purified rabbit anti-cathepsin L IgG and chicken anti-CTLA-2α IgY were generated. In brief, antiserum against cathepsin L was obtained by immunizing rabbit against recombinant cathepsin L. Antiserum against CTLA-2α was obtained by immunizing chicken against recombinant CTLA-2α (Camenisch et al., 1999). Immunization and preparation of antiserum were performed as previously described (Takahashi et al., 1993). The polyclonal anti-cathepsin L antibody against rabbit cathepsin L protein and anti-CTLA-2α antibody against chicken CTLA-2α protein were obtained through affinity chromatography column with recombinant cathepsin L and CTLA-2α conjugated resins respectively. The specificity of the purified antibodies was characterized as previously described (Bui et al., 2015)

2.3. Double immunofluolencence microscopy

Sections were deparaffinized and hydrated in a consecutive series of xylene and ethanol to phosphate-buffered saline (0.01 M PBS-pH 7.4). Endogenous peroxidase activity was blocked by immersing the tissue sections in a solution of 0.3% v/v hydrogen peroxide in distilled water for 30 min at room temperature (RT) and then washed (3 × 5 min) in PBS. Afterwards, the sections were blocked with 10% goat normal serum for 30 min at RT to avoid nonspecific labeling. The sections were incubated with a mixture of primary antibodies containing both cathepsin L and CTLA-2α (1:500) IgG and IgY in PBS, pH 7.4 for 24 h in a dark, humid chamber at 4 °C. For negative control, 10% goat normal serum was applied to some sections in place of primary antibody. Sections were then washed (3 × 5 min) in PBS followed by incubation with a mixture of Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (FITC) and Alexa Fluor® 594-conjugated goat anti-chicken IgY (TRITC) at a dilution of 1:100 (Molecular Probes, Inc. Eugene, USA) for 1 h at RT. At the end of incubation, the sections were washed (3 × 5 min) in PBS and mounted. Immunoreactivity was examined using the BZ-9000E HS all-in-one Fluorescence Microscope (KEYENCE, Japan). Morphological structures refer to the neuron-anatomical atlas from Paxinos and Franklin (2001) (Table 1).

3. Results

3.1. Colocalization pattern of cathepsin L with CTLA-2α protein in various structures of the mouse brain

Immunofluorescence evaluation of cathespin L colocalization with CTLA-2α was performed on sagittal and coronal sections of the mouse brain. Cathepsin L with CTLA-2α displayed a region-specific colocalization, being strongly present in some brain structures but not detectable in others. Strong labeling was observed in the external capsule (ec); corpus callosum (cc); fimbria of hippocampus (fi); interneurons in Cornu Ammonis 2, 3 fields of hippocampus; stria medullaris (sm); fibres of mammillothalamic tract (mt) and anterior commissure (ac). Moderate labeling was detected in neocortex; intermediate part of lateral septal nucleus (LSI) and in majority of thalamic nuclei including anterodorsal thalamic nucleus (AD); central part of mediodorsal thalamic nucleus (MDC) and medial preoptic area (MPA) in sagittal section (Fig. 1).

3.2. Detailed analysis of cathepsin L colocalization with CTLA-2α protein in coronal sections from various regions of the mouse brain

3.2.1. Cerebral cortex and hippocampus

Consistent double labeling for cathepsin L and CTLA-2α proteins was observed in the primary motor cortex, secondary motor cortex and somatosensory cortex. High density of colocalization signals was detected in the corpus callosum (cc) which is a structure that connect the two hemispheres of the brain; the Cornu Ammonis 2, 3 fields of hippocampus; alveus of the hippocampus (Alv); in neuron cell body of stratum pyramidale (Py) and fimbria of hippocampus(fi) (Fig. 2a,b).

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