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Protective role of macrophage-derived ceruloplasmin in inflammatory bowel disease

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Abstract

Objective—Intestinal microflora and inflammatory cell infiltrates play critical roles in the pathogenesis of acute colitis. Ceruloplasmin is an acute-phase plasma protein produced by hepatocytes and activated macrophages, and has ferroxidase with bactericidal activities. The goal is to understand the role of ceruloplasmin in colitis progression in a genetically modified murine model.

Design—Experimental colitis was induced in ceruloplasmin null (Cp^{-/-}) and wild-type (WT) mice by dextran sulphate sodium administration. The role of ceruloplasmin was further evaluated by transplantation of WT macrophages into Cp^{-/-} mice.

Results—Cp^{-/-} mice rapidly lost weight and were moribund by day 14, while WT mice survived at least 30 days. Colon culture supernatants from Cp^{-/-} mice exhibited elevated levels of TNFα, KC and MCP-1, indicative of increased inflammation and neutrophil and macrophage infiltration. Elevated leucocytes and severe histopathology were observed in Cp^{-/-} mice. Elevated protein carbonyl content was detected in colons from Cp^{-/-} mice suggesting ceruloplasmin antioxidant activity might contribute to its protective function. Unexpectedly, intraperitoneal administration of human ceruloplasmin into Cp^{-/-} mice did not afford protection. Bone marrow transplantation from

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WT mice or injection of isolated peripheral blood monocytes markedly reduced severity of colitis and morbidity in $Cp^{-/-}$ mice.

Conclusion—Macrophage-derived ceruloplasmin contributes importantly to protection against inflammation and tissue injury in acute and chronic experimental colitis. The findings suggest that defects in ceruloplasmin expression or processing may influence the onset or progression of inflammatory bowel disease in patients.

Ulcerative colitis is a chronic idiopathic inflammatory disease of the gastrointestinal mucosa that affects the rectum and extends proximally through the colon. Ulcerative colitis is characterised by localised inflammation and immune cell infiltration in upper regions of the colon mucosa.¹ The relapsing and remitting course of the disease is known for its variable severity, and 15% of ulcerative colitis patients develop an ‘acute severe’ phenotype during their disease course.² Acute severe ulcerative colitis is defined as six or more stools per day and body temperature of more than 37.8°C, a pulse rate of more than 90 beats per minute, severe colonic bleeding, haemoglobin level less than 10.5 g/dl, or erythrocyte sedimentation rate more than 30 mm/h.³

Dextran sodium sulphate (DSS)-induced experimental colitis is a widely used mouse model of colitis elicited by extensive damage to the epithelial cells that involves supplementation of drinking water with DSS. The induced colitis can be acute or chronic depending on the DSS administration schedule, continuous or alternating with DSS-free water, respectively. DSS-induced colitis causes a robust inflammatory response and severe epithelial injury limited to the colonic mucosa. Recent studies suggest that macrophages infiltrating into the intestinal mucosa play an important role in maintaining homeostasis by negatively regulating immune responses triggered by commensal bacteria. In contrast, macrophages may also be partly responsible for excessive inflammatory response to intestinal microbiota that results in chronic intestinal inflammation.⁴⁵

Ceruloplasmin is an acute-phase plasma protein made principally by hepatocytes and activated monocytes and macrophages.⁶⁷ The plasma level of ceruloplasmin nearly doubles in response to inflammation, trauma, or infection.⁸ Myeloid cell expression of ceruloplasmin is induced by interferon- γ (IFN- γ) and by tumour necrosis factor α (TNF α).⁹ The ferroxidase activity of ceruloplasmin that converts Fe^{2+} to Fe^{3+} plays an important role in iron metabolism and transport and consequently in erythropoiesis.^{10–12} In addition, the ferroxidase activity of ceruloplasmin inhibits ferrous ion-mediated production of reactive oxygen species and thus ceruloplasmin possesses a potent antioxidant activity. Ceruloplasmin also exhibits ferroxidase-dependent bactericidal activity.⁶¹³

Aceruloplasminaemia is a rare disorder generally caused by mutations inducing premature termination of ceruloplasmin messenger RNA translation, a complete absence of plasma ceruloplasmin, and ultimately pathological iron accumulation in the brain, liver, spleen and other tissues.^{14–17} In a murine model generated by ceruloplasmin gene disruption, ceruloplasmin null ($Cp^{-/-}$) mice are normal at birth, but reveal progressive liver and spleen iron accumulation accompanied by mild anaemia.¹⁶ Despite its role as an acute-phase reactant and its oxidant-related activities, there are no reports of inflammatory abnormalities due to reduced ceruloplasmin, and the role of ceruloplasmin in inflammation remains unclear.

To test the hypothesis that ceruloplasmin plays a protective role in innate immune-mediated disease, experimental colitis was induced in $Cp^{-/-}$ mice by the administration of 3% DSS. We observed that $Cp^{-/-}$ mice are highly susceptible to DSS challenge and develop colon inflammation accompanied by tissue oxidation and extensive epithelial lining injury. Remarkably, the major source of anti-inflammatory activity affording protection against

experimental colitis is not liver-derived plasma ceruloplasmin, but rather macrophage-derived ceruloplasmin.

MATERIALS AND METHODS

Mice

Mice with targeted ceruloplasmin gene deletion¹⁴ were backcrossed at least 17 generations into C57BL/6J background. Cp^{-/-} and wild-type (WT) C57BL/6J controls (Jackson) were housed in specific pathogen-free conditions with free access to food and water.

DSS-induced experimental colitis

To induce experimental colitis, 8–12-week female mice were permitted free access to 3% DSS (w/v, MW 40 000 kDa; MP Biomedicals, Solon, Ohio, USA) in drinking water for up to 30 days in acute colitis experiments. Chronic colitis was induced by three cycles of 7-day DSS administration followed by 7 days of DSS-free water.

Scoring of histopathology and colonic bleeding

Colon segments were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5 µm, and stained with H&E for histopathological detection of smooth muscle cell hyperplasia, epithelial damage, inflammatory cell infiltrate and submucosal swelling. Scoring of histopathology and colonic bleeding was done by an individual blinded to the treatment.¹⁸

Immunohistochemistry

Formalin-fixed, paraffin-embedded colon sections were deparaffinised in xylene and rehydrated with alcohol. Samples were boiled for 10 min in antigen retrieval solution (DAKO, Carpinteria, California, USA) and left at room temperature for 30 min. Before incubation with primary antibody to CD3, F4/80, mouse neutrophils (all from AbD Serotec, Raleigh, North Carolina, USA), or ceruloplasmin (Accurate Chemicals, Westbury, New York, USA), slides were blocked with peroxidase inhibitor (DAKO), protein block (DAKO), and biotin/avidin blocking solutions (Vector Laboratories, Burlingame, California, USA). After incubation with biotin-conjugated secondary antibody (Abcam, Cambridge, Massachusetts, USA) and streptavidin-horseradish peroxidase (Vector), signals were visualised by DAB kit (BioGenex, Fremont, California, USA) and counterstained with Mayer's haematoxylin (SigmaAldrich, St. Louis, Missouri, USA).

Cytokine and chemokine production by whole colon culture

Colon tissue (100–200 mg) from rectum to caecum was washed in cold phosphate-buffered saline (PBS) supplemented with penicillin and streptomycin. Dissected segments were cultured in 12-well, flat-bottom culture plates (BD Falcon, BD Biosciences, Sparks, Maryland, USA) in serum-free RPMI medium with antibiotics. After 24 h at 37°C, samples were centrifuged and supernatants stored at –80°C. KC, monocyte chemoattractant protein 1 (MCP-1) and TNFα were determined by ELISA (R&D Systems, Minneapolis, Minnesota, USA).

Haematological analysis

Following cardiac puncture of killed mice, blood was drawn into EDTA-containing tubes (Sarstedt, Nümbrecht, Germany). After vigorous mixing, 0.1 ml of blood was diluted with 0.2 ml of 3% bovine serum albumin in PBS, and complete blood count determined in an Advia 120 haematology system using mouse-specific settings. For monocyte quantification,

the fraction within the white blood cell (WBC) population was determined by blood smear, and corrected by WBC count.

DAB-enhanced Prussian blue determination of colon iron

Formalin-fixed and paraffin-embedded colon and liver sections were deparaffinised in xylene and rehydrated with alcohol. Following preincubation with peroxidase block (DAKO), sections were washed with deionised water and incubated for 1 h in 10% potassium ferrocyanide (w/v) and 20% hydrochloric acid (v/v). Prussian blue signal was enhanced with DAB substrate kit (BioGenex) and counterstained with Mayer's haematoxylin (Sigma).

Commensal depletion by antibiotic treatment

Mice (6–8 weeks old) were treated with ampicillin (1 g/l; Sigma), vancomycin (500 mg/l; Sigma), neomycin sulphate (1 g/l; Gibco, Invitrogen, Grand Island, New York, USA), and metronidazole (1 g/l; Sigma) freely available in drinking water for 4 weeks. For determination of commensal depletion, stool (approximately 50 mg) was ground in 1.5 ml PBS, diluted 10× with PBS, and fixed with formalin. Fixed bacteria were filtered through 0.2 mm filter (Whatman Anodisc 25, VWR, Whatman, Piscataway, New Jersey, USA) and bound bacteria incubated with SYBR Green 1 nucleic acid gel stain (Invitrogen, Grand Island, New York, USA).¹⁹ The filters were dried, transferred to a slide, covered with mounting medium, and microflora quantified by fluorescence microscopy.

Bone marrow transplantation and ceruloplasmin injection

Bone marrow from 5–6-week-old mice was transferred by retroorbital sinus injection into 6-week-old, lethally irradiated recipients. To ensure appropriate myeloid cell engraftment, the blood genotype was established after 6 weeks. In some experiments, purified human ceruloplasmin (500 µg; Vital Products, Concord, Ontario, Canada) was injected intraperitoneally into 10-week-old mice 2 days before DSS administration and then every other day for 30 days.

Peritoneal macrophage isolation and transfer

Peritoneal macrophages were collected by lavage 4 days after intraperitoneal injection of thioglycollate (1 ml, 4%; Sigma). Cells were washed with PBS and subjected to positive selection using biotinylated antibody to mouse F4/80 and anti-biotin magnetic microbeads (Miltenyi Biotec, Auburn, California, USA). Purity was determined by flow cytometry (Cytomics FC500, RXP software; Beckman Coulter, Indianapolis, Indiana, USA) with anti-F4/80-FITC and CD11b-PE antibodies (ABD Serotec). Donor macrophages (10^7 cells) were injected intraperitoneally into recipient mice on days 0, 5 and 10 of DSS treatment.

Analysis of colon protein carbonyl content

Colon was dissected from killed mice, cut longitudinally to remove lumen content, washed with PBS, and frozen in liquid nitrogen. Frozen colon was granulated and homogenised through a 22-gauge needle in RIPA buffer and centrifuged for 10 min at low speed. The supernatant protein carbonyl content was determined as per the manufacturer's instructions (Cayman Chemical, Ann Arbor, Michigan, USA).

Real-time quantitated PCR of ceruloplasmin mRNA

To quantitate ceruloplasmin mRNA, first-strand complementary DNA was synthesised with Superscript III reverse transcriptase (Invitrogen) using total RNA (2 µg) extracted with Trizol (Invitrogen), and amplified with SYBR Green PCR master mix (Applied Biosystems, Carlsbad, California, USA) in an ABI PRISM 7000. β -Actin mRNA was used as

normalisation control. Primers for 302-nt ceruloplasmin PCR product were: GATCGAGTTAAGGATCTCTATAGTGGGCTAATAGG (forward) and CCATTCCCATCACATACCAGTTGACTTCATC (reverse); primers for 133-nt β -actin product were ACGGATGTCAACGTCACACT (forward) and GGTCACTACTATTGGCAACG (reverse).

Statistical analysis

Survival experiments were shown as Kaplan–Meier plots and differences evaluated by two-tailed log-rank test. Differences in parametric data were evaluated by the Student's two-tailed t test. Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Increased susceptibility to experimental colitis in $Cp^{-/-}$ mice

Heterozygous mice with targeted ceruloplasmin gene deletion in C57BL/6 background were intercrossed to generate $Cp^{-/-}$, $Cp^{+/-}$ and $Cp^{+/+}$ littermates. Experimental colitis was induced by the administration of 3% DSS freely available in drinking water for up to 30 days. $Cp^{-/-}$ mice lost weight more rapidly than $Cp^{+/-}$ or $Cp^{+/+}$ littermates, with statistically significant differences after day 7 (figure 1A). All $Cp^{-/-}$ mice were moribund by day 15, while 70% and 90% of the $Cp^{+/-}$ and $Cp^{+/+}$ littermates, respectively, survived for at least 30 days ($p < 0.0001$, figure 1B). Immunostaining with anti-ceruloplasmin IgG revealed ceruloplasmin localisation in and around blood vessels in healthy mouse colon. DSS treatment increased ceruloplasmin in submucosal swellings surrounding inflammatory infiltrates, reaching a maximum at day 9 (figure 1C). Ceruloplasmin was not detected in $Cp^{-/-}$ mice colon.

Haematological parameters in progeny of $Cp^{-/-}$ intercrosses were compared with WT mice in the same background. Excessive weight loss (figure 2A) and diminished survival ($p < 0.0001$, figure 2B) were observed in $Cp^{-/-}$ mice, as above. Bloody stool appeared in both groups between days 3 and 5; however, bloody diarrhoea was more severe in $Cp^{-/-}$ mice (figure 2C). Colorectal bleeding in WT animals subsided and was stable for at least 20 days. Unmanipulated $Cp^{-/-}$ mice were slightly anaemic with approximately 10% lower haematocrit compared with age and sex-matched controls, consistent with mildly impaired iron homeostasis.¹²²⁰ Nonetheless, $Cp^{-/-}$ mice exhibited a greater relative decrease in haematocrit, red blood cells and haemoglobin during the acute colitis course (figure 2D). By day 9 of DSS administration, the colon of $Cp^{-/-}$ animals was significantly shorter than WT controls ($p < 0.01$), and when moribund the entire colon and caecum were filled with loose, bloody stool (figure 2E, F).

Elevated inflammatory response of $Cp^{-/-}$ mice

Colon architecture of untreated $Cp^{-/-}$ mice was indistinguishable from WT mice by H&E staining (figure 3A). At day 5 of DSS treatment, the compact epithelial cell-lined mucosal crypts were equally perturbed in both groups. However, at day 7 $Cp^{-/-}$ colons exhibited substantially greater epithelial damage, mucosal and mesenchymal cell hyperplasia, submucosal swelling and inflammatory cell infiltration compared with controls (figure 3B). By day 9, the epithelial barrier was highly eroded and crypts were barely detectable in $Cp^{-/-}$ colons.

A dramatic increase in WBC was observed in DSS-treated $Cp^{-/-}$ mice at day 7, with concurrent increases in blood neutrophils, lymphocytes and monocytes (figure 3C). The cellular content of the cell infiltrate was determined by immunohistochemistry. Before DSS administration, both mouse genotypes had comparable, low levels of neutrophils,

macrophages and CD3⁺ Tcells in the middle colon lamina propria (not shown). Neutrophil and macrophage influx, an indicator of colonic inflammation, was evident in both genotypes on day 5 (not shown), but was substantially and specifically increased at day 9 in Cp^{-/-} mice (figure 3D). CD3⁺ T-cell infiltration was less dramatic and comparable in both genotypes (not shown). Infiltrate inflammatory activity was determined as chemokine and cytokine secretion by colon cultures. At day 7, TNF α , keratinocyte chemoattractant (KC), and MCP-1 were elevated in Cp^{-/-} colon culture supernatants (figure 3E). Together, these experiments show that Cp^{-/-} mice are highly susceptible to colonic inflammation accompanied by near-complete epithelial erosion and extensive inflammatory cell infiltration.

Microbiota-independent activity of ceruloplasmin in experimental colitis

Ceruloplasmin exhibits ferroxidase-dependent bactericidal activity in vitro.¹³ Inappropriate response to gut microflora is an important factor contributing to the pathogenesis of human inflammatory bowel disease (IBD).²¹ Likewise, commensal bacteria play a major role in the initiation of inflammation in DSS-induced colitis models.²² We considered the possibility that severe colitis in Cp^{-/-} mice might result from elevated microflora in the absence of ceruloplasmin bactericidal activity. Cp^{-/-} and WT mice were treated with antibiotics freely available in drinking water for 4 weeks. Depletion of gut microflora was greater than 99.5% effective in both groups as determined by SYBR green staining of homogenised stool. Following antibiotic treatment, mice were challenged with 3% DSS. Microbiota-depleted WT mice lost weight gradually and became moribund between days 16 and 18 of DSS treatment (figure 4A, B), consistent with a previous finding that depletion of gut microbiota reduces regeneration of intestinal epithelia and leads to mortality of WT mice upon continuous DSS administration.²³ Cp^{-/-} mice lost weight more rapidly and became moribund 8 days earlier than WT controls. Haematological analysis on day 9 revealed a greater decrease in haematocrit and higher WBC counts in Cp^{-/-} mice, indicating more extensive colonic bleeding and inflammation, respectively (figure 4C, D). Histological analysis of colon sections revealed mild inflammation in WT mice, whereas epithelial damage and inflammatory cell infiltration in Cp^{-/-} colons were severe and comparable with Cp^{-/-} mice with normal microflora (figure 4E). Therefore, DSS-induced damage and inflammation in Cp^{-/-} mice are microbiota independent and suggest ceruloplasmin bactericidal activity does not play a major role in disease progression.

Increased protein oxidation in colon of Cp^{-/-} mice

Pathological iron accumulation is common to several hereditary diseases of ageing, particularly neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease and aceruloplasminaemia.²⁴ Because ceruloplasmin deficiency leads to iron accumulation in certain tissues, for example, liver, spleen and brain, we tested the possibility that iron accumulates abnormally in inflamed colon of Cp^{-/-} mice. Iron was not detectable in untreated (not shown) and DSS-treated (figure 5A) colon from Cp^{-/-} and WT mice as determined by DAB-enhanced Prussian blue stain; as a positive control, marked iron accumulation was observed in Cp^{-/-} mouse liver.¹²

Ceruloplasmin ferroxidase activity oxidises toxic ferrous iron to the ferric form for binding and transport by transferrin, and by this mechanism ceruloplasmin can reduce free radical-mediated tissue injury in the nervous system.²⁵ Similarly, ceruloplasmin could exert antioxidant activity in the colon and limit the extent of free radical-induced oxidation at inflammation sites. We tested this hypothesis by determining protein carbonylation following colitis induction. Protein carbonyl content in homogenised colon supernatants was determined by spectrophotometric detection of hydrazone produced by reaction between 2,4-dinitrophenylhydrazine and protein carbonyl groups. Inflamed colon of DSS-treated

Cp^{-/-} mice on day 9 had approximately a threefold higher protein carbonyl content than WT controls (figure 5B), which suggests ceruloplasmin might reduce experimental colitis by inhibiting protein oxidation.

Bone marrow-derived macrophage ceruloplasmin reduces severity of experimental colitis in Cp^{-/-} mice

Purified human ceruloplasmin intraperitoneally administered into Cp^{-/-} mice has a half-life of approximately 30 h, and repeated injection at 48-h intervals substantially restores serum iron, transferrin saturation and iron homeostasis.¹²²⁶ Moreover, injected ceruloplasmin localises in intestinal tissues. We attempted to rescue Cp^{-/-} mice from experimental colitis by repeated injection of purified human ceruloplasmin approximately reflecting liver synthesis and release of ceruloplasmin. Unexpectedly, restoration of circulating ceruloplasmin in Cp^{-/-} mice failed to reduce DSS colitis severity as shown by severe weight loss and morbidity (figure 6A).

We considered the possibility that colon ceruloplasmin might be derived from monocyte/macrophages, the principal extrahepatic source of ceruloplasmin,²⁷⁻²⁹ recruited to the inflamed colon. Bone marrow cells from Cp^{-/-} and WT donors were transferred by retroorbital sinus injection into lethally irradiated Cp^{-/-} and WT recipients in a crossover experimental design. After 6 weeks, chimeric mice were screened for genotype transference; blood cells of WT mice exhibited Cp^{-/-} genotype and the cells from Cp^{-/-} mice exhibited WT genotype, indicating engraftment of transferred bone marrow and complete replacement of host nucleated blood cells. After recovery, mice were treated with DSS. Remarkably, experimental colitis severity in chimeric mice was determined strictly by donor bone marrow cell genotype. Cp^{-/-} recipients given bone marrow from WT donors exhibited diminished weight loss and morbidity similar to WT recipients given bone marrow from WT donors (figure 6B), and to WT mice not subjected to transplantation. In contrast, WT recipient mice administered bone marrow from Cp^{-/-} donors lost weight rapidly and nearly all were moribund by day 14. Interestingly, the latter result confirms the results of the ceruloplasmin injection experiment as the recipients have WT levels of liver-derived plasma ceruloplasmin, but remain susceptible to DSS challenge. Complete blood analysis of day 9 chimeric mice showed that Cp^{-/-} recipients given WT bone marrow had a small decrease in haematocrit and low WBC counts compared with WT recipients given Cp^{-/-} bone marrow (figure 6C).

Bone marrow contains non-myeloid cells that possibly secrete ceruloplasmin. We therefore determined whether immunopurified macrophages rescue Cp^{-/-} mice from severe experimental colitis. Thioglycollate-elicited peritoneal macrophages from WT and Cp^{-/-} mice were enriched for F4/80 surface antigen by magnetic bead separation. Flow cytometry for F4/80 and CD11b surface antigens showed greater than 97% cell purity from both WT and Cp^{-/-} mice. Cp^{-/-} recipient mice injected with macrophages from Cp^{-/-} donors rapidly lost weight and became moribund by day 14, while Cp^{-/-} mice injected with WT macrophages survived DSS challenge despite endogenous Cp^{-/-} macrophages (figure 6D). The expression of colon ceruloplasmin by transferred macrophages was determined by quantitated reverse transcription PCR of RNA. Low-level ceruloplasmin mRNA was detected in mucosal isolates from untreated WT mice that increased approximately 10-fold upon induction of colitis ($p < 0.0001$, figure 6E). As expected, ceruloplasmin expression in Cp^{-/-} mice colon was essentially undetectable before and after colitis induction, but was reconstituted following injection of WT macrophages, indicating infiltrating macrophages as the principal ceruloplasmin source ($p < 0.01$).

Protective role of ceruloplasmin in chronic colitis

Chronic experimental colitis was induced by alternating 3% DSS freely available in drinking water with DSS-free water. $Cp^{-/-}$ mice lost weight more rapidly than WT controls (figure 7A). Approximately 40% of $Cp^{-/-}$ mice were moribund by the second cycle of DSS administration (day 21); in contrast, all WT controls survived the entire challenge ($p < 0.01$, figure 7B). After termination of the experiment, the colon of $Cp^{-/-}$ mice was significantly shorter than WT controls ($p < 0.01$, figure 7C), and exhibited much greater epithelial damage with barely detectable crypts, submucosal swelling and inflammatory cell infiltrates (figure 7D).

To investigate the possible involvement of ceruloplasmin in human IBD, ceruloplasmin was determined in colon from Crohn's disease, ulcerative colitis and control (non-IBD) subjects. As observed in mouse experimental colitis, ceruloplasmin expression in colon from controls was low and localised primarily in blood vessels. In contrast, ceruloplasmin expression in Crohn's disease and ulcerative colitis subjects was elevated, and found in inflammatory infiltrates as well as in blood vessels (figure 7E).

DISCUSSION

We show a protective role for ceruloplasmin in experimentally induced colitis, and that macrophages recruited to the inflammation site are the primary colon ceruloplasmin source. Our findings suggest that the antioxidant activity of macrophage-derived ceruloplasmin contributes to protection from chemically induced experimental colitis, and its absence is responsible for the severe inflammatory phenotype observed in $Cp^{-/-}$ mice subjected to sublethal doses of DSS.

The increase in plasma levels of ceruloplasmin during the acute-phase reaction suggests a possible anti-inflammatory function. At least three reported activities of ceruloplasmin are consistent with this function, namely, antioxidant, bactericidal and ferroxidase activities. The latter might reduce the availability of free iron, a limiting factor for bacterial growth. Our studies do not provide evidence for ceruloplasmin bactericidal or ferroxidase function in reducing experimental colitis. However, protein carbonylation, a measure of protein modification by reactive oxygen species,³⁰ is markedly elevated in $Cp^{-/-}$ mice, suggesting an important role of ceruloplasmin antioxidant activity. Elevated colon protein carbonylation in $Cp^{-/-}$ mice subjected to experimental colitis is consistent with the finding that antioxidants can reduce the onset and severity of ulcerative colitis.³¹ Our report is the first to show a role for ceruloplasmin antioxidant activity in colon pathology; however, substantial evidence for a protective antioxidant function of ceruloplasmin in other organs has been reported, particularly in the brain. A membrane-bound, glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by central nervous system astrocytes.³² Glycosylphosphatidylinositol-linked ceruloplasmin is encoded by an alternatively spliced variant of the ceruloplasmin gene, and thus is absent in $Cp^{-/-}$ mice and aceruloplasminaemic patients. Elevated protein carbonyl content has been observed in the brain of an aceruloplasminaemic patient suggesting that protein oxidation in astrocytes as a consequence of pathological accumulation of iron might contribute to neuronal injury.³³ Likewise, aceruloplasminaemic patients have increased lipid peroxidation in brain cortex and putamen.³⁴ Moreover, ceruloplasmin antioxidant activity might play an important role in preventing ceruloplasmin gene-independent neurodegenerative disorders, for example, Parkinson's and Alzheimer's diseases.⁶²⁵³⁵

We found that mice heterozygous for ceruloplasmin deletion, which have approximately half-normal plasma ceruloplasmin concentration,³⁶³⁷ exhibited slightly greater weight loss and statistically insignificant lower survival compared with WT controls upon induction of

experimental colitis. Homozygous ceruloplasmin-deficient mice have exacerbated inflammatory response; however, the disease does not develop spontaneously and requires induction by DSS. In our 10-year experience with $Cp^{-/-}$ mice, they do not spontaneously develop colitis or other inflammatory disorders. Despite massive iron accumulation in the brain, liver and other organs, there are no reports of spontaneous colitis in homozygous aceruloplasminaemic patients, explained partly by the extreme rarity of the genetic disorder.³⁸ More importantly, our studies indicate ceruloplasmin deficiency by itself is insufficient to induce colitis, but a primary injury such as that induced by DSS challenge, is required. As expected, heterozygous ceruloplasmin-deficient patients with half-normal plasma ceruloplasmin are generally asymptomatic or occasionally develop mild neurological dysfunction.^{39,40} Decreased serum ceruloplasmin is characteristic of Wilson's disease patients, an autosomal recessive genetic disorder caused by mutations in the Wilson's disease gene (*ATP7B*) required for copper loading into ceruloplasmin.⁴¹ Although 5% of Wilson's disease patients exhibit plasma ceruloplasmin concentrations within the normal range (0.2–0.5 g/l, mean 0.3 g/l), approximately half exhibit very low plasma ceruloplasmin concentrations, less than 0.05 g/l.^{42,43} Overrepresentation of ulcerative colitis in Wilson's disease patients has not been reported.⁴⁴ However, in one case report, a Wilson's disease patient with a ceruloplasmin level of 0.029 g/l developed severe ulcerative colitis intractable to prednisolone and salazosulfapyridine, suggesting ceruloplasmin deficiency might adversely contribute to the inflammatory process.⁴⁵ Clinical studies are consistent with the concept that ceruloplasmin deficiency alone is insufficient to induce colitis, but complete or near-complete absence exacerbates the inflammatory status of the gut, and accelerates colitis progression following triggering by other factors, for example, toxicity, stress, or infection.

Acute DSS-induced colitis is generally accepted to be T-cell independent as indicated by a study in severe combined immunodeficient mice.⁴⁶ Ceruloplasmin is thus unlikely to exert anti-inflammatory activity via modulation of a T-cell response. Moreover, immunohistochemical staining for CD3+ T cells did not reveal elevated infiltration in $Cp^{-/-}$ mice colons throughout the acute phase of DSS-induced colitis (not shown). In contrast, experiments by us and others suggest that macrophages play an important protective role in colitis. Macrophage-depleted mice exhibit more severe DSS-induced colitis compared with mice with an intact macrophage population.⁴⁷ Moreover, treatment with granulocyte macrophage colony-stimulating factor, which stimulates innate immunity by inducing macrophage polarisation, lessens DSS colitis severity in mice.^{48,49} Our transplantation experiments confirm the anti-inflammatory property of macrophages in experimental colitis. IFN- γ (Ito *et al*;⁵⁰ our data not shown) and TNF α (figure 3E) are elevated in colon culture supernatants of DSS-challenged mice; both cytokines induce macrophage ceruloplasmin expression⁹ and are likely mediators of ceruloplasmin expression in experimental colitis.

In summary, our results indicate that ceruloplasmin synthesised by infiltrating macrophages plays an important anti-inflammatory role in IBD. According to one plausible mechanism, ceruloplasmin generated by macrophages recruited to the intestinal inflammation site contributes to the prevention of tissue oxidation and damage. Analogous to our results, bone marrow transplantation or transfer of in-vivo and in-vitro-stimulated macrophages effectively reduce experimental colitis.^{48,51–53} However, ours is the first study to show that the transfer of unstimulated WT macrophages into a genetically deficient recipient can fully restore the anti-inflammatory function of monocytic cells in experimental colitis, and should be considered as a potential therapeutic modality in human IBD.

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Significance of this study

What is already known about this subject?

- Macrophages infiltrating into the intestinal mucosa maintain homeostasis by negatively regulating immune responses triggered by commensal bacteria.
- The plasma level of ceruloplasmin, an acute-phase plasma protein made principally by hepatocytes, but also by activated myeloid cells, nearly doubles in response to inflammation, trauma, or infection.
- Ceruloplasmin exerts a potent antioxidant activity by means of its ferroxidase activity that diminishes ferrous ion-mediated production of reactive oxygen species.

What are the new findings?

- $Cp^{-/-}$ mice subjected to sublethal doses of DSS develop severe inflammatory phenotype.
- The antioxidant activity of macrophage-derived ceruloplasmin contributes to protection from chemically induced experimental colitis.
- Injection of ceruloplasmin into a ceruloplasmin-deficient recipient does not restore protection against experimental colitis; however, transfer of unstimulated, WT macrophages into $Cp^{-/-}$ mice fully restores protective activity.

How might they impact on clinical practice in the foreseeable future?

- Ceruloplasmin deficiency should be considered a potential risk factor in human ulcerative colitis.
- Macrophage transfer represents a potential therapeutic modality in the treatment of severe ulcerative colitis.

Figure 1.

Severe experimentally induced colitis in ceruloplasmin-deficient mice. Ceruloplasmin null ($Cp^{-/-}$), $Cp^{+/-}$ and $Cp^{+/+}$ mice in C57BL/6J background were continuously administered 3% dextran sodium sulphate (DSS) freely available in drinking water. (A) Normalised weight loss (n=5 mice per time point, * $p=0.006$, ** $p=0.02$, # $p<0.001$, compared with $Cp^{+/+}$ control; Student's t test). (B) Percentage survival (n=14 mice per group, * $p<0.0001$ compared with $Cp^{+/+}$ control, ** $p<0.001$, log-rank test). (C) Immunohistochemical analysis of ceruloplasmin in colon rolls from wild-type (WT) mice (middle and distal colon) and $Cp^{-/-}$ (distal only) on days 0 and 9 of DSS administration (50 $\times$ magnification).

Figure 2.

Blood parameters in ceruloplasmin null (Cp^{-/-}) mice following experimentally induced colitis. Cp^{-/-} and wild-type (WT) mice were freely administered 3% dextran sodium sulphate. (A) Normalised weight loss 0 (n=10 mice per time point per group, *p=0.005, **p<0.001, Student's t test). (B) Percentage survival (n=20 mice, *p<0.0001 by log-rank test). (C) Colonic bleeding score (n=5 mice per time point, *p<0.001, Student's t test). (D) Percentage change in haematocrit (left), red blood cell count (centre), and serum haemoglobin (normalised to day 0, left) (n=3 per time point, *p<0.05, Student's t test). (E) Representative images of Cp^{-/-} and WT colon on day 9. (F) Colon length of Cp^{-/-} and WT mice on day 9 (mean±SEM, n=5 mice, p<0.01 by Student's t test. p values are given for differences between Cp^{-/-} mice and WT controls.

Figure 3.

Increased inflammation in experimentally-induced colitis in ceruloplasmin null ($Cp^{-/-}$) mice. (A) Representative photomicrographs showing epithelial damage and colonic inflammation (H&E stain, 50× magnification) in wild-type (WT) and $Cp^{-/-}$ mice at days 0, 5, 7 and 9 of dextran sodium sulphate (DSS) administration. (B) Histopathological analysis of H&E stained colon tissue sections showing mucosal mast cell (MMC) hyperplasia, submucosal swelling, inflammatory infiltrate, and epithelial injury (mean±SEM, n=3 mice per time point per group, * $p<0.05$, Student's t test). (C) White blood cell (WBC), neutrophil, lymphocyte and monocyte counts in blood of WT and $Cp^{-/-}$ mice following DSS administration (mean±SEM, n=5 mice per time point, * $p<0.05$, Student's t test). (D) Immunohistochemical analysis of neutrophils (anti-neutrophil allotypic marker) and

macrophages (anti-F4/80) in WT and $Cp^{-/-}$ colon rolls on day 9 of DSS administration (50× magnification). (E) KC, monocyte chemoattractant protein 1 (MCP-1) and tumour necrosis factor α (TNF α) in colon culture supernatants (mean $\pm$ SEM, n=5 samples per time point, *p<0.05, Student's t test). p Values are given for differences between $Cp^{-/-}$ mice and WT controls.

Figure 4.

Depletion of intestinal microflora in ceruloplasmin null (Cp^{-/-}) mice does not prevent dextran sodium sulphate (DSS)-induced colitis. Cp^{-/-} and wild-type (WT) mice were treated with antibiotics for 4 weeks to generate microflora-depleted ^{dep}Cp^{-/-} and ^{dep}WT mice, respectively, and then with DSS. (A) Normalised weight change (n=5 per time point, * p<0.05). (B) Percentage survival (n=10 mice, *p<0.0001 for ^{dep}Cp^{-/-} compared with ^{dep}WT mice by log-rank test). (C) Change in haematocrit (HCT, normalised to day 0, n=5 mice, p=0.003 by Student's t test) and (D) white blood cell (WBC) count in WT and Cp^{-/-} mice at day 9 (mean±SEM, n=5 mice, p=0.004 by Student's t test). (E) H&E staining of WT and Cp^{-/-} colon sections on day 9 of DSS administration treatment (50× magnification). p Values indicate differences between microflora-depleted Cp^{-/-} mice and microflora-depleted WT controls.

Figure 5. Colon iron and protein carbonyl content in ceruloplasmin null (Cp^{-/-}) mice. (A) Prussian blue stain of wild-type (WT) and Cp^{-/-} colon sections on day 9 of dextran sodium sulphate administration. Liver sections were used as positive controls (100× magnification). (B) Protein carbonyl content in the colon of WT and Cp^{-/-} mice upon induction of experimental colitis (mean±SEM, n=4 per time point n=5 mice, *p<0.001 by Student's t test).

Figure 6.

Rescue of experimental colitis in ceruloplasmin null ($Cp^{-/-}$) mice by transplantation with wild-type (WT) macrophages. (A) Normalised weight loss (left) and survival (right) of WT and $Cp^{-/-}$ mice injected with human ceruloplasmin and subjected to 3% dextran sodium sulphate (DSS) administration (mean $\pm$ SEM, n=12, *p<0.05; **p<0.001, Student's t test; #p<0.0001, log-rank test). p Values represent differences between ceruloplasmin-injected $Cp^{-/-}$ mice compared with ceruloplasmin-injected WT controls. (B) Normalised weight loss (left, mean $\pm$ SEM, n=10 mice per group) and survival (right, n=10 mice per group, *p<0.05, **p<0.001, Student's t test; #p<0.02, by log-rank test) of lethally irradiated WT and $Cp^{-/-}$ mice reconstituted with bone marrow (BM)-derived cells. p Values calculated for differences between WT mice with $Cp^{-/-}$ bone marrow compared with $Cp^{-/-}$ mice with WT bone marrow. (C) Change in haematocrit (HCT) (left) and white blood cell

(WBC) count (right) in bone marrow-transplanted mice at day 9 (mean±SEM, n=5 mice, *p<0.05, Student's t test). (D) Weight loss (left) and survival (right) of Cp^{-/-} mice injected with purified thioglycollate-elicited peritoneal macrophages from Cp^{-/-} and WT (mean ±SEM, n=10 mice per group, *p<0.05, **p<0.001, Student's t test; #p<0.002 by log-rank test). p Values indicate differences between WT mice injected with Cp^{-/-} macrophages compared with Cp^{-/-} mice injected with WT macrophages. (E) Ceruloplasmin mRNA level determined by quantitated reverse transcription PCR and normalised to β-actin in colon mucosa of Cp^{-/-} and WT mice on days 0 and 9 of DSS administration (mean±SEM, n=10 mice, *p<0.0001, **p<0.001, Student's t test). PBS, phosphate-buffered saline.

Figure 7.

Role of ceruloplasmin in mouse chronic experimental colitis and ceruloplasmin expression in human inflammatory bowel disease (IBD) subjects. (A) Ceruloplasmin null (Cp^{-/-}) and wild-type (WT) mice were administered three 7-day cycles of 3% dextran sodium sulphate freely available in drinking water alternated with DSS-free water. Normalised weight loss (n=16 mice, *p<0.05 by Student's t test). (B) Survival of mice described in (A) (*p<0.01, log-rank test). (C) Colon length of Cp^{-/-} and WT mice (*p<0.01, Student's t test). (D) H&E stain of representative WT and Cp^{-/-} mice described in (A). p Values represent Cp^{-/-} mice compared with respective time point or curve of WT controls. (E) Immunohistochemical analysis of colon ceruloplasmin in ulcerative colitis, Crohn's disease and control subjects (50× magnification).