Targeted expression of baculovirus p35 caspase inhibitor in oligodendrocytes protects mice against autoimmune-mediated demyelination

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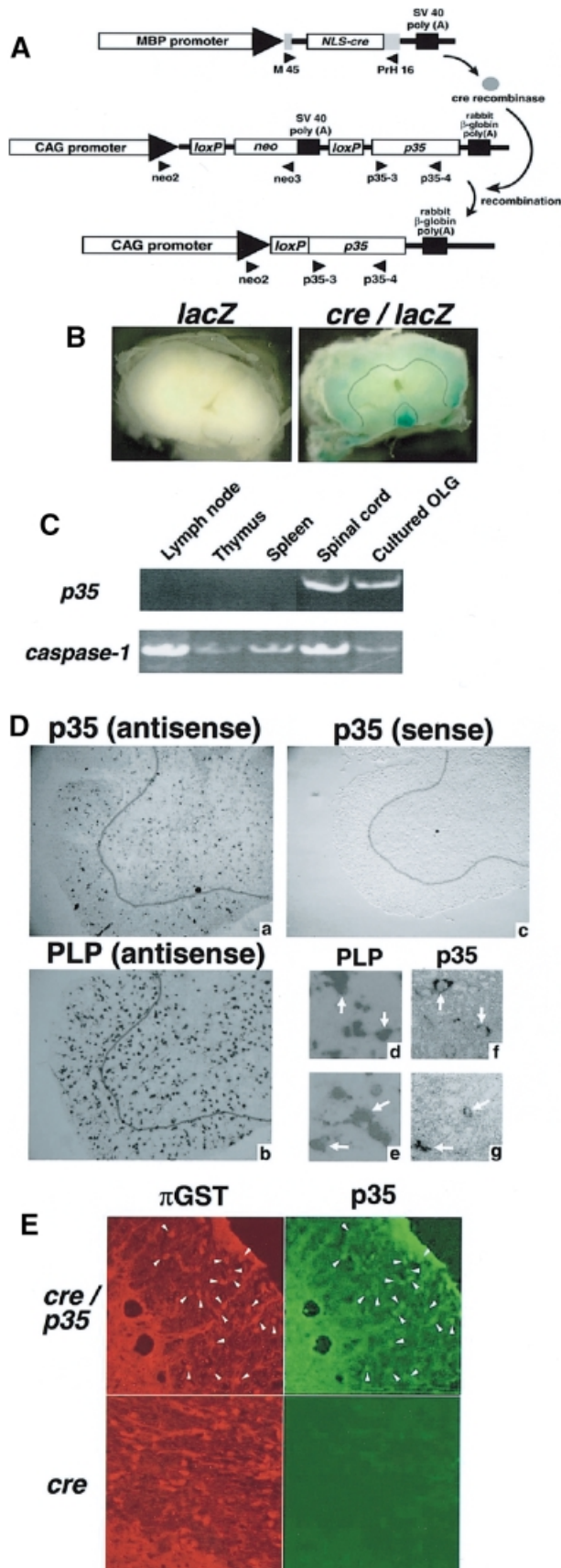e-mail: mmiura@nana.med.osaka-u.ac.jp

The mechanisms underlying oligodendrocyte (OLG) loss and the precise roles played by OLG death in human demyelinating diseases such as multiple sclerosis (MS), and in the rodent model of MS, experimental autoimmune encephalomyelitis (EAE), remain to be elucidated. To clarify the involvement of OLG death in EAE, we have generated transgenic mice that express the baculovirus anti-apoptotic protein p35 in OLGs through the Cre-*loxP* system. OLGs from *cre/p35* transgenic mice were resistant to tumor necrosis factor-α-, anti-Fas antibody- and interferon-γ-induced cell death. *cre/p35* transgenic mice were resistant to EAE induction by immunization with the myelin oligodendrocyte glycoprotein. The numbers of infiltrating T cells and macrophages/microglia in the EAE lesions were significantly reduced, as were the numbers of apoptotic OLGs expressing the activated form of caspase-3. Thus, inhibition of apoptosis in OLGs by p35 expression alleviated the severity of the neurological manifestations observed in autoimmune demyelinating diseases.

Keywords: apoptosis/baculovirus p35/caspase/ demyelination/oligodendrocyte

Introduction

Experimental autoimmune encephalomyelitis (EAE), which is induced in rodents, is an animal model for multiple sclerosis (MS), an inflammatory demyelinating disease of the central nervous system (CNS) mediated in part by T cells. In both EAE and MS the myelin and myelin-forming cells, the oligodendrocytes (OLGs), are targets of autoimmune attack. Since the destruction of OLGs results in the loss of both myelin and neuronal function, considerable efforts have been made to identify the mechanisms responsible for this damage. Clinical observations and studies using animal models, and tissue and cell culture, have led to the proposal that pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) play an important role in mediating OLG death in MS and EAE (Ruddle *et al*., 1990; Simmons and Willenborg, 1990; D'Souza *et al*., 1996; Ledeen and Chakraborty, 1998). Markedly elevated Fas expression has been detected in OLGs within MS lesions (Selmaj and Raine, 1988; Selmaj *et al*., 1991; Louis *et al*., 1993; D'Souza *et al*., 1995) and infiltrating Th1 CD4^{+} cells express Fas ligand (FasL). *In vitro*, OLG death can be induced by TNF-α, IFN-γ, lymphotoxin (LT) and FasL (Selmaj and Raine, 1988; Selmaj *et al*., 1991; Louis *et al*., 1993; D'Souza *et al*., 1995; Hisahara *et al*., 1997).

OLGs represent ~15% of the TUNEL-positive cells in acute MS plaques (60% are macrophages and microglia, 20% are astrocytes) and 25–40% of the dying cells in chronic MS plaques (Dowling *et al*., 1997). These reports support the concept that OLGs undergo apoptosis in MS and that their death plays an important role in the development and progression of this disease. However, some apparently conflicting results have also been reported. In MS lesions, the massive death of invading T cells, but not of OLGs is observed (for a review, see Raine, 1997). To clarify the precise role of OLG death in the progression of EAE, we generated transgenic mice with targeted expression of the baculovirus anti-apoptotic, caspase-inhibitory protein p35 (for a review, see Villa *et al*., 1997) in their OLGs and examined the susceptibility of these animals to a demyelinating form of EAE provoked by immunization with myelin oligodendrocyte glyco-protein (MOG). Since prevention of caspase activity in the early embryo could result in an embryonic lethal phenotype, we have generated mice carrying a *p35* gene that can be expressed ubiquitously but is not expressed under normal conditions because its open reading frame (ORF) has been disrupted by the insertion of a DNA segment flanked by loxP sites, the Cre recognition sites. Expression of Cre, which mediates recombination of two loxP sites into a single site, with concomitant removal of the DNA segment they flank, restores the p35 ORF,

Fig. 1. (**A**) Schematic representation of OLG-specific *p35* expression in the Cre-*loxP* ON/OFF system. The OLG-specific MBP promoter is located 5′ of the NLS-containing *cre* structural gene, followed by the SV40 polyadenylation signal. The *neo* gene is under the transcriptional control of the CAG promoter, and this gene is flanked with loxP sites. The baculovirus caspase inhibitory gene, *p35*, is placed 3′ of the loxP-*neo* cassette. After crossing these transgenic mice, excision of the *neo*-stuffer sequence and OLG-specific *p35* expression occurred in the *cre/p35* transgenic mice. The gray boxes indicate the β-globin exon. The location of each primer used for PCR and RT–PCR is indicated by an arrowhead. (**B**) β-galactosidase staining of the lumbar spinal cord from a *lacZ* and a *cre/lacZ* mouse. *MBP-cre* transgenic mice were crossed with *CAG-loxP-CAT-loxP-lacZ* homozygous mice. β-galactosidase staining was performed with 21-day-old *cre/lacZ* mice and their *lacZ* littermates. The spinal cord was sectioned axially at the lumbar level. A line has been drawn to separate the gray (outside) and white matter (inside) (right panel). (**C**) Tissue expression of *p35* in a MOG-peptide-injected *cre/p35* transgenic mouse. RT–PCR was performed using primers p35-3 and p35-4, indicated by the arrowheads in (A). We also observed the expression of *caspase-1* as an internal control of cDNA synthesis. (**D**) *p35* mRNA is expressed in *PLP* mRNA-positive cells in the *cre/p35* mouse. *In situ* hybridization was performed with *p35* and *PLP* probes in the lumbar spinal cord of a *cre/p35* mouse. Many *p35*-positive and *PLP*-positive cells were located in the white matter. (a, f and g) *p35* antisense; (b, d and e) *PLP* antisense. (d), (f), (e) and (g) are representative high-magnification views of the white matter (consecutive thin sections) indicating that *p35* mRNA is expressed in *PLP* mRNA-positive cells (arrow). (c) sense probe of *p35* in wild type. Lines have been drawn to separate the gray (outside) and white matter (inside) (a–c). Original magnification: (a–c) 100×; (d–g) 400×. (**E**) Immunohistochemical analysis of the lumbar spinal cords from a *cre* transgenic and a *cre/p35* transgenic mouse. Most of the p35-expressing cells (green) were also [pi]GST-positive OLGs (red) in the white matter of the spinal cord in the *cre/p35* transgenic mouse (arrowheads). p35 signal was not detected in the *cre* transgenic mouse. Original magnifications 400×.

thereby allowing production of p35. We have generated transgenic mice in which *cre* is expressed under the control of OLG specific promoter myelin basic protein (MBP) gene. By crossing *p35* transgenic mice with *cre* transgenic mice, *p35* can be expressed in OLGs. OLGs from *cre/p35* transgenic mice were resistant to TNF-α-, anti-Fas antibody- and IFN-γ-induced cell death. When EAE is induced in mice by immunizing with MOG peptide, the numbers of infiltrating T cells and macrophages/microglia in the EAE lesions were significantly reduced, as were the numbers of apoptotic OLGs expressing the activated form of caspase-3 in *cre/p35* transgenic mice. Consistent with these observations, the incidence and severity of the progression of EAE were significantly reduced in *cre/p35* transgenic mice. Thus, inhibition of apoptosis in OLGs by p35 expression alleviated the severity of the neurological manifestations observed in EAE.

Results

Expression of anti-apoptotic protein p35 in oligodendrocytes

We have generated transgenic mice bearing a *p35* gene whose ORF was disrupted by the insertion of a DNA segment flanked by loxP sites, which are Cre recognition sites. To express Cre recombinase in OLGs, we generated transgenic mice expressing Cre recombinase under the control of the MBP gene promoter, whose expression is OLG specific (Miura *et al.*, 1989; Gow *et al.*, 1992) (Figure 1A). The genetic background of both *p35* and *cre* transgenic mice was C57BL/6. The *cre* transgenic mice

(line #29) expressed *cre* only in the brain and testis, as determined by RT–PCR (data not shown). To visualize the site of recombination *in vivo*, *cre* transgenic mice were crossed with *loxP-lacZ* transgenic mice (Sakai and Miyazaki, 1997). In *cre/lacZ* transgenic mice, β-galactosidase activity was detected mainly in the white matter of the spinal cord (Figure 1B). After crossing *cre* and *p35* transgenic mice, excision of the *neo*-stuffer sequence and OLG-specific *p35* expression could occur in the *cre/p35* transgenic mice. The mice expressing the *cre/p35* transgenes showed no apparent developmental or behavioral abnormalities. We confirmed by RT–PCR that *p35* mRNA was expressed in the spinal cord and cultured OLGs, but not in organs of the immune system, such as the lymph nodes, thymus and spleen derived from *cre/p35* transgenic mice (Figure 1C). Next, we examined the expression of *p35* in serial thin sections of the lumbar spinal cord of the *cre/p35* transgenic mice by *in situ* hybridization, probing with *p35* and *proteolipid protein* (*PLP*), an OLG marker. Many *PLP*-positive cells expressed *p35* mRNA in the white matter of the spinal cord (Figure 1D). We also performed immunohistochemistry using anti-[pi] glutathione *S*-transferase (GST) antibody, a specific marker of the cell body of mature OLGs (Tansey and Cammer, 1991), and anti-p35 antibody. Most of the p35-expressing cells were also positive for [pi]GST in *cre/p35* transgenic mice (Figure 1E). These results indicated that p35 was indeed expressed in the spinal cord of OLGs in the *cre/p35* transgenic mice.

Oligodendrocytes expressing p35 are resistant to TNF-α-, Fas- and IFN-γ-induced cell death

To examine the anti-apoptotic functions of the *p35* transgene in OLGs, we tested the sensitivity of OLGs from *cre/p35* transgenic mice to TNF-α and IFN-γ, because cytotoxic cytokines such as TNF-α and IFN-γ are thought to play a critical role in OLG apoptosis both *in vitro* and in EAE/MS (Sharief and Hentges, 1991; Stoll *et al.*, 1993; Hisahara *et al.*, 1997). OLGs from *cre/p35* transgenic mice were significantly more resistant to TNF-α- and IFN-γ-induced cell death than OLGs from wild-type mice (Figure 2). It has also been reported that Fas is upregulated on OLGs in CNS tissue from MS lesions and that human CNS-derived OLGs are sensitive to FasL (D'Souza *et al.*, 1996). To examine the effects of p35 on Fas-mediated OLG death, anti-Fas antibody (Jo2) was administered to the OLG culture. OLGs from *cre/p35* mice were also significantly resistant to anti-Fas antibody-induced cell death (Figure 2B). These results suggest that p35 can prevent OLG death by various cytotoxic cytokines that may stimulate cell death in EAE/MS.

Progression of EAE is prevented in cre/p35 transgenic mice

The EAE susceptibility of *cre/p35* mice compared with their littermate controls (*cre* and *p35*) and wild-type mice was determined by immunizing them with the rat MOG35-55 peptide. Most of the *cre*, *p35* or wild-type mice developed severe hind limb paralysis 11–20 days after immunization (Figure 3A). The incidence of EAE in control mice was ~88% (11 out of 12 in wild-type and 11 out of 13 in *cre* or *p35* mice, respectively), with a mean peak of clinical severity of 4.25 ± 0.281 in wild-type and
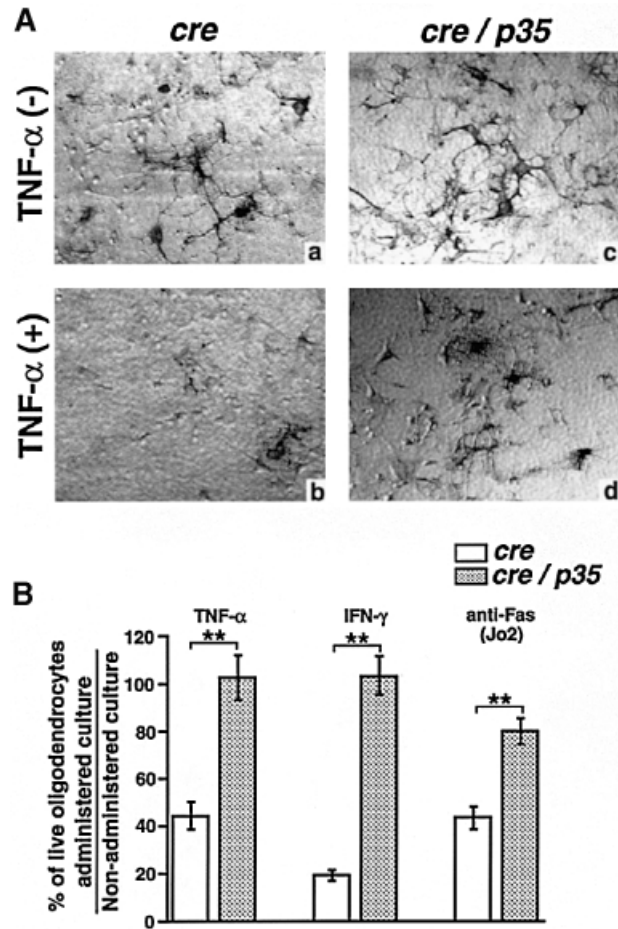

Fig. 2. (**A**) Primary cultures of OLGs from transgenic mice were incubated for 72 h with or without 200 ng/ml TNF-α, then fixed and stained with anti-MBP antibody. (a and c) No administration; (b and d) TNF-α administration. Many MBP-positive (live) OLGs from *cre/p35* transgenic mice were observed, even in the presence of TNF-α (d). Original magnification 200×. (**B**) Proportions of live OLGs, calculated by counting MBP-positive OLGs. TNF-α (200 ng/ml), IFN-γ (200 U/ml) or anti-Fas antibody (Jo2) (200 ng/ml) were added to OLG cultures, then each culture was fixed and stained 72 h later. In each experiment, the OLGs collected from each independent well were counted, and relative viability was expressed as the mean ± SEM. Data were collected from three independent experiments. **$p < 0.01$ (Student's *t*-test).

3.97 ± 0.402 in *cre* or *p35* mice. In contrast, the incidence and severity of EAE in the *cre/p35* transgenic mice (5 out of 10; 1.5 ± 0.641) were significantly reduced ($p < 0.01$) compared with the control mice (Figure 3A).

Oligodendrocyte apoptosis is prevented in the EAE lesion of cre/p35 transgenic mice

To study changes in the number of OLGs in the spinal cord of EAE and control mice, we performed *in situ* hybridization using a *PLP* probe. The lumbar spinal cords of *cre* and *cre/p35* transgenic mice 20 days after EAE induction, as well as of age- and sex-matched uninjected wild-type mice, were analyzed. In EAE *cre* mice, the number of *PLP*-positive OLGs was markedly reduced in the white matter of the spinal cord, compared with uninjected wild-type mice (Figure 3B, a and b). There were many infiltrating cells in the area where OLGs were decreasing, as revealed by Hoechst 33342 nuclear staining
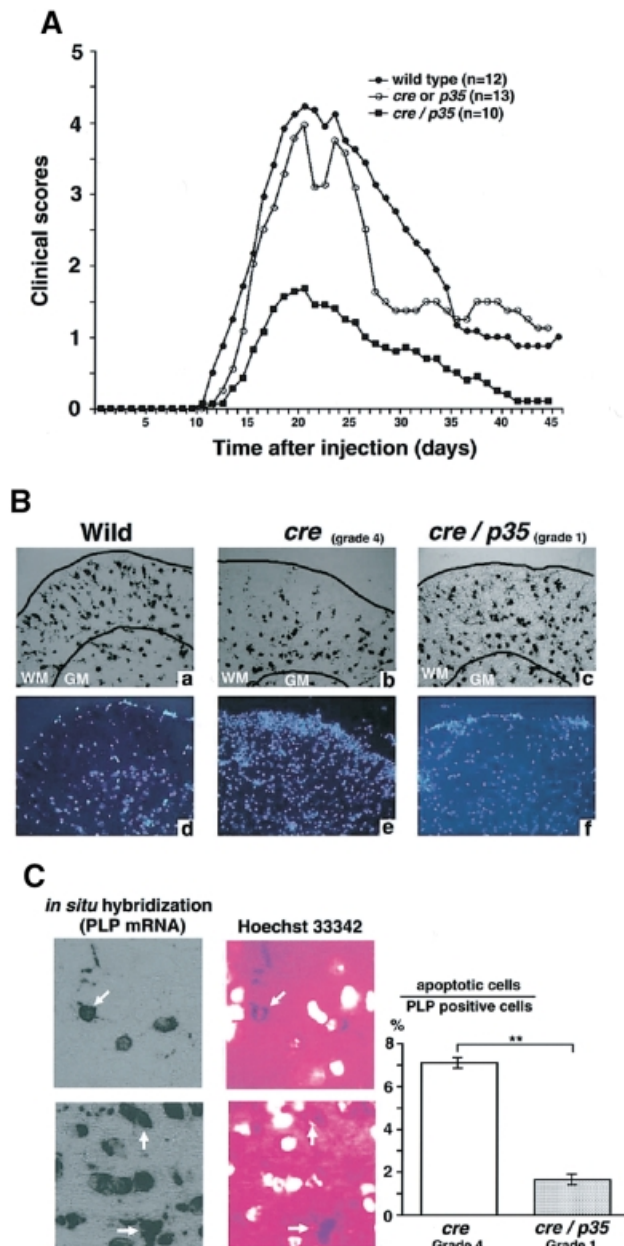
Fig. 3. (**A**) Daily mean clinical course and severity of the EAE induced by MOG 35-55 peptide for each genotype. Clinical signs of the disease were monitored daily beginning on day 7 and were graded on a scale from 0 to 6. (**B**) Pattern of *PLP* mRNA expression (a–c) and Hoechst 33342 nuclear staining (d–f) in the lumbar spinal cord as detected by *in situ* hybridization in a wild-type mouse (a and d), a *cre*-transgenic mouse with grade 4 EAE (b and e), and a *cre/p35* transgenic mouse with grade 1 EAE (c and f). The spinal cord of both transgenic mice was isolated at 20 days after MOG peptide injection. *PLP* mRNA was heavily expressed in the white matter (WM) and to a lesser extent in the gray matter (GM) in the wild-type mouse (a). Note that the *PLP* mRNA-expressing OLGs were decreased in the white matter of the grade 4 EAE mouse (b). Many infiltrating cells were found in these areas (e). The *cre/p35* transgenic mouse did not show a decrease in *PLP* mRNA-expressing OLGs (c, f). Original magnifications 200×. (**C**) Nuclear staining with Hoechst 33342 in *in situ* hybridization samples in the *cre* transgenic mouse (grade 4 EAE). *PLP* mRNA-positive OLGs that showed condensed and fragmented chromatin (arrow). The right graph shows the ratio of apoptotic OLGs in lumbar spinal cord of EAE mice. Data are expressed as the percentage of apoptotic cells out of the total number of *PLP*-positive cells (mean ± SEM). Original magnifications 400×. **p <0.01 at each distance (Student's *t*-test).

(Figure 3B, d and e). In contrast, in *cre/p35* transgenic mice only a few infiltrating cells were observed in the white matter, and the number of OLGs was only slightly lower than in uninjected animals (Figure 3B, c and f). In addition, the number of apoptotic OLGs with fragmented and condensed nuclei was significantly lower in the white matter of the *cre/p35* mice compared with *cre* mice (Figure 3C). The average number of apoptotic OLGs was <2% of the total *PLP*-positive OLG population in *cre/p35* mice compared with ~7% in *cre* mice. These results indicate that apoptosis and loss of OLGs are inhibited in *cre/p35* mice.

Since one of the hallmarks of apoptosis is the activation of caspase-3, we performed immunohistochemistry with an antibody that specifically recognizes activated caspase-3 (Kouroku *et al.*, 1998) on the spinal cords from an EAE grade 4 *cre* mouse, an EAE grade 1 *cre/p35* mouse and an EAE grade 1 wild-type mouse. We found that the number of OLGs expressing activated caspase-3 was reduced in the spinal cord of the *cre/p35* mouse compared with the grade 4 EAE mouse (Figure 4A). Most nuclei of the OLGs expressing activated caspase-3 were fragmented and condensed, indicating that these cells were undergoing apoptosis (data not shown). While more than half of the OLGs expressed activated caspase-3 in the *cre* mouse, only 15% of the OLGs in the *cre/p35* mouse expressed activated caspase-3 (Figure 4B). Moreover, the number of infiltrating cells such as macrophages/microglia and T cells was markedly decreased in the spinal cord of the *cre/p35* mouse (Figure 4C). The degree of infiltration in the *cre/p35* mouse was comparable to that observed in the wild-type mouse with grade 1 EAE. These results suggest that expression of *p35* in OLGs resulted in the inhibition of OLG apoptosis, as well as of infiltration by T cells and macrophages in EAE.

Discussion

A broad-range inhibitor of the caspase family, baculovirus p35, blocks apoptosis induced by TNF, Fas, gluco-corticoids, radiation, DNA-damaging agents and nerve growth factor withdrawal (for a review, see Villa *et al.*, 1997), and inhibits caspases-1, -2, -3, -4, -6, -7, -8 and -10 *in vitro* (Bump *et al.*, 1995; Zhou *et al.*, 1998). *In vivo*, transgenic mice expressing p35 in their thymocytes show inhibition of the major histocompatibility complex (MHC) class II-restricted negative selection induced by staphylococcal enterotoxin B (SEB) superantigen (Izquierdo *et al.*, 1999). These results support the idea that transgenic mice carrying p35 can be used to study the involvement of caspases in degenerative diseases including EAE/MS. There are some inherent difficulties in producing *p35* transgenic mice. Inhibition of caspase activity in the embryo could prevent survival because the knockout of certain caspase family members (caspases -3, -8 and -9) causes embryonic lethality due to the CNS abnormality or cardiac failure (for a review, see Los *et al.*, 1999). Thus, to address the role of effector or executioner caspases in OLGs on the induction of EAE, we specifically expressed p35 in OLGs using a Cre-*loxP* ON/OFF system in transgenic mice (Kanegae *et al.*, 1995; Sakai and Miyazaki, 1997).

Pro-inflammatory cytokines such as TNF-α and IFN-γ,
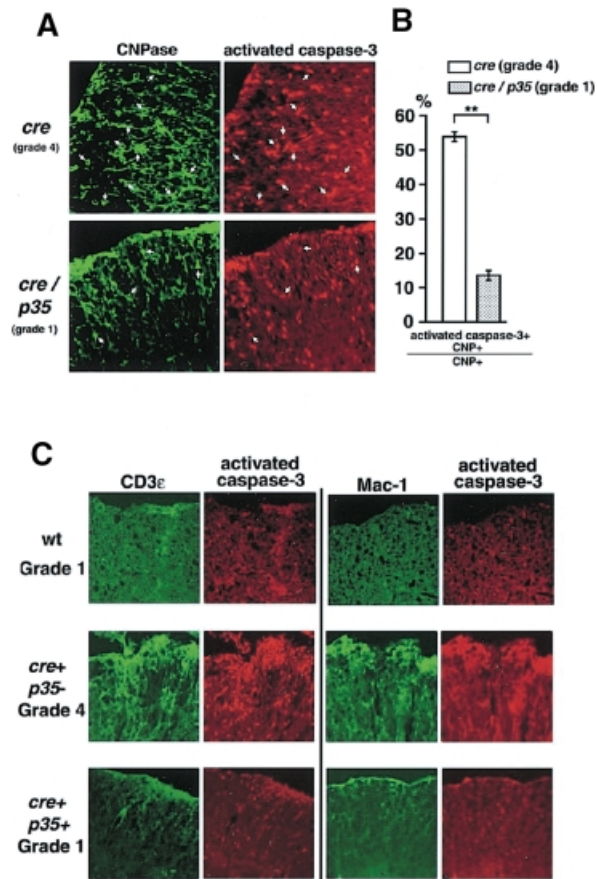
Fig. 4. (**A**) Immunohistochemical analysis of the lumbar spinal cord from a *cre* transgenic (grade 4 EAE) and a *cre/p35* transgenic (grade 1 EAE) mouse. Each panel shows sections stained with anti-CNPase (green), a specific marker of mature OLGs, and activated caspase-3 (red). Arrows indicate the OLGs that express both CNPase and activated caspase-3. Little expression of activated caspase-3 was detected in the lumbar spinal cord of the *cre/p35* transgenic mouse. Original magnifications 400×. (**B**) Relative number of both CNPase and activated caspase-3-positive OLGs in the white matter of the lumbar spinal cord from both the *cre* and *cre/p35* transgenic mouse. Data are expressed as the percentage of apoptotic cells out of the total number of *PLP*-positive cells (mean ± SEM). Note that double-stained OLGs are markedly decreased in the *cre/p35* transgenic mouse. **$p < 0.01$ at each distance (Student's *t*-test). (**C**) Double immunohistochemical staining with anti-CD3ε (a marker of T cells) and with anti-Mac-1 (a marker of macrophages/microglia) antibodies (green) and anti-activated caspase-3 antibody (red) in a wild-type (grade 1 EAE), *cre* transgenic (grade 4 EAE) and *cre/p35* transgenic (grade 1 EAE) mouse. In the grade 4 EAE mouse, infiltrating T cells and macrophages/microglia in the EAE lesions of the lumbar spinal cord expressed high levels of activated caspase-3. There were fewer T cells and macrophages/microglia in wild-type and *cre/p35* transgenic mice. Note that in wild-type mice with grade 1 EAE some of the cells that were negative for CD3ε and Mac-1 were positive for caspase-3. Fewer cells expressing caspase-3 could be detected in *cre/p35* transgenic mice with the same grade of EAE. Original magnifications 400×.

and Fas play an important role in mediating OLG death in MS and EAE (Selmaj and Raine, 1988; Ruddle *et al.*, 1990; Simmons and Willenborg, 1990; Selmaj *et al.*, 1991; Louis *et al.*, 1993; D'Souza *et al.*, 1995, 1996; Ledeen and Chakraborty, 1998). These agents exert their pro-apoptotic function through the activation of caspases. Activated TNF receptor I can bind to adapter molecule TRADD, which in turn binds to another adapter molecule, FADD. Activated Fas recruits FADD. In both cases,

caspase-8 is recruited to FADD, then activated in this protein complex (for a review, see Nagata, 1997). In activated T cells, Bcl-2 provides little protection against Fas-induced cell death, but CrmA can prevent Fas-induced T cell death (Strasser *et al.*, 1995). Bcl-2 has differential effects on Fas-induced cell death, which has been explained by the presence of two Fas signaling pathways (Scaffidi *et al.*, 1998). In so-called type I cells, activated caspase-8 propagates the apoptotic signal by activating downstream caspases. In so-called type II cells, activated caspase-8 first cleaves BID, a pro-apoptotic member of the Bcl-2 family, then truncated BID translocates to the mitochondria, where it induces cytochrome *c* release (Li *et al.*, 1998; Luo *et al.*, 1998). Bcl-2 can prevent Fas-induced cell death in type II cells but not in type I cells (Scaffidi *et al.*, 1998). These reports suggest that the inhibition of caspase activity by p35 may be more effective than Bcl-2 overexpression for preventing the cell death that occurs through the activation of death receptors. IFN-γ-induced cell death is mediated through the upregulation of caspase-1 (Chin *et al.*, 1997). Our results also support the involvement of caspases in IFN-γ-induced OLG death. Taken together, these results indicate that p35 expression could efficiently prevent OLG death by various cytotoxic cytokines that may stimulate OLG injury in the induction of EAE/MS.

Non-Fas- and non-TNF-dependent mechanisms might also be involved in OLG injury in EAE. Cytotoxic T lymphocytes (CTL) rapidly kill target cells using both the granule exocytosis and Fas–Fas ligand pathways. The Fas–Fas ligand-mediated cell death pathway is dependent on caspases (for a review, see Nagata, 1997). Granzyme B can also activate several caspases including caspase-3 (Darmon *et al.*, 1995), and granule-dependent CTL-mediated cell death may be the direct result of granzyme B-mediated caspase-3 activation. While granzyme B seems to initiate cell death via caspase-3 activation, there have been reports that CTL granule exocytosis may be independent of caspases (Sarin *et al.*, 1997; Trapani *et al.*, 1998). Perforin knockout mice show more severe EAE compared with wild type, but there are no major differences in the extent of demyelination and OLG death in the spinal cord in the acute phase of the disease (Malipiero *et al.*, 1997). The regulatory role of CD8^{+} T cells in EAE involves a perforin-dependent pathway that may operate in the killing of antigen-presenting cells. However, the expression of MHC molecules has not been detected in OLGs *in situ* (Lee and Raine, 1989). Thus, CTL granule cytolysis does not seem to play a direct role in promoting OLG death *in situ*.

Caspase-1 plays crucial roles in the cleavage and secretion of pro interleukin-1β (IL-1β) and interferon-γ-inducing factor (IGIF) (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992; Ghayur *et al.*, 1997; Gu *et al.*, 1997). The production of IGIF and IFN-γ in OLGs has not been reported, but IL-1β is constitutively expressed in rat OLGs (Blasi *et al.*, 1999). The major sources of these cytokines in EAE are inflammatory cells, activated microglia and astrocytes (Bauer *et al.*, 1993; Stoll *et al.*, 1993; Wildbaum *et al.*, 1998), not OLGs. It is possible that the expression of p35 prevents IL-1β production in OLGs, but the amount of OLG-derived IL-1β relative to the total IL-1β observed in EAE lesions must be quite low. The expression of p35

in OLGs reduced OLG death and the infiltration of inflammatory cells in the spinal cord. Thus, the expression of p35 in OLGs could also affect the secretion of various cytokines from inflammatory cells in EAE indirectly.

Our study revealed that the inhibition of OLG death by p35 expression results in a significant reduction in both cellular infiltration and demyelination following the induction of EAE by MOG. Interestingly, the clinical severity of EAE was not completely inhibited in all *cre/p35* mice. There are several possible explanations for this observation. First, the inhibition of caspase activity depends on p35 expression, and the p35 expression level in the OLGs of these mice may have been insufficient to block the caspase activity completely. Secondly, the activation of caspase family members may be dose dependent. By increasing the antigen peptide dose, it is conceivable that a member of this family could overwhelm the activity of the p35.

Whereas OLGs are a target of the inflammatory response in EAE, the contribution of the OLGs has been unclear. Some apparently conflicting results have been reported. In MS lesions, the massive death of invading T cells, but not of OLGs is observed (for a review, see Raine, 1997). In contrast, apoptotic OLGs were reported in the spinal cords of EAE lesions (Pender *et al.*, 1991). OLGs comprise 15% of the TUNEL-positive cells (60% are macrophages and microglia, 20% are astrocytes) in the acute plaques of MS (Dowling *et al.*, 1997). In the rat EAE model, 64% of the apoptotic cells are T lymphocytes and 9% are OLGs (Gold *et al.*, 1997). In our study, few healthy mature 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)-positive OLGs were detected in the acute plaques of EAE, which were filled with infiltrating cells such as T lymphocytes, macrophages and microglia (S.Hisahara and M.Miura, unpublished data). Apoptotic OLGs could be identified in the regions surrounding the plaques. These OLGs expressed activated caspase-3 and showed nuclear fragmentation and condensation (S.Hisahara and M.Miura, unpublished data). Our results suggest that OLGs located around the plaques undergo apoptosis due to exposure to cytotoxic cytokines secreted by the infiltrating cells. The reduction in the number of *PLP* mRNA-positive OLGs and the increase in the number of apoptotic OLGs in the regions surrounding the plaques also support the idea that OLG death is tightly associated with the pathology of EAE. These results indicate that OLG death may be partly responsible for the progression of EAE. Our findings also support the idea that decreasing the antigenic myelin fraction by inhibiting OLG death may weaken T cell activation and turn off the ensuing vicious circles. We propose that the inhibition of caspases in target cells might eventually be applicable to reducing the progression of autoimmune diseases, including MS, in humans.

A number of *cre* transgenic mice or *cre* knock-in mice have been reported to express Cre in certain tissues, e.g. the insulin promoter for pancreatic β-cells, the myosin light chain 2v locus for cardiac ventricular muscle, and the Ca^{2+}/calmodulin-dependent protein kinase IIa promoter for the pyramidal cells of the hippocampal CA1 region (Tsien *et al.*, 1996; Hirota *et al.*, 1999; Kulkarni *et al.*, 1999). By crossing these mice with *p35* mice, we can express p35 in many tissues of interest. Our *p35* transgenic mice could provide a useful model for examin-

ing the involvement of caspases in development and/or in various models of degenerative diseases *in vivo*.

Materials and methods

Transgenic mice

pMPV302, a plasmid containing the MBP promoter, was a kind gift from Dr M.Kimura (Kimura *et al.*, 1989). The MBP 5′ region (*Hin*dIII fragment, 5 kb) was isolated from cosBJAB5 (Okano *et al.*, 1991), then cloned into the *Hin*dIII site of pMPV302. The resulting plasmid was named pM138. NLS-cre/pBlue II, a nuclear localizing signal (NLS)–Cre recombinase gene in the pBluescript II vector (a kind gift from Dr M.Hashimoto), was digested with *Xba*I, blunt-ended with Klenow, then an *Eco*RI linker was added to both ends. The fragment was digested with *Eco*RI and inserted into the *Eco*RI site of pM138. The resulting plasmid was named pH80. The transgene containing 6.3 kb of the MBP promoter, NLS-cre, and a polyadenylation signal was isolated from pH80 by digestion with *Kpn*I and was used to generate transgenic mice. Anti-apoptotic *p35* gene was a kind gift from Dr V.M.Dixit (Beidler *et al.*, 1995), and was cloned into the *Swa*I site of pCALNLw (a kind gift from Dr I.Saito) (Kanegae *et al.*, 1995). A fragment containing the CAG promoter, *loxP-neo-loxP*, *p35* coding sequence and a polyadenylation signal was cloned into pBluescript and named pA23. This fragment was used to generate *p35* transgenic mice. Each transgene was injected into fertilized C57BL/6 eggs. DNA from each transgenic mouse was extracted from tail biopsies as described (Laird *et al.*, 1991) and genotyped by Southern blotting. Genomic DNA was digested with *Eco*RI and probed with the *Eco*RI fragment of pH80 for *cre* transgenic mice, and probed with the *Spe*I–*Hin*dIII fragment of pA23 for *p35* transgenic mice. The *CAG-loxP-CAT-loxP-lacZ* mouse has been described previously (Sakai and Miyazaki, 1997).

PCR and RT–PCR

To detect the *p35* transgene in mice, we performed PCR with primers of neo2 and neo3 or p35-3 and p35-4 as follows: forward (neo2) 5′-TCTGACTGACCGCGTTACTC-3′; reverse (neo3) 5′-TATTCG-GCAAGCAGGCATCG-3′; forward (p35-3) 5′-TGGATGGATTCCA-CGATAGC-3′; reverse (p35-4) 5′-TGCACACTCTCCACGTAAGC-3′. DNA was denatured at 94°C for 2 min, annealed at 55°C for 1.5 min and elongated at 72°C for 1 min with 30 cycles. The primers used for PCR and RT–PCR to detect *cre* expression in *MBP-NLS-cre* mouse were as follows: forward (M45) 5′-GGATCCTGAGAACTTCAGG-3′; reverse (PrH16) 5′-GGGAATTCACTATCCAGGTTACGGAT-3′. Total RNAs were isolated from thymus, spleen, lymph nodes and spinal cord of *cre/p35* transgenic mouse 20 days after EAE induction. Total RNA samples (1 μg) were treated with 1 U of DNase I (Gibco-BRL) at 25°C for 1 h. We confirmed the complete digestion of genomic DNA in each RNA solution by PCR using p35-3 and p35-4 primers (data not shown), then cDNAs were synthesized as described (Hisahara *et al.*, 1997). RT–PCR (25 cycles) using p35-3 and p35-4 primers was performed to detect *p35* expression. We also investigated caspase-1 expression by RT–PCR (25 cycles) with specific primers as follows: forward (M47) 5′-TCCAGGAGGGAATATGTGG-3′; reverse (M48) 5′-CTTGTTT-CTCTCCACGGCA-3′. To detect the CAG-*loxP-CAT-loxP-lacZ* transgene by PCR, we used CAT 1 and CAT 2 primers (Sakai and Miyazaki, 1997).

β-galactosidase staining

The spinal cords were dissected from 8-week-old male *cre/lacZ* mice and from their male littermates after the animals were killed under diethyl ether and perfused with 2% paraformaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS). The lumbar cord was cut into pieces ~1–2 mm in length and incubated with 15% sucrose in PBS at 4°C overnight. These samples were washed with PBS three times, then incubated with β-galactosidase staining solution (0.08 M sodium phosphate, 8 mM KCl, 1 μM $MgCl_2$, 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe(CN)_6]$, 0.1% Triton X-100, 0.05% X-gal) at 37°C for 14–18 h. Specimens were examined by microscopy (Olympus IX70).

In situ hybridization

A *cre/p35* mouse and one of its littermate *cre* mice were perfused under anesthesia through the left ventricle with 4% paraformaldehyde in PBS, then the cervical/thoracic and lumbar spinal cords were dissected. For *in situ* hybridization, cryostat sections (7 μm) were cut and affixed to glass slides pre-coated with 3-aminopropyltriethoxysilane (APS). Slides

were post-fixed with 3.7% formaldehyde in PBS pH 7.4 for 5 min, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine–HCl pH 8.0 for 10 min, and dehydrated in graded alcohol solutions. Mouse *PLP* cDNA was a kind gift from Dr Ikenaka (Kagawa *et al.*, 1994). Antisense and sense RNA probes were prepared by *in vitro* transcription with SP6 (sense)/T7 (antisense) RNA polymerase for the *PLP* probes and T7 (sense)/SP6 (antisense) RNA polymerase for *p35*. Hybridization was performed with 80 ng/ml RNA probes at 68°C in hybridization buffer (50% formamide, 5× SSPE, 5% SDS, 1 mg/ml yeast tRNA) overnight. The next day, the slides were washed with three incubations in washing solution (50% formamide, 2× SSC) at 65°C for 30 min, then incubated with blocking solution [1% skimmed milk in TBST (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.2% Tween)] at room temperature for 1 h. The samples were then incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (ALP) (1:1000 dilution) (Boehringer Manheim, Mannheim, Germany) overnight at 4°C. After washing with TBST, signals bound to the antibody were detected with an ALP buffer (0.1 M Tris–HCl pH 9.5, 0.1 M NaCl, 0.1% polyoxyethylene sorbitan monolaurate, 0.05 M $MgCl_2$) containing bromo-chloro-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) at room temperature for 3–12 h. Nuclear staining was performed with Hoechst 33342 (10 µM) simultaneously. *PLP* mRNA-positive OLGs were examined with a fluorescence microscope (Zeiss Axiophot2). The proportion of apoptotic *PLP* mRNA-positive OLGs was determined by counting the number of cells. Data were collected in three independent visual fields in a blind fashion.

Primary cultures

The methods for OLG primary culture and analysis of cell death with cytotoxic cytokines were described previously (Hisahara *et al.*, 1997). Briefly, embryonic day 17–18 pups were decapitated and their brains were minced and placed in 25-cm² delta (Δ)-treated flasks (Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS). After 7–9 days in culture, the flask was shaken by hand. Cells that became detached were collected and plated in poly-L-lysine-coated dishes in DMEM containing 2% FBS and Bottenstein and Sato's (BS) supplement (Hall *et al.*, 1996). Mature OLGs were observed after several days, at which time 200 ng/ml TNF-α, 200 U/ml IFN-γ (Genzyme) and 200 ng/ml anti-Fas antibody (Jo2) (Pharmingen) were added. After a 72 h incubation, cells were fixed with 4% paraformaldehyde in PBS for 10 min. Samples were incubated with rabbit polyclonal anti-MBP antibody and ALP-conjugated anti-rabbit IgG antibody (Dako). Proteins were detected with an ALP buffer containing BCIP and NBT as substrates. We performed three independent experiments. In each, the number of live OLGs collected from each independent well was counted, and the relative viability was expressed as the mean ± SEM. Total RNA of cultured OLGs for RT–PCR in *cre/p35* mouse was isolated as described previously (Hisahara *et al.*, 1997).

Induction and assessment of EAE

The Animal Welfare Guidelines of Osaka University Medical School were followed for all studies and experiments with mice. The procedure for inducing EAE was as described previously (Liu *et al.*, 1998). Briefly, rat oligodendrocyte glycoprotein (MOG) 35-55 (MEVGWYRSPFSRVV-HLYRNGK) peptide was synthesized by Biologica, Japan. Eight-week-old transgenic and wild-type mice received a subcutaneous injection into the footpads of bilateral hind feet of 100 µg of peptide in 0.1 ml of sterilized PBS emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 4 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco). Each mouse also received 300 ng of pertussis toxin (List Biological Laboratories) in 0.25 ml of PBS, intravenously, immediately and 48 h after the immunization. Clinical signs of disease were graded on a scale of 0–6, with 0.5 points for immediate clinical findings as follows: 0 = normal; 1 = weakness of the tail; 2 = complete loss of tail tonicity or abnormal gait; 3 = partial hind limb paralysis; 4 = complete hind limb paralysis; 5 = forelimb paralysis or moribund; 6 = death. To eliminate any diagnostic bias, genotyping of transgenic mice was performed 30–40 days after injection.

Immunohistochemistry

Tissue preparation was as described above. Specimens were incubated with anti-[pi]GST antibody (1:200 dilution; MBL, Nagoya, Japan), a marker of the mature cell body of OLGs, and polyclonal anti-p35 antibody (1:50 dilution). Polyclonal antibody against p35 was produced by injecting a recombinant GST–p35 fusion protein into mice. We confirmed the specificity of this antibody by Western blotting and immunocytochemistry using COS7 cells expressing p35 (data not shown).

The fluorescent signal of p35 was enhanced using a Tyramide Signal Amplification (TSA™-Direct) kit (NEN Life Science Products). For investigation of caspase family expression in the spinal cord lesion, samples were incubated with a rabbit polyclonal anti-activated caspase-3 antibody that recognizes only the processed form of caspase-3 (Kouroku *et al.*, 1998). Samples were also incubated with anti-CNPase antibody (1:100 dilution; Sigma), a marker of mature OLGs, anti-Mac-1 antibody (1:100 dilution; Boehringer Mannheim), a marker of macrophages/microglia, or with an anti-CD-3ε antibody (1:100 dilution; Pharmingen), a marker of T cells. The specimens were examined with a fluorescence microscope (Zeiss LSM-510). Counting and analysis of the proportion of activated caspase-3-positive OLGs were performed in three independent visual fields at a magnification of 400×, in a blind fashion.

Statistical methods

Results were expressed as the mean ± SEM. The probability of statistical differences between experimental groups was determined by Student's *t*-test, as indicated.

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