Deletion of protease-activated receptor 2 prolongs survival of scrapie-inoculated mice

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Protease-activated receptor 2 (PAR2) has recently been identified to be a possible modulator of neurodegeneration. To investigate whether PAR2 plays a role in prion infection, we inoculated PAR2-deficient (PAR2−/−) and wild-type (WT) mice intracerebrally with the Rocky Mountain Laboratory strain of scrapie. PAR2−/− mice demonstrated a delayed onset of clinical symptoms, including weight loss, and demonstrated moderate but highly significant prolongation of survival over WT controls. Concomitantly, no apparent differences in brain pathology, infectivity or features of brain prion protein between deceased WT and PAR2−/− mice were found. Our study suggests that PAR2 deletion modulates dynamics of the disease without gross perturbation of its pathogenesis.

Protease-activated receptor 2 (PAR2) is a ubiquitous surface molecule participating in many biological processes. It belongs to the family of G protein-coupled receptors activated via site-specific proteolysis by trypsin or certain other proteinases, leading to the exposure of tethered ligand sequences within the amino terminus of the protein (Osovskaya & Bunnett, 2004). PAR2 participates in tissue growth and differentiation, regeneration and repair, inflammatory-response regulation and malignant transformation (Adams et al., 2011; Hansen et al., 2008). PAR2 activation not only causes the enhancement of different intracellular mechanisms linked to coupling with the G protein, but is also suggested to have a role as a non-specific enhancer of membrane internalization, sorting and transcellular transport or in the degradation of various extracellular or membranous materials (Soh et al., 2010). In the brain, PAR2 seems to play both protective and damaging roles (Luo et al., 2007). It has recently been shown that PAR2 affects the survival and the death of brain cells in certain experimental models of neurodegenerative disorders, including Alzheimer’s disease and multiple sclerosis (Afkhami-Goli et al., 2007; Noorbakhsh et al., 2006). PAR2 activation may be caused by the leakage of various proteinases (trypsin, mast-cell tryptase, FVIIa, FXa) through the blood–brain barrier in certain pathological conditions (injury, inflammation, irradiation, intoxication) (Olejar et al., 2002; Osovskaya & Bunnett, 2004). In addition, tissue-specific trypsin and trypsin-like proteinases are commonly expressed in brain and play roles in neural development, plasticity, neurodegeneration and neuroregeneration (Wang et al., 2008).

The exact mechanism of neurodegeneration in prion diseases remains unclear. However, it is generally accepted that an insoluble and proteinase-resistant form of misfolded prion protein (PrPSEN) accumulates in neurons and, in cooperation with cellular prion protein (PrPC), causes fatal cellular damage (Soto & Satani, 2011). The involvement of PARs in the pathophysiology of prion diseases, in contrast to other neurodegenerative diseases, has not yet been studied.

To evaluate the role of PAR2 in the pathogenesis of prion disease in vivo, we carried out a transmission study using homozygous PAR2-knockout (PAR2−/−) mice (B6.Cg-F2rl1tm1 Mslb/FVB; Jackson Laboratories) and wild-type (WT) mice with the same genetic background (C57BL/6; Jackson Laboratories). PAR2−/− mice were generated on a mixed C57BL/129/SvJ background (Schmidlin et al., 2002), bred with F2RL1-transgenic FVB/N mice and, upon arrival at Jackson Laboratories, selected for PAR2−/− and back-crossed for six generations to C57BL/6J mice. The study was approved by the Charles University Committee on the Ethics of Animal Experiments. Cohorts of 9-week-old female mice (n=12) were inoculated intracerebrally (i.c.) with 30 μl homogenate of brain infected with the Rocky Mountain Laboratory (RML) strain of scrapie (1% in PBS). The mouse-adapted scrapie isolate RML5 was provided by...
Adriano Aguzzi (Institute of Neuropathology, University of Zurich, Switzerland) and propagated in CD1 mice in our laboratory. Four PAR2\textsuperscript{−/−} control mice were inoculated with PBS only. The mice were observed three times a week to monitor the development of clinical signs of scrapie and sacrificed at the terminal stage of the disease, characterized by severe lethargy and profound weight loss. The first scrapie symptoms (dishevelled appearance and decreased motor activity) were observed 18 and 21 weeks after inoculation of WT and PAR2\textsuperscript{−/−} mice, respectively. Similarly, PAR2\textsuperscript{−/−} mice demonstrated a significant delay in the onset of weight loss, an objective sign of the disease progress (Fig. 1a). Finally, PAR2\textsuperscript{−/−} mice exhibited highly significant prolongation of survival over WT mice [166 ± 8 vs 150 ± 6 days, mean ± SD, P<0.001 (unpaired t-test and one-way ANOVA; SigmaStat 3.5)] (Fig. 1b). At autopsy, the brain was removed and the right cerebral hemisphere was fixed in 3.5% formaldehyde then embedded in paraffin, whilst the left cerebral hemisphere was frozen and stored at −80°C. Serial sections (5 μm thick) from paraffin-embedded blocks were stained with haematoxylin–eosin (HE) or incubated with a polyclonal antibody against glial fibrillary acidic protein (GFAP; Dako) or mAbs against PrP 12F10 (Cayman Chemical) and 6H4 (Prionics AG). Before PrP\textsuperscript{TSE} immunostaining, the sections were subjected sequentially to proteinase K (PK) digestion (10 μg ml\textsuperscript{−1}, 25°C, 10 min) and guanidine thiocyanate treatment (2 M, 25°C, 30 min). Immunoreactions were visualized with the ARK detection system (Dako) using diaminobenzidine as the chromogen. Two experienced pathologists carried out unblinded semiquantitative evaluation of the distribution of spongiform changes, PrP\textsuperscript{TSE} deposits and level of astrogliosis in the brains of six WT and six PAR2\textsuperscript{−/−} mice by consensus. Frontal cortex, nucleus caudatus, posterior hippocampus, mesencephalon and cerebellar cortex regions were scored on a scale of 0–3. Typical spongiform changes, ranging from variably sized individual vacuoles to vacuolar clusters, were observed in both groups of animals (Fig. 2a, b). Similarly, both groups exhibited diffuse synaptic PrP\textsuperscript{TSE} positivity, with areas of a pronounced patch/perivascular type of immunoreactivity (Fig. 2c, d) and occasionally the formation of small, plaque-like structures (not shown). GFAP labelling demonstrated pronounced astrogliosis in all studied regions of brains in both groups of mice (Fig. 2e, f). Comparison of the semiquantitative data revealed only slight, statistically insignificant differences between the PAR2\textsuperscript{−/−} and WT mice (Fig. 2g–i). The scrapie status of all mice and the electrophoretic pattern of PrP\textsuperscript{TSE} were evaluated by Western blotting as described previously (Julak et al., 2011). Briefly, aliquots of 1% brain homogenate were treated with 5 or 50 μg PK ml\textsuperscript{−1}, resolved on a 12% polyacrylamide gel, blotted and developed with the mAb AH6 (TSE Resource Centre, Roslin Institute, Edinburgh, UK) alone or in a mix with mAb 6D11 (Covance). The density of bands on blots was quantified using a MiniLumi gel documentation system utilizing GelQuant densitometer software (DNR Bio-Imaging Systems Ltd). No apparent differences in the overall quantity of PrP\textsuperscript{TSE}, its sensitivity to PK digestion or electrophoretic mobility were observed (Fig. 3a, b). The level of RML infectivity in PAR2\textsuperscript{−/−} and WT brains was compared by a scrapie cell assay as described by Mahal et al. (2008). Briefly, aliquots of brain homogenates of six PAR2\textsuperscript{−/−} or six WT infected mice were pooled, serially diluted in sterile PBS and incubated with CAD5 cells (provided by Charles Weissmann, Department of Infectology, Scripps Research Institute, FL, USA). The cells were split at a 1:10 ratio every 3 days and, after the third passage, 20,000 cells were plated per well (n=12) of ELISPOT plates. Plates were dried and treated with PK, and proteins were then denatured using 3 M guanidine isothiocyanate. The membranes were washed and blocked, then they were developed with the mAb 6D11, alkaline phosphatase-conjugated donkey secondary antibody and a Bio-Rad AP conjugate substrate kit. The number of stained cells per well was counted using a CTL ImmunoSpot Series 3A Analyser, and the means and SD from two independent experiments were calculated using GraphPad Prism.

Fig. 1. Survival and weight loss of scrapie-infected PAR2\textsuperscript{−/−} mice and WT controls. (a) PAR2\textsuperscript{−/−} mice inoculated i.c. with the RML strain of scrapie displayed a delayed onset of clinical signs including weight loss. Mean weight of PAR2\textsuperscript{−/−} (○) and WT (△) mice 146 days after inoculation was 22.0 ± 2.4 g (n=12) and 18.9 ± 2.6 g (n=9), respectively (*P<0.01). The weights of control, sham-inoculated, PAR2\textsuperscript{−/−} mice (CTRL, ●, n=4) are shown for comparison. (b) Infected PAR2\textsuperscript{−/−} mice (dashed line) demonstrated significantly longer survival times (166 ± 8 days, n=12) than inoculated WT controls (solid line; 150 ± 6 days, n=10, P<0.001). The survival time spread was 21 days for PAR2\textsuperscript{−/−} mice and 20 days for WT mice.
software. Both PAR2−/− and WT brain homogenates displayed similar levels of scrapie infectivity, slightly lower than that present in the standard RML brain homogenate propagated in CD1 mice (Fig. 3c).

Our study demonstrates that the deletion of PAR2 delays the onset of clinical symptoms, including weight loss, and prolongs the survival of mice after i.c. inoculation with a high level of RML prions. Concomitantly, no apparent differences in brain pathology or features of brain PrP TSE between deceased WT and PAR2−/− mice were found, suggesting that the deletion of PAR2 affected dynamics of the disease without gross perturbation of its pathogenesis. The possibility that this effect was caused by differences in the genetic background of WT and PAR2−/− mice is unlikely. The mice, after six-back-cross breeding, are statistically 98.4% congenic and, in addition, all mouse strains contributing to the original mixed PAR2−/− genetic background (C57, 129SvJ, FVB/N) demonstrate a similarly short incubation time after i.c. inoculation with RML prions (Tamgüney et al., 2008). The exact mechanism of the survival prolongation remains to be elucidated, but a plausible explanation may lie in an i.p. PAR2 signalling contribution to the speed of neuronal death. Activation of PAR2 led to shortened survival of rat hippocampal neurons in vitro (Smith-Swintosky et al., 1997) and PAR2 deficiency reduced neuroinflammation and severity of disease in experimental mouse autoimmune encephalomyelitis (Noorbakhsh et al., 2006). Similarly, PAR2 deletion was neuroprotective against amyloid beta toxicity in a mouse model of Alzheimer’s disease by limiting the inflammatory response of glial cells (Afkhami-Goli et al., 2007). These results suggest that PAR2 deficiency can protect neurons either directly or by alleviating neuroinflammation. The role of brain inflammation in prion-triggered neurodegeneration remains unclear (Soto & Satani, 2011). We did not find differences in the overall level of astrogliosis between PAR2−/− and WT

Fig. 2. Scrapie-associated vacuolization, astrogliosis and PrP TSE accumulation in the brains of PAR2−/− and WT mice. (a, b) Representative images of the spongiform changes observed in PAR2−/− (a) as well as in WT (b) mice, which range from variably sized individual vacuoles to vacuolar clusters (cortex, HE staining, original magnification ×400). (c, d) Representative images of immunohistochemical staining of the hippocampal formation in PAR2−/− (c) and WT (d) mice after labelling with prion 6H4 mAb (original magnification ×400). (e, f) Representative images of immunohistochemical staining of the cerebellum in PAR2−/− (e) and WT (f) mice after labelling with anti-GFAP polyclonal antibody (original magnification ×100). (g–i) Semiquantitative vacuolization score (g), PrP TSE deposition score (h) and astrogliosis density (i) in different brain areas of terminally sick PAR2−/− mice (▲, mean ± SEM, n = 6) and WT mice (○, mean ± SEM, n = 6). FX, Frontal cortex; NC, nucleus caudatus; PS, posterior hippocampus; MN, mesencephalon; CX, cerebellar cortex.
infected mice; however, morphological observation is not necessarily the best indicator of the cells’ physiological function. Suppression of PAR2 expression in neurons of brains from Alzheimer’s patients was reported (Afkhami-Goli et al., 2007), but its expression pattern in brains of patients with prion diseases remains to be elucidated. A systematic global gene-expression study in prion-infected mice did not detect any significant change in PAR2 mRNA expression in the brain (Hwang et al., 2009); however, the level of protein expression does not always correlate with its mRNA level, as we demonstrated recently for PrPC in differentiating erythroid cells (Panigaj et al., 2011). Obviously, further studies are needed to clarify the precise role of PAR2 in the neurodegeneration triggered by prion infection.

Our study, in accord with the above-mentioned studies, identifies PAR2 as a potential pharmacological target in the development of therapeutic strategies for neurodegenerative diseases. Several specific inhibitors of PAR2 have been described already (Severino et al., 2009; Sevigny et al., 2011), and their use for the treatment of various CNS disorders is being contemplated (Bushell, 2007).

Acknowledgements

We would like to thank Ms Alena Hudakova for technical help, Professors Adriano Aguzzi and Charles Weissmann for donation of materials and the TSE Resource Centre (Roslin Institute, Edinburgh, UK) for donation of mAb AH6. This work was supported by the following grants from the Czech Science Foundation: GACR 309/09/P204, GACR 310/09/P260, GACR 310/08/0878 and GACR P303/12/1791; and by a grant from the Ministry of Education, Youth and Sports of the Czech Republic: PRVOUK-P24/LF1/3. O.J. was supported by Charles University project UNCE 204022.

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