Gastrointestinal, Hepatobiliary, and Pancreatic Pathology

Endogenous Prion Protein Attenuates Experimentally Induced Colitis

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Although the cellular prion protein (PrP^C) is expressed in the enteric nervous system and lamina propria, its function(s) in the gut is unknown. Because PrP^C may exert a cytoprotective effect in response to various physiologic stressors, we hypothesized that PrP^C expression levels might modulate the severity of experimental colitis. We evaluated the course of dextran sodium sulfate (DSS)-induced colitis in hemizygous Tga20 transgenic mice (approximately sevenfold overexpression of PrP^C), *Prnp*^{-/-} mice, and wild-type mice. On day 7, colon length, disease severity, and histologic activity indices were determined. Unlike DSS-treated wild-type and *Prnp*^{-/-} animals, PrP^C overexpressing mice were resistant to colitis induction, exhibited much milder histopathologic features, and did not exhibit weight loss or colonic shortening. In keeping with these results, pro-survival molecule expression and/or phosphorylation levels were elevated in DSS-treated Tga20 mice, whereas pro-inflammatory cytokine production and pSTAT3 levels were reduced. In contrast, DSS-treated *Prnp*^{-/-} mice exhibited increased BAD protein expression and a cytokine expression profile predicted to favor inflammation and differentiation. PrP^C expression from both the endogenous *Prnp* locus or the Tga20 transgene was increased in the colons of DSS-treated mice. Considered together, these findings demonstrate that PrP^C has a previously unrecognized cytoprotective and/or anti-inflammatory function within the murine colon. (*Am J Pathol* 2011, 179:2290–2301; DOI: 10.1016/j.ajpath.2011.07.025)

The physiologic functions of the normal cellular prion protein (PrP^C) remain poorly understood despite the well-characterized role of the misfolded molecule in the pathogenesis of neurodegenerative diseases such as bovine

spongiform encephalopathy and Creutzfeldt-Jakob disease. Although mice lacking PrP^C are viable,¹ results from studies of transgenic mice either lacking or overexpressing PrP^C,^{2,3} together with the prion gene (*Prnp*) being highly conserved within vertebrate species,^{4,5} strongly suggest an important role for this protein. *Prnp* encodes a widely expressed 253-amino acid membrane-anchored glycoprotein that is particularly abundant in the central nervous system and lymphoid organs.⁴ Copper binding to PrP^C induces endocytosis of the copper-PrP^C complex via clathrin-coated pits, implying that PrP^C is involved in copper homeostasis.⁶ In addition, PrP^C functions as a cell surface receptor with the capacity to modulate downstream intracellular signaling pathways.^{7–9} In the gut, PrP^C has been localized to components of the enteric nervous system^{10,11} and gut-associated lymphoid tissue,¹² and it is also expressed in epithelial cells.¹³

Several lines of evidence suggest a fundamental role for PrP^C during states of pathophysiologic stress. For example, PrP^C was one of the two most up-regulated proteins during the recovery phase after traumatic brain injury,¹⁴ and the level of PrP^C expression is a significant factor not only in determining responses to experimental cerebral ischemia¹⁵ but also in murine models of Alzheimer's disease.^{16,17} Furthermore, *Prnp* deficiency exacerbates neuroinflammation during murine experimental autoimmune encephalomyelitis,^{18,19} dysregulates iron and copper homeostasis,^{20,21} and sensitizes cells to ischemia and oxidative stress.^{3,22} Thus, via molecular mechanism(s) that remain ill defined, PrP^C is able to exert cytoprotective functions.

The pathogenesis of inflammatory bowel disease, a chronic relapsing and remitting disease of the gut that includes both Crohn's disease and ulcerative colitis, remains to be determined.^{23,24} Oxidative stress, destabili-

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zation of host mucosal defenses, and impaired regulation of innate immune responses to nonpathogenic commensal enteric bacteria likely have a role in disease pathogenesis.²³ Given the distribution and function of PrP^C in various components of the innate and adaptive immune system and in elements of the enteric nervous system,^{4,11} we postulated a role for this molecule in the pathogenesis of inflammatory bowel disease.

PrP^C has a number of potential protective roles during the course of inflammatory bowel disease. For example, it has been recently demonstrated that PrP^C is involved in modulating T-cell activation and proliferation^{18,25} and the phagocytic activity of macrophages.^{26,27} Moreover, depletion of PrP^C skews T cells toward the helper T cell type 1 (Th1) and type 17 (Th17) phenotypes.^{18,19} This is of significance because these lineages produce a variety of proinflammatory cytokines that are up-regulated in the intestinal mucosa in individuals with inflammatory bowel disease.²⁸ Another possibility is that PrP^C functions as an antimicrobial peptide. Pasupuleti et al²⁹ have demonstrated that intact recombinant PrP^C has antibacterial activity at normal and low pH and that synthetic peptides derived from the N-terminal region of PrP^C are cytotoxic to several bacterial species including *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*.

Although acute colitis can accelerate the onset of disease after oral challenge with infectious prions,³⁰ a physiologic role for PrP^C in colitis has not been reported. We, therefore, examined the role of PrP^C in mice either lacking or overexpressing this protein in the dextran sulfate sodium (DSS)-induced model of experimental colitis. Although this model does not fully replicate human inflammatory bowel disease, it does share a number of pathologic features with this disease.^{31,32} Because PrP^C deficiency exacerbates oxidative stress and neuroinflammation, we hypothesized that PrP^C might demonstrate protective and/or anti-inflammatory effects in DSS-induced colitis.

Materials and Methods

Animals

These studies were conducted in accordance with the guidelines established by the Canadian Council of Animal Care, and all protocols were approved by the Health Sciences Animal Care Committee at the University of Calgary. Male mice matched by age and body weight (20 to 25 g) were fed standard laboratory chow, had access to water *ad libitum*, and were maintained in a barrier unit in a room with controlled temperature (22°C ± 1°C), humidity (65% to 70%), and light cycle (12 hours light and 12 hours dark).

Male hemizygous Tga20 mice (approximately sevenfold overexpression of PrP^C),³³ Zurich I *Prnp*-deficient mice,¹ and age-matched (8 to 10 weeks of age) wild-type (WT) littermates (all on a C57Bl/6 background) were administered 3% DSS in their drinking water for 7 days. A second group was given 3% DSS for 3 days. Mice were monitored daily to assess fluid consumption, change in

body weight, stool consistency and for the presence of gross blood in the feces.

Disease Activity Index

Mice were euthanized at day 3 or 7 after initiation of treatment using DSS or tap water, and their colons were quickly removed. The severity of colitis was assessed using an established Disease Activity Index (DAI) demonstrated to be relatively representative of clinical measures for assessment of inflammatory bowel disease.³⁴ The DAI score was derived as follows: for weight loss, 0 = none, 1 = 1% to 5%, 2 = 5% to 10%, 3 = 10% to 15%, and 4 = greater than 15%; for stool consistency, 0 = normal, 2 = loose, and 4 = watery diarrhea; and for colonic bleeding, 0 = none, 2 = slight, and 4 = gross. The scores were added to produce a DAI ranging from 0 (unaffected) to 12 (severe colitis).

Histologic Activity Index

Colonic samples (3 to 4 cm distal to the cecum) were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained using either H&E or periodic acid-Schiff. Damage was assessed using a previously described histologic activity index (HAI),³⁴ as follows: for epithelial damage, 0 = none, 1 = minimal loss of goblet cells, 2 = extensive loss of goblet cells, 3 = loss of crypt cells and extensive loss of goblet cells, and 4 = extensive loss of crypt cells and goblet cells; and for infiltration, 0 = none, 1 = crypt base, 2 = muscularis mucosa, 3 = extensive muscularis mucosa and edema, and 4 = submucosal.

Intestinal Permeability

Intestinal permeability was determined in healthy untreated mice by measuring levels of plasma fluorescein isothiocyanate (FITC)-labeled dextran³⁵ after administration via gavage of 60 mg/100 g body weight of FITC-conjugated dextran (average molecular mass 4000 Da; Sigma-Aldrich Corp., St. Louis, MO) in PBS (pH 7.4). Blood was obtained by cardiac puncture 4 hours after administration of FITC-conjugated dextran and centrifuged for 10 minutes (5000 rpm at 4°C). The plasma (100 µL) was added to a 96-well dark microplate, and the concentration of fluorescein was determined using spectrophotofluorometry (Fluoroskan Ascent; Thermo LabSystems, Inc., Beverly, MA) with an excitation wavelength of 485 nm and an emission wavelength of 527 nm, using serially diluted samples of the marker as standard.

Immunoblotting

To determine PrP^C expression, colonic homogenates (10% w/v) were prepared in extraction buffer (0.15 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton-X 100, 10 mmol/L Tris-HCl [pH 7.4]) with the addition of a protease inhibitor cocktail (Complete; Roche Diagnostics GmbH, Mannheim, Germany). Protein concentrations were determined using the Bradford

assay. Proteins (30 μ g) were separated on 12% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Lysates were prepared from mouse brain for use as a quality control measure. Membranes were blocked in 5% skim milk-TBST (Tris-buffered saline and Tween 20) and incubated with anti-PrP monoclonal antibodies SAF-32 (1:4000 dilution; Cayman Chemical Co., Inc., Ann Arbor, MI) or BAR-221 (1:4000 dilution; SPIbio, BioNovus Life Sciences, Cherrybrook, NSW, Australia) for 24 hours at 4°C. For determination of PrP^C expression in the colon, the primary antibody used was SAF-32. A horseradish peroxidase-conjugated sheep anti-mouse secondary antibody was used. Bands were visualized using a chemiluminescence substrate (SuperSignal West Pico; Thermo Scientific Pierce Protein Research Products, Rockford, IL), and densities were assessed using a calibrated imaging densitometer equipped with Quantity One software (Bio-Rad, Mississauga, ON, Canada). Filters were then stripped and re-probed using an anti- β -actin antibody (Sigma-Aldrich Corp.).

To determine pro-survival and pro-apoptotic signaling molecule expression, methods were duplicated as described above with modifications. Proteins (50 μ g) from each sample were separated on 10% SDS-polyacrylamide gels, and after transfer, membranes were blocked in 5% skim milk-TBST, washed three times in TBST, and incubated overnight at 4°C in 5% bovine serum albumin with the polyclonal antibodies p44/42 (1:1000), phospho-p44/42 (1:500), phospho-Akt (1:500), phospho-STAT3 (1:500), p38 (1:1000), and phospho-p38 (all from Cell Signaling Technology, Inc., Beverly, MA).

Immunohistochemistry

The mouse colons (3 to 4 cm distal to the cecum) were removed, opened along the midline, and placed in PBS containing 1 μ mol/L nifedipine to fully relax the smooth muscle. After 5 minutes, these segments were pinned flat, fixed in 10% formalin for 48 hours, embedded in paraffin, and sectioned (6 μ mol/L). After deparaffinization in xylene and rehydration in graded ethanol, endogenous peroxidase activity was blocked via incubation with 3% hydrogen peroxide. Tissue sections were then heated at 100°C in 10 mmol/L citrate buffer (pH 6.0) to retrieve antigens, and were preincubated with normal goat serum (10%). The primary antibody used for PrP^C staining was mouse monoclonal anti-SAF-32 (1:500; overnight incubation at 4°C; Cayman Chemical Co., Inc.), and the secondary antibody used was donkey anti-mouse CY3 antibody (1:100; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The tissues were subsequently mounted and visualized using an AxioImager A2 fluorescence microscope in conjunction with an AxioCam MR3 digital monochrome camera and AxioVision 4 LE software (all from Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Micrographs (40 $\times$ magnification) were obtained under identical conditions.

Figure 1. Overexpression of PrP^C reduced weight loss and colonic shortening during DSS-induced colitis. **A:** All mice receiving DSS demonstrated significant reductions in body weight when compared with mice given tap water. However, weight loss was attenuated in mice that overexpressed PrP^C (*P < 0.05 versus DSS-treated *Prnp*^{0/0} and WT mice). **B:** Treatment with DSS for 7 days induced colonic shortening in *Prnp*^{0/0} and WT mice but not in Tga20 mice (*P < 0.05 for untreated WT or DSS-treated Tga20 mice relative to DSS-treated *Prnp*^{0/0} and WT mice). Given are mean $\pm$ SE values in seven or more mice.

RNA Extraction and Analysis

Real-time RT-PCR was used to examine transcriptional changes of several cytokines, as well as regulators of cytokine expression that have demonstrated a role in colitis. These genes included *Il1b*, *Il6*, *Il10*, *Il17A*, *Tgfb*, *Tnfa*, *Ifng*, *Socs3* and *Nos2*. Colonic tissues from control and DSS-treated mice were placed in TRIzol reagent (Gibco-BRL, Invitrogen Corp., Carlsbad, CA), and total cellular RNA was extracted according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μ g total RNA in a 25- μ L reaction volume. Duplicate independent quantitative real-time PCR was performed using the LightCycler System (Roche Diagnostics GmbH). SYBR Green I (Roche Diagnostics GmbH) was used to visualize and monitor the amplified product in real time. Gene-specific oligonucleotide primers were designed for the genes of interest. β -Actin was amplified as an internal control. The change in fluorescence of SYBR

Figure 2. Overexpression of PrP^C reduced colonic damage in DSS-treated mice. Mice were treated with either 3% DSS or tap water for 7 days. **A:** No DSS – tap water only. Representative images of colonic tissue damage induced by DSS treatment in WT (**B**), Prnp^{-/-} (**C**), or Tga20 (**D**) mice. **E:** Clinical assessment of colitis using a DAI based on stool consistency, bleeding, and weight loss. DSS treatment increased DAI scores in all groups when compared with control mice given tap water (**P < 0.001). However, the DAI in DSS-treated Tga20 mice was significantly reduced when compared with both DSS-treated Prnp^{-/-} and WT mice (*P* < 0.01). Given are mean ± SE values in seven or more mice. **F:** Although the HAI in all mice receiving DSS was elevated (**P < 0.001; **P < 0.01 versus untreated DSS group), colonic damage in Tga20 mice was markedly reduced (*P* < 0.01). Given are mean ± SE values in six or more mice. Scale bar = 1 cm.

Green I dye in each cycle was determined using the LightCycler system software (Roche Diagnostics GmbH), and the threshold cycle above background for each reaction was calculated. The Ct value of β -actin was subtracted from that of the gene of interest to obtain a Ct value. The Ct value of an arbitrary calibrator (eg, WT control group) was subtracted from the Ct value of each sample to obtain a Ct value. The transcription level relative to the calibrator was expressed as 2^{-Ct} .

Real-Time Primer Sequences

Primers used were β -actin (forward, 5'-AGAGGGAAATCGTGCGTGAC-3'; reverse, 5'-CAATAGTGATGACCTGGCCGT-3'), IFN- γ (forward, 5'-TCAAGTGGCATAGATGTGGAGAA-3'; reverse, 5'-TGGCTCTGCAGGATTTTCATG-3'), iNOS (forward, 5'-CAGCTGGGCTGTACAAACCTT-3'; reverse, 5'-CATTGGAAGTGAAGCGTTTCG-3'), IL-1 β (forward, 5'-CAACCAAGTGATATTCTCCATG-3'; reverse, 5'-GATCCCACTCTCCAGCTGCA-3'), IL-6 (forward, 5'-TCCAGTTGCCCTTCTGGGAC-3'; reverse, 5'-GTGTAATTAAGCCTCCGACTTG-3'), IL-10 (forward, 5'-GGTTGCCAAGCCTTATCGGA-3'; reverse, 5'-ACCTGCTCCACTGCCTTGCT-3'), IL-17 (forward, 5'-GAAGCTCAGTGCCGCCA-3'; reverse, 5'-TTCATGTGTGGTCCAGCTTT-3'), SOCS3 (forward, 5'-GGGTGGCAAGAAAAGGAG-3'; reverse, 5'-GTTGAGCGTCAAGAC-

CCAGT-3'), TGF- β (forward, 5'-TGACGTCAGTGGAGTTGTACGG-3'; reverse, 5'-GGTTCATGTGATGGATGGTGC-3'), and TNF- α (forward, 5'-CATCTTCTCAAAATTCGAGTGA-3'; reverse, 5'-TGGGAGTAGACAAGGTACAAC CC-3').

Statistical Analysis

Results are expressed as mean ± SE. Comparisons among groups of data were made using one-way analysis of variance followed by a *post hoc* Tukey's test. *P* < 0.05 was considered significant.

Results

PrP^C Overexpression Attenuates DSS-Induced Weight Loss and Colonic Shortening

Treatment with DSS significantly reduced body weight in all groups when compared with untreated mice (Figure 1A). However, weight loss was decreased at 5 to 7 days after initiation of treatment in PrP^C overexpressing mice when compared with either Prnp^{-/-} or WT mice. These overexpressing mice also demonstrated attenuation of colonic shortening when compared with Prnp^{-/-} or WT mice (Figure 1B).

Figure 3. Histologic damage is reduced in colonic tissues of DSS-treated Tga20 mice. H&E staining was used to visualize colonic samples from healthy and DSS-treated mice. No histologic abnormalities were observed in untreated mice (**left panels**). With DSS treatment, epithelial damage, depicted by loss of goblet cells, crypt cells, and brush border, was exacerbated in WT and *Prnp*^{-/-} mice as compared with Tga20 mice. In addition, infiltration was reduced in Tga20 mice as compared with the other groups. Periodic acid-Schiff staining of sections from DSS-treated mice revealed preservation of colonic brush border (**black arrowheads**) and goblet cells (**yellow arrowheads**) in Tga20 mice as compared with WT and *Prnp*^{-/-} mice. Scale bar = 100 μm.

PrP^C Overexpression Reduces Damage during DSS-Induced Colitis

Colonic morphologic damage induced by DSS was markedly reduced in mice that overexpressed PrP^C (Figure 2, A–D). DSS treatment increased colonic damage in all groups when compared with control mice given tap water, however, DAIs from Tga20 mice were significantly reduced relative to *Pmp*^{-/-} or WT mice (Figure 2E). Although there were elevations in HAI in all mice receiving DSS, the degree of damage in mice that overexpressed PrP^C was decreased relative to WT or *Pmp*^{-/-} mice (Figure 2F). In healthy colonic tissue, DAI and HAI values for *Pmp*^{-/-} and Tga20 mice were identical to those for healthy WT mice (data not shown). No structural colonic abnormalities were observed among the various groups of healthy mice (Figure 3). However, there were marked reductions in DSS-induced colonic epithelial damage and crypt cell

loss in Tga20 mice relative to similarly treated WT and *Pmp*^{-/-} mice (Figure 3). Moreover, leukocyte infiltration, muscle thickening, and edema were markedly reduced in Tga20 mice when compared with either *Pmp*^{-/-} or WT mice. Periodic acid-Schiff staining revealed that brush border membrane and goblets cell losses were attenuated in the colonic tissues of DSS-treated Tga20 mice when compared with similarly treated WT and *Pmp*^{-/-} mice. These losses of brush border membrane and goblet cells seemed to be exacerbated in *Pmp*-deficient mice.

PrP^C Expression Is Increased during DSS-Induced Colitis

Immunoblotting was performed to determine the levels of PrP^C expression in the colon of WT, *Pmp*^{-/-}, and Tga20

Figure 4. Colonic PrP^C expression is increased in DSS-treated mice. **A:** PrP^C expression as determined via SAF-32 and BAR 221 antibody staining in brain tissues from untreated WT, *Prnp*^{-/-}, and Tga20 mice. **B:** PrP^C expression (anti-PrP monoclonal SAF-32) in colonic tissues of untreated WT, *Prnp*^{-/-}, and Tga20 mice. PrP^C levels in the colon of Tga20 mice were greater (7.4-fold) as compared with those in WT mice. **C:** DSS-induced colitis led to increased PrP^C expression from both the endogenous locus (1.4-fold relative to WT mice) and the Tga20 transgene (10.7-fold compared with WT mice, and 1.6-fold relative to untreated Tga20 mice). β -Actin was used as the lane loading control for all immunoblot analyses.

mice, both before and after induction of colitis. Initially, PrP^C expression was verified using brain lysates as a positive control and immunoblotting with two different antibodies: SAF-32, which recognizes an epitope within

the octarepeat region, and BAR-221, which recognizes an epitope located in the C-terminus (Figure 4A). In healthy mice, PrP^C levels were significantly greater in *Prnp*^{+/-} Tga20 mice (7.4-fold increase relative to untreated WT mice) and absent in colonic tissues of the prion-deficient mice (Figure 4B). In contrast, mice treated with DSS exhibited marked elevations in PrP^C expression, both from the endogenous locus (1.4-fold increase relative to WT mice) and the Tga20 transgene (10.7-fold increase compared with WT mice, and 1.6-fold increase relative to untreated Tga20 mice) (Figure 4C).

Using immunohistochemistry, we next evaluated PrP^C expression both before and during DSS-induced colitis. PrP^C expression was evident in the colonic tissue of healthy WT mice (Figure 5A), and, as expected, was not detectable in tissue from *Prnp*^{-/-} mice (data not shown). Although signals were higher in the Tga20 samples, the tissue pattern of PrP^C expression was similar in colons of vehicle-treated WT and Tga20 mice (data not shown). PrP^C immunoreactivity was localized to various levels of the gut including the myenteric plexus, nerve processes, and circular smooth muscle. Consistent with previous reports,^{10,36} we also observed high levels of PrP^C immunoreactivity in sites within the mucosa, which were suggestive of enteroendocrine cells. Moreover, in all of these mentioned regions, colonic inflammation markedly increased the intensity of PrP^C immunoreactivity in both WT (Figure 5B) and Tga20 (Figure 5D) mice. As expected, PrP^C was not detected in DSS-treated *Prnp*^{-/-} mice (Figure 5C). There was thickening of the circular muscle layer in the colonic samples from WT mice, and this was increased in the colons of DSS-treated *Prnp*^{-/-} mice.

PrP^C Expression Does Not Affect Intestinal Permeability in Untreated Mice

Basal intestinal permeability, possibly regulated by the presence or absence of PrP^C, could have led to either increased or decreased access to enteric bacterial anti-

Figure 5. Immunohistochemical localization of PrP^C in DSS-induced colitis. **A:** PrP^C expression was evident in the colonic tissues of healthy WT mice. Prion protein was not detectable in *Prnp*^{-/-} tissues, but was localized in a similar fashion when vehicle-treated WT and Tga20 mice samples were compared (data not shown). The colonic inflammation induced by DSS increased PrP^C immunoreactivity intensity in both WT (**B**) and Tga20 (**D**) mice. **C:** PrP^C was not detected in DSS-treated *Prnp*^{-/-} mice. In WT and Tga20 mice, prion protein was localized to regions within the gut, consistent with the myenteric plexus (white arrowheads), enteroendocrine cells (yellow arrowheads), and nerve processes in the circular smooth muscle layer (red arrowheads). Note thickening of the circular muscle layer in the colonic samples from WT and *Prnp*^{-/-} mice. Scale bars = 100 μ M.

Figure 6. PrP^C deficiency promotes proinflammatory cytokine transcript expression during colitis. **A:** Cytokine expression on day 3 of DSS administration. IL-6, TNF- α , and SOCS3 expression were elevated in colonic tissues of PrP^C-deficient mice relative to untreated mice. Moreover, colonic expression of IL-6 and TNF- α was increased in *Pmp*^{-/-} mice compared with similarly treated Tga20 mice. TGF- β expression was increased in DSS-treated groups relative to untreated mice. **B:** After 7 days of treatment using DSS, *Pmp*^{-/-} mice demonstrated significantly elevated expression of IL-6, IL-1 β , SOCS3, IL-17, iNOS, IL-10, and TNF- α relative to untreated WT mice. In addition, colonic tissue IL-6, IL-1 β , and IL-17 levels in *Pmp*-deficient mice were increased as compared with those in Tga20 mice. RFC, relative fold-change. Bars represent the mean $\pm$ SE values of four or more mice (day 3) or seven or more mice (day 7). *P < 0.05, **P < 0.01, ***P < 0.001 versus untreated WT mice; †*P* < 0.01 versus Tga20 mice.

gens. To test whether there were differences in barrier function, healthy untreated WT, Tga20, or *Pmp*^{-/-} mice underwent gavage using FITC-conjugated dextran. Plasma concentrations of fluorescein, which provided an index of the degree of gut permeability, suggested that there were no differences in gut permeability between the animal groups before DSS treatment: WT, 621.3 $\pm$ 95.7 nmol/L; *Pmp*^{-/-}, 516 $\pm$ 38.5 nmol/L; and Tga20, 521.5 $\pm$ 27.6 nmol/L (*n* = 4 or more mice in each group).

PrP^C Modulates Proinflammatory Cytokine Expression in DSS-Treated Mice

Having established that PrP^C expression influences the degree of damage induced by DSS, we next examined whether altered proinflammatory cytokine expression was involved to explain this effect. By day 3, IL-6, TNF- α , and SOCS3 transcripts in the colonic tissues of DSS-treated *Pmp*^{-/-} mice were significantly elevated

as compared with those in untreated mice (Figure 6A). Moreover, IL-6 and TNF- α levels in *Pmp*^{-/-} mice were increased when compared with levels in similarly treated Tga20 mice. In *Pmp*^{-/-} mice treated with DSS for 7 days, there were marked increases in iNOS transcripts and in transcripts encoding several cytokines including IL-6, IL-1 β , SOCS3, IL-17, IL-10, and TNF- α (Figure 6B). In particular, colonic RNA transcripts for IL-6, IL-1 β , and IL-17 in *Pmp*-deficient mice were markedly increased relative to Tga20 mice. There were no differences in cytokine expression transcripts in colonic tissue samples from healthy untreated *Pmp*^{-/-}, WT, or Tga20 mice (data not shown).

PrP^C Affects Pro-Survival and Pro-Apoptotic Molecule Expression

Inasmuch as the presence or absence of PrP^C had the potential to regulate a variety of cell-signal transduction pathways, and finding that DSS-induced colitis markedly

Figure 7. Effects of PrP^C expression on pro-apoptotic and pro-survival factors in DSS-treated mice. **A:** There were no basal level differences in either pro-survival or pro-apoptotic signaling molecules among groups. **B:** Representative immunoblots of cell survival factors from colonic tissues of mice at 7 days after treatment with DSS. **C:** Colonic expression of activated STAT3 was reduced in DSS-treated Tga20 mice relative to *Prnp*^{-/-} or WT mice (*P < 0.05 or ***P < 0.001 versus untreated WT mice; †*P* < 0.01 versus Tga20 mice). **D:** Phospho-Akt expression was increased in mice that overexpressed PrP^C relative to all other groups (***P < 0.001). Levels were also elevated in DSS-treated WT mice when compared with untreated control mice (*P < 0.05). **E:** Colonic expression of activated p44/42 MAPK was also increased in Tga20 mice relative to all other groups examined (*P < 0.05 versus DSS-treated WT and *Prnp*^{-/-} mice; ***P < 0.001 versus untreated mice). Bars represent the mean ± SE values of five or more mice.

increased PrP^C expression, we subsequently tested whether signaling molecules known to be involved in the regulation of cell survival were affected. There were no measurable differences in the colonic basal expression levels of phospho-Akt, p44/42, p38, or STAT3 among the untreated groups (Figure 7A). However, Tga20 mice that received DSS for 7 days exhibited significantly increased levels of phospho-p44/42 mitogen-activated protein kinase (MAPK) and phospho-Akt along with a trend to reduced phospho-p38 relative to similarly treated WT or *Prnp*^{-/-} mice (Figure 7, B, D, and E). Moreover, phospho-STAT3 levels were markedly reduced in the Tga20 mice when compared with either *Prnp*-deficient or WT mice (Figure 7C).

Given that as phospho-Akt and phospho-p44/42 MAPK, molecules involved in signal transduction pathways that promote cell survival, were elevated in the colon of DSS-treated PrP^C overexpressing mice, we next examined downstream apoptosis regulator proteins of the Bcl-2 family that are known to be regulated by these molecules (Figure

8A). There were no significant differences in the basal levels of anti-apoptotic Bcl-2 (Figure 8B) or pro-apoptotic Bax (Figure 8C) expression among the various groups of mice. However, in DSS-treated *Prnp*-deficient mice, BAD expression (Figure 8D) was significantly elevated, whereas the expression level of anti-apoptotic phospho-BAD (ser136) was reduced (Figure 8F) relative to all other groups of mice. Conversely, phospho-BAD expression levels in DSS-treated overexpressing PrP^C mice, both at Ser112 (Figure 8E) and Ser136 (Figure 8F), were markedly elevated relative to the other similarly treated mice. No increase in activated caspase-3 or poly (ADP-ribose) polymerase cleavage was observed among the various DSS-treated groups (data not shown).

Discussion

Although the physiologic function(s) of PrP^C remains unclear, evidence suggests that this molecule possesses

Figure 8. PrP^C expression modulates apoptosis-regulating proteins during DSS-induced colitis. **A:** Representative immunoblots of Bcl-2 family proteins sampled from mice treated with DSS for 7 days. There were no differences in Bcl-2 (**B**) or Bax (**C**) expression among treated groups. **D:** Expression of BAD was significantly elevated in DSS-treated *Prnp*-deficient mice relative to all other groups (**P < 0.01). **E** and **F:** In contrast, phospho-BAD expression (at Ser112 and Ser136) in DSS-treated overexpressing PrP^C mice was elevated relative to vehicle- or DSS-treated groups (*P < 0.05, **P < 0.01, ***P < 0.001 versus DSS-treated Tga20 mice). The expression of Ser136 was significantly reduced in *Prnp*-deficient mice when compared to all groups (**P < 0.01). Bars represent the mean ± SE values of four or more mice.

potent cytoprotective properties.³⁷ For example, *Prnp*-deficient mice exhibited increased sensitivity to oxidative stress,^{3,38} suggesting that this protein somehow promotes antioxidant defenses that might have a role in attenuating inflammatory responses. In view of these cytoprotective properties, mice that either lacked *Prnp* or overexpressed PrP^C were subjected to an experimental model of colitis. We observed that overexpression of PrP^C provided protection against DSS-induced colitis. In contrast, mice lacking this molecule demonstrated marked elevations in several proinflammatory cytokine transcripts and pro-apoptotic regulatory proteins, factors predicted to exacerbate colitis. As reported by Sigurdson et al,³⁰ who demonstrated increased PrP^C expression in the inflamed colons of *Salmonella*-infected mice, we observed that DSS-induced colitis increased PrP^C expression from the endogenous locus. Expression of PrP^C from the Tga20 transgene was also up-regulated, which suggests that the necessary regulatory elements were present in the *Prnp* promoter fragment used in the vector.³³ The colons of mice that overexpressed PrP^C demonstrated reduced levels of proinflammatory cytokine transcripts and evidence of pro-survival signaling pathway activation. Collectively, our findings demonstrate that PrP^C has previously unrecognized cytoprotective and/or anti-inflammatory properties within the gut.

PrP^C deficiency exacerbates both oxidative stress and inflammation, factors that are involved in the pathogene-

sis of inflammatory bowel disease. In the present study, we observed that symptoms like those of inflammatory bowel disease (ie, diarrhea, bloody stools, and weight loss) occurred earlier in *Prnp*^{-/-} and WT mice as compared to Tga20 mice. Although there were elevations in the HAI in all mice that received DSS, there were marked reductions in colonic damage indices in mice that overexpressed PrP^C. Moreover, microscopic examination clearly demonstrated that DSS treatment, which markedly increased DAIs in PrP^C-deficient and WT mice, was dramatically attenuated in Tga20 mice. Moreover, DSS-treated PrP^C-deficient mice exhibited reduced brush border membrane and goblet cell numbers relative to similarly treated WT mice.

Basal intestinal permeability, potentially affected by either the presence or absence of PrP^C, could have altered susceptibility to enteric bacterial invasion and, thus, exacerbated immune responses in mice lacking this molecule. Our results demonstrate that there were no differences in overall gut permeability among the various groups of mice. This was notable because it demonstrated that intestinal barrier function was not altered under normal physiologic conditions and, thus, was unlikely to be the initial factor responsible for the observed differences in disease severity.

PrP^C appears to be involved in the regulation of cellular immunity.^{18,25} For example, the severity of murine experimental autoimmune encephalomyelitis, a model of mul-

tiple sclerosis, was significantly increased in PrP^C-deficient mice,^{18,25} whereas overexpression of PrP^C conferred protection against induction of disease.¹⁹ The increased neuroinflammation observed in PrP^C deficiency was attributed to the presence of increased levels of IL-17-producing T cells.^{18,25} These observations are interesting in the context of the present study because the cytokine expression profile in the mice lacking *Prnp*, together with the marked increase in phospho-STAT3, a molecule required for the ontogeny of Th17 cells, would be predicted to favor Th17 cell differentiation. This is of interest because transcripts for Th17-related cytokines are increased in particular in ulcerative colitis, and to a lesser degree in Crohn's disease.³⁹ Furthermore, IL-17A expression is increased in the intestinal mucosa of patients with inflammatory bowel disease, and although controversial, there are indications that Th17-driven immune responses may be involved in the pathogenesis of inflammatory bowel disease.^{28,40}

The IL-6/gp130/STAT3 pathway promotes disease progression by promoting cytokine and growth factor production, hyperplasia, and fibrosis,²⁴ whereas SOCS3 is part of the JAK/STAT negative feedback loop in which SOCS3 acts to suppress STAT3-modulated inflammatory reactions driven by cytokines of the IL-6-family.⁴¹ Furthermore, increased phospho-STAT3 and SOCS3 levels have been detected in mice with experimental ileitis⁴² and in patients with ulcerative colitis or active Crohn's disease.²⁴ We observed that phospho-STAT3 levels were markedly reduced in Tga20 mice as compared with either *Prnp*-deficient or WT mice. This is significant because genome-wide association studies have indicated that the gene encoding STAT3, a factor involved in Th17 differentiation, is associated with susceptibility to inflammatory bowel disease.⁴³ We also observed that SOCS3 expression was attenuated in mice that overexpressed PrP^C when compared with the other DSS-treated groups. This finding was consistent with the observation that DSS-treated Tga20 mice exhibited diminished IL-6 transcript levels and STAT3 activation.

The anti-inflammatory cytokines IL-4 and IL-10 may also have a role in prion diseases, inasmuch as it has been recently demonstrated that IL-10-deficient mice are highly predisposed to prion disease development and reduced incubation times.⁴⁴ Conversely, Stoeck et al⁴⁵ demonstrated that IL-10 was significantly elevated in the cerebrospinal fluid in patients with variant Creutzfeldt-Jakob disease and in patients with various other neurodegenerative diseases. In the present study, although DAI, HAI, and proinflammatory cytokine production was increased in *Prnp*^{-/-} mice, there was also an increase in IL-10 expression above that seen in all other DSS-treated groups. This was likely the result of the increased inflammation present in the colon of these animals and the ability of macrophage-derived IL-10 to act as a counter-regulatory measure during inflammation.

Because the presence or absence of PrP^C has the potential to affect various cell-signaling pathways such as the phosphatidylinositol 3-kinase-Akt axis,^{7,15} we examined signal transduction molecules known to be involved in the regulation of cell survival. Within the colon of

the Tga20 mice, we observed significant elevations in phospho-p44/42 MAPK and Akt, and a trend toward a decreased level of phospho-p38 in response to DSS as compared with treated WT or *Prnp*^{-/-} mice. This was interesting in view of reports that PrP^C seems to be involved in the activation of p44/42 MAPK and Akt during macrophage phagocytosis.^{26,27} Hence, modulation of macrophage p44/42 MAPK and Akt by PrP^C might be of significance in colitis. The higher levels of pro-survival molecules such as phospho-Akt in the inflamed colons of Tga20 mice were consistent with previous observations of phosphatidylinositol 3-kinase pathway activity regulation by PrP^C,¹⁵ and could account, at least in part, for the milder colonic disease manifested by these PrP^C overexpressing animals.

A frequently cited cellular function of PrP^C is its survival-promoting effect on neuronal and non-neuronal cells. These protective functions are reportedly mediated by the anti-apoptotic or anti-oxidative properties of PrP^C; however, relatively little is known about the proximal mechanisms responsible for these effects.⁴⁶ We observed that phospho-Akt and phospho-p44/42 MAPK levels were markedly increased in mice that overexpressed PrP^C. Increased levels of either of these signaling molecules leads to the phosphorylation of BAD, at either Ser112 (by p44/42)⁴⁷ or Ser136 (by pAkt).⁴⁸ Phosphorylation initiates BAD homodimerization, which in turn would leave Bcl-2 free to inhibit Bax-triggered apoptosis.⁴⁹ Thus, Akt or p44/42 MAPK-induced phosphorylation of BAD, as we observed in the colons of PrP^C overexpressing mice, may well have promoted cell survival. In contrast, dephosphorylated BAD, which heterodimerizes with Bcl-2,⁴⁹ could effectively counteract the protective effects of Bcl-2 and, hence, promote cell death. Increased levels of dephosphorylated BAD, as were observed in the colons of DSS-treated *Prnp*^{-/-} mice, may have promoted BAD/Bcl-2 heterodimerization, thus attenuating the anti-apoptotic ability of Bcl-2. However, we detected no evidence of activated caspase-3 or cleaved poly (ADP-ribose) polymerase in DSS-treated colonic tissues. Perhaps a longer term model of colitis would reveal differences in the levels of apoptotic cells between the various genotypes.

In the gut, we and others have observed that the cells of the enteric nervous system, the proliferation of which increases during intestinal inflammation,⁵⁰ express high levels of immunoreactive PrP^C.¹¹ In view of the ability of enteric glial cells to produce IL-6,⁵¹ these cells may have provided an additional source of this cytokine. In addition, transcripts encoding IL-1 β and IL-6, which were increased during DSS-induced colitis, have been shown to regulate cytokine production by enteric glial cells.⁵¹ Although no differences were observed in basal intestinal permeability to FITC-conjugated dextran, the possibility remains that permeability differences might be present under inflammatory conditions.

It has been proposed that PrP^C may function as an antimicrobial peptide. One characteristic of antimicrobial peptides is that they have cationic peptide motifs (hence they are also called cationic antimicrobial peptides) and heparin-binding peptide sequences that enable them to act on both gram-positive and gram-negative bacteria.⁵²

Because PrP^C has exposed cationic and heparin-binding regions at the N-terminus, this molecule has been proposed as antimicrobial peptide candidate. Indeed, Pasupuleti et al²⁹ have demonstrated that both intact PrP^C and N-terminal peptides derived from this molecule exerted membrane-disrupting effects on bacteria similar to those observed with the human antimicrobial peptide LL-37. Thus, if PrP^C were functioning as an antimicrobial peptide, it might, at least in part, account for the attenuation of colitis in Tga20 mice.

In conclusion, while overexpression of PrP^C greatly attenuated DSS-induced colitis, mice lacking this molecule experienced more severe clinical disease, accompanied by increased expression of several transcripts encoding proinflammatory cytokines and changes in cell-signaling factors. In contrast, PrP^C overexpression was associated with reduction in proinflammatory cytokine levels and the up-regulation of pro-survival pathways. Our results are of potential relevance in human inflammatory bowel disease, given the interindividual and interspecies variations observed in PrP^C expression.^{53–56} In addition, multiple polymorphic sites in human *PRNP* could potentially affect the function of this molecule.⁵⁷ Thus, in the context of inflammatory bowel disease, variations in *PRNP* or sequence might plausibly have a modulating effect on disease susceptibility or clinical course.

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