Cathepsin B-dependent motor neuron death after nerve injury in the adult mouse

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There are significant differences in the rate of neuronal death after peripheral nerve injury between species. The rate of neuronal death of motor neurons after nerve injury in the adult rats is very low, whereas that in adult mice is relatively high. However, the understanding of the mechanism underlying axotomy-induced motor neuron death in adult mice is limited. Cathepsin B (CB), a typical cysteine lysosomal protease, has been implicated in three major morphologically distinct pathways of cell death; apoptosis, necrosis and autophagic cell death. The possible involvement of CB in the neuronal death of hypogrossal nucleus (HGN) neurons after nerve injury in adult mice was thus examined. Quantitative analyses showed the mean survival ratio of HGN neurons in CB-deficient (CB−/−) adult mice after nerve injury was significantly greater than that in the wild-type mice. At the same time, proliferation of microglia in the injured side of the HGN of CB−/− adult mice was markedly reduced compared with that in the wild-type mice. On the injured side of the HGN in the wild-type adult mice, both pro- and mature forms of CB markedly increased in accordance with the increase in the membrane-bound form of LC3 (LC3-II), a marker protein of autophagy. Furthermore, the increase in CB preceded an increase in the expression of Noxa, a major executor for axotomy-induced motor neuron death in the adult mouse. Conversely, expression of neither Noxa or LC3-II was observed in the HGN of adult CB−/− mice after nerve injury. These observations strongly suggest that CB plays a critical role in autophagy-induced motor neuron death in adult mice.

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1. Introduction

There are significant differences in the rate of neuronal death after a peripheral nerve injury between species. The rate of neuronal death of motor neurons after nerve injury in the adult rats is very low, whereas that in adult mice is relatively high [8,12,19]. Despite intensive research efforts, the understanding of the mechanism underlying motor neuron death after nerve injury in adult mice is still limited. Cathepsin B (CB, EC 3.4.22.1), a typical cysteine lysosomal protease, has been implicated in the three major morphologically distinct pathways of cell death; apoptosis, necrosis and autophagic cell death [9,10,14,17]. In the normal central nervous system (CNS), a granular immunoreactivity for CB is most prominent in the neuronal cell bodies and proximal dendrites, whereas the CB immunoreactivity in glial cells is below detection levels at the light microscopic level [11]. In the aging and pathological CNS, increased CB immunoreactivity is observed in neurons as coarse aggregates. Furthermore, extra-neuronal granular CB immunoreactivity was found mainly in activated microglia and partially in reactive astrocytes [6,11]. In the present study, we have attempted to elucidate the possible involvement of CB in the cell death of hypogrossal nucleus (HGN) neurons after nerve injury in adult mice. We found that slowly progressive neuronal death and microglial proliferation were markedly reduced. Both pro- and mature forms of CB markedly increased in the injured HGN neurons of the adult wild-type mice in advance with the expression of Noxa, a major executor for axotomy-induced motor neuron death in the adult mouse [8] and autophagy also increased after nerve injury in HGN of adult CB-deficient (CB−/−) mice. These observations strongly suggest that CB plays a critical role in autophagy-induced motor neuron death in adult mice.

2. Materials and methods

2.1. Animals and surgeries

This study was approved by the Animal Research Committee of Kyushu University. Every effort was made to minimize the number of animals used and their suffering. The adult male CB−/− and
wild-type C57BL/6 mice (7-week old) were used for the various experiments. Animals were anesthetized with sodium pentobarbital (40 mg/kg, ip) and the right hypoglossal nerves were transected with scissors. The skin incision was repaired, and then the animals were returned to their cages after awakening from the anesthesia.

2.2. Histological and immunohistochemical analyses

Animals that received sham surgery, and representative animals that received the real surgery, were randomly selected for evaluation on the day of surgery, or on days 3, 7, 14 and 28 after nerve injury (number of animals = 3/group). The animals, which were designated D0, D3, D7, D14 and D28, respectively, were anesthetized with sodium pentobarbital (40 mg/kg, ip) and then killed by intracardiac perfusion with isotonic saline. The soluble fractions obtained from the HGN homogenates by differential centrifugation were electrophoresed in 15% or 18% SDS-polyacrylamide gels. The proteins on SDS gels were transferred electrophoretically to nitrocellulose membranes and then incubated with 10% normal goat or donkey serum for 4 h at room temperature. The sections were stained with the following combinations of antibodies for 3 days at 4 °C: rabbit or goat polyclonal anti-CB IgG (1:200, Upstate, Lake Placid, NY, USA) and mouse monoclonal anti-NeuN IgG (1:500, Chemicon International, Temecula, CA, USA), goat polyclonal anti-CB IgG (1:100, Santa Cruz Biotech, Santa Cruz, CA, USA) and rabbit polyclonal anti-Iba1 IgG (1:500, Wako Chemical Industries Ltd., Osaka, Japan). After washing with PBS, the sections were incubated with a mixture of 0.5% Alexa488 anti-rabbit or mouse IgG (Molecular Probes) and Cy3 anti-rabbit or goat IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) for 4 h at room temperature. After washing with PBS, the sections were mounted in the anti-fading medium Vectashield (Vector Laboratories, Burlingame, CA, USA) and then were examined with a confocal laser-scanning microscope (CLSM) (LSM510MET, Carl Zeiss, Jena, Germany).

2.3. Immunoblot analyses

The HGN was prepared from animals in each group (D0, D3, D7 and D14, n = 3/group) that were first anesthetized with sodium pentobarbital (40 mg/kg, ip) and then killed by intracardiac perfusion with isotonic saline. The soluble fractions obtained from the HGN homogenates by differential centrifugation were electrophoresed in 15% or 18% SDS–polyacrylamide gels. The proteins on SDS gels were transferred electrophoretically to nitrocellulose membranes and then they were incubated for 18 h at 4 °C under gentle agitation with anti-CB IgG (1:1000, Upstate), anti-LC3 IgG (1:1000, Medical and Biological Lab., Nagoya, Japan), anti-Noxa IgG (1:1000, Abcam, Tokyo, Japan) or anti-actin IgG (1:5000, Santa Cruz Biotech). After washing, the membranes were incubated with 0.5% horseradish peroxidase (HRP)-labeled donkey anti-rabbit IgG (1:1000, Amersham Phramacia Biotech, Buckinghamshire, UK) for 2 h at room temperature. Subsequently, the membrane bound HRP-labeled antibodies were detected by the enhanced chemiluminescence detection system (ECL kit, Amersham) with an image analyzer LAS-1000 (Fuji Photo Film, Tokyo, Japan), and the protein bands were analyzed densitometrically.

Fig. 1. Differential survival ratio and microglial responses upon nerve injury in the HGN of the wild-type (+/+) and CB−/− mice. (A) Nissl staining of HGN neurons in the wild-type and CB−/− mice on D14. Dotted lines indicate the boundary of the HGN. Co, control side; Ax, axotomized side. Scale bar = 100 μm. (B) The survival ratio curves of HGN neurons in the wild-type (+/+) and CB−/− mice following nerve injury. The percentage of surviving HGN neurons on the axotomized side compared with that on the control side was calculated. Each point and vertical bar represents the mean and SEM of three experiments. The asterisks indicate a significant difference in comparison to the results of the wild-type mice (p < 0.01, Student’s t-test). (C) Immunofluorescent CLSM images for Iba1 (red) and NeuN (green) in HGN on D3, D7, and D14 after nerve injury in the wild-type and CB−/− mice scale bar = 50 μm.
3. Results

3.1. The survival ratio of HGN neurons in wild-type and CB−/− mice after nerve injury

The survival ratio of injured HGN neurons in the CB−/− and the wild-type mice was examined after nerve injury. As shown in Fig. 1A and B, the mean survival ratio of the injured HGN neurons in CB−/− mice was significantly higher than that in the wild-type mice. In the wild-type mice, approximately 30% and 80% of the HGN neurons had died on D7 and D14, respectively. The immunoreactivity for NeuN markedly decreased, whereas that for Iba1-positive cells markedly increased in the injured side of the HGN on D7 and D14 after nerve injury (Fig. 1C). Thick processes of microglia tightly surrounded HGN neurons that showed only faint immunoreactivity for NeuN.

In contrast, only approximately 40% of the HGN neurons had died by D28 after nerve injury in the CB−/− mice (Fig. 1A and B). The immunoreactivity for NeuN was moderately decreased, and the number of Iba1-positive cells was moderately increased in the injured side of HGN on D7 and D14 after nerve injury in these mice (Fig. 1C). Thin processes of microglia frequently attached to the surface of HGN neurons that showed a relatively strong immunoreactivity for NeuN.

3.2. Changes in the cellular localization and amount of CB in the HGN after nerve injury

To further address the role of CB in axotomy-induced motor neuron death, changes in the amount and localization of CB in the HGN after nerve injury were analyzed. CB was observed mainly as a propeptide with an apparent molecular mass of 41 kDa, which corresponded well with that of pro-CB. Nerve injury induced increased synthesis and processing of pro-CB in the HGN of the wild-type mice. The processed single chain form of CB with a molecular weight of 29 kDa markedly increased on D3 and D7 and tended to decline on D14 (Fig. 2A). The proteolytic processing of the single-chain enzyme as well as pro-CB takes place in the lysosomes. The membrane-bound form of LC3 (LC3-II) became visible on D3 and peaked on D7 in the injured side of the HGN, indicating the induction of autophagy in injured HGN neurons. Furthermore, Noxa, a major executor for axotomy-induced motor neuron death in adult mice, became visible in the injured side of the HGN on D3 and peaked on D7. After D7, Noxa moderately increased in expression and localization of CB in the HGN after nerve injury. (A) Immunoblots from SDS–polyamide gel electrophoresis of the HGN extracts from the sham and axotomized wild-type (+/+ ) and CB−/− mice developed with antibodies specific for CB, LC3 and Noxa. (B) Immunofluorescent CLSM images of CB (red) and NeuN (green) in the HGN on D3. Scale bars = 100, 25 μm. (C) Immunofluorescent CLSM images of CB (red), Iba1 (green) and their merge in the HGN on D3. Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
increased even in the contralateral side. In CB−/− mice, however, only a faint band corresponding to either LC3-II or Noxa was observed in the injured side of the HGN even on D7. On D3 after nerve injury, the granular immunoreactivity for CB was found to mainly have increased in the HGN neurons and partially in activated microglia. High magnification images show the granular immunoreactive products for CB markedly to mainly increase in the cytoplasm of the HGN neurons on D3 after nerve injury, thus indicating their lysosomal localization (Fig. 2B). On the other hand, activated microglia with shortened processes and a large cell body showed a relatively strong immunoreactivity for CB (Fig. 2C).

4. Discussion

The present study indicates that CB plays an essential role in the death of HGN neurons following nerve injury in adult mice. We have previously reported the mean survival ratio of facial motor neurons in cathepsin S-deficient mice with a DBA background to be significantly smaller than that in wild-type mice at 30 and 60 days after nerve injury [6]. The mean expression level of CB was observed to have significantly increased in the facial motor nucleus of the wild-type mice, but not in the cathepsin S-deficient mice at 7 days after nerve injury. These observations also support the present finding that CB is one of major causative factors for axotomy-induced motor neuron death. Further studies will be necessary to elucidate the changes in CB in adult rats, in which the rate of motor neuron death after nerve injury is very low. There are at least four different possible roles that CB may have in axotomy-induced motor neuron death: (1) leakage of CB may trigger a neuronal death pathway, (2) CB may activate molecules that can induce neuronal death without leakage of CB itself, (3) CB secreted by activated microglia may induce neuronal death after nerve injury, and (4) increased CB may induce enhanced autophagy, which can lead to neuronal death.

Recently, much attention has been paid to the lethal effects of CB in the cytosol. The cytosolic release of CB was first reported to exert an executioner effect for the TNF-α-mediated apoptosis of hepatocytes [5]. The release of CB from lysosomes triggers death signaling pathways including the mitochondrial release of cytochrome c and subsequent caspase activation [5]. The present observations clearly showed that increased expression and maturation of pro-CB preceded the neuronal death and the marked increase in Noxa, a major executor for axotomy-induced motor neuron death in the adult mouse [8]. Noxa is known to be a crucial executor in neuronal death dependent on p53, which can induce lysosomal destabilization [7]. In the present study, Noxa was also found to moderately increase on the contralateral side of HGN following nerve injury. We previously reported that both the frequency and amplitude of the spontaneous excitatory postsynaptic currents recorded from the dorsal vagal motor neurons in the control side significantly increased after nerve injury in C57BL/6 mice, probably due to a compensation mechanism for marked neuronal death which occurred on the injured side [19]. Therefore, a compensatory hyperexcitability of the contralateral HGN neurons following nerve injury may be responsible for the moderate expression of Noxa observed on the contralateral side.

However, the first mechanism (induction of cell death by leakage of CB) is unlikely since we did not observe any evidence of the cytosolic localization of CB. Furthermore, no active caspase-3-immunoreactive cells were detected in the adult axotomized facial nucleus [1,18]. The second mechanism (activation of cell death-related molecules by CB) is also unlikely since a deficiency in caspase-1, the pro-form of which is proteolytically processed by CB [16], increases the motor neuron death induced by facial nerve lesions [1].

Gan et al. [4] conducted genomic studies which identifies CB as a gene that is transcriptionally induced by amyloid-β in microglia. They have also further shown that inhibition of CB expression in microglia using either siRNA or a specific inhibitor of CB, CA074, leads to a decrease in the neurotoxic effects of amyloid-β-activated microglia. We have also recently reported that CB was intensely expressed in activated microglia in advance of severe pyknotic changes of the cerebellar granule cells following chronic treatment with methylmercury [13]. The coadministration of CA074 significantly inhibited the severity of pyknotic changes of the cerebellar granule cells. In the present study, a relatively strong immunoreactivity for CB was found in activated microglia, which had shortened processes with a large cell body, on D3 after nerve injury. On the other hand, perineuronal microglia showed only a faint immunoreactivity for CB. This is consistent with our previous observation that CB only increased in the phagocytic microglia, but not in the perineuronal microglia after nerve injury [6]. These observations strongly suggest that an increased level of CB is likely associated with the enhanced lysosomal mechanism of activated microglia. Furthermore, CB is released from primary cultured microglia mainly as its pro-form (41 kDa) and only partially as mature forms (29 and 26 kDa) after treatment with chromogranin A, which is released from the injured neurons [16]. Therefore, in order to verify the third mechanism (CB secreted by activated microglia), it necessary to examine whether the amount of mature CB secreted from activated microglia is enough to kill neurons.

As for the fourth mechanism (induction of excessive autophagy by CB), we observed the formation of the membrane-bound form of LC3 (LC3-II) on the injured side of the HGN, indicating the induction of autophagy, which was consistent with the previous study [3]. Although autophagy is generally considered to be a cytoprotective response, excessive autophagy has been proposed to induce neuronal death under certain circumstances [2]. In HGN neurons after nerve injury, an increase in LC3-II was first detected on D3. This observation may support a causative role of excessive autophagy in axotomy-induced mortor neuron death. Although the precise reason for the marked inhibition of the LC3-II expression in the HGN of CB−/− mice following nerve injury is still unknown, cathepsin L is considered to be involved, specifically in the autolysosomal degradation of LC3-II [15]. We previously reported the expression of cathepsin L in primary cultured microglia prepared from CB−/− mice to increase by approximately 6-fold in comparison to the wild-type mice after cellular activation [16]. Therefore, it is likely that cathepsin L is increasingly expressed in the injured HGN neurons of CB−/− mice after nerve injury, thus resulting in a marked degradation of the induced LC3-II following nerve injury.

Taken together, the involvement of increased CB in excessive autophagy leading to neuronal death after nerve transection is the most likely explanation for the present observations. However, the present study does not allow us to completely exclude any of the other mechanisms. Further studies will thus be needed to clarify the precise role of CB in axotomy-induced mortor neuron death in adult mice.

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