Differences in MUC2 Gene Expression Based on the Clinical Severity of Colitis and the Degree of Histopathological Damage to the Colonic Mucosa in Colitis-induced Rat

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Abstract

BACKGROUND: Inflammatory bowel disease (IBD), such as Crohn’s disease (CD) and ulcerative colitis (UC), is characterized by intestinal inflammation and epithelial damage. Impaired mucosal barrier function mainly associated with thinning of the mucin layer may be the initial events underlying injury and inflammation in UC. Impaired expression of specific mucins is closely associated with IBD. MUC2 is a gene that produces mucin, which is predominant in the colon in humans and rats.

METHODS: This study is an experimental study with a posttest-only design. The sample comprised 16 colitis-induced rats. Induction of colitis was done by giving a solution of dextran sodium sulfate (DSS) 2.5% 1 mL/day orally for 7 days. MUC2 gene expression was measured by rTPCR. The clinical severity of colitis was classified based on the disease activity index (DAI) score. The degree of histopathological damage was classified based on the score of colonic histology observations. The statistical analysis was done by the Shapiro–Wilk normality test and continued with an independent samples t-test.

RESULTS: There were differences in MUC2 gene expression in mild and moderate colitis (1.81 vs. 2.99) but the difference was not significant (p > 0.05). MUC2 gene expression also differed in mild and severe histopathological damage degrees (2.32 vs. 2.1) but the difference was not significant (p > 0.05).

CONCLUSION: It was concluded in this study that MUC2 gene expression did not have significant differences based on the clinical severity of colitis and the degree of histopathological damage to the colonic mucosa in colitis-induced rats.

Introduction

Inflammatory bowel disease (IBD), such as Crohn’s disease (CD) and ulcerative colitis (UC), is a chronic and recurrent disorder of the gastrointestinal tract characterized by intestinal inflammation and epithelial damage. Both diseases are associated with significant morbidity and can have a major impact on a person’s quality of life and ability to work [1].

This disease is still a global health problem, especially in gastroenterology. The number of IBD patients continues to increase every year. It is estimated that there are over 160,000 patients with UC (approximately 100 per 100,000) and 40,000 patients with CD (approximately 27 per 100,000) [2]. The prevalence of IBD in Asian countries is lower, but it continues to increase with high urbanization. The highest incidence occurs in East Asia (Korea, Japan, China, and Hong Kong) and South Asia (India) [3].

Inflammatory bowel disease as chronic and recurrent can cause physical and psychosocial weakness in the patients and affect the burden on society through absenteeism from activities or work and high health costs. Over 2 million Europeans and 1.5 million North American residents suffer from IBD, with most health-care costs being spent on treatment [4].

Inflammatory bowel disease is a disease that shows hyperreactivity of the gastrointestinal mucosal immune system to its mucosal microbiota [5]. In genetically predisposed individuals, the interaction between exogenous factors (such as the composition of normal gut microbiota) and host endogenous factors (such as intestinal epithelial cell barrier function, innate, and adaptive immune function) causes a chronic condition in the form of impaired mucosal immune function which is further influenced by environmental factors (such as smoking and enteropathogens) so that IBD is considered an inappropriate immune response to the commensal microbiota in the gut, with or without an autoimmune component [6].
The epithelial layer and mucus in the intestines form a physical barrier that prevents toxins or harmful agents from damaging the underlying tissue. In this case, goblet cells have a significant role in producing mucus that helps in intestinal protection. This protective layer consists of a complex mixture of large and highly glycosylated proteins (mucins) and glycolipids that cover intestinal epithelial cells and is the site of attachment for bacteria that colonize the gut, as well as with lactic acid bacteria (LAB) which help promote human and animal health [7]. Among the human mucin genes, MUC2 and MUC3 are the predominant genes in the colon [8].

Impaired expression of specific mucins is closely related to gastrointestinal diseases such as Crohn's disease and ulcerative colitis, indicating the importance of these proteins in the gastrointestinal tract [8]. Impaired mucosal cell barrier function which causes permeability to macromolecules, increased bacterial invasion, and/or translocation is mainly associated with thinning of the mucin layer and may be the initial event of injury and inflammation in UC [9]. Based on the above background, the researchers wanted to see the differences in MUC2 gene expression based on the severity of colitis and histopathological features of the colon.

Materials and Methods

Animal model and experimental design

Male white rats (Rattus norvegicus) weighing between 170 and 240 g were used as experimental animals. Rats were placed in cages lined with husks to absorb feces. The cages were placed in a well-ventilated room at a temperature of 20–26°C. The cages are cleaned daily. Food is given 40–50 g/day/head and drinking is given ad libitum. Animals were adapted for 1 week before treatment. Induction of colitis was done by giving a 2.5% solution of dextran sulfate sodium (DSS) 1 mL orally every day for 7 days. After 7 days of DSS administration, the rats were then killed by pressing the neck while pulling it anteriorly (atlanto-occipital dislocation). Then, a laparotomy was immediately performed and colon tissue samples were taken for the examination of MUC2 gene expression and histopathological examination.

RNA isolation

As much as, 50–100 mg of rat colon tissue were put into an Eppendorf tube containing 500 L of triazol fluid. Then, 200 L of chloroform was added. The tube was inverted and incubated for 5 min at room temperature. 12,000 g was centrifuged at 4°C for 15 min. A total of 150 L of supernatant were transferred to a new sterile tube and 300 L of isopropanol was added, then incubated for 10 min at room temperature. Centrifugation was carried out again at 12,000 g, at 4°C for 10 min. Then, the supernatant was discarded, and the pellet was then washed with 350 L of 70% ethanol solution. 7500 g was recentrifuged at 4°C for 5 min. The supernatant portion was discarded and the pellet was dried for 10 min. The pellet was then added with 40 L of RNAse liquid. The isolated samples were stored at −20°C for further analysis.

cDNA synthesis

Before cDNA synthesis, the RNA concentration was measured from isolated samples using the nanodrop tool. After the RNA concentration is obtained, the volume of the sample to be used is calculated. In this study, 10 L of RNA samples were used, then 4 L of 5× enzyme, 1 L of reverse transcriptase, and 5 L of nuclease-free water (NSFW) were added. Then, cDNA was synthesized at a temperature of 25°C for 10 min, 42°C for 15 min, and 85°C for 5 min.

Real-time PCR

RT-PCR test was performed using Two-Step RT-PCR with SYBR® Green RT-PCR Reagents Kit (Biosystem). MUC2 Rat Forward Primer: 5’-ACC ACC ATT ACC ACC ACC TCA G-3’ and MUC2 Rat Reverse Primer: 5’-CGA TCA CCA CCA TTG CCA CTG-3’ (the temperature used was 95°C for 2 min for 1 cycle, followed by 95°C for 5 s, 60–65°C for 10 s, and 72°C for 5–20 s for 40 cycles).

Histopathological analysis

Histopathological examination was carried out on the colon of rats that had been induced with DSS for 7 days. Colonic tissue was fixed with 10% neutral buffered formaldehyde and continued with the process of dehydration, clearing, and paraffin embedding. Paraffin blocks were cut with a thickness of 4 μm and continued by staining with hematoxylin-eosin and ending with mounting. Then, the degree of mucosal damage and the presence of inflammatory cell infiltration were assessed. Mucosal damage was given a score of 0 if there was no damage/normal, a score of 1 if the damage was mild, a score of 2 if the damage was moderate, and a score of 3 if the damage was extensive. The presence of inflammatory cell infiltration was given a score of 0 if normal, a score of 1 if there was mild infiltration, a score of 2 if there was moderate infiltration, and a score of 3 if there was extensive infiltration. All scores were summed and then categorized as mild (score ≤2) and severe (score >2).

Evaluation of disease activity index (DAI)

In calculating the DAI score, an assessment
of weight loss, stool consistency, and the presence or absence of blood in the stool is carried out on the 7th day. Weight loss is calculated as 0 if there is no decrease or there is an increase in body weight, a score of 1 if a decrease of 1–5%, a score of 2 if a decrease of 5–10%, a score of 3 if a decrease of 10–15%, and a score of 4 if a decrease of >15%. Stool consistency was assessed as a score of 0 if formed and hard, a score of 1 if the stool was formed but soft, a score of 2 if the stool was soft, a score of 3 if there was mild (watery) diarrhea, and a score of 4 if there was real diarrhea. Rectal bleeding was calculated as a score of 0 if there was no blood, a score of 2 if occult blood was found, and a score of 4 if significant bleeding was found. All scores were added up and then categorized into mild (score ≤2) and severe (score >2).

**Study ethics**

This research has passed the ethical test and has been approved by the Ethics Committee of the Faculty of Medicine, Andalas University, Padang, with registration number: 575/UN.16.2/KEP-FK/2021.

**Statistical analysis**

Data are presented in the form of mean and elementary. Statistical analysis is used by the computer. The data normality test was performed using the Shapiro–Wilk method. Then, an independent sample t-test was analyzed to determine the differences in MUC2 gene expression based on the clinical severity of colitis and the degree of histopathological damage to the colon.

**Results**

**MUC2 gene expression in colitis-induced rats**

MUC2 gene expression in colitis-induced rats is shown in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>MUC2</th>
<th>Mean (SD)</th>
<th>Shapiro–Wilk</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2.25 (1.55)</td>
<td>0.66</td>
<td></td>
</tr>
</tbody>
</table>

From Table 1, it can be seen that the normality test using the Shapiro–Wilk method shows that the MUC2 gene expression data are normally distributed (p > 0.05). The average MUC2 gene expression in colitis-induced mice was 2.25.

The clinical severity of colitis based on the DAI score and the degree of histopathological damage to the colon was calculated and classified into mild and severe degrees. The results of histopathological observations of the rat colon induced by colitis, as shown in Figure 1, the degree of mucosal damage and inflammatory cell infiltration was calculated to be classified into mild and severe degrees.

**Differences of MUC2 gene expression based on the clinical severity of colitis**

The differences in MUC2 gene expression based on the clinical severity of colitis are shown in Table 2.

<table>
<thead>
<tr>
<th>Colitis grade (n)</th>
<th>MUC2 gene expression</th>
<th>Mean (SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild (10)</td>
<td>1.81 (1.1)</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Severe (6)</td>
<td>2.99 (2.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2 shows that there was a difference in MUC2 gene expression in mild and severe colitis, but the difference was not significant (p > 0.05).

**Differences in MUC2 gene expression based on the degree of histopathological damage to the colonic mucosa**

The differences in MUC2 gene expression based on the degree of histopathological damage to the colonic mucosa are shown in Table 3.

<table>
<thead>
<tr>
<th>Histopathological grade (n)</th>
<th>MUC2 gene expression</th>
<th>Mean (SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild (11)</td>
<td>2.32 (1.73)</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Severe (5)</td>
<td>2.10 (1.22)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3 shows that there was a difference in MUC2 gene expression in mild and severe histopathological damage of colonic mucosa, but the difference was not significant (p > 0.05).

**Differences in the degree of histopathological damage to the colon according to the clinical severity of colitis**

We analyzed the differences in the degree of histopathological damage to the colon according to the clinical severity of colitis using the Chi-square method.
Table 4: Differences in the degree of histopathological damage to the colon according to the clinical severity of colitis

<table>
<thead>
<tr>
<th>Colitis grade</th>
<th>Histopathological grade</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mild (n%)</td>
<td>Severe (n%)</td>
</tr>
<tr>
<td>Mid</td>
<td>7 (70%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>Severe</td>
<td>4 (66.7%)</td>
<td>2 (33.3%)</td>
</tr>
</tbody>
</table>

as shown in Table 4 and Figure 2.

Table 4 shows that the frequency of mild colitis is higher than that of severe colitis. In this study, there was no significant difference in the degree’s frequency of histopathological damage in the two degrees of colitis (p > 0.05).

Figure 2: Frequency of colitis grade and histopathological grade. (a) Colitis grade based on DAI score. (b) Histopathological grade based on mice with

Discussion

The intestinal epithelium is covered by a viscoelastic mucous layer whose main functions are to build a protective barrier against the harsh luminal environment (containing digestive enzymes), facilitate the passage of food, and prevent the strong attachment of bacteria to the epithelial cells preventing the entry of bacteria into the lamina propria. By limiting the interaction and penetration of bacteria, a healthy mucous layer plays an important role in preventing inflammation and infectious disease. Mucus in the gastrointestinal tract is produced by goblet cells in the intestinal epithelium and consists mainly of mucin [8].

In IBD, particularly ulcerative colitis, there is a discontinuity of the colonic mucosal barrier. Unlike healthy individuals, most patients with IBD have bacteria in the inner lining of the mucus, showing a breakdown of the barrier [10].

Differences of MUC2 gene expression based on the clinical severity of colitis

Mucins are high-molecular-weight glycoproteins and are divided into two groups: Secreted mucins (encoded by the MUC2, MUC5AC, MUC5B, and MUC6) genes which handle the formation of the mucous layer; and transmembrane mucins (MUC1, MUC4, MUC13, and MUC16) whose function is still little known but may be involved in signaling pathways. Among the human mucin genes, MUC2 and MUC3 are the predominant genes in the colon [8].

In this study, an analysis of differences in MUC2 gene expression was carried out based on the clinical severity of colitis assessed from the DAI score. We found that MUC2 gene expression was higher in severe than mild colitis (2.99 vs. 1.81), but the difference we found was not significant (p > 0.05). Research conducted by Wenzel et al. on mice with MUC2 deficiency showed that MUC2−/− mice would spontaneously develop symptoms such as changes in stool consistency and perianal swelling similar to symptoms of ulcerative colitis in humans [11].

Another study conducted by Sluis et al. compared wild-type mice (MUC2+/+), MUC2−/− mice, and MUC2−/− mice that were given DSS and then assessed colitis with DAI scores. They found that at the beginning of DSS administration, there was no significant difference in DAI scores between MUC2−/− mice and MUC2−/− mice, but on days 7 and 8, MUC2+/− mice showed significantly higher DAI scores than MUC2−/− mice. MUC2−/− mice had significantly higher DAI scores than MUC2−/− mice and MUC2−/− mice since the start of DSS administration [12].

A study by Peterson et al. found that during the induction of DSS colitis, mucin thickness decreased at the same time, the mice showed an increase in DAI scores. MUC2−/− mice will develop severe colitis rapidly. The DAI score of MUC2−/− mice was also significantly higher than wild-type mice [13]. This suggests that MUC2 deficiency can lead to the rapid onset of more severe colitis.

In our study, MUC2 expression was found to be higher at higher DAI scores or severe colitis compared to lower DAI scores or mild colitis. This finding is similar to that of Renes et al. who reported increased MUC2 expression in the proximal colon in colitis-induced mice with DSS [14]. In addition, in this study, there are more rats with a lower DAI score (10) than rats with a higher DAI score (6).

The loss of mucus barrier causes bacteria in the lumen of the colon to penetrate the lamina propria and even to the mesenteric lymph node (MLN). As a result of the penetration of these bacteria, inflammatory cell infiltration occurs. Infiltration of inflammatory cells causes an increase in pro-inflammatory cytokines which then lead to the presence of symptoms of inflammation in the colon [11]. DSS causes a decrease in goblet cells in the colonic mucosa, which may trigger an increase in MUC2 expression as a gut defense mechanism. If the disease progresses, MUC2 expression will decrease and the inflammation continues in UC [14].

Differences of MUC2 gene expression based on the degree of histopathological damage to the colonic mucosa

Research conducted by Wenzel et al.
comparing the histological changes of MUC2−/− mice with MUC2+/− mice found that MUC2−/− mice had a distorted and flattened epithelial structure, loss of lamina propria structure, cell infiltration, and superficial epithelial erosion that more protrudes to the distal of the colon [11]. Sluis et al. found that MUC2−/− mice were given DSS showed significantly higher histological damage scores than MUC2+/− mice based on crypt loss, infiltration, and ulcerations. This indicates that partial MUC2 deficiency may be a predisposing factor for colonic inflammation. MUC2−/− mice showed histopathological differences compared to MUC2+/− mice and MUC2−/− mice which were characterized by the presence of crypt abscess. It can be concluded that the loss of MUC2 in the gut causes abnormal morphological changes. It is characterized by increased thickness of the intestinal mucosa, flattening and ulceration of epithelial cells, general loss of architecture, mild increase in inflammatory cells, increased proliferation, and decreased differentiation of cells in the colon. Therefore, changes in mucus composition caused by MUC2 deficiency, can lead to colon inflammation, and contribute to the onset of IBD [12].

In this study, we found that there was no significant difference between MUC2 gene expression in mild histopathological damage and severe histopathological damage (p > 0.05), which were 2.32 versus 2.10, respectively. This may be due to the greater number of mice with mild histopathological damage than those with severe damage, which were 11 versus 5, respectively.

**Differences in the degree of histopathological damage to the colon according to the clinical severity of colitis**

The mechanism of DSS-induced colitis is thought to be due to disruption of the intestinal epithelial layer which causes luminal bacterial infiltration into the mucosa and causes colonic tissue inflammation. Various histological studies were conducted to determine changes in the structure of the colonic tissue due to DSS induction. In a study conducted by Xiaojuan et al., it was found that there were pathological changes in the colitis rat group which were characterized by damage to the goblet cell architecture in the colon, infiltration of inflammatory cells into the submucosal tissue [15]. In this study, we also found colonic mucosal damage and inflammatory cell infiltration in colitis-induced rats using DSS.

In this study, we analyzed the differences in the degree of histopathology according to the clinical severity of colitis. We categorized the histopathological score consisting of the degree of mucosal damage as well as inflammatory cell infiltration into two, mild grade with a total score of 2 or below and severe grade with a total score of >2. We found that mild histopathology had more frequency in both mild and severe colitis.

A study by Allenspach et al. compared histopathological scores with the Canine Inflammatory Bowel Disease Activity Index (CIBDAI) scores. There was a significant relationship between CIBDAI and histology scores in the colon of dogs diagnosed with idiopathic IBD. However, another study showed that improvement in clinical signs (CABDAI scores) in treated dogs with IBD was not accompanied by significant changes in histologic results. A separate study by Munster et al. did not find a strong correlation between treatment response (CABDAI clinical score) and the severity of the histopathological lesions of IBD [16].

**Conclusion**

From this study, it was concluded that MUC2 gene expression did not have a significant difference in the clinical severity of colitis and the degree of histopathological damage to the colonic mucosa in colitis-induced rats with DSS. In addition, the damage observed from the histopathological results will not always have an impact on the appearance of clinical symptoms, clinical symptoms may appear late, or even the clinical improvement appears before the histopathological improvement.

**References**


