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Carboxypeptidase N-deficient mice present with polymorphic disease phenotypes on induction of experimental autoimmune encephalomyelitis

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ABSTRACT

Carboxypeptidase N (CPN) is a member of the carboxypeptidase family of enzymes that cleave carboxy-terminal lysine and arginine residues from a large number of biologically active peptides and proteins. These enzymes are best known for their roles in modulating the activity of kinins, complement anaphylatoxins and coagulation proteins. Although CPN makes important contributions to acute inflammatory events, little is known about its role in autoimmune disease. In this study we used CPN$^{-/-}$ mice in experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis. Unexpectedly, we observed several EAE disease phenotypes in CPN$^{-/-}$ mice compared to wild type mice. The majority of CPN$^{-/-}$ mice died within five to seven days after disease induction, before displaying clinical signs of disease. The remaining mice presented with either mild EAE or did not develop EAE. In addition, CPN$^{-/-}$ mice injected with complete or incomplete Freund's adjuvant died within the same time frame and in similar numbers as those induced for EAE. Overall, the course of EAE in CPN$^{-/-}$ mice was significantly delayed and attenuated compared to wild type mice. Spinal cord histopathology in CPN$^{-/-}$ mice revealed meningeal, but not parenchymal leukocyte infiltration, and minimal demyelination. Our results indicate that CPN plays an important role in EAE development and progression and suggests that multiple CPN ligands contribute to the disease phenotypes we observed.

Introduction

Carboxypeptidase N (CPN) is a zinc-dependent metalloprotease and a member of one of two groups of mammalian carboxypeptidases that includes CPH/E, CPM, CPD, and CPZ. The active form of these enzymes is secreted or membrane-bound and cleaves the carboxy-terminal arginine or lysine residues from a wide array of proteins, peptides or prohormones (reviewed in Koomen et al. 2005; Matthews et al. 2004; Skidgel 1996; Skidgel and Erdos 2007). CPN substrates include kinins (bradykinin, kallidin and met-lys-bradykinin), complement anaphylatoxins (C3a and C5a), creatine kinase MM and cell surface proteins that bind plasminogen (so-called plasminogen receptors) (Matthews et al. 2004; Skidgel and Erdos 2007). Through these cleavages, CPN broadly modulates a number of biologically important functions including inflammation, chemoattraction, leukocyte activation and trafficking, and plasmin-dependent extracellular matrix degradation. Complete CPN deficiency has not been documented, however an individual with partial CPN deficiency presented with angioedema and elevated histamine levels, supporting the importance of CPN in controlling kinin- and complement-mediated inflammation (Mathews et al. 1980, 1986). The full range of CPN biological functions in human health and disease remains unknown.

The successful generation of CPN$^{-/-}$ mice provided the opportunity to begin careful dissection of the biological roles of this enzyme in greater detail (Mueller-Ortiz et al. 2009). Under routine husbandry, CPN$^{-/-}$ mice presented with no overt phenotype, however, they were hypersensitive to anaphylactic shock due to complement activation in a C5a-dependent fashion. Furthermore,

Abbreviations: CPN, carboxypeptidase N; CVF, cobra venom factor; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein.

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C5a-induced histamine release was primarily responsible for the lethality observed in these studies (Mueller-Ortiz et al. 2009). More recently, we examined the role of CPN in experimental cerebral malaria (ECM), the animal model for cerebral malaria, the most fatal form of this parasitic disease (Darley et al. 2012). CPN$^{-/-}$ mice survived longer than wild type mice, but ultimately succumbed to ECM. These findings were surprising in that deletion of CPN in an infectious disease setting, where complement activation is occurring, was at least transiently protective and clearly did not exacerbate disease onset and severity. These results prompted us to examine the role of CPN in experimental autoimmune encephalomyelitis (EAE), the animal model for the human demyelinating disease, multiple sclerosis. Using MOG_{35-55} peptide-induced EAE, we found that CPN$^{-/-}$ mice presented with multiple phenotypes including lethal adjuvant-induced shock, mild EAE or the failure to develop EAE. CPN$^{-/-}$ mice that developed EAE generally had a monophasic course of disease followed by remission. These results combined with previous studies confirm that CPN biology in inflammatory diseases is highly complex.

Materials and methods

Mice

CPN$^{-/-}$ mice were generated as described (Mueller-Ortiz et al. 2009). CPN1$^{-/-}$ mice develop normally, display no gross abnormalities, are comparable in size to wild type C57BL/6 mice and are fertile. CPN$^{-/-}$ mice used in these studies (male and female) were crossed more than ten times to the C57BL/6 background. Inbred C57BL/6 mice from our own colony were used as controls for all experiments. All studies were performed with approval from the UAB IACUC.

Induction of active and adoptive transfer EAE

For active EAE, wild type and CPN$^{-/-}$ mice were immunized with MOG $peptide_{35-55}$ as previously described (Bullard et al. 2007). In some experiments, mice were immunized with $PLP_{139-151}$. MOG and PLP peptides were synthesized by standard 9-fluorenylmethoxycarbonyl chemistry and were >95% pure as determined by reversed phase-HPLC (Biosynthesis, Lewisville, TX). Onset and progression of EAE clinical signs was monitored daily (30 days) using a standard clinical scale ranging from 0 to 6 as follows: 0, asymptomatic; 1, loss of tail tone; 2, flaccid tail; 3, incomplete paralysis of one or two hind limbs; 4, complete hind limb paralysis; 5, moribund; 6, dead. Only mice with a score of at least 2 (flaccid tail) observed for 2 or more consecutive days were judged to have onset of EAE. A cumulative disease index (CDI) was calculated from the sum of the daily clinical scores observed between day 7 and day 30. For transferred EAE, spleens of wild type donors were removed two to three weeks following induction of active EAE, and prepared as previously described (Szalai et al. 2002). Transferred EAE was induced by injecting $\sim 5 \times 10^6$ purified T cells into wild type or CPN$^{-/-}$ mice as described in the Results section. Mice were evaluated daily for 19 days using the scoring system described above.

Histopathology

Mice with actively induced EAE were sacrificed at 30 days p.i. by CO_2 inhalation, and spinal columns were removed, fixed in 10% buffered-formalin, and paraffin embedded. Sections (5 μm thick) from the cervical, thoracic, and lumbar spinal cord were cut and either stained with hematoxylin and eosin for overall lesion evaluation and characterization of inflammatory responses or with Luxol fast blue for evaluation of demyelination. The extent of inflammation and demyelination was scored based on lesion size (0–4) and lesions were evaluated for lymphocyte accumulation, neutrophil infiltration, demyelination, axonal degeneration, and gliosis (0–4). Tissues were evaluated without identification as to experimental group. Severity scores were calculated as the mean over all segments of the products of the intensity scores multiplied by the extent scores for each lesion characteristic (inflammation, axonal degeneration, gliosis, and demyelination). The means of the individual lesion characteristic severity scores were summed to give the overall severity score.

Table 1
Adjuvant-induced mortality in CPN$^{-/-}$ mice.

Adjuvant/peptide components	Mortality (%)
CFA + MOG_{35-55}	7/17 (41%)
CFA + $PLP_{139-151}$	1/4 (25%)
MTB + $PLP_{139-151}$	1/4 (25%)
Oil + $PLP_{139-151}$	2/4 (50%)
	4/12 (33%)

CFA, complete Freund's adjuvant; PLP, proteolipid protein; MTB, inactivated mycobacterium tuberculosis.

Isolation and flow cytometric analysis of leukocytes from spinal cords

Spinal cords were removed from control and CPN$^{-/-}$ mice with active EAE (15 days post-induction) after perfusion with PBS, ground through a cell strainer, washed in PBS, resuspended in 40% Percoll and layered on 70% Percoll. After centrifugation at 2000 rpm (RT, 25 min.), cells at the interface were removed and washed in PBS and stained as described. Cells obtained from spinal cords were incubated with anti-CD16/32 (24G2, FcR block) to prevent non-specific staining. Spinal cord leukocytes were stained with anti-CD4-FITC ($GK1_{[CR1]}$.5), anti-CD8-PE (53–6.7), anti-IFN-γ-FITC (XMG1.2) or anti-CD25-PE all from eBiosciences (San Diego, CA). Some samples were also permeabilized using the eBioscience regulatory T cell staining kit and then stained with anti-Foxp3-APC. Stained cells and forward scatter were analyzed using a FACSCalibur and the data analyzed using CellQuest software (BD Biosciences, San Jose, CA).

Statistics

Statistical significance between wild type and CPN$^{-/-}$ mice for active EAE experiments was calculated using the Wilcoxon signed rank test, while for disease onset and max clinical score, Student's *t*-test was used. Results of evaluation for inflammation and demyelination were analyzed using analysis of variance for main effects and Tukey's test for pair-wise mean comparisons.

Results

CPN$^{-/-}$ mice are sensitive to adjuvant-induced lethal shock on EAE induction; survivors have attenuated disease

We first analyzed and compared the EAE phenotype in CPN$^{-/-}$ and wild type mice to determine any differences in initiation and progression of disease. We found that of the seventeen CPN$^{-/-}$ mice induced for EAE, seven died between days five and seven (41% mortality) post-disease induction, before onset of clinical signs of disease (Table 1). No wild type mice died in the same post-disease induction time period. To determine if this exceptionally high mortality rate might be adjuvant-induced, we injected wild type and CPN$^{-/-}$ mice with complete Freund's adjuvant containing $PLP_{139-151}$, a myelin-derived peptide that does not induce EAE