Involvement of two different cell death pathways in retinal atrophy of cathepsin D-deficient mice

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Abstract

To understand the mechanisms of retinal atrophy in cathepsin D-deficient mice, the postnatal development of their retinas was analyzed. TUNEL-positive cells appeared abundantly in the outer nuclear layer (ONL) and slightly in the inner nuclear layer (INL). Nitric oxide synthase (NOS) was induced in microglial cells which invaded retinal layers and phagocytosed dead cell debris, while NOS inhibitors prevented cell death in the INL but not in the ONL. Caspases 9 and 3 were activated only in the ONL after P15. Moreover, no atrophic change was detected in the retina of mice deficient in cathepsin B or L. These results suggest that cathepsin D is essential for the metabolic maintenance of retinal photoreceptor cells and that its deficiency induces apoptosis of the cells, while the loss of INL neurons is mediated by NO from microglial cells.

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Introduction

Neuronal ceroid lipofuscinoses (NCLs) are a group of autosomal recessive diseases and are clinically characterized by seizures, dementia, and blindness, eventually resulting in death (Rider and Rider, 1988; Rider et al., 1992; Carpenter, 1988). Classical forms of NCLs are subdivided into four major types and their variant forms, the pathological features of which are lysosomal storage diseases (Boustany et al., 1988). Biochemical investigations have revealed that subunit c of mitochondrial ATP synthase accumulates in the lysosomes of neurons in the central nervous system (CNS), except for infantile type of NCLs in which sphingolipid activator proteins (saposins) accumulate (Palmer et al., 1989; Fearley et al., 1990; Hall et al., 1991; Kominami et al., 1992). Recent genetic approaches suggest that eight NCL forms result from 100 different mutations on genes CLN1 to CLN8 causing different phenotypes (Wisniewski, 2001). We previously demonstrated that CNS neurons in cathepsin D-deficient (CD−/−) mice show a new form of lysosomal accumulation disease with a phenotype resembling that of NCLs (Koike et al., 2000). A form of NCLs caused by the CD deficiency has not clinically been identified, but Swedish sheep lacking CD activity have been shown to have a severe loss of neurons in the cortical area and hippocampus and an accumulation of protein like-storage materials resembling NCLs (Tyynelä et al., 2000).
Two major abnormalities in pathology of NCLs which explain the clinical symptoms are the massive loss of cortical neurons and photoreceptor cells in the retina, which has been suggested to be apoptosis (Lane et al., 1996). Our previous studies using CD−/− mice have shown that retinal atrophy can be attributed mainly to the loss of photoreceptor cells in the outer nuclear layer (ONL) (Koike et al., 2000), while neuronal death in CNS tissues is due to nitric oxide (NO) production via inducible NO synthase (iNOS) in activated microglial cells and is prevented by NOS inhibitors (Nakanishi et al., 2001). In the course of the study using CD−/− mice, we noted that treatment with L-N^G^-nitroarginine methylester (L-NAME), a competitive NOS inhibitor or S-methylisothiourea hemisulfate (SMT), an iNOS inhibitor, which rescues neuronal death in CNS, had no effect on photoreceptor cell death but, interestingly, prevented the loss of neurons in the inner nuclear layer (INL), indicating that neuronal death in the INL and ONL is mediated by the different cell death pathways.

The present study therefore examined the precise courses of neuronal death in retinal tissue of CD−/− mice and found that neuronal death occurred drastically in the ONL and, to a lesser extent, in the INL after the retinal layers had morphologically been formed. Photoreceptor cell death was characterized by positive staining of TUNEL, internucleosomal cleavage of genomic DNA, the activation of caspases 9 and 3, and phagocytosis by activated microglial and Müller cells, indicating characteristics of apoptosis. Moreover, no atrophic change was detected in the retina of mice deficient in cathepsin B or L. The results suggest that CD is essential for the metabolic maintenance of photoreceptor cells and that its deficiency induces apoptosis of the neurons in mouse retina.

Results

Retinal atrophy in CD−/− mice is associated with apoptosis in the outer nuclear layer

Since subunit c of mitochondrial ATP synthase accumulates in the retinal neurons of CD−/− mice and retinal atrophy becomes distinct near the terminal stage of the mice (P25) (Koike et al., 2000), precise morphological alterations in the CD−/− retina were analyzed. In semithin sections, thickness of CD−/− retinae at P25 was strongly reduced, compared to that of control littermate retinae (Figs. 1A and 1B). When examined at the level of the optic nerve horizon, the retinal thickness of the mice at P25 was nearly the half that of the control littermates (Fig. 1C). Electron microscope observations revealed that the reduction in thickness of CD−/− mouse retina at P25 was due largely to the loss of cells from the ONL and, to a lesser extent, to that from the INL (Figs. 1D and 1E); the photoreceptor layer was largely abolished and the number of cells per one vertical row was one to three in the ONL. Similar to neuronal cells in CNS tissues, those in ONL, INL, and the ganglion cell layer (GCL) contained numerous autophagosome-like bodies and dense granular osmiophilic deposits, while fingerprint profiles were, in some cases, observed in retinal pigment epithelial cells (Figs. 1E and 1F). Microglial cells, the cytoplasm of which were occupied with vacuolar structures containing cellular debris, were frequently found in the ONL, INL, and GCL. As opposed to CNS tissues of CD−/− mice, typical apoptotic cells which had nuclei with densely condensed chromatin frequently appeared in the ONL (Fig. 1D). Nuclei of dying cells in the INL, however, differed from those in the ONL and contained dispersed dense chromatin masses (Fig. 1E). These results suggest that, although CD is present in neuronal cells of the ONL, INL, and GCL, neurons with apoptotic characteristics appear mostly in the ONL of the CD−/− mouse retina, leading to retinal atrophy.

CB or CL deficiency does not morphologically affect postnatal development of mouse retina

It is well known that CB and CD are localized in pigment epithelial cells and neuronal cells of ONL, INL, and GCL (Bernstein et al., 1989; Yamada et al., 1990; Rakoczy et al., 1999). In addition to these proteinases, immunoreactivity for CL was also detected in neuronal cells and pigment epithelial cells in each layer of control littermate mouse retinae (Figs. 2A-2C). To assess the roles of CB and CL in the retina, tissues obtained from mice deficient in CB and CL, respectively, were morphologically analyzed and no clear-cut alterations in retinal morphology could be observed (Figs. 2D and 2E), indicating that these lysosomal cysteine proteinases are dispensable and compensated by other proteinases in lysosomes of retinal neurons.

Postnatal changes in retinal morphology

To determine postnatal progress of retinal atrophy in CD−/− mice, retinal tissues obtained from CD−/− and control littermate mice were examined at the light microscope level. Hematoxylin—eosin-stained retinal sections which included the optic nerve disc and ora serrata indicated no clear-cut structural difference between CD−/− and control littermate mice up to P12 when each layer of the retina, including the photoreceptor layer, was formed (Fig. 3A). The retinal thickness of ONL decreased drastically between P12 and P18 and this decrease continued in the ONL up to P25 and also became distinct in the INL after P20. To confirm the decrease in retinal thickness, the number of nuclei per one vertical row in the ONL and INL was counted under light microscopy (Fig. 3B). When the number of nuclei per one vertical row in layers of the control littermate retina was estimated as 100%, the rates of the decrease in retinal thickness were found to be 60% at P18 and 85% at P25 in ONL, while they were 11% and 33% in INL, respectively. These results indicate that retinal atrophy...
Fig. 1. Morphological demonstrations of retinal atrophy in CD−/− mice at P25. (A, B) Light microscopy of control littermate (CD+/−) (A) and CD−/− mouse retinae (B). Retinal thickness is reduced drastically in CD−/− mice, compared to CD+/− mice. Toluidin blue staining. (C) Vertical thickness across the retinae of CD+/− and CD−/− mice. The measurements were performed at 10 fields within a 2-mm-distance superior and inferior from the optic nerve head (ONH) in both right and left sides. (D-F) Electron micrographs of retinae from CD−/− mice. The photoreceptor layer (PR) largely disappears and cells having dark round or irregularly-shaped nuclei with condensed chromatin (arrowheads) are discernible in the outer nuclear layer (ONL) (D, E). In addition to pigment granules, numerous large and small vacuoles are present in pigment epithelial cells (D) and a fingerprint profile can be seen in the cytoplasm of the cells (D, inset). Large microglial cells (M), which are occupied with vacuolar structures in the cytoplasm, are detected in the ONL and inner nuclear layer (INL) (D, E). Numerous autophagosomes and granular osmiophilic deposits (GO) are seen in the cytoplasm of neurons in the INL and ONL (D-F). In the INL, a neuron which has a nucleus with small dispersed chromatin masses is detectable near the outer plexiform layer (OPL) (arrow) (E). IPL, inner plexiform layer; PE, pigment epithelial layer. Scale bars are 25 μm in (A) and (B), 5 μm in (D) and (E), 0.5 μm in inset of (D), and 2 μm in (F).
appeared rather intact, compared to non-treated CD−/− and control retinae (Fig. 4A). By TUNEL staining, positive nuclei were found to be abundant in the ONL from both the treated and the nontreated retinae, whereas they were rarely detected in the INL from the treated retinae or, even though detected, much less in number than in the INL from the nontreated retinae (Fig. 4B). The number of TUNEL-positive nuclei was 3.5 ± 1.38 (mean ± standard deviation) in the INL and 151.83 ± 24.19 in the ONL from L-NAME-treated CD−/− mice (n = 3), while it was 17.5 ± 3.27 in the INL and 161.6 ± 23.48 in the ONL from nontreated CD−/− mice (n = 3). The difference in the number was highly significant (P < 0.001) in the INL between the two groups, but no significant difference was detected in the ONL. No difference was detected in retinal morphology between treated and nontreated control littermate mice (data not shown). When the number of nuclei per one vertical row in the INL was counted, no significant difference was detected between L-NAME or SMT-treated CD−/− mice and nontreated control mice, although the number was significantly different between nontreated CD−/− mice and nontreated control or treated CD−/− mice (Fig. 4C, data for SMT were not shown). Moreover, the number of nuclei per one vertical row in the ONL from treated CD−/− mice was similar to that from nontreated CD−/− mice (Fig. 4C).

Since the loss of neurons in the INL of CD−/− retinae appeared to be significantly prevented by L-NAME or SMT, it becomes crucial to understand the behavior of microglial cells in retinal tissue. Immunohistochemical staining with F4/80, which specifically recognizes microglial cells, revealed that immunopositive cells appeared to be more intense and larger in number in the case of CD−/− retinae examined than in control retinae where only a few positive cells were detected (Fig. 5A). In serial sections of CD−/− retinae at P20, immunoreactivity for iNOS appeared in F4/80 immunopositive cells (Figs. 5B and 5C). Western blot analysis showed that a protein band immunoreactive for iNOS appeared in CD−/− retinal tissue at P20, immunoreactivity for iNOS appeared in F4/80 immunopositive cells (Figs. 5B and 5C). Western blot analysis showed that a protein band immunoreactive for iNOS appeared in CD−/− retinal tissue at P20, but not in control retinal tissue where a band for CD was detected (Fig. 5D). These results suggest that death modes of neurons were different between the ONL and the INL in CD−/− retinae, while neuronal death in the INL can be attributed to NO released from activated microglial cells, similar to that in the CNS tissues of the mice (Nakanishi et al., 2001).

Progressive photoreceptor degeneration due to typical apoptosis

As demonstrated above, neuronal death in the ONL of CD−/− mouse retinae was accompanied by apoptotic figures and was not prevented by NOS inhibitors. Therefore, the death mode of neurons was examined in CD−/− mouse retinae by TUNEL staining and genomic DNA electrophoresis.

As shown in Fig. 6A, positive TUNEL staining was

started postnataally and proceeded at a rapid rate after the retinal layers had been morphologically formed.

NOS inhibitors do not affect cell death in ONL of CD−/− retinae but prevent that in INL.

We have previously shown that the loss of neurons is due to NO released from activated microglial cells which appear in the thalamic region of CD−/− mouse brains and can be prevented by NOS inhibitors (Nakanishi et al., 2001). We therefore examined the issue of whether L-NAME, a competitive NOS inhibitor, or SMT, an iNOS inhibitor, inhibits neuronal loss in the CD−/− retina. The retinal thickness of CD−/− mice treated with and without L-NAME or SMT was largely reduced only in the case of the ONL (Fig. 4A). The thickness of the INL from treated retinae, however,
observed in the INL of both CD−/− and control littermate mouse retinae from P5 to P14, and was most abundant at P8, indicating that physiological neuronal death occurs postnatally in the INL (Young, 1984). Different from physiological neuron death in the INL, TUNEL-positive nuclei appeared in the innermost layer of the ONL from CD−/− mouse retinae at P8 and became distinct at P12 (Fig. 6A). TUNEL-positive nuclei were consistently localized in the inner part of the ONL facing the outer plexiform layer after P12, and were even detected in the ONL at P25 when the number of nuclei in one vertical row became one to three. In the INL, several TUNEL-positive nuclei, dispersed in the layer, were observed, while positively stained nuclear masses which were longitudinally arrayed were present (Figs. 6A and 6B). Longitudinally running processes which were continuous to the row of positive nuclei often extended into the inner plexiform layer, suggesting that these nuclear masses seemed to be phagocytosed by Müller cells (Fig. 6B). When the numbers of TUNEL-positive nuclei in each retinal layer of CD−/− mice were counted, they were found to increase steeply from P12 to P18 in the case of ONL, reaching a peak at P18 (Fig. 6C). Since the number of nuclei per one vertical row almost linearly decreased from P12 to P18 in the ONL (Fig. 3B) and that of TUNEL-positive nuclei inversely linearly increased during this period (Fig. 6C), the number of dead neurons per 2 days during this period may occur evenly, indicating that the number of TUNEL-positive nuclei during this period included those not only from newly dead neurons but also from dead ones in the former phases which were not phagocytosed and, even if phagocytosed, not digested by phagocytic cells. On the other hand, in the INL of the mice, a small peak was observed around P7, which did not increase in value thereafter (Fig. 6C). Similar to the case of the INL of CD−/− mice, a small peak was detected around P7 in the INL of control littermate mice, but the positive nuclei could not be counted after P14. To further confirm DNA fragmentation into oligonucleosomes, genomic DNA from CD−/− and control retinae was examined by electrophoresis. Accompanying physiological neuron death which occurred in the INL, a DNA ladder was detected in both CD−/− and control retina at P7 and P9, whereas it was distinctly formed only in CD−/− retina after P15 (Fig. 6D).

Since the decrease in retinal thickness can largely be attributed to apoptosis of ONL cells, the caspases responsible for the execution of apoptosis in these cells were then examined by immunohistochemistry and enzyme assay. By
immunohistochemistry using two types of antibodies (anti-m9D368 for caspase 9 and anti-p20/p17 for caspase 3) which recognize only the active forms of caspases 9 and 3, positive signals for these caspases were observed in INL neurons in control littermate retinas and of INL and ONL neurons in CD−/− retinae at P10 (Fig. 7A). Positive staining for these caspases was detected in ONL neurons of CD−/− retinae at P18 and P24, whereas no positive staining for caspase 9 and caspase 3 was observed in INL neurons after P14 (Fig. 7A). Staining for these caspases distinctly showed a diffuse pattern in INL and ONL neurons (Fig. 7, insets). To confirm the immunohistochemical results showing the participation of caspases 9 and 3 in the apoptosis of photoreceptor cells, the activities of these enzymes were measured using AC-LEHD-MCA and AC-DEVD-MCA as substrates for caspases 9 and 3, respectively (Fig. 7B). The proteolytic activities of both enzymes were significantly more increased in CD−/− retinae than in control retinae at P15. These results indicate that retinal atrophy in CD−/− mice can be attributed mainly to apoptosis of photoreceptor cells which is executed by the activation of caspases 9 and 3.

Phagocytosis of dead cells by activated microglial and Müller cells in CD−/− retinae

It is well known that apoptosis is characterized by the appearance of apoptotic figures, internucleosomal cleavage of genomic DNA, and the phagocytosis of dead cells or apoptotic bodies by macrophages/microglial cells. We therefore examined the issue of whether activated microglial cells in the retinal layers of CD−/− mice phagocytose dead neurons. Double staining analysis demonstrated that F4/80-immunopositive microglial cells containing TUNEL-positive nuclei were frequently present in the ONL, as well as in the INL of CD−/− retinae (Fig. 8A). Since TUNEL-positive nuclei were longitudinally arrayed in CD−/− retinal sections, we further examined the issue of whether Müller cells are able to phagocytose dead neurons. In control retinal sections obtained at P12, P14, and P25, positive staining for GFAP was detected only in the end-feet of Müller cells located in the innermost layer of the retina (Fig. 8D). On the contrary, positive signals for GFAP were localized not only in the end-feet but also in longitudinally running cytoplasm of Müller cells as well in CD−/− retinae at P12, while its immunoreactivity considerably increased in the cytoplasm of the cells as retinal atrophy proceeded (Fig. 8D), indicating that an astrocytosis-like reaction also

is not seen in the INL of a nontreated control littermate mouse (left) and rarely in that of a treated CD−/− mouse (middle), but it appears in the INL of a nontreated CD−/− mouse (right) and in the ONL of treated and nontreated CD−/− mice. (C) The number of nuclei per one vertical row in the ONL and INL of retinae from treated and nontreated CD−/− mice and nontreated CD+/− mice. P < 0.01, *P < 0.05 in t test. Scale bar in (B) is 30 μm for (A) and (B).
occurs in Müller cells of the retinae. Double staining by TUNEL and GFAP revealed that GFAP-positive Müller cells often contained TUNEL-positive nuclei (Fig. 8B). Moreover, electron microscope observations revealed that a certain type of cells which possessed polygonal nuclei with relatively dense homogeneous karyoplasm and several large heterophagosomes containing cellular debris and nuclei with condensed chromatin appeared in the INL (Fig. 8C). Since the nuclei of the cells were located in the INL and the ultrastructure of the cells was different from that of microglial cells, which had heterochromatin in the periphery, and compatible with that of Müller cells reported by Strettoi and Masland (1995), these cells could be identified as Müller cells which phagocytosed dead cells. These results suggest that dead neurons in CD−/− retinae are phagocytosed by activated microglial and Müller cells and retinal atrophy results in Müller cell gliosis.

Discussion

The present study demonstrates the following: (1) CD deficiency induced retinal atrophy after the morphological formation of retinal layers. (2) This atrophy can be attributed largely to the loss of ONL neurons and, to a lesser extent, to that of INL neurons; the former was apoptosis executed by caspases 9 and 3, while these caspases were not detected in the latter which was prevented by NOS inhibitors and mediated by NO released from activated microglial cells. (3) CB or CL deficiency did not induce any alteration of retinal morphology.

In the postnatal development of mouse retina, it has been shown that cell death occurs in a sequential manner from the inner side of the retina to the outer side. It appears in ganglion cells and amacrine cells within the first week after birth, followed by the INL around P8, while it occurs in the ONL but the number is low, compared to that in the INL (Young, 1984). The present study, using TUNEL staining, revealed that cell death occurs largely in the INL from P5 to P14, peaking at P6 and P7, in both CD−/− and control littermate mouse retinae, although it is also found in the inner side of the retinae before P5. TUNEL-positive neurons in the ONL were rarely found in control littermate retinae, as reported previously (Young, 1984; Johnson et al., 1999). As demonstrated under Results, however, CD−/− mouse retinae showed a drastic neuronal loss after physiological cell death had ceased. These results suggest that the loss of neurons in CD−/− mouse retinae, which results in retinal atrophy and proceeds mainly after the retinal layers have been morphologically formed, differs from naturally occurring cell death.

Apoptosis is characterized by morphological and biochemical characteristics such as cell shrinkage, nuclear chromatid condensation, and the intermucrosomal cleavage of genomic DNA, followed by phagocytosis by phagocytic cells (Kerr et al., 1972; Wyllie, 1980). In the cell death pathway, mitochondria play a pivotal role in the execution of apoptosis by releasing cytochrome c into the cytoplasm which binds, together with dATP, to Apaf-1; activated...
Apaf-1 activates caspase 9 which activates caspase 3 and activated caspase 3 further activates DNase by cleaving its inhibitor (see reviews: Nagata, 2000; Wang, 2001). The present study using sitespecific antibodies against the active forms of caspases 9 and 3, respectively (Koroku et al., 1998; Fujita et al., 2000), displayed positive staining of these caspases in INL neurons, in addition to positive TUNEL staining and internucleosomal cleavage of genomic DNA obtained from CD−/− mice and control littermate mouse retinas before P15. Since these caspases play critical roles in the execution of apoptosis in CNS tissues during development (Kuida et al., 1996, 1998), it is likely that naturally occurring cell death which occurs postnatally in mouse retina is executed by the activation of caspases 9 and 3 via the mitochondrial pathway.

CD−/− mice have previously been shown to manifest seizures and blindness and accumulate subunit c/erosid lipofoecin-containing lysosomes in CNS neurons and peripheral tissue cells which possess granular osmiophilic deposits and fingerprint profiles, thus demonstrating that the loss of...
Fig. 7. Presence of the active forms of caspases 9 and 3 in retinal tissues. (A) Immunohistochemical staining with anti-m9D368 for caspase 9 and anti-p20/p17 for caspase 3 which recognize only the active forms of caspases 9 and 3. At P10, positively stained cells for caspase 9 and 3 are present in the inner nuclear layer (INL) of both CD+/− and CD−/− mice, while in the outer nuclear layer (ONL), they are detected only in CD−/− mice. At P18 and P24, positive cells for these caspases are detected only in the ONL of CD−/− mice but not in the INL. Note that positive staining for caspases 9 and 3 are diffuse in INL and ONL cells (insets). (B) Proteolytic activity of caspases 9 and 3 at P15. The activities of these enzymes are significantly more increased in retinal tissues from CD−/− mice than in those from CD+/− mice. OPL, outer plexiform layer. Scale bar in (A) is 20 μm; scale bars in insets are 5 μm.
the CD generation in mice is associated with NCL phenotypes (Koike et al., 2000). The present electron microscope study revealed that ONL, INL, and GCL neurons contain numerous autophagosome-like bodies and granular osmiophilic deposits, while fingerprint profiles were, in some cases, detected in retinal pigment epithelial cells. Retinal neurons in NCLs show specific ultrastructures including granular osmiophilic deposits, curvilinear profiles, and fingerprint profiles (Traboulsi et al., 1987; Goebel et al., 1988, 1998; Bensaoula et al., 2000). In animal models, these ultrastructures characteristic of NCLs have been demonstrated in retinal neurons of dogs and do not accompany retinal atrophy, whereas sheep and mouse models of NCLs manifest both morphological features and retinal atrophy, which appears at a relatively late onset (Goebel, 1992; Chang et al., 1994). Recently, mice deficient in various
CLN genes have been produced, but their precise phenotypes in the retinae have not yet been reported, except for one report concerning mice deficient in CLN3 in which the degree of retinal degeneration up to age of 20 months is not extensive, although accumulation of autofluorescent material and intracellular inclusions are increased in neurons located in retinal layers (Mitchison et al., 1999; Katz and Johnson, 2001; Gupta et al., 2001; Seigel et al., 2002). These lines of evidence indicate that the histopathological features of CD−/− retinae, which show rapidly progressing retinal atrophy with loss of photoreceptor cells, are similar to those in NCLs.

Retinal atrophy in CD−/− mice is due largely to the loss of photoreceptor cells which starts at P8, steeply increasing in number from P12 to P18 and continuing up to the terminal stage of the mice. Inherited retinal degeneration, known as retinitis pigmentosa, is characterized by the progressive loss of photoreceptor cells, while animal models of these diseases show a similar phenotype in retinae (see reviews: Travis, 1998; Clarke et al., 2000), indicating that retinal atrophy in CD−/− mice belongs to this category. Photoreceptor cell death in these human hereditary diseases and animal models has been shown to be apoptosis (Chang et al., 1993; Travis, 1998) and to occur in a caspase 3-dependent manner (Liu et al., 1999; Jomary et al., 2001). In the cases of NCLs and related animal models, photoreceptor cell death has also been shown to be apoptotic, but its executioners have yet to be identified. The present data demonstrate that photoreceptor cell death in CD−/− retinae is accompanied by the typical morphology of apoptosis, positive TUNEL staining, internucleosomal cleavage of genomic DNA, increases in the proteolytic activities of caspasas 9 and 3-like proteinases, and positive staining for the active forms of these proteinases, followed by phagocytosis of ONL cells by microglial cells. The present double staining with TUNEL and GFAP and electron microscopy suggest that phagocytosis of dead neurons is also mediated by Müller cells. Moreover, retinal atrophy in CD−/− mice resulted in Müller cell gliosis, as has been suggested in an animal model of NCLs (Goebel, 1992). These data suggest that photoreceptor cell death in the retina of CD−/− mice, which results in retinitis pigmentosa, is apoptosis executed by caspasas 9 and 3 via the mitochondrial pathway. Moreover, the present study also examined retinal morphology of CB−/− and CL−/− mice and found no alterations present, although these enzymes were present in retinal neurons, suggesting that these lysosomal cysteine proteinases are dispensable and are compensated by other proteinases in the lysosomes of retinal neurons during postnatal development. Collectively, different from CB or CL deficiency, CD deficiency induces a perturbation in lysosomal degradation, thus leading photoreceptor cell death.

Among cysteine proteinases in lysosomes of retinal pigment epithelial cells, cathepsin S has been shown to play an important role in the maintenance of normal retinal function by participating in the degradation of outer segments of photoreceptor cells (Lai et al., 2000). Downregulation of cathepsin S using antisense transcripts by recombinant adenoviruses suppresses de novo CD synthesis in ARPE 19 cells and the expression of this antisense transcripts in rat retina induces the shortening of photoreceptor outer segments and intense autofluorescent debris accumulation, suggesting that cathepsin S is involved in the retinal function by regulating CD activity in pigment epithelial cells (Rakoczy et al., 1998; Lai et al., 2000). Moreover, cystatin C an inhibitor of lysosomal cysteine proteinases and a secretory protein, is abundantly present in retinal pigment epithelial cells of mouse, rat, and human retinae and it has been speculated that it participates in the regulation of photoreceptor degradation (Paraoan et al., 2000; Wasselius et al., 2001). Thus, CD, in cooperation with cathepsin S, in retinal pigment epithelial cells may be involved in the degradation of outer segments of photoreceptor cells, and this suggests that the loss of the CD generation in the cells may also contribute to the abolishment of the photoreceptor layer in CD−/− mouse retina.

Microglial cells in the CNS tissues of CD−/− mice appear in regions where neurons are largely damaged and phagocytose these neurons (Koike et al., 2000; Nakanishi et al., 2001). Moreover, they release NO which is produced via cellular iNOS and treatment with NOS inhibitors prevents neuronal death in the thalamic region of the mice (Nakanishi et al., 2001). Western blot analysis demonstrated the presence of iNOS in retinal tissue of CD−/− mice where iNOS-immunopositive microglial cells were present, corresponding to progressive retinal atrophy. The loss of neurons in the CD−/− retinae was 85% in ONL and 33% in INL when the number of nuclei per one vertical row in each layer was counted at P25, while treatment with L-NNAME or SMT had no effect on the loss of neurons in the ONL but significantly prevented that in the INL. These results strongly support the hypothesis that NO production via the iNOS activity of microglial cells is involved in the loss of INL neurons. To date, numerous mechanisms for NO neurotoxicity and death machineries involved in each cell death cascade after exposure to NO have been proposed (Brown and Cooper, 1994; Leist et al., 1997; Tamatani et al., 1998; Bal-Price and Brown, 2001). At present, the precise molecular mechanisms for neuronal death in the INL of CD−/− retinae after P15 remains to be solved, although the present study demonstrated the involvement of NO as a possible inducer of this neuronal death. However, the present data showed that immunostaining for the active forms of caspasas 9 and 3 was negative in INL neurons after P15, but was positive in ONL cells, while the nuclei in dying INL neurons possessed small dispersed chromatin masses, similar to the nuclei in dying CNS neurons (Koike et al., 2000; Nakanishi et al., 2001) and different from those in dying ONL cells. From these lines of evidence, it seems likely that cell death in the INL of CD−/− mouse retinae resembles that in the thalamic region of the mouse brain which results
from secondary damage by NO released from microglial cells, and its pathway differs from that in ONL neurons.

The evidence provided above indicates that it is likely that photoreceptor cell death in CD−/− mouse retinae is associated with a disorder in lysosomal degradation which is the direct results of CD deficiency, although the precise molecular mechanisms for this remain unexplained. This photoreceptor cell death is apoptosis executed via the activation of caspases 9 and 3 and is different from neuronal death in the INL which results from secondary damage induced by NO production via iNOS activity in microglial cells. These events further indicate that CD is essential for the metabolic maintenance of retinal photoreceptor cells.

Experimental methods

Animals

Cathepsin D-deficient mice: Heterozygous (+/−) mice (Saftig et al., 1995) were transferred to the Institute of Experimental Animal Sciences (IEXAS), Osaka University Graduate School of Medicine, and kept in conventional facilities on a 12-h light/dark cycle. Selection of CD−/− mice from littermates obtained by heterozygous coupling was performed according to the method of Saftig et al., 1995) were transferred to the Institute of Graduate School of Medicine, and kept in conventional facilities. Animals

Animals were produced and purified by affinity chromatography. Monoclonal antibodies against mouse microglial cells (F4/80) (Serotec, Oxford, UK), gliarial fibrillary acidic protein (GFAP) (Dako Japan, Kyoto, Japan), and iNOS (Transduction Laboratories, Lexington, KY) were obtained commercially.

Morphological analysis

Sampling

CD−/− and CD+/− littermates obtained every day from P0 to P25, CB−/− and CB+/+ littermates at P25, and CL−/− and CL+/+ littermates at P25 (n = 9 for each stage and each genotype) were deeply anesthetized with pentobarbital (25 mg/kg, ip) and fixed by cardiac perfusion with 2% paraformaldehyde-2% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2) (PB) for ordinary electron microscopy (n = 3 for each), and with 4% paraformaldehyde buffered with PB containing 4% sucrose for light microscopy and immunohistochemistry (n = 6 for each). CD−/− mice treated with L-NAME, SMT, or saline for 13 consecutive days from P12 to P25 were also processed at P25 for light microscopy and immunohistochemistry (n = 5 for each). For electron microscopy, retinal samples were excised from the mice, cut into small pieces, and further immersed in the same fixatives at 4°C overnight. After thorough washing with the same buffer containing 7.5% sucrose, samples were postfixed with 1% OsO4 in the same buffer containing 7.5% sucrose, at 4°C for 1 h and then block-stained with a 2% aqueous solution of uranyl acetate for 1 h. The tissues were dehydrated with a graded series of ethanol and embedded in Epon 812.

For light microscopy, ocular and brain tissues were quickly removed from the mice and further immersed in the same fixative for 2 h. Samples processed for paraffin em-
bedding were cut into 5-μm sections with a microtome and placed on silan-coated glass slides. Samples for cryosections were embedded in OCT compound (Miles, Kankakee, IL) after cryoprotection with 15 and 30% sucrose solutions, cut into 10-μm sections with a cryostat (Leica, CM3050/VT1000S, Germany), placed on silan-coated glass slides, and stored at −80°C until use.

Ordinary light and electron microscopy

For light microscope observations, deparaffinized sections were stained with hematoxylin-eosin (HE), while semithin sections were cut at 1 μm with an ultramicrotome (Reichert Ultracut, Nissei, Japan) and stained with toluidin blue. For electron microscopy, silver sections were cut with an ultramicrotome, stained with lead citrate and uranyl acetate, and observed with a Hitachi H-7100 electron microscope.

Immunohistochemistry for light microscopy

For the detection of cathepsins B, D, and L, F4/80, GFAP, iNOS, and active forms of caspases 9 and 3, deparaffinized or frozen sections were used and immunostained according to a previously described method (Koike et al., 2000). Briefly, the samples were treated with 0.3% H2O2 in methanol for 30 min and incubated with 2% normal goat serum for 20 min at room temperature (RT). They were then incubated at 4°C with the following first polyclonal and monoclonal antibodies for 1 to 3 days: anti-cathepsin B (2 μg/ml), anti-cathepsin L (2 μg/ml), anti-cathepsin D (10 μg/ml), antiactive form of caspase 9 (1:100), antiactive form of caspase 3 (1:100), anti-GFAP (8 μg/ml), F4/80 (20 μg/ml), and anti-iNOS (2 μg/ml). Further incubations were performed with biotinylated goat anti-rabbit IgG for polyclonal antibodies, with biotinylated goat anti-mouse IgG for monoclonal antibodies and with biotinylated goat anti-rat IgG for F4/80 for 1 h, and, finally, with peroxidase-conjugated streptavidin (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) for 1 h at RT. After each step, the sections were rinsed thoroughly in 0.1 M phosphate-buffered 0.5 M saline (pH 7.2), containing 0.1% Tween 20 (TPBS) (Sigma, St. Louis, MO). Staining for peroxidase was performed using 0.0125% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.002% H2O2 in 0.05 M Tris-HCl buffer (pH 7.6) for 10 min. For the demonstration of lysosomal proteinasises, immunofluorescent staining was also performed. After reacted with anti-cathepsins B, D, and L, respectively, the sections were further incubated with Alexa 594-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 1 h at RT. The sections were mounted with an antifade solution and examined by a confocal laser microscope (Olympus, LSM-GB200).

TUNEL staining

To detect nuclear DNA fragmentation, TUNEL staining was applied to deparaffinized sections of each ocular tissue using the modified method of Gavrieli et al. (1992) (Nitatori et al., 1995). Briefly, retinal tissues were incubated with 100 U/ml terminal deoxynucleotidyl transferase (TdT) and 10 nmol/ml biotinylated 16-2′dUTP (Boehringer-Mannheim-Yamanouchi, Osaka, Japan) in TdT buffer (100 mM sodium cacodylate, pH 7.0, 1 mM cobalt chloride, 50 μg/ml gelatin) in a humid atmosphere at 37°C for 1 h, followed by further incubation with peroxidase-conjugated streptavidin for 1 h at RT. Staining for peroxidase was performed by the same manner as described above.

Double staining

Since CD−/− mouse tissue cells show massive autofluorescence due to the accumulation of subunit c in lysosomes (Koike et al., 2000), visualization of double staining was performed using peroxidase/alkaline phosphatase-conjugated streptavidin. Immunohistochemistry performed prior to TUNEL staining was first visualized with peroxidase-conjugated streptavidin, followed by the DAB reaction as described above. The sections were then treated with alkaline phosphatase-conjugated streptavidin (Vectastain ABC kit) and visualized using alkaline phosphatase substrate kit III (blue substrate) (Vectastain ABC kit), according to the manufacturer’s recommended protocol.

For control experiments, deparaffinized and frozen sections were incubated with nonimmunized rabbit serum diluted to 1:1000, followed by the respective second antibodies. Some sections were directly incubated with the second antibodies without pretreatment with the first antibodies.

Quantification of thicknesses of the retina, ONL, INL, and TUNEL-positive cells

To measure the vertical thickness across the retina, HE-stained retinal sections of CD−/− and control littermate mice at P25 (n = 3 each), made along the vertical meridian, were used. Sixty measurements of the retina were made at 20 contiguous fields around the retinal section (10 fields within a 2-mm distance superior and inferior from the optic nerve head (ONH) and 3 measurements per field per animal). The mean value of each field was plotted as a distribution of thickness across retina.

The thickness of the ONL and INL in CD−/− and control littermate mouse retinae after P8 (n = 3 for each) and that of mice treated with L-NAME or SMT were measured using a previously described method (Ogilvie et al., 2000). Briefly, HE-stained retinal sections that included the ora serrata and the optic nerve were used and two regions of each retinal section, 100 μm on either side of the ONH, were randomly selected for quantitative analysis. The thickness of the ONL or INL was determined by averaging data from three animals, for each of which, three counts from each region of the number of ONL or INL nuclei were performed in a vertical column adjoining a single grid line on a reticule.

To quantify dead neurons in the ONL and INL from CD−/− and control littermate mouse retinae after birth and
from retinae of L-NAME-treated and nontreated CD−/− mice at P25, retinal sections (n = 3 for each) that included the ora serrata and the optic nerve disc were subjected to TUNEL staining using previously described methods (Young, 1984; Portera-Cailliau et al., 1994). The number of TUNEL-positive nuclei in ONL and INL, from optic disc to ora serrata, was counted by microscopy, respectively (three TUNEL-positive nuclei in ONL and INL, from optic disc to ora serrata and the optic nerve disc were subjected to DNA gel electrophoresis’.

Retinal and various brain regions from CD−/− and littermate control mice at P7, P9, P15, P18, P23, and P24, and those treated with L-NAME or SMT at P24, were littermate control mice at P7, P9, P15, P18, P23, and P24, respectively (three independent homogenates of CD−/− and nontreated CD−/− retinae and various brain regions from CD−/− and control littermate mice at P15 (n = 3 for each) were used for measurements of caspase activity following methodology described elsewhere (Cheng et al., 1998). Briefly, retinal samples from each mouse were independently homogenized in a lysis buffer containing 10 mM N-2-hydroxyethylpiperazine-N’-ethanesulfonic acid (Hepes), pH 7.5, 42 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol (DTT), 0.5% 3-[3-cholamidopropyl]dimethylammonio-1-propane-sulfonate (CHAPS), 1 mM PMSF, and a protease inhibitor cocktail (Boehringer Mannheim). After the amount of total protein in each sample was determined as described above, 100 μg of total protein in each sample was diluted in a reaction buffer containing 25 mM Hepes, 1 mM EDTA, 3 mM DTT, 0.1% CHAPS, 10% sucrose, and a protease inhibitor cocktail (Boehringer Mannheim). The activities of caspase 3- and caspase 9-like proteinases were then assessed using N-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin and N-acetyl-Leu-Glu-His-Asp-aminomethylcoumarin (Peptide Institute, Inc., Minoo, Japan), as fluorogenic substrates of caspase 3 and caspase 9, respectively. The fluorescence intensity (excitation, 365 nm; emission, 465 nm) was monitored by using a Bioduet 960 microplate reader (Molecular Dynamics, CA). The initial rate of change was regarded as the activity of the given enzymes. The accumulation of fluorescence was linear for at least 45 min. Experiments were repeated three times with samples from each retina and data from three animals in each group were averaged.

**Measurement of caspase activities**

Retinal tissues which were isolated from CD−/− and control littermate mice at P15 (n = 3 for each) were used for measurements of caspase activity following methodology described elsewhere (Cheng et al., 1998). Briefly, retinal samples from each mouse were independently homogenized in a lysis buffer containing 10 mM N-2-hydroxyethylpiperazine-N’-ethanesulfonic acid (Hepes), pH 7.5, 42 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol (DTT), 0.5% 3-[3-cholamidopropyl]dimethylammonio-1-propane-sulfonate (CHAPS), 1 mM PMSF, and a protease inhibitor cocktail (Boehringer Mannheim). After the amount of total protein in each sample was determined as described above, 100 μg of total protein in each sample was diluted in a reaction buffer containing 25 mM Hepes, 1 mM EDTA, 3 mM DTT, 0.1% CHAPS, 10% sucrose, and a protease inhibitor cocktail (Boehringer Mannheim). The activities of caspase 3- and caspase 9-like proteinases were then assessed using N-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin and N-acetyl-Leu-Glu-His-Asp-aminomethylcoumarin (Peptide Institute, Inc., Minoo, Japan), as fluorogenic substrates of caspase 3 and caspase 9, respectively. The fluorescence intensity (excitation, 365 nm; emission, 465 nm) was monitored by using a Bioduet 960 microplate reader (Molecular Dynamics, CA). The initial rate of change was regarded as the activity of the given enzymes. The accumulation of fluorescence was linear for at least 45 min. Experiments were repeated three times with samples from each retina and data from three animals in each group were averaged.

**DNA gel electrophoresis**

Retinal tissues and various brain regions from CD−/− and littermate control mice at P7, P9, P15, P18, P23, and P24, and those treated with L-NAME or SMT at P24, were isolated by dissection and genomic DNA fragmentation was examined according to the method described by Daniel et al. (1999). Briefly, each isolated tissue was separately homogenized gently in 1 ml of a lysis buffer consisting of 4 M guanidine thiocyanate and 0.1 M Tris-HCl, pH 7.0, and incubated at RT for 10 min. Then 10 μl of QIAEX II Suspension (QIAGEN, Inc., CA) was added to each sample. Samples were vortexed, incubated at RT for 10 min and centrifuged at 12500g for 2 min. The pellets were washed by resuspending them in a washing solution three times, followed by resuspension in a TE buffer consisting of 10 mM Tris/ HCl and 1 mM EDTA, pH 8.0. Samples were incubated at RT for 10 min and centrifuged at 12500g for 2 min. The supernatants were collected, approximately 3 μg of DNA from each sample was electrophoresed by 2.0% agarose gel electrophoresis, and the resulting gels were visualized by staining with ethidium bromide.

**Western blotting analysis**

Retinal tissues obtained from CD−/− and control littermate mice at P10, P15, P18, and P20 (n = 3 for each) were independently homogenized in 100 μl of 0.05 M Tris-buffered 0.15 M saline (TBS) containing 1% Triton X-100 and a protease inhibitor cocktail (Boehringer Mannheim, IN) using a Politron homogenizer at 80% maximal speed. After being centrifuged twice at 10,500g for 10 min at 4°C, the protein concentrations in the supernatants were measured using the BCA protein assay system (Pierce, Rockford, IL) and the remainder was boiled for 5 min in 100 μl of a loading buffer containing 100 mmol/liter dithiothreitol and 0.1% SDS. They were then analyzed by 7.5% SDS-PAGE. The electrophoretic transfer of proteins from polyacrylamide gels to a PVDF membrane (Immobilon-P, Millipore Co., Tokyo, Japan) was performed, as described previously (Koike et al., 2000). The sheets were soaked in a chemiluminescent ECL kit (Amersham International plc, Buckinghamshire, UK), according to the manufacturer’s recommended protocol.

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