INFLAMMATORY BOWEL DISEASE

Crucial role of the melanocortin receptor MC1R in experimental colitis

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Gut 2006;55:1415–1422. doi: 10.1136/gut.2005.083634

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Revised version received
26 February 2006
Accepted for publication
28 February 2006
Published online first
16 March 2006

Background and aims: α -Melanocyte stimulating hormone (α MSH) is known to exert anti-inflammatory effects, for example in murine DSS (dextran sodium sulphate induced) colitis. The anti-inflammatory functions of α MSH are mediated by the melanocortin1-receptor (MC1R) in an autoregulatory loop. The aim of this study was therefore to determine whether a breakdown of the α MSH–MC1R pathway leads to worsening of disease.

Methods: Experimental colitis was induced in mice with a frameshift mutation in the MC1R gene (MC1Re/e), C57BL/6 wild type mice, and MC1Re/e-C57BL/6 bone marrow chimeras. The course of inflammation was monitored by weight loss, histological changes in the colon, and myeloperoxidase activity. In addition, MC1R expression was analysed in intestinal epithelial cells.

Results: While the colon of untreated MC1Re/e appeared normal, the course of DSS-colitis in MC1Re/e mice was dramatically aggravated, with a significantly higher weight loss and marked histological changes compared to C57BL/6WT. The inflammation eventually led to death in all MC1Re/e, while all C57BL/6WT survived. Similar observations were detected in a transmissible murine colitis model induced by *Citrobacter rodentium*. Infected MC1Re/e showed delayed clearance of infection. To determine whether missing haematopoietic cell expressed MC1R was responsible, DSS colitis was induced in MC1Re/e-C57BL/6 bone marrow chimeras. MC1Re/e mice receiving MC1R+ bone marrow showed a similar course of inflammation to non-transplanted MC1Re/e. Likewise, transplantation of MC1R bone marrow into C57BL/6WT mice did not lead to any worsening of disease.

Conclusions: This is the first study to show a functional role of MC1R in intestinal inflammation. The data suggest a pivotal role of non-haematopoietic cell expressed MC1R in the host's response to pathogenic stimuli.

Alpha melanocyte stimulating hormone (α MSH) is an endogenous neuroimmunomodulatory tridecapeptide that is derived from the cleavage of a larger precursor molecule, pro-opiomelanocortin (POMC).¹ While it has originally been discovered to be expressed in the pituitary gland, it was subsequently detected in various different cell types—for example, melanocytes, monocytes, B cells, natural killer cells, a subset of cytotoxic T cells, and epithelial cells.^{2–9} Although named for its influence on pigmentation, recent studies have revealed a broad spectrum of functions as an antipyretic, antimicrobial, anti-inflammatory, and immunomodulatory peptide,^{10–13} interacting with various types of cells (macrophages, neutrophils, and epidermal cells) in down-regulating either the production or the action of proinflammatory cytokines such as interleukin 1 (IL1), tumour necrosis factor α (TNF α), and IL6.^{11–17} Regarding intestinal inflammation Rajora *et al* were able to show that α MSH has the ability to modulate dextran sodium sulphate (DSS) induced colitis, a murine model of inflammatory bowel disease, in an anti-inflammatory fashion in mice¹⁸, attributing the effect in part to a marked decrease in TNF α and nitric oxide production.

The actions of α MSH are known to be transmitted by activating seven transmembrane domain G-protein coupled receptors, of which five different subtypes have been identified so far, termed melanocortin receptor MC1–5R.¹⁹ Many of the anti-inflammatory and immunomodulatory activities of α MSH are thought to be mediated through binding to the MC1R.^{15–17–20–21} Among all cloned melanocortin receptors, the MC1 receptor has the highest affinity for

α MSH.²² It has been shown to be expressed by a variety of cells—for example, macrophages and monocytes,¹⁴ a subset of cytotoxic T cells,⁸ dendritic cells,²¹ neutrophils,¹⁷ endothelial cells,²³ mast cells,²⁴ fibroblasts,²⁵ and goblet cells in the duodenum.²⁶ Several studies have suggested that α MSH, after binding to its receptor, exerts its anti-inflammatory and immunomodulating effects by inhibiting nuclear transcription factor- κ B (NF κ B) activation^{27–30} and by protection against I κ B α degradation.³¹ These intracellular events would produce a reduction in the expression of proinflammatory cytokines and adhesion molecules, thereby affecting the humoral and cellular phases of inflammation.^{32–34} In this context, the inflammatory activity of cells such as macrophages and glial cells seems to be mediated through an endogenous circuit that depends on α MSH and melanocortin receptors, as these cells express both the peptide and receptor.³⁵ Despite the vast amount of convincing data on MC1R expression and function in studies carried out in vitro, relatively little has been demonstrated in vivo. So far it has been shown that a functional MC1 receptor is not required under normal circumstances for survival, as mice with a non-functional MC1 receptor due to a frameshift mutation between transmembrane domain IV and V appear to have a normal phenotype apart from having a yellow coat.³⁶

Abbreviations: α MSH, α -melanocyte stimulating hormone; DSS, dextran sodium sulphate; EPEC, enteropathogenic *Escherichia coli*; MC1R, melanocortin 1 receptor; MPO, myeloperoxidase POMC, pro-opiomelanocortin

Using this mouse model, we addressed the question of whether a breakdown of the autoregulatory loop of α MSH and its cognate MC1 receptor, while apparently not required for the healthy intestinal immune system, could lead to a worsening of disease.

METHODS

Animals

The mice used in this model were C57BL/6 wild type mice (C57BL/6 WT)—obtained from Charles River, Germany—and melanocortin-1-receptor mutant mice (MC1Re/e) on a C57BL/6 background. MC1Re/e mice have a frameshift mutation between exon 4 and 5 and are lacking a functional melanocortin-1-receptor.³⁶ The MC1Re/e mice were a generous gift of Roger D Cone, Vollum Institute and the Center for Weight Regulation and Associated Disorders, Oregon Health and Science University, Portland, Oregon, USA. The mice for the experiments described here were bred/raised by Charles River Laboratories, Germany. In initial experiments no major sex specific differences could be detected in the course of the colitis or its severity, so the experiments described here were done using female mice for practical reasons only. For bone marrow transplantation, C57BL/6 Ly5.1 mice (obtained from Charles River, Belgium) were used to allow chimera control. All animals were female and six to eight weeks of age at the beginning of the studies. They were kept under pathogen-free conditions at 24°C with a controlled 12 h day-night cycle and free access to standard diet and drinking water. All animal studies were approved by the local animal subjects committee, University of Muenster (permit G92/2002).

Bone marrow chimeras

C57BL/6 Ly5.1 and MC1Re/e mice were killed and the femora and tibiae removed. Bone marrow was recovered with Hank's balanced salt solution without calcium and magnesium (BSS, Invitrogen Corporation, UK). This cell suspension was washed three times with BSS. C57BL/6 Ly5.1 and MC1Re/e recipient mice were exposed to a total irradiation of 11 Gy divided into four radiation cycles during two subsequent days. MC1Re/e mice received C57BL/6 Ly5.1 bone marrow cells, while C57BL/6 Ly5.1 mice received MC1Re/e bone marrow cells. The transplantation was undertaken one day after the last radiation by injection of the bone marrow cell suspension (2×10^6 cells/mouse) into the tail vein. Chimera control by FACS (fluorescent antibody cell sorting) using Ly5.1 antibodies (BD Biosciences Pharmingen, San Diego, California, USA) verified transplantation efficacy. The success of the irradiation was also verified by detecting the number of leucocytes on days 4, 14, 28, and 35 after transplantation. Leucocytes were counted after staining with Turk's solution in a Neubauer counting chamber.

The chimeric mice in the text are named as follows: C57BL/6 Ly5.1 mice with haematopoietic MC1Re/e cells are called WT $\times$ MC1R and MC1Re/e mice with C57BL/6 Ly5.1 haematopoietic cells are called MC1R $\times$ WT.

Only mice with a chimerism of greater than 75% were used for experiments. The transplanted animals and two further control groups were kept under pathogen-free conditions and received cotrimoxazol (200 mg/l) as antibiotic in their drinking water for three consecutive weeks. To rule out transplantation specific alterations of disease, autologous transplants were used as control groups, where MC1Re/e bone marrow was transplanted into MC1Re/e mice, named MC1R $\times$ MC1R, and C57BL/6 bone marrow was transplanted into C57BL/6 mice, named WT $\times$ WT in the respective passage. The chimeras were created using the same protocol as described above for bone marrow chimeras.

Figure 1 (A) Weight loss in response to dextran sodium sulphate (DSS). Both MC1Re/e and C57BL/6 WT mice received 2.5% wt/vol DSS in their drinking water, and inflammation was monitored by daily measurement of individual weights. Data are means with SD, $n = 11$ for C57BL/6 WT mice and $n = 10$ for MC1Re/e mice at the beginning of the experiment. The experiment was repeated three times with similar results. Overall weight loss between the two groups was significantly different ($p < 0.001$; two way ANOVA). Asterisks depict significant differences ($p < 0.05$) between groups at given days of treatment. (B) Lethality in response to DSS. In the same setting as for (A), DSS colitis led to increased lethality in MC1Re/e mice, while all C57BL/6 WT mice survived the treatment with DSS (2.5% for six days). Evaluation with a log rank test showed a significantly increased risk of death in the MC1Re/e mice (* $p < 0.001$).

DSS colitis

C57BL/6 WT, MC1Re/e, WT $\times$ MC1R, and MC1R $\times$ WT mice were given 2.5% dextran sodium sulphate salt (DSS, ICN Biomedicals Inc, Eschwege, Germany) in drinking water for six days. Consumption of drinking water was monitored for each group. To assess the disease activity, mice were controlled daily for body weight and rectal bleeding using standard haemoccult tests (Roche Diagnostics, Germany). On day 6 of DSS treatment all mice were killed and the colon removed. Colon length was measured.

The organs were opened longitudinally, embedded as "swiss rolls" in OCT compound (Tissue-Tek, Sukura Fine Tek Europe, Netherlands) and kept frozen at -80°C until further use. Sections of 5 μm thickness were stained with haematoxylin and eosin. Histological analysis—which was carried out by two investigators blinded to the experimental group from which the given tissue section was derived (CM

Figure 2 Histological analysis of colonic tissue. Untreated MC1Re/e mice (C) showed a normal colonic phenotype, as did C57BL/6 mice used as control (A). After the induction of acute dextran sodium sulphate (DSS) colitis, a strong epithelial disintegration, multiple ulcerations, immune cell infiltrates, oedema, and epithelial denudation could be detected in MC1Re/e mice (D). In comparison, histological changes in the colon of C57BL/6 WT mice were less severe, showing fewer ulcerations and denudations, and a less pronounced inflammatory cell infiltrate (B). A representative example of each group is shown, with $n=11$ for C57BL/6 WT and $n=5$ for MC1Re/e per group.

and KK)—focused on epithelial denudations, ulcerations, oedema, and leucocyte infiltrates.

Analysis of myeloperoxidase levels

Myeloperoxidase (MPO) activity, as an indicator of leucocyte accumulation, was measured in samples of colonic tissue taken from the distal colon. After snap freezing in liquid nitrogen, tissue samples were homogenised and afterwards resuspended in 500 μ l of 100 mM NaCl, 20 mM Tris pH 7.5, and 0.1% Triton X-100 (Sigma Chemical Co, Germany). The homogenates were centrifuged at 15 300 $\times g$ for 20 minutes and the supernatant removed for MPO assay; 10 μ l of supernatant were added to 200 μ l of 50 mM phosphate buffer, pH 6.0, containing 0.4 mg/ml of substrate O-phenylenediamide (Sigma Chemical Co, St Louis, Missouri, USA) and 10 μ l H_2O_2 . After 20 minutes 50 μ l of 0.4 mM H_2SO_4 were added to stop the reaction. Absorbance was measured at 460 nm and enzyme activity calculated using a standard curve.

Immunoblotting

For preparation of intestinal epithelial cells the colon was removed at the end of the experiment, opened longitudinally, the intestinal contents carefully removed, and the colon then cut into small sections which were placed into CMF solution (0.2% fetal calf serum, 50 mM HEPES, 50 mM $NaHCO_3$, pH 7.2). Tissue samples were washed in CMF solution at least four times, after which the epithelial layer was removed by incubation in EDTA solution (10% fetal calf serum, 50 mM HEPES, 50 mM $NaHCO_3$, and 50 mM EDTA, pH 7.2) for 30 minutes at 37°C on a rocking table, followed by 30 seconds of rapid shaking. The supernatant was then transferred, centrifuged, and the pellet resuspended in 3 ml 40% Percoll, which was carefully placed on 2 ml 100% Percoll. The suspension was then centrifuged at 15°C for 15 minutes at 1700 $\times g$, after which the top phase containing the epithelial

cells was removed, washed, and pelleted. The epithelial cell pellet then was lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X (all Sigma) on ice and sonicated for 15 seconds. Lysates were cleared by centrifugation, and total protein concentration determined by Bradford assay (BioRad, Hercules, California, USA). After this, 30 μ g of total cellular protein were size separated on 7.5% SDS-PAGE gel, blotted onto nitrocellulose membrane (Amersham Pharmacia Biotech, Arlington Heights, Illinois, USA), and blocked with blocking buffer (phosphate buffered saline (PBS) containing 10% (wt/vol) non-fat dry milk and 1% (wt/vol) bovine serum albumin (BSA)). Blots were incubated with polyclonal rabbit anti-murine MC1R antibody (Chemicon, Temecula, California, USA) overnight at 4°C. Immunodetection was carried out using biotinylated goat anti-rabbit antibody (BD Biosciences Pharmingen) for one hour at room temperature, followed by streptavidin horseradish peroxidase and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Citrobacter rodentium induced colitis

Citrobacter rodentium induced transmissible murine colitis was induced as described recently.³⁷ Briefly, *C. rodentium* was grown overnight in Luria-Bertani broth (LB) at 37°C, harvested by centrifugation, and resuspended in fresh LB medium at a concentration of 2.5×10^9 /ml.

C57BL/6 WT and MC1Re/e mice were infected with 200 μ l of the bacterial suspension (5×10^8 bacteria) by oral gavage. Fecal pellets were collected from individual mice, weighed, and homogenised in 1 ml sterile PBS (PAA Laboratories GmbH, Austria). Serial dilutions of the homogenates were plated onto MacConkey Agar. After overnight incubation at 37°C, colonies of *Citrobacter* were counted.

The identity of representative colonies was verified by polymerase chain reaction (PCR). Bacterial colonies were picked with a sterile inoculating loop and suspended in 50 μ l

Figure 3 Myeloperoxidase activity in colonic tissue. After a 6-day treatment course with 2.5% dextran sodium sulphate (DSS), mice were killed and colonic tissue was removed. The colonic tissue was divided into several small parts, which were used for total protein isolation. Myeloperoxidase (MPO) activity determined in these samples of colonic tissue showed a significantly higher MPO activity in MC1Re/e than in C57BL/6 WT mice (* $p=0.011$; Student's t test; $n=11$ for C57BL/6 WT; $n=5$ for MC1Re/e). In contrast, no significant difference could be detected comparing MPO activity in samples of colonic tissue from non-DSS treated C57BL/6 and MC1Re/e animals.

water; 5 μ l of this suspension were directly added to standard PCR, containing the primers 5'-AAG TCT GTC AAT ACC GCC TC-3' (sense) and 5'-AAT GTG CCA ACT GTC TCA TC-3' (anti-sense). These primers amplify a 95 bp PCR product of the *C. rodentium espB* gene. The amplification profile was 35 cycles of one minute denaturation at 95°C and 2.5 minutes annealing and extension at 53°C. The resulting amplicons were separated on a 1% agarose gel and the amplified bands viewed through DNA binding to ethidium bromide under ultraviolet light.

Statistical analysis

For statistical analysis of body weight, average body weight and standard deviation were determined for each day. To test for significance of the results Student's t test and analysis of variance (ANOVA) were applied where suitable. Survival of animals was evaluated with the help of the log rank test. Probability (p) values of less than 0.05 were considered significant.

RESULTS

Aggravated course of DSS induced colitis in MC1Re/e v C57BL/6 WT mice

Murine colitis was induced by application of 2.5% DSS in drinking water for six days. The average body weight of the animals (mean (SD)) was 18.0 (1.26) g at the beginning of the experiment before starting DSS. Progressive weight loss was observed, starting on day 4 of DSS application for MC1Re/e mice and on day 5 for C57BL/6 WT mice. The overall weight loss between the two groups was significantly different as determined by two way ANOVA ($p<0.001$; fig 1A). Haematochezia was observed starting on day 2 of DSS application, with a slight but not significant predominance in MC1Re/e mice. During the last two days of the experiment all MC1Re/e mice had macroscopic signs of rectal bleeding, while C57BL/6 WT mice did not.

With respect to lethality, one MC1Re/e mouse died on day 5 and four other animals from this group on day 6 of the experiment, while all C57BL/6 WT mice survived the DSS treatment (fig 1B). Evaluation of lethality with log rank tests showed a highly significant increased risk of death in the MC1Re/e mice ($p<0.001$).

Figure 4 Course of dextran sodium sulphate (DSS) colitis in MC1Re/e-C57BL/6 bone marrow chimeras. MC1Re/e-C57BL/6 bone marrow chimeras were generated as described in Methods. Mice were treated with 2.5% wt/vol DSS in drinking water for a total of six days, and the course of inflammation was monitored by daily measurement of individual weights. Data are means with SE; $n=5$ mice per group and time point. Overall weight loss was significantly different between groups ($p<0.05$, two way ANOVA) with exception of WTxWT v WTxMC1R. Analysis of differences between groups at given days indicate statistical significance ($p<0.05$) between WTxWT and MC1RxWT, and between MC1RxWT and WTxMC1R from day 3 onwards; between MC1RxMC1R and WTxWT and between MC1RxMC1R and WTxMC1R from day 4 onwards; and between MC1RxMC1R and MC1RxWT on day 6 only.

Figure 5 Myeloperoxidase (MPO) activity in colonic tissue of MC1Re/e-C57BL/6 bone marrow chimeras. Total protein was isolated from distal segments of colonic tissue of the mice used for the experiment shown in fig 4, and subjected to analysis of MPO activity. There were no significant differences between the bone marrow chimeras and their respective controls (MC1RxMC1R v MC1RxWT and WTxWT v WTxMC1R; $p>0.05$), while MPO activity was significantly increased in MC1RxWT mice compared with WTxMC1R bone marrow chimeras (* $p<0.05$; one way ANOVA).

After six days of DSS treatment all animals were killed. Further histological analysis of colonic tissue on day 6 after start of DSS treatment in MC1Re/e mice revealed a strong epithelial disintegration with ulcerations, oedema, immune cell infiltrates, muscular thickening, and wide areas of epithelial denudation (fig 2). By comparison, colonic tissue from C57BL/6 WT mice showed fewer signs of epithelial damage with less ulceration, cellular infiltration, and oedema.

To characterise the inflammatory process further we determined MPO concentration as a marker for neutrophil accumulation (fig 3). In accordance with the histological

findings of increased and more severe intestinal inflammation in MC1R^{e/e} mice, the activity of myeloperoxidase was significantly increased about fourfold in MC1R^{e/e} mice. Importantly, MPO activity in non-inflamed colon of MC1R^{e/e} mice was comparable to the activity in non-inflamed colon of C57BL/6 WT mice (C57BL/6, 2.0 (0.8) U/l; MC1R^{e/e}, 2.2 (1.2) U/l; $n = 4$ per group (NS)).

Protective role of mesenchymal cell expressed MC1R

To assess whether the reduced host defence of MC1R^{e/e} mice in DSS colitis is mediated by mesenchymal rather than haematopoietic cells, we created WT^xMC1R and MC1R^xWT chimeras (for definitions see Methods). Determination of leucocyte numbers verified that irradiation was sufficient. Leucocyte counts before irradiation were 2767 (450)/ μ l cells in C57BL/6 Ly5.1 mice, and 3317 (1207)/ μ l cells in MC1R^{e/e} mice. Four days after irradiation and transplantation of bone marrow cells, leucocyte counts had decreased to 59 (57)/ μ l in WT^xMC1R mice and 100 (81)/ μ l in MC1R^xWT mice. By day 35 after the transplantation, leucocyte numbers had returned to 1400 (270)/ μ l in WT^xMC1R chimeras, and 2200 (435)/ μ l in MC1R^xWT mice. FACS analysis for Ly5.1 showed a chimerism of more than 75% in all animals used in the experiments. Murine colitis was induced by 2.5% DSS in drinking water for a total of six days. The two groups of bone marrow chimeras, as well as the autologous control animals used to rule out transplantation specific alterations of disease (see Methods), had an average body weight of 19.0 (1.4) g at the start of DSS application. By day 4 of DSS application, body weight in both MC1R^xMC1R and MC1R^xWT animals started to decrease progressively (fig 4). WT^xWT and WT^xMC1R animals had a detectable loss of body weight starting at day 5 of DSS application. By day 6 of the experiment, body weight in MC1R^xMC1R control mice had decreased to 81.8 (3.4)%, while that of WT^xWT control mice was still at 91.9 (2.0)%. Body weight of MC1R^xWT chimeras had decreased to 76.52 (1.0)% of their initial body weight, while that of WT^xMC1R chimeras was 89.5 (4.3)%. There were no significant differences between WT^xWT control mice and WT^xMC1R chimeras, while weight loss between MC1R^xWT chimeras and MC1R^xMC1R control animals was only significant on day 6 ($p < 0.05$).

By day four of DSS treatment haematochezia was observed in all animals, with a slightly stronger prevalence in MC1R^xMC1R control animals and MC1R^xWT chimeras.

Compared with untreated animals, colon length had decreased to 66.4 (3.3)% in MC1R^xMC1R control animals, while it was 84.4 (3.9)% in WT^xWT control animals. MC1R^xWT chimeras had 67.3 (3.3)% of the original colon length, while WT^xMC1R chimeras had 75.0 (6.4)%. The colon

length of MC1R^xMC1R control mice decreased significantly more than that of WT^xWT control mice ($p = 0.001$). There was no significant difference in colon length between bone marrow chimeras and non-chimeric animals ($p = 0.72$ for MC1R^{e/e} and $p = 0.07$ for WT animals). Furthermore, in accordance with the weight curve, analysis of MPO activity in colonic tissue showed no significant difference between MC1R^xWT and MC1R^xMC1R control animals on the one hand and WT^xMC1R and WT^xWT control animals on the other, while MPO activity between MC1R^xWT and WT^xMC1R animals turned out to be significant (fig 5).

These data therefore suggest that non-haematopoietic cell-expressed MC1 receptor is crucial for its role in intestinal host defence. To identify potential MC1R expressing non-haematopoietic cells, epithelial cells were isolated from murine colonic tissue and MC1R expression detected by immunoblot. As shown in fig 6, murine colonic epithelial cells constitutively expressed MC1R protein. Furthermore, this expression appeared to be unaffected in intestinal epithelial cells of mice subjected to DSS colitis as no significant changes in expression levels could be detected. This is in accordance with our findings for human intestinal epithelial cell lines—for example, Caco-2, HT29, and T84—which all show constitutive MC1R mRNA as well as protein expression, which remain unchanged in response to stimulation with proinflammatory cytokines such as interleukin 1 β , tumour necrosis factor α , and interferon γ (data not shown).

Citrobacter rodentium induced colitis is prolonged in MC1R^{e/e} mice

After showing a crucial role for MC1R in a murine model of inflammatory bowel disease, we next addressed the question whether mucosal MC1R expression is also required for defence against intestinal bacterial infection. We thereby took advantage of the *C. rodentium*-induced transmissible murine colitis, a murine model of human enteropathogenic *Escherichia coli* (EPEC) infection. For this, C57BL/6 WT and MC1R^{e/e} mice were infected with 5×10^8 bacteria by oral gavage, after analysis of the feces in all mice had ensured that none of the animals was infected with *C. rodentium* before the start of the experiment. Five days after infection with *C. rodentium* all animals showed bacterial colonisation of the colon. The number of colony forming units (CFU)/g feces at this time point was 1.12×10^7 for C57BL6/wt and 2.08×10^8 for MC1R^{e/e} mice (fig 7A). By day 14, bacterial counts reached peak levels of 6.3×10^8 for C57BL/6 WT and 2.1×10^9 for MC1R^{e/e} mice, showing the expected course of infection, as described recently.³⁷ By day 18 after inoculation, C57BL/6 WT mice had no detectable levels of *C. rodentium* in their feces, while we detected 8.26×10^5 CFU/g in the fecal pellets of MC1R^{e/e} mice. This significant difference continued up to day 26, after which *C. rodentium* excretion levels fell below the detection level.

Supporting the difference in excretion of *C. rodentium*, histological analysis of colonic tissue in the same set of mice revealed a more severe crypt hyperplasia and mucosal oedema in the MC1R deficient mice (fig 7B). It therefore appears that intestinally expressed MC1R generally takes part in the host's defence against intestinal inflammation.

DISCUSSION

Melanocortin receptors have become a focus of intensive investigation in various fields of medicine in recent years. Binge eating has been shown to be the major phenotype of mutations of the melanocortin-4 receptor,³⁸ while normal signalling through MC4R appears to stimulate anorexigenic neural pathways on the one hand and to inhibit orexigenic pathways on the other. These findings are supported by murine studies showing that a targeted disruption of the

Figure 6 MC1R is constitutively expressed by intestinal epithelial cells. Intestinal epithelial cells from mice treated with 2.5% wt/vol dextran sodium sulphate (DSS) for five days and from control mice were isolated as described in Methods; 30 μ g total protein from each preparation were size separated on a 7.5% SDS-PAGE gel and MC1R expression (35 kDa protein) was detected using a polyclonal rabbit anti-murine MC1R antibody. Epithelial cells thereby show a constitutive MC1R expression with no significant changes in expression in response to DSS induced colitis (optical density (software image J): control group, 0.503 (0.121); DSS group, 0.806 (0.104), $p = 0.129$; Student's *t* test; $n = 3$ mice/group).

Figure 7 *Citrobacter rodentium* is more severe in MC1Re/e mice. (A) MC1Re/e and C57BL/6 mice were infected orally with 5×10^8 *C. rodentium*, and bacterial numbers in the stool were detected as indicated. Bacterial counts (colony forming units, CFU) thereby reached peak levels by day 14 post-inoculation. By day 18, C57BL/6 WT mice had cleared the infection, while *C. rodentium* was detectable up to day 26 in MC1Re/e mice. Data are means with SD; $n = 5$ mice per group and time point. (B) Colonic tissues from the same set of mice as in (A) were removed on day 26 and haematoxylin and eosin sections were subjected to histological analysis by two blinded investigators. MC1Re/e mice showed a significantly increased colonic hyperplasia and mucosal oedema compared with C57BL/6 mice. A representative example of each group is shown; (A) = C57BL/6, (B) = MC1Re/e; $n = 5$ mice per group.

melanocortin-4 receptor leads to obesity in mice.³⁹ In contrast, mice with a frameshift mutation in the MC1 receptor gene leading to a non-functioning receptor with the exception of a fair colour otherwise appear to have a normal phenotype. In this study we now describe the first functional role for MC1R in intestinal disease. A breakdown of the MC1 receptor signalling pathway leads to worsening of colitis in mice. This appears to be independent of the cause of colitis as both the chemically induced DSS colitis and infectious colitis induced by *C. rodentium* had a more severe course in the MC1R non-functioning mice.

Interestingly, studies with bone marrow chimeras suggest a major role of non-haematopoietic cell expressed MC1 receptor. This is supported by in vitro studies showing MC1R expression by endothelial cells²³ and goblet cells,²⁶ and by the expression by intestinal epithelial cells in the intestinal mucosa shown in the present study. However, macrophage/monocytic cells,^{14–15} lymphocytes with antigen presenting and cytotoxic functions,⁸ neutrophils,¹⁷ and peripheral blood derived dendritic cells²¹ were likewise found to express MC1R. One possible reason why mesenchymal cells play a

major role might be different MC1R expression levels. For example, flow cytometry studies showed that MC1R is expressed by in vitro activated monocytes/macrophages and by the THP-1 monocytic cell line at ratios of approximately one third to one fifth of that of melanoma cells. These studies, however, have not so far been done for intestinally expressed MC1R.

As a breakdown of the MC1R signalling pathway leads to worsening of intestinal inflammation one might speculate that treatment of intestinal inflammatory conditions such as inflammatory bowel disease with MC1 receptor agonists could lead to an improvement of disease. One potential agonist could be its natural agonist, the tridecapeptide α MSH. Supporting this idea Rajora *et al* were able to show an anti-inflammatory effect of α MSH in the DSS colitis model,¹⁸ a finding that we were able to reproduce. As many of the anti-inflammatory effects of α MSH are thought to be transmitted through the C-terminal tripeptide Lys-Pro-Val (KPV),^{40–41} this tripeptide could be an even less expensive and potentially less toxic alternative. Moreover, Szardenings *et al* have described two further potential MC1R agonists, termed

MS05 and MS09.⁴² These appear to downregulate the expression and secretion of endothelial cell selectin (E-selectin), vascular adhesion molecule (VCAM), and intracellular adhesion molecule (ICAM) in human dermal vascular endothelial cells treated with tumour necrosis factor α . The MC1R might therefore be an attractive target for new anti-inflammatory treatments in the intestine. However, recent data from Slominski *et al* on hair pigmentation should be taken into account as well when discussing potential MC1R ligands, as these workers were able to show that eumelanin hair pigmentation is preserved in proopiomelanocortin deficient mice on a nonagouti (a/a) genetic background.⁴³ These data would suggest that the mouse melanocortin receptor 1 might have sufficient basal activity to trigger and sustain melanogenesis in vivo or that in addition there are non-melanocortin pathways that are able to compensate in cases of melanocortin deficiency. Further studies are needed to evaluate whether similar regulations and pathways are involved in the anti-inflammatory potential of the melanocortin system as well.

Furthermore the MC1 receptor gene is a major determinant of human pigmentation, and specific allelic variants have been associated with red hair and sun sensitive skin types^{44–46} as well as an increased skin cancer risk in white individuals.^{47–48} As an MC1R frameshift mutation, as demonstrated in this study, leads to a significant aggravation of intestinal inflammation in intestinal inflammation models, one might speculate that specific allelic variants of the MC1 receptor gene might also be associated with a greater risk of developing inflammatory bowel disease. This is especially intriguing as the MC1 receptor gene is located on human chromosome 16, the same chromosome for which a definitive susceptibility gene for Crohn's disease—namely the NOD2/CARD15 gene—has been identified.^{49–50} It therefore appears to be advisable to study MC1 receptor mutations as well as allelic variants in the context of association with inflammatory bowel disease.

The participation of the melanocortin system in the intestinal host response is a good example of how hormone-like peptides can play a relevant role in the intestinal immune system. Future studies, however, are required to further define the actual role of this system in the pathogenesis of inflammatory bowel disease in humans.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Eli and Edythe L Broad Foundation (IBD-0038). We thank S Jansen and E Weber for expert technical support and B Sonntag for statistical support.

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Conflict of interest: None declared.

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EDITOR'S QUIZ: GI SNAPSHOT

Weight loss and an abdominal mass after resection of a lung carcinoma

Robin Spiller, Editor

Clinical presentation

A 47 year old man underwent a right upper lobectomy with resection of the chest wall for pleomorphic carcinoma of the lung in July 2003. In January 2004, he had abdominal pain and anorexia. Gastrointestinal endoscopy showed no remarkable findings. On admission, body temperature was 37.3°C. The abdomen was flat but tenderness was present in the lower abdomen. There was no rebound tenderness or muscle guarding. Faecal occult blood test was negative. Laboratory studies showed a white blood cell count of 17600/mm³, haemoglobin 5.8 g/dl, and C reactive protein 10 mg/dl. Tumour markers were within normal limits. Abdominal computed tomography (CT) revealed an intra-abdominal lesion 8.2×7.9 cm in size (fig 1). No recurrence of lung cancer was observed on a chest CT. Intravenous cefmetazole sodium 4 g/day and catheter drainage were performed. Contrast study from the drainage tube revealed a connection between the mass and the small bowel. Cytological examination of abscess fluid was benign.

Question

What is the diagnosis?

See page 1528 for answer

This case is submitted by:

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Figure 1 Abdominal computed tomography. The size of the mass was 8.2×7.9 cm. It contained fluid and air.

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doi: 10.1136/gut.2005.090035