Role of Macrophage Microaggregation in the Diagnosis of Inflammatory Colitis

Makrofaj Mikroagregasyonunun İnflamatuvar Kolit Tanısında Rolü

Özet
Amaç: Son dönemde yapılan çalışmalar, makrofaj mikroagregasyonunun (MMA) Crohn koliti (CC) tanısında kriter olabileceğini desteklemektedir. Bu çalışmada MMA'nın ülseratif kolit (UC) ve CC'nin ayrımını rollüne araştırık. Gereç ve Yöntem: Bu çalışmada 29 UC hastası, 26 CC hastası ve 22 infiamatuvar barsak hastalığı olmayan sağlıklı bireyden MMA'nın rolünü araşturduk. Biyopsi materyalleri MMA için immünohistokimyasal olarak boyandı. Ayrıca serum örneklerinden ELISA yöntemi ile perinükleer antinötrofil sitoplazmik antikor (pANCA) ve anti-Saccharomyces Cerevisiae antikor (ASCA) bakıldı. Bulgular: MMA değeri CC ve UC hastalarında kontrol grubundan yüksek bulundu (% 46.2, % 42.3, % 9.1). Hastalarla kontrol grubundan histopatolojik olarak Helicobacter pylori (H. pylori) varlığına bağıldı. H. pylori positivity was determined in 41.3% of MMA patients with CC, in 75% of patients with UC, and in 50% of healthy subjects. There was no significant difference between the three groups (p=0.344). Discussion: MMA positivity increases in patients with both CC and UC. In patients with inflammatory colitis, H. pylori existence, pANCA and ASCA positivity was similar to healthy subjects.

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Abstract
Aim: Recent studies have advocated that the presence of macrophage microaggregations (MMA) may be a criterion in the diagnosis of Crohn's colitis (CC). In our study we aimed to investigate the role of MMA to differentiate ulcerative colitis (UC) and (CC). Material and Method: We analyzed the role of MMA in 29 patients with UC, 26 patients with CC and 22 healthy subjects without diagnosis of inflammatory bowel disease. For all subjects, esophagogastroduodenoscopy was performed. Biopsies were taken from non-lesion regions of stomach and duodenum. Biopsy materials underwent immunohistochemical staining for the microscopic investigation of the presence of MMA. Also, determination of Perinuclear Anti-Neutrophil Cytoplasmic Antibodies (pANCA) and Anti-Saccharomyces Cerevisiae Antibodies (ASCA) (Immunoglobulin G and A) was done with ELISA in serum samples. In patient and control groups, presence of Helicobacter pylori (H. pylori) positivity was histopathologically evaluated. Results: MMA was higher in patients with both CC and UC compared with control groups (46.2%, 41.3%, and 9.1% respectively). There was statistically significant difference between the three groups (p=0.344). Discussion: MMA positivity increases in patients with both CC and UC. In patients with inflammatory colitis, H. pylori existence, pANCA and ASCA positivity was similar to healthy subjects.

Keywords
Macrophage Microaggregations; Ulcerative Colitis; Crohn's Disease; Esopha-gastroduodenoscopy; Helicobacter Pylori

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Introduction
Crohn’s disease (CD), and ulcerative colitis (UC) are among the idiopathic inflammatory bowel diseases (IBDs) that are characterized by inflammation and with no known etiologies [1], such as infections, medications, ischemia, or radiation. The characteristics and mechanisms of IBD’s have not been defined, yet. Studies indicate that the following factors may be involved in the etiology of these diseases: Genetic factors, autoimmune disorders (dysfunction of cellular immunity, modified neutrophil and T-cell suppressor activity, and monocyte macrophage system dysfunction), infectious agents (bacteria, especially Mycobacteria and viruses) [2], dietary habits [3], smoking [4,5], use of oral contraceptives [5,6], measles infection and live measles vaccine [7], appendectomy [8], and psychological factors [9]. Although the etiology of IBDs is not known, mechanism of tissue injury and nonspecific inflammatory mediator profiles are quite similar in these two diseases [1].

For diagnosis of IBD’s, especially differentiation between CD and UC, serum perinuclear anti-neutrophil cytoplasmic antibody (p-ANCA) and anti-saccaromyces cerevisiae antibody (ASCA) may be useful [10]. But, the sensitivity and specificity of these tests are not very high. Recently, the presence of macrophage microaggregations (MMA), determined by endoscopic biopsy, in the gastric and duodenal mucosa of patients with CC appears to be a promising method for the diagnosis of CC. MMA were determined in 54.5% of 22 patients with CC, but it was not found in any of the 23 UC patients. However, there are a few studies in the literature investigating the MMA on gastroduodenal mucosa in IBD’s [11]. The present study aims to investigate the presence of MMA, pANCA and ASCA in patients with IBD's and to determine their roles in the differentiation of CC from UC.

Material and Method
Patients
In this study, we evaluated patients diagnosed as UC and CC in our hospital between September 2001 and August 2005. Age of disease, history of previous treatment and concomitant diseases, smoking status, gastrointestinal symptoms including diarrhea, frequency of defecation, abdominal pain and discomfort, and weight loss were recorded. Physical activity, serum biochemistry and complete blood count were noted. Patients were diagnosed with clinical, radiological, lower gastrointestinal (GI) endoscopic, and histopathological findings. Patient population consisted of patients with UC and CC. The control group, however, consisted of 22 individuals who underwent colonoscopic examinations for various complaints and were not diagnosed with IBDs. Upper GI endoscopy with a Pentax FG-32X EPK 1000 video endoscopy was performed on all the participants. To investigate the presence of MMA and to perform histopathological analyses, at least 3 biopsies were taken with Pentax biopsy forceps from each of corpus-antrum and duodenum where no lesion exist. Ethical approval was obtained from local ethics committee of Gazi University School of medicine.

Histopathological Evaluation
Samples were formalin fixed for 24 hours and paraffin embedded by conventional techniques. Biopsy samples were fixed in formalin for 24 h. Sections in 5-μ-size were taken after a routine follow-up. Histopathological analyses were performed in hematoxylin-eosin (HE) sections. The identification of Helicobacter pylori (H.pylori) was evaluated in HE sections. The organisms were present in the superficial mucus layer and among the microvillus of the gastric epithelial cells.

Immunological Method
We took biopsies from antrum, corpus and duodenum and prepared them as 5-μ-sections in order to determine the presence of CD68 positive MMA (macrophage marker, clone KP1, mouse monoclonal antibody) positive cell immunohistochemically. Immunochemical staining was performed using the 3-stage indirect streptavidin-biotin immunoperoxidase method. Before application, the antibody was diluted 1:100 in PBS. The secondary antibody, streptavidin-biotin, and DAB (diaminobenzidintetrachlorid; LabVision Neomarkers, USA ) used in the study were all received as commercial kits. Thin sections (3-4 μ) were deparaffinized in an incubator at 56 °C for 12 h, kept in xylene for 30 min, dehydrated in 100 % 95 %, and 90 % alcohol for 5 min each, and then washed with tap water. In order to block the endogenous peroxidase, they were kept in 3 % hydrogen peroxide for 10 min at room temperature. The sections were washed with PBS buffer (pH 7.6) for 5 min and kept in protease at 37 °C for 25 min, and then washed with distilled water 3 times. Next, the sections were left in non-immune protein blocking serum for 20 min, and they were immersed into primary antibodies Ki67 (mouse IgG1, clone: MB67, Neomarkers) bcl-2a (Mouse IgG, clone: 100/D5, Neomarkers) and left at room temperature for 2 h. Later, they were washed with PBS twice for 5 min each. Then they were incubated in secondary antibodies (Multi-species Ultra Strepavidin detection system-HRP, Signet, Massachusetts, USA) for 20 min at room temperature, washed with PBS twice for 5 min each, treated with streptavidin-biotin complex for 30 min (neomarkers), and washed with PBS twice for 5 min each. In order to get an image by staining, they were incubated with DAB for 10 min, and washed with distilled water for 10 min. They were then stained with Mayer’s Hematoxylin using background quick staining technique, immersed in 90%, 95%, and 100% alcohol for 5 min each for dehydration, deparaffinized with xylene, and mounted in Entellan. As positive and negative tissue controls, tonsil tissues were used for CD68. All of the preparations were reexamined by two pathologists independently. Slight differences in interpretation were resolved by simultaneous viewing.

Serological Investigation Methods
1. p-ANCA and ASCA
There are 2 types of antibodies directed against human neutrophils: c-ANCA and p-ANCA. It was reported that myeloperoxidase(MPO) is the most important antigen for p-ANCA. In this study, p-ANCA levels were determined with the Trinity Biotech Captia TM (USA) MPO IgG, A and M ELISA kit. MPO is a lysosomal enzyme found in human neutrophils. ELISA kit which we used, determines the positivity of IgG, IgA and IgM antibodies against MPO. Patient serum was put into the wells coated with MPO antigen in 1:21 dilution, and left at room temperature for 30 min. Per instructions of the manufacturer for ELISA test, horseradish-peroxidase (HRP) conjugate and chromogen tetramethylbenzidine (TMB) were added in that
order. Optical densities (OD) were determined 3 times using a spectrophotometer (Bio-Tek EL x 800, USA) at 450 nm wavelength.

The average value of the calibrators was calculated using their mean OD values. Verification value for each test was obtained from the calibrator well. Cut off calibration value was obtained by multiplying the verification value with the average calibrator value. Index value for each sample was obtained by dividing its OD to the cut off calibration value. The results were calculated according to index value as <0.9, 0.91-1.09 and >1.10 which were negative, intermediate and positive, respectively.

ASCA values were determined using with Aeskulisa Crohn’s Check TM ELISA kit (USA), which measures the antibodies of IgA and IgG (ASCA) against the mannan obtained from Saccharomyces cerevisiae. Patients’ serums were kept in 1:101 diluted and mannan coated pits for 30 min at room temperature. Then the ELISA protocol was followed according to the instructions of manufacturers’ by adding HRP conjugate and TMB substrate. The pits were measured 3 times using spectrophotometer at 450 nm wavelength. Standard curve was plotted using each calibrator OD value (y axis) and the corresponding 7 U/ml value (x axis). To this end, for each patient’s OD value, a corresponding U/ml value was calculated. Any value ≤20 U/ml was accepted as negative, and > 20 U/ml was positive [12-14].

Statistical Analyses
Chi-square test was used to compare the patient groups and MMA positivity. To compare the groups, One-Way ANOVA and Tukey tests were used. The analyses were performed using SPSS 12.0 for Windows. The results were considered as significant if p value was < 0.05.

Results
The present study included 29 patients with UC and 26 patients with CC and the control group consisted of 22 healthy subjects without colitis and IBDs. The characteristics of the patients were indicated in Table 1.

Of the patients with UC, 18 were male and 11 female. The average age of patients with UC was 41 (20-62) years. The average age of the disease was 66 months (1-151 months). To assess clinical activity, Rachmilevitz’s clinical and endoscopic activity index was used [15], fourteen of the UC patients were found to be clinically active. Of the 26 patients with CC, 15 were male and 11 were female with an average age of 39 (20-49). The average disease age of the CC patients was 62 months (1-134 months). According to the Crohn's Disease Activity Index (CDAI), only 1 patient had active CC. The control group consisted of 12 male and 10 female participants with an average age of 36 (20-51).

The microscopic images of gastrointestinal tissue obtained from our patients were demonstrated in figure 1-3. The percents of MMA among the groups were shown in Figure 4. MMA was higher in patients with both CC and UC compared with the controls. The p values were significant if p value was < 0.05.

Table 1. The characteristics of the patients

<table>
<thead>
<tr>
<th></th>
<th>UC (n=29)</th>
<th>CC (n=26)</th>
<th>Controls (n=22)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age</td>
<td>41 (20-62)</td>
<td>59 (20-49)</td>
<td>36 (20-51)</td>
<td>0.276</td>
</tr>
<tr>
<td>Age of disease</td>
<td>66 (1-151)</td>
<td>62 (1-134)</td>
<td>(-)</td>
<td>0.507</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>18/11</td>
<td>15/11</td>
<td>12/10</td>
<td>0.118</td>
</tr>
<tr>
<td>CD68 positivityMMA</td>
<td>41.3%</td>
<td>46.2%</td>
<td>9.1%</td>
<td>0.007</td>
</tr>
<tr>
<td>pANCA</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td></td>
</tr>
<tr>
<td>ASCA IgA positivity</td>
<td>37.9%</td>
<td>38.4%</td>
<td>4.5%</td>
<td>0.009</td>
</tr>
<tr>
<td>ASCA IgG positivity</td>
<td>41.3%</td>
<td>46.1%</td>
<td>18.8%</td>
<td>0.104</td>
</tr>
<tr>
<td>H. pylori positivity in MMA (CD 68)</td>
<td>75%</td>
<td>41.3%</td>
<td>50%</td>
<td>0.344</td>
</tr>
</tbody>
</table>

UC: ulcerative colitis; CC: Crohn’s colitis; CD: cluster differentiation; MMA: macrophage microaggregation; p ANCA: perinuclear-antineutrophil cytoplasmic antibody; ASCA: anti-Saccharomyces Cerevisiae Antibodies (ASCA); Ig: immunoglobulin; H. pylori: Helicobacter pylori.
control groups (46.2%, 41.3%, and 9.1% respectively). Therefore, there was a significant relationship between the control group and CC and UC in terms of MMA but the same relation was not present between CC and UC groups (Table 1; p=0.007).

For ASCA IgG positivity, there was no significant relationship between three groups (p = 0.104). But, ASCA IgA positivity was different between the patient groups (p= 0.009). While the percentage of ASCA IgA positivity was 4.5% in control groups, it was 38.4% and 37.9% in patients with CC and UC. pANCA was negative for all patient groups.

H.pylori positivity was determined in 41.3% of MMA positive patients with CC, in 75% of patients with UC, and in 50% of healthy subjects. There was no significantly difference between 3 groups (p=0.344).

**Discussion**

Intestinal macrophages are an important part of mononuclear phagocytic system which contains acid phosphatase, non-specific enolase and CD 68. They are localized at the entrance point of antigens. Periethelital region of the small bowel and sub-epithelial dome of the Peyer’s plaques are some of the places that they are located. In these localizations, the first defense mechanism is formed against the pathogens found in the colon lumen [16,17]. In CC cases, considering the immune response system’s systematic response in which cellular unit plays a leading role, presence of inflammatory cells found somewhere (e.g. gastric and/or duodenal mucosa) other than the location where the disease is primarily localized must be checked out and their presence in such locations will support the diagnosis of the disease [16,17].

Recently, it has been reported that MMA found in gastroduodenal mucosa by immunochemical methods supports the diagnosis of CD. In the study, the authors showed that macroaggregate and granuloma formation occurred only in CD patients. While 54.5% of the 22 CC cases were diagnosed histologically, neither macrophage aggregates nor epithelioid cell granuloma were observed in none of the 23 UC patients. In the patients with colitis, presence of microaggregates was observed in non-inflamed gastroduodenal mucosa rather than the colon. It is remarkable that MMA were found higher than epithelioid cellular granuloma, which is assumed to be an important finding in differentiation of CD from UC [11].

In our study, we found that the rate of MMA positivity was higher in both CC and UC. But the rate was 9.1% of healthy people. For patients with CC, our results were similar to those of study of Yao et al [11]. The authors had no MMA positivity for UC patients, but we found 41.3% MMA positivity for patients with UC. In another study, 67% of the CC patients with endoscopically normal gastroduodenal mucosa were histologically shown to have microaggregates. According to these findings, researchers reported that in CD patients, noticeable inflammation or ulcer should be investigated in noninflamed gastroduodenal mucosa that is not close to the colon to provide minimal and specific findings, and they also reported that, especially in differentiating UC patients from CD patients, normal mucosa biopsies could be used [18]. In the mentioned study, the authors stated that MMA positivity might be useful histological marker for differentiating CC from UC. But, the results of our study showed that MMA positivity increases in patients with IBDs-associated colitis, but its value was limited for differential diagnosis of IBDs.

In the present study, all of the 45 patients (14 CD, 13 UC, and 18 healthy controls) were found to be pANCA and ASCA negative. On the other hand, ASCA, when evaluated with IgA and IgG subgroups, did not distinguish the two diseases. While ASCA IgG did not distinguish between the patient groups, ASCA IgA was found to be higher in the group with inflammation compared to the control group. However, it did not distinguish both CD and UC groups. As reported in literature, in CC patients, the rate of pANCA and ASCA negativity is very high. In our study, in the patients with pANCA and ASCA negativity and MMA in the gastroduodenal mucosa were found 42.8% in CD and 46.1% in UC. Microaggregates were not determined in the control group. In seronegative patients, formation of microaggregates distant to inflammation zones occurs, but it does not appear to be a distinguishing feature.

In the literature, pANCA positivity varies from 26 to 84% in patients with UC and from 4% to 25% in the CD patients [19]. The difference in the frequency of ANCA could be a result of methodology, localization of diseases, or fluctuations of ANCA [20]. The role of pANCA and ASCA evaluation in IBD diagnosis and differentiation is still imprecise and controversial [21]. The frequency of ASCA ranges between 39% and 61% in CD cases, and 6% and 15% in UC cases. Frequently, ANCA negativity and ASCA positivity indicate CD, but ANCA positivity and ASCA negativity point out UC [22]. On the other hand, we found that p-ANCA and ASCA were not useful for differentiating CC from UC.

In another study, similar to the present study, upper GI endoscopic biopsies were examined in patients with 75 CD, 7 UC, and 200 healthy controls. Diffuse CD68/CD68R histocyte infiltration was observed in the H.pylori positive control group. All of the UC patients, however, were H.pylori negative [23]. In our study, H.pylori positivity was determined in the mucosal biopsies of 41.3% of the CD patients with microaggregates, and 75% of the UC patients with microaggregates. In the control group, 1 of the 2 patients with microaggregates was H.pylori positive, and also 1 of the 12 H.pylori positive patients in the same group had microaggregates. Considering the patient groups in the present study, it is possible that H.pylori has no role in the formation of microaggregates. However, it is still not clear yet, and more
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There are some limitations of our study. Firstly, the sample size of our study is insufficient. Secondly, the timing of endoscopic evaluation of patients was very homogenous. But, the most importantly, the best time of endoscopic evaluation has not been described. It is unclear whether the targeted population for endoscopic evaluation is patients with active colitis and whether biopsy is performed from normal or inflamed tissue.

In conclusion, the results of our study showed that the percent of H. pylori was higher in patients with both CC and UC colitis than those healthy subjects. But the test was no surrogate marker for differentiation of IBDs-associated colitis. This study does not support the findings of very few studies reporting that macrophage aggregates in gastroduodenal mucosa indicate CD. This difference could be a result of several factors, such as geographical status, diet, environment, microbial agents, and H. pylori infection, which are not explained in the present study.

Further studies involving more participants and other related factors are needed to interpret this issue better.

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Competing interests
The authors declare that they have no competing interests.

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