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Clinical Trials Study

Melanocortin 3,5 receptors immunohistochemical expression in colonic mucosa of inflammatory bowel disease patients: A matter of disease activity?

Gravina AG *et al.* MC_3R and MC_5R in IBD

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Abstract

BACKGROUND

Melanocortin 3 and 5 receptors (i.e., MC_3R and MC_5R) belong to the melanocortin family. However, data regarding their role in inflammatory bowel diseases (IBD) are currently unavailable.

AIM

This study aims to ascertain their expression profiles in the colonic mucosa of Crohn's disease (CD) and ulcerative colitis (UC), aligning them with IBD disease endoscopic and histologic activity.

METHODS

Colonic mucosal biopsies from CD/UC patients were sampled, and immunohistochemical analyses were conducted to evaluate the expression of MC_3R and MC_5R. Colonic sampling was performed on both traits with endoscopic scores (Mayo

endoscopic score and Crohn's disease endoscopic index of severity) consistent with inflamed mucosa and not consistent with disease activity (i.e., normal appearing mucosa).

RESULTS

In both CD and UC inflamed mucosa, MC_3R (CD: +7.7 fold *vs.* normal mucosa, $p < 0.01$; UC: +12 fold *vs.* normal mucosa, $p < 0.01$) and MC_5R (CD: +5.5 fold *vs* normal mucosa, $p < 0.01$; UC: +8.1 fold *vs* normal mucosa, $p < 0.01$) were significantly more expressed compared to normal mucosa.

CONCLUSION

MC_3R and MC_5R are expressed in the colon of IBD patients. Furthermore, expression may differ according to disease endoscopic activity, with a higher degree of expression in the traits affected by disease activity in both CD and UC, suggesting a potential use of these receptors in IBD pharmacology.

Key Words: Melanocortin 3 receptor; Melanocortin 5 receptor; Ulcerative colitis; Crohn's disease; Inflammatory bowel disease

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Core Tip: This study sought to examine the expression levels of Melanocortin 3 and 5 receptors (MC_3R and MC_5R) in the colons of individuals diagnosed with Crohn's disease (CD) and ulcerative colitis (UC). Analysis of tissue samples obtained from both inflamed and non-inflamed sections of the colon revealed a notable increase in the expression of both receptors within inflamed regions compared to non-inflamed areas,

with the extent of expression suggesting a potential association with the severity of disease activity. These findings imply that MC_3R and MC_5R may serve as potential targets for pharmacological interventions in the context of inflammatory bowel diseases.

INTRODUCTION

The etiology of inflammatory bowel diseases (IBD), predominantly encompassing Crohn's disease (CD) and ulcerative colitis (UC), remains enigmatic, primarily due to the intricate interplay of various factors and molecular pathways. Inhibitors of the tumor necrosis factor (TNF)-induced inflammatory pathway continue to constitute a significant component of the treatment protocols employed in current clinical practice [1,2]. In any case, various lines of evidence have demonstrated that the modulation of the melanocortin system can influence inflammatory pathways, thereby unveiling the therapeutic potential of melanocortins [3–6].

The melanocortin system constitutes a sophisticated and evolutionarily antique network of peptides, encompassing α, β, γ-Melanocyte-Stimulating-Hormone (MSH), and adrenocorticotropic hormone (ACTH). These peptides stem from a common protein precursor called pro-opiomelanocortin (POMC). [7]. It functions *via* five melanocortin receptors ($MC_{1-5}R$) that belong to the family of G protein-coupled receptor proteins, distributed diversely in both animals and humans. They are promiscuously activated by [Nle^4, $DPhe^7$]-α-MSH, α-MSH, β-MSH, γ-MSH, ACTH, and agouti-related protein [3]. The MC_1R receptor exhibits expression within a heterogeneous spectrum of cell types, encompassing fibroblasts, melanocytes, keratinocytes, neutrophils, monocytes, dendritic cells, B lymphocytes, gliocytes, endotheliocytes, and additionally, neoplastic cells. In the context of melanogenesis, it primarily contributes to the synthesis of eumelanin by activating the enzyme tyrosinase. Additionally, its expression is observed in the colon, where it plays a role in gut inflammation in rats [8,9].

In contrast, MC_2R, primarily expressed in the adrenal cortex and adipocytes, is implicated in steroid synthesis [7,10] and does not have a purported role in inflammation.

MC_3R displays a comprehensive expression profile within the central nervous system and immune cells, specifically in B lymphocytes and macrophages. Additionally, its presence extends to diverse anatomical locations in the rat, including the gut, heart, and placenta [7,11]. Its primary roles are associated with metabolic control and inflammatory response. MC_4R is located within the vagal nerve afferents to the stomach and small intestine, participating in postprandial functions in mice [7,12].

MC_5R exhibits a primarily widespread distribution and is implicated in the immunomodulation of B-T lymphocytic responses, along with the regulation of secretions from exocrine glands [7,13–19], with no clear available information on its localization and role at the intestinal level. The extent of expression of MC_3R and MC_5R in the colon and their roles in the intestinal microenvironment of IBD are not well understood.

This study aims to assess the immunohistochemical expression of MC_3R and MC_5R in the colons of patients with IBD and to determine whether there is a greater immunohistochemical expression in segments affected by the disease compared to those with apparently normal mucosa.

MATERIALS AND METHODS

Study design and setting

Patients exhibiting symptoms consistent with and suspected of having IBD (e.g., increased bowel movements, rectal bleeding, abdominal pain) and receiving a specialist recommendation for colonoscopy, subsequently diagnosed with IBD (either CD or UC), were included in the study. The study was carried out at the Hepatogastroenterology Division of the University of Campania Luigi Vanvitelli from January to December 2020. The research adhered to the principles of the Declaration of Helsinki and received approval from the Ethics Committee of the University of Campania Luigi Vanvitelli (protocol code 795 on December 23, 2019).

Inclusion and exclusion criteria

Several inclusion and exclusion criteria were established. IBD participants aged 18 or older, who were treatment-naïve for any IBD, were included. Specifically, patients were required not to have received conventional therapy (e.g., mesalazine, budesonide, conventional immunosuppressants like azathioprine, systemic-acting steroids), advanced therapies such as biologics (e.g., infliximab, adalimumab, ustekinumab), or small molecules (e.g., tofacitinib, filgotinib, upadacitinib).

Furthermore, to prevent study exclusion, all IBD diagnoses had to be confirmed by a pathologist in accordance with current guidelines [20].

Hospitalized patients or those with clinically significant infections (e.g., tuberculosis, SARS-CoV-2, HIV, *Clostridioides difficile*) within the preceding six months, or with clinical or instrumental evidence of neoplasm, were excluded. Additionally, patients with subsequent endoscopic and/or histological diagnoses of ischemic colitis, radiation colitis, microscopic colitis, undetermined colitis, colonic dysplasia, and benign or malignant colonic neoplasms were also excluded from our study. Patients presenting conditions that, according to the investigators' assessment, could potentially introduce bias into the study (for instance, individuals with decompensated comorbidities or those currently experiencing severe acute conditions such as decompensated diabetes or severe cardiovascular disease) were also excluded. Moreover, patients with other digestive diseases (e.g., celiac disease, autoimmune atrophic gastritis) were excluded. Individuals with known psychiatric disorders, legal incapacity to provide informed consent, or, lastly, those with any contraindication to undergoing colonoscopy were omitted.

Collected variables and colonic sampling

The following clinical-demographic variables were recorded: age (in years), gender, weight (in kg), height (in cm), body mass index (BMI, in kg/m^2), smoking status (i.e., active smoker or non-smoker), IBD type (i.e., CD or UC), and comorbidity.

During a colonoscopy (conducted for diagnostic purposes, as mentioned above, unrelated specifically to this study), in addition to the standard biopsies required by

current clinical practice (i.e., two segmental biopsies in the cecum, ascending, transverse, descending, sigmoid colon, and rectum) [21], additional mucosal biopsy samples were obtained for the study. Specifically, two biopsies were taken in colonic/rectal tracts displaying clear signs of mucosal inflammatory involvement (i.e., inflamed mucosa), and two in tracts with endoscopically normal-appearing mucosa. Consequently, for the study's sampling criteria, only patients with a Montreal classification for CD of L2 (colic) or L3 (ileocolonic) were included. In contrast, only patients with E1 (proctitis) or E2 (left colitis, distal colitis) were admitted for UC.

The difference between inflamed and normal-appearing mucosa was made using validated endoscopic scores to evaluate IBD [22]. In detail, in the case of suspected UC (continuous inflammatory involvement without skip lesions and with rectal involvement), the colonic mucosa was assessed using the Mayo endoscopic subscore at the endoscopic examination [23]. We performed biopsies on Mayo endoscopic subscore 0 compatible traits and Mayo endoscopic subscore ≥ 1 compatible tracts. Colonic mucosa was evaluated using the CD endoscopic index of severity (CDEIS) [24] for suspected CD (i.e., segmental colitis and ileum involvement at retrograde ileoscopy). Biopsies were conducted on tracts with CDEIS < 3 (compatible) and tracts with CDEIS ≥ 3 (consistent). The endoscopic procedures were carried out by a gastroenterologist experienced in IBD digestive endoscopy with extensive casuistry. The histological diagnosis of IBD (i.e., CD or UC) was established based on histological criteria validated by current European guidelines [20].

Samples management and immunohistochemistry evaluations

As previously mentioned, supplementary histological and immunohistochemical investigations were conducted on the latter. In addition to routine assessments on the same samples, these investigations aimed to identify the expression profiles of MC_3R and MC_5R. Following the collection of biopsy samples, they were fixed in formalin and subsequently embedded in paraffin. Sections of 5 µm were cut and then stained with haematoxylin-eosin for morphological evaluation. Two slides were chosen for each

patient, one representing healthy tissue and the other depicting the site of the inflammatory process. Immunohistochemical staining for anti-MC_3R and anti-MC_5R antibodies was carried out on these slides. For this purpose, sections with a thickness of 4-5 µm were prepared, and paraffin was removed using a xylene substitute (Hemo-De; Thermo-Fisher Scientific, Darmstadt, Germany). The immunohistochemistry procedure was conducted using the BenchMark Automated IHC/ISH slide staining system, following the manufacturer's instructions (BenchMark Ventana, Tucson, AZ, USA) [25].

In brief, tissue sections underwent sequential rehydration with ethanol gradient washes, pre-heating, and staining with haematoxylin and eosin. Citrate antigen retrieval was conducted by placing slides in citrate buffer (0.1 M citric acid monohydrate and 0.1 M sodium citrate; pH 6) in a water-filled steamer for 20 minutes. Endogenous peroxidase activity was quenched in a 3% hydrogen peroxide aqueous solution for 15 minutes, and non-specific antibody binding was inhibited by one-hour incubation at room temperature in a blocking solution (1% BSA, 0.2% powdered skim milk, 0.3% Triton-X 100 in PBS).

Sections were incubated with specific anti-MC3R (1:100 in blocking solution; ab140864, Abcam, UK) and anti-MC5R (1:100 in blocking solution; sc-28994, Santa Cruz, USA) antibodies, washed with PBS, incubated with biotin-conjugated secondary antibodies and avidin-biotin-peroxidase complex (DBA, Milan, Italy), and 3,3 diaminobenzidine (DAB) reaction was employed to visualize the specific antigens in each section [25]. Slides were counterstained with haematoxylin. Immunostaining analysis was conducted by an expert pathologist (intraobserver variability 5%). As no existing data on the immunohistochemical expression of MC_3R and MC_5R in colonic mucosa are available in the literature, all staining was assessed, with particular attention to the inflammatory infiltrate. The data are expressed as a percentage ± standard error of the mean (S.E.M.) of MC_3R or MC_5R positive cells relative to the total cells counted.

Statistical analysis

Descriptive statistics were employed for data presentation. Continuous variables were reported as the median (interquartile range). The receptor expression profile was presented as a percentage ± S.E.M. Data distribution was assessed to determine whether parametric or non-parametric tests were more appropriate. The comparison between ordinal and continuous variables across groups was conducted using the Mann-Whitney U-test.

The accepted level of statistical significance was set at a two-tailed p-value less than 0.05. IBM® SPSS® was utilized as the software for data analysis, while GraphPad Prism 9® was used for graph processing.

RESULTS

Characteristics of included patients

Forty-six patients underwent initial screening for inclusion criteria to be enrolled in the study; however, twenty did not subsequently meet the inclusion criteria, resulting in a final enrolment of twenty-six patients overall. Among the twenty excluded patients, the reasons for exclusion were as follows: three patients had a diagnosis of significant comorbidity (gastric cancer in one case and systemic sclerosis in two cases), in five cases, exclusion was dictated by the diagnosis of colonic dysplasia, in two cases by the diagnosis of non-specific colitis, in one case of ischemic colitis, and finally, nine patients had a completely negative colonoscopy.

Of the 26 patients finally enrolled, 13 (50%) had CD, and 13 (50%) had UC. The overall median age was 49.5 (40.75 – 69.5) years. Table 1 dysplays the clinical and demographic characteristics of included patients, and categorized on IBD type.

Microscopic examination of specimens with haematoxylin-eosin staining

In patients with CD, haematoxylin-eosin staining revealed surface erosion and de-epithelialisation in the colonic mucosa, particularly on the right side. This was accompanied by a dense chronic inflammatory infiltrate and histological activity

featuring cryptitis. Additionally, non-necrotising epithelioid granulomas were observed.

Conversely, in patients with UC, the colonic mucosa displayed erosions of the surface epithelium, characterized by a significant reduction in the glandular muciparous quota and distortion of crypt architecture. Within the lamina propria, a severe chronic inflammatory infiltrate exhibited histological activity, including cryptitis and cryptic microabscesses.

In all examined samples from individuals with IBD, no signs of epithelial dysplasia were evident (Figures 1-4).

Immunohistochemical expression of MC_3R and MC_5R is evident in the colon affected by IBD and could potentially vary based on endoscopic disease activity.

All patients, 100% (26/26), tested positive for MC_3R, and positivity was observed in all colonic samples within the segments affected by the disease (Figures 1 and 2). Interestingly, this positivity was not observed in the tracts where the disease was inactive (Figures 1 and 2).

Furthermore, immunohistochemistry for MC_3R in CD samples exhibited cytoplasmic staining at the level of both mononuclear and polymorphonuclear inflammatory infiltrates, with a significant prevalence in "pathological" (i.e., inflamed mucosa) slides compared to healthy tissue obtained from normal-appearing mucosa (fold: +7.7, $P < 0.01$ *vs* normal mucosa) (Figure 1).

Similarly, MC_3R labelling in UC samples was significantly higher in inflamed mucosa (fold: +12, $P < 0.01$ *vs* normal mucosa), particularly at the level of the rectal and sigmoidal tracts (Figure 2).

MC_5R tested positive in 22 out of 26 patients (84%), and like MC_3R, it exhibited positivity in tracts affected by both CD (Figure 3) and UC (Figure 4).

Similar to MC_3R, immunohistochemistry for MC_5R displayed cytoplasmic staining at the mononuclear and polymorphonuclear inflammatory infiltrate levels, with a significant prevalence in "pathological" slides compared to healthy tissue. Moreover, in

favorable cases, MC_5R demonstrated a higher intensity staining than MC_3R (CD fold: +5.5, $P < 0.01$ *vs* normal mucosa. UC fold: +8.1, $P < 0.01$ *vs* normal mucosa).

DISCUSSION

The present study suggests a distinctive expression pattern of MC_3R and MC_5R in the colorectum of patients with IBD. It proposes that their expression could be hypothetically linked to disease activity, indicating a heightened presence in segments of the large bowel affected by histological damage. To the best of our knowledge, such a profile of immunohistochemical expression has not been previously reported [7].

The current evidence on the expression profile of melanocortin receptors at the level of body tissues is, in fact, not yet wholly conclusive [7]. Still, it needs to be studied, and in particular, the evidence on the relationship between MC_3R and MC_5R receptors and IBD is scarce [9]. However, our results do not fully clarify the role of these two types of receptors in specific inflammatory pathogenesis. A mechanistic breakthrough is necessary to assess the mechanisms through which these receptors may exert immunomodulatory effects.

Numerous studies have previously highlighted the participation of MC_3R in inflammation. Furthermore, various molecules with the capability to engage with MC_3R have shown anti-inflammatory characteristics linked to this receptor. Specifically, fragments of ACTH that activate MC_3R have demonstrated the ability to inhibit cytokine synthesis in peritoneal macrophages, thereby indirectly impeding neutrophilic diapedesis [26]. Furthermore, at the cardiac level, where macrophages express MC_3R, the application of agonists during instances of acute myocardial infarction in mice has exhibited a protective function, even amidst reperfusion. This demonstration revealed that the protection was associated with a reduction in systemic and local inflammatory markers, including interleukin-1 and myeloperoxidase [27]. In other mice models, the same protective effects were identified in gouty arthritis [28]. The anti-inflammatory function of MC_3R has been substantiated in the context of metabolic syndrome in mice lacking MC_3R, regardless of weight gain [29].

The MC_5R receptor is predominantly linked to the modulation of immune-mediated inflammation and the initiation of the JAK2-mediated pathway [30]. This latter pathway has been effectively utilized pharmacologically for the treatment of UC [31].

MC_5R has been associated with ocular immunity, although its involvement in inflammatory responses remains incompletely understood. Selective agonists of MC_5R have demonstrated promising positive effects in conditions characterized by immune dysregulation [32].

Earlier studies have attempted to assess the pharmacological role of certain melanocortin receptors in IBD. This includes PL-8177 (a selective MC_1R ligand), which, in a study involving induced murine colitis, demonstrated the ability to reduce bowel inflammation with effects comparable to sulfasalazine [9].

Upon observing a noteworthy expression of the receptors in the impaired tissue, it is tempting to speculate that, from a translational standpoint, the expression of MC_3R and MC_5R may not correspond to an activity adequate for resolving colonic inflammation. Therefore, substantial stimulation with specific agonists may be necessary to overcome the burst of inflammation underlying IBD. This considers their reviewed role in fighting inflammation in several conditions [9,32–35].

The capacity of these receptors to respond to various endogenous agonists such as [Nle^4, $DPhe^7$]-α-MSH, α-MSH, and γ-MSH may underscore the importance of regarding these receptors as crucial responders to inflammation. However, Montero-Melendez [36], in a systematic review, has emphasized the concept that α-MSH can serve as an excellent illustration of "endogenous-based pro-resolving therapy." Unlike biological drugs targeted against a single entity, this molecule can simultaneously modulate interleukin-1β, prostaglandins, TNF-α, cell adhesion molecules, and inflammatory cells such as monocytes, macrophages, and neutrophils, as previously elucidated.

Given a well-determined receptor expression higher in patients with CD and UC samples, one would argue that new MC_3R-MC_5R drugs could be molecules in rectal and gastro-resistant oral formulations. However, this is a remote hypothesis that should be considered only after further preclinical studies demonstrate mechanistically a genuine

anti-inflammatory potential for IBD. It is known that a proportion of IBD patients experience primary failure or secondary loss of response to several lines of biological therapy. Therefore, as a consequence, the exploration of new therapeutic agents and, particularly, new therapeutic mechanisms for IBD is desirable. Presently, there are specific MC_3R and MC_5R agonists already developed and documented in the literature, which could be regarded as potential therapeutic agents. Specifically, DTrp-α-MSH and the macrocyclic peptide PG911 are well-established agonists at hMC_3R and hMC_5R, respectively [37,38].

In CD, the data in this study are, by its design, restricted to patients with disease extension L2 or L3 according to the Montreal classification and, in any case, exclusively within the colonic microenvironment. Consequently, it is important to consider this observation and the non-applicability of the data to ileal or ileojejunal locations or other non-colonic disease localizations. Therefore, a greater understanding of the expression of these receptors in the small intestine is absolutely necessary as an additional piece in this already limited research context. A preliminary study by Gantz *et al* [39] suggested MC_3R intestinal expression (specifically in the duodenum) through northern blot hybridization and polymerase chain reaction. However, subsequently, there haven't been robust studies that thoroughly detail the expression profiles of these two receptors in different gastrointestinal segments.

This study possesses several strengths. The majority of investigations concerning the melanocortin system and IBD have been conducted using pre-clinical cell models or mouse models [7]. The assessment of MC_3R-MC_5R expression profiles was conducted in treatment-naïve patients diagnosed with IBD for the first time and highly selected for comorbidities. This implies that the evaluation was carried out in patients with a diminished risk of bias attributable to prior treatments or comorbidities. However, this study is subject to several limitations. Firstly, the patient sample needs to be expanded to encompass individuals expressing varying levels of these receptors. Additionally, the correlation between these expression profiles and the IBD therapies undertaken by patients should be explored in future investigations. Moreover, it is noteworthy that

this study, despite its novelty, did not incorporate assessments through polymerase chain reaction or Western blot analysis. Subsequent studies should consider incorporating these techniques to thoroughly evaluate the expression of MC_3R and MC_5R. Nevertheless, the high specificity demonstrated by the MC_3R-MC_5R kits employed in our study leads us to presume that analyses of this nature would likely corroborate the observed expression profile at the colonic level.

Moreover, it could be intriguing from a translational standpoint to investigate whether there exists a correlation between MC_3R-MC_5R expression and endoscopic disease activity, assessed using validated tools and scores [22], aiming to identify a potential direct association. However, it is crucial to highlight that the sample size within our dataset impedes the undertaking of correlation analyses essential for revealing statistically significant findings.

Therefore, we await the execution of future, more extensive studies and randomized double-blind trials to test new melanocortin agonists and verify their immunomodulatory potentialities.

CONCLUSION

In conclusion, this study delves into the distinctive immunohistochemical expression patterns of MC_3R and MC_5R in the colorectum of patients with IBD. The observed expression of these receptors potentially appears to be linked to disease activity, with heightened presence in segments of the large bowel affected by histological damage. This unique expression profile has not been previously reported, indicating a novel avenue for exploration in the context of IBD and the melanocortin system.

However, the study has limitations, such as a small sample size and a lack of correlation analyses with endoscopic disease activity. Future investigations with larger cohorts, incorporating mechanicistics and molecular analyses like PCR and Western blot, could provide a more comprehensive understanding. Additionally, the observed expression patterns prompt consideration for the development of specific MC_3R and MC_5R ligands

as potential therapeutic agents for IBD, necessitating further exploration in preclinical and clinical settings.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), present complex etiologies. The modulation of the melanocortin system, involved in inflammatory pathways, offers therapeutic potential.

Research motivation

Understanding the immunohistochemical expression of melanocortin 3 and 5 receptors ($MC_{3,5}R$) in colonic mucosa of IBD patients is crucial for unraveling potential immunomodulatory roles.

Research objectives

To assess immunohistochemical expression patterns of MC_3R and MC_5R in colons of IBD patients, exploring associations with disease activity.

Research methods

A study involving treatment-naïve IBD patients was conducted. Biopsies were taken from inflamed and normal mucosa, and immunohistochemical staining for MC_3R and MC_5R was performed.

Research results

Both MC_3R and MC_5R exhibited significant positivity in inflamed mucosa compared to normal mucosa, suggesting a potential correlation with disease activity. The expression pattern was distinct in CD and UC samples.

Research conclusions

The study proposes a unique expression profile of MC_3R and MC_5R in IBD, indicating potential links to disease activity. However, limitations include a small sample size and a lack of correlation analyses with endoscopic disease activity.

Research perspectives

Future investigations with larger cohorts and mechanistic analyses, such as polymerase chain reaction and Western blot, are necessary for a comprehensive understanding. The observed expression patterns suggest potential avenues for developing specific MC_3R and MC_5R ligands as therapeutic agents for IBD, warranting exploration in preclinical and clinical settings.

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