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Intramucosal Calprotectin Expression in Inflammatory Bowel Disease (IBD) and Non-IBD Colorectal Inflammation

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Abstract

BACKGROUND: Inflammatory bowel disease (IBD) diagnosis remains a challenge accompanied with high numbers of misdiagnosis causing suboptimal management. Tons of trials have been conducted to improve the diagnostic accuracy, one of which is the use of biomarker such as calprotectin. Calprotectin can be detected in tissue (intramucosal) and is becoming a potential marker of IBD.

AIM: This study aims to determine intramucosal calprotectin expression in IBD, non-IBD colitis, and control.

METHODS: This analytic retrospective study included consecutively sampled IBD and non-IBD colitis colorectal biopsy specimens, and control group obtained from Cipto Mangunkusumo Hospital registered from 2017 to 2019. Cases were included in the study if specimens were indicative of IBD and non-IBD clinically and histopathologically and no abnormality were found histopathologically in the control group. Specimens with non-adequate data from the hospital medical records or with missing tissue slides were excluded from the study. Calprotectin immunostaining was conducted to evaluate mean intramucosal calprotectin expression (cell/HPF) in each group.

RESULTS: Most of the samples from IBD and non-IBD group (45 samples each) showed mild active inflammation. Mucosal calprotectin expression in aforementioned groups was higher than that of control group ($p < 0.001$). Subjects with active inflammation showed higher calprotectin expression compared to those with inactive inflammation ($p < 0.001$). Calprotectin expression was also related to activity grade.

CONCLUSION: Higher calprotectin expression showed significant association with the presence of inflammation and disease activity. However, the application of intramucosal calprotectin immunohistochemistry test to determine inflammatory etiology (IBD vs. non-IBD) still needs to be further evaluated.

Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing condition that can greatly affects patients' quality of life if not being properly assessed and treated. The diagnosis of IBD requires integration of clinical data, endoscopic, radiological, and laboratory findings. Difficulties to obtain the aforementioned information are due to limited access to medical facilities, unaffordable cost or other reasons, as well as lack of multidisciplinary coordination pose a huge challenge in the diagnostic attempt, not infrequently result in diagnostic error and suboptimal management [1], [2], [3].

Histopathology examination of colorectal biopsy specimen is one of the valuable diagnostic modalities. However, often the examination fails to conclude a specific diagnosis, due to diverse features and overlapping morphology between colitis of various etiologies [1], [2].

Histopathologic findings in IBD are related to disease onset, disease activity and ongoing treatment at the time of biopsy, hence the importance of clinical and endoscopic correlation, supported by other related ancillary tests. Unfortunately, we are regularly faced with cases of colitis with unavailable clinical information, complicating the diagnostic process [3].

Lots of trials have been conducted to diagnose IBD. Recently, one of the potential diagnostic modalities that have been under the spotlight is calprotectin test. Calprotectin is a biomarker expressed in inflammatory conditions including IBD. Calprotectin made up 50-60% of neutrophil intracytoplasmic proteins and is also detected in smaller amount in the macrophage and eosinophils. Calprotectin has proinflammatory, bactericidal, and immunoregulatory properties and plays a role as endogenous ligand for tissue damage [4], [5], [6], [7]. In inflammation, it is released by the inflammatory cells and can be detected directly in the associated tissue (i.e., intramucosal calprotectin), in blood (i.e., serum calprotectin) and also secreted through feces (i.e., faecal calprotectin). Fecal calprotectin is considered as a non-invasive and representative test to distinguish between organic

diseases, such as IBD, from functional disorder such as irritable bowel syndrome (IBS). Fecal calprotectin has become one of routine laboratory tests for patients with gastrointestinal complaints in some countries. However, fecal calprotectin has its own limitation since its value varies between individuals [4], [5], [8], [9], [10], [11], [12], [13], [14], [15], [16].

Intramucosal calprotectin can be detected in tissues using immunohistochemistry. The study of calprotectin expression directly within the tissue helps us to localize the foci of inflammation. Another advantage is that intramucosal calprotectin has a lower variability between individuals compared to fecal calprotectin. In addition to diagnostic value, it has a prognostic value in predicting long-term outcome in IBD patients [5].

To date, there is only a small number of studies regarding intramucosal calprotectin. Although the difference of expression of calprotectin between IBD and normal colorectal mucosa is already stated in some literature, it is yet to be measured in non-IBD colitis. This study aims to determine intramucosal calprotectin expression in IBD, non-IBD colitis, and control colorectal biopsy specimens.

Methods

Sampling method

This retrospective study included Formalin-Fixed, Paraffin-Embedded (FFPE) specimens registered in the archives of Anatomical Pathology Department, Cipto Mangunkusumo National Referral Hospital, Jakarta, Indonesia. The IBD and non-IBD colitis samples are biopsy specimens obtained from colon with or without ileum and rectum specimens. For IBD group, we collected clinical data and picked initial biopsy specimen registered from 2017 to 2019. Non-IBD samples were collected from colon biopsy specimens diagnosed as chronic active or inactive colitis registered in 2019. The control group consisted of the visually normal area of resected colorectal cancer specimens registered in 2019. The specimens were consecutively picked and evaluated for adequacy and inflammatory activity.

Immunohistochemistry procedure

We used primary monoclonal mouse anti-human macrophage antigen clone MAC387 antibody [AbCam], protein G purified, that can detect calprotectin molecule comprising 12kDa α-chain and a 14kDa β-chain, with 1:750 dilution. Positive control was Hodgkin lymphoma specimen.

Intramucosal calprotectin expression evaluation

Evaluation was carried out in 6 high power fields in mucosal areas with highest calprotectin expression. Positive expression defined as cytoplasmic staining of inflammatory cells regardless of the intensity. Positive cells in the specified areas were counted and divided by 6 to generate mean value (in cells/high power fields).

Results

Patient demographics

This retrospective study included 45 IBD samples, 45 non-IBD colitis samples, and 29 control samples (with one sample control group being dropped out due to specimen inadequacy). The patient demographics and inflammatory activity are presented in Table 1.

Table 1: Patient demographics and inflammatory activity

Variable	Inflammatory colitis		Control (n = 29)
	IBD (n = 45)	Non-IBD (n = 45)	
Patient demographics			
Age (years old, median [min-max])	48 (20–84)	48 (18–76)	53 (24–78)
<30	8 (17.8%)	7 (15.6%)	2 (6.9%)
31–40	6 (13.3%)	8 (17.8%)	4 (13.8%)
41–50	11 (24.4%)	10 (22.2%)	7 (24.1%)
51–60	12 (26.7%)	9 (20.0%)	6 (20.7%)
61–70	7 (15.6%)	9 (20.0%)	7 (24.1%)
>70	1 (2.2%)	2 (4.4%)	3 (10.3%)
Gender			
Male	15 (33.3%)	24 (53.3%)	17 (58.6%)
Female	30 (66.7%)	21 (46.7%)	12 (41.4%)
Morphology			
Inflammatory activity			
Active	27 (60.0%)	36 (80.0%)	n/a
Inactive	18 (40.0%)	9 (20.0%)	n/a
Activity grade*			
Mild	22 (81.5%)	28 (77.8%)	n/a
Moderate	2 (7.4%)	4 (11.1%)	n/a
Severe	3 (11.1%)	4 (11.1%)	n/a

*In population with active inflammation. IBD: Inflammatory bowel disease.

Most subjects showed active inflammation (60% in IBD group and 80% in non-IBD group) with mild activity grade. Histologic findings in IBD group varied, most of them were associated with chronic colitis and characterized by crypt distortion and/or atrophy. One IBD sample showed fibrosis. Non-IBD colitis group showed chronic active and inactive colitis, most of them with no known specific etiology. One sample showed granulomatous lesion and was diagnosed as tuberculous colitis. Amoeba infection was found in four samples.

Intramucosal calprotectin expression in IBD, non-IBD colitis, and control populations

Calprotectin was detected in cytoplasm of inflammatory cells, mostly in neutrophils, macrophages, and eosinophils (Figure 1)

Mean intramucosal calprotectin expression varied between the groups, with the highest number observed in non-IBD colitis group (Table 2 and Figure 2).

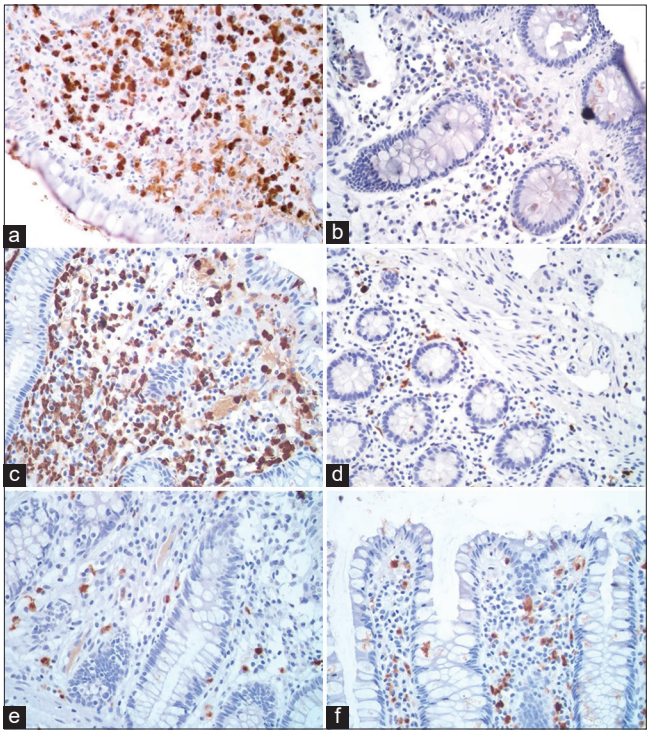

Figure 1: Intramucosal calprotectin expression. (a) IBD with active inflammation, (b) IBD with inactive inflammation, (c) non-IBD colitis with active inflammation, (d) non-IBD colitis with inactive inflammation, and (e and f) control group, H&E ×400

The mean differences of intramucosal calprotectin between three groups are summarized in Table 3.

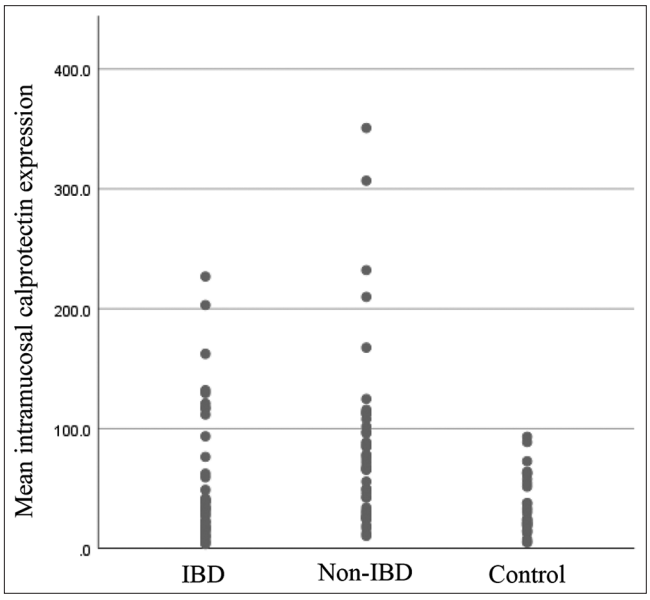

Figure 2: Mean intramucosal calprotectin expression in IBD, non-IBD colitis, and control group

The value differences IBD versus non-IBD colitis group, and non-IBD colitis versus control group were statistically significant.

Table 2: Mean intramucosal calprotectin expression in IBD, non-IBD colitis and control group

Variable	Inflammatory colitis		Control (n = 29)	p value
	IBD (n = 45)	Non-IBD (n = 45)		
Mean intramucosal calprotectin expression (cells/HPF)	53.1±53.7	83.5±72.2	35.6±24.5	<0.001*

HPF: High power field.

Table 3: Mean difference between three groups

	Mean difference	Confidence interval 95%		p value
		Minimum	Maximum	
IBD versus non-IBD colitis	−30.4	−0.46	−0.07	0.004
IBD versus control	17.5	−0.14	0.31	1.000
Non-IBD colitis versus control	47.9	0.13	0.57	<0.001

Mean intramucosal calprotectin expression in active and inactive colitis

In IBD and non-IBD colitis, calprotectin was expressed in both active and inactive inflammation, with higher expression recognized in subjects with active inflammation (Table 4 and Figure 3).

Table 4: Mean intramucosal calprotectin expression in active and inactive colitis

Variable	Active colitis (n = 63)	Inactive colitis (n = 27)	p value
Mean intramucosal calprotectin expression (cell/HPF)	89.0±67.7	19.9±9.8	<0.001*

HPF: High power field.

Some cases of inactive colitis had higher expression than the others. From seven (25.9%) cases of inactive colitis with higher intramucosal calprotectin expression (>26 cells/HPF), five belonged to IBD group and only two of them belonged to non-IBD colitis group. Such cases showed diffuse lymphoplasmacytic inflammatory infiltrate in lamina propria with dense eosinophil aggregates. Some cases presented with crypt distortion and one case showed melanosis (Figure 4).

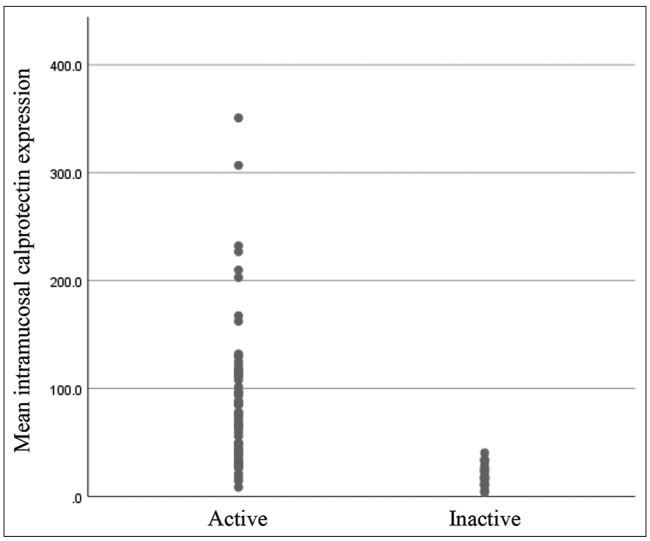

Figure 3: Mean intramucosal calprotectin expression in active and inactive colitis

Calprotectin expression in IBD and non-IBD colitis divided to subgroups based on activity grade is summarized in Table 5.

Table 5: Intramucosal calprotectin expression and activity grades

	Inactive	Active, activity grade		
		Mild	Moderate	Severe
IBD	16.8 (3.5–40.3)	39.8 (8.5–162.3)	214.9 (203.0–226.8)	129.5 (116.5–132.0)
Non-IBD	24.2 (10.3–34.0)	76.0 (26.5–167.5)	161.3 (42.5–306.8)	166.9 (66.2–350.8)

IBD: Inflammatory bowel disease.

Calprotectin was expressed lowest in inactive group and increased in concordance with activity grade, except in IBD group, in which moderate activity group had higher expression than severe activity group.

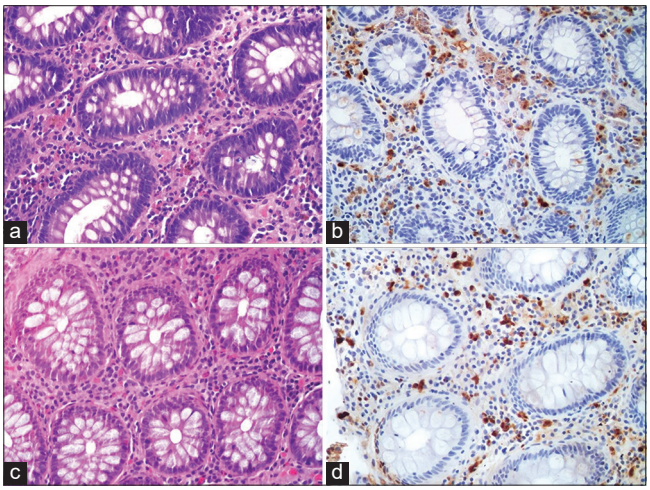

Figure 4: Melanosis in IBD case, (a) H&E ×400, (b) calprotectin immunohistochemistry, ×400; diffuse dense lymphoplasmacytic and eosinophilic infiltration in non-IBD colitis, (c) H&E ×400, and (d) calprotectin immunohistochemistry ×400

Discussion

Histopathologic features

The histologic findings expected from initial biopsy that is the morphology of the early IBD, with either specific or non-specific depending on disease duration. Biopsy taken within 0–15 days from onset usually shows non-specific findings, with neither transmucosal inflammatory cell infiltration nor crypt architectural abnormality [17], [18]. The only supporting feature that might be present is basal plasmacytosis (in 38–63% of cases). In most cases, biopsies which are collected in more than 6 weeks from onset, reveal chronic features like crypt distortion, crypt atrophy, or surface irregularity [17], [19], [20], [21].

In this study, 60% of IBD initial biopsy presented with active inflammation with 81.5% of cases showing mild activity. Most samples displayed crypt architectural abnormality suggesting that most initial biopsies in this study were taken after 15 days from onset. The presence of fibrosis in initial biopsy might indicate the difficulty in establishing IBD diagnosis in our country. Since that all samples included in this study were obtained from a national referral hospital, which is the final stop for patients with unsolved diagnosis and unsuccessful treatment, the samples might represent patients who had experienced the symptoms for months or even years and had looked for help to several peripheral health facilities with limited diagnostic tools. Thus, diagnosis confirmation become impossible in some cases [1]. In addition, some patients already underwent therapy before they were referred to a central hospital, where initial biopsy was performed. This discovery may raise a question about the true initial biopsy and diversity of the study samples, but the obstacles we faced in this study emphasized how difficult it was to establish IBD diagnosis resulting in treatment delay especially in remote healthcare facilities, hence the importance of a simple and available biomarker test.

A study evaluating histomorphology of IBD biopsy samples in Cipto Mangunkusumo National Referral Hospital in 2019 stated that the most common findings were crypt distortion and basal lymphoid aggregates, which were signs of chronicity. Basal plasmacytosis, which is a more common finding in the early IBD, was absent in all samples. This result might be caused by sample inadequacy and suboptimal specimen orientation [22]. In our study, the adequacy of biopsy samples was also varied: some samples were considered representative and taken from all colon segments, while others were not. Some specimens were poorly oriented as well.

Non-IBD colitis samples showed chronic colitis morphology with unknown specific etiology, due to lack of clinical information and endoscopy findings.

Intramucosal calprotectin expression

In this study, intramucosal calprotectin expression in IBD population was quite high (53.1 cells HPF). Another study by Guirgis *et al.* [5] showed similar results: Calprotectin in IBD group without histological remission was expressed in 50.6 cells/HPF (median), compared to 3.5 cells HPF in IBD group with histological remission.

Mean intramucosal calprotectin expression in non-IBD colitis was 83.5 cells/HPF. Within our knowledge, there is no study regarding intramucosal calprotectin expression in non-IBD colitis so far. Some literature stated that fecal calprotectin is increased in both IBD and non-IBD colitis with no cut off value to distinguish the two entities [23].

Intramucosal calprotectin expression in control group was 35.6 cells/HPF, which became another unexpected result in this study. In a study by Guirgis *et al*. [5] intramucosal calprotectin expression in the control group was less than 20 cells/HPF. Such finding in the control group could be explained by the possibility that calprotectin expression might be affected by demographic, dietary, socioeconomic, environmental factors. Older age, obesity, and physical inactivity are known to have association with higher calprotectin expression since these factors are related with continuous asymptomatic mild inflammation represented by increasing calprotectin expression. On the contrary, fiber-rich diet and vegetable consumption have negative association with calprotectin expression [24].

Calprotectin also is known to be increased in patients with colorectal cancer and can be detected in feces. However, fecal calprotectin could not be used to point out the exact location of inflammation [25], [26]. We find it interesting that while our sample for control group was obtained from the considered "normal" areas in colorectal cancer resection specimen, the evidence of higher calprotectin expression may actually indicate the presence of local and systemic inflammatory activity even in the normal-looking areas, which needs to be further elaborated.

Calprotectin was detected in cytoplasm of neutrophils, macrophages, dendritic cells, and eosinophils found in lamina propria, with higher expression found in IBD and non-IBD colitis compared to control group. Calprotectin has proinflammatory, bactericidal, and immunoregulatory properties. It plays an important role in recruiting macrophages to inflammatory site. It also serves as an endogenous ligand for tissue damage (damage-associated molecular pattern/DAMP). In inflammatory conditions such as IBD and non-IBD colitis, there is an increase in calprotectin synthesis following the release of inflammatory stimulus, causing continuous loop of recruitment of macrophages carrying even more calprotectin in their cytoplasm [4], [6], [27], [28]. The increasing calprotectin expression indicates ongoing inflammatory process and can be used to distinguish true inflammatory condition from functional disorder [5], [28], [29].

In this study, mean calprotectin expression in non-IBD colitis was higher than in IBD. Some literatures state both intramucosal and fecal calprotectin to be non-specific and should not be used to define inflammatory etiology. However, one study showed that very high level of fecal calprotectin has predictive value for IBD or food intoxication [23]. Considering the Inhomogeinity of the samples in this study and result difference from the study above, perhaps further research is needed to determine the characteristic expression of calprotectin in more uniform samples (subgroup analysis between non-IBD colitis of known specific etiology, subgroup analysis of IBD, and non-IBD colitis of same activity grade, etc.).

Increased intramucosal calprotectin was also observed in amoebic colitis and tuberculous colitis. No study has evaluated intramucosal calprotectin in these cases; however, one literature showed increased fecal calprotectin level in patient with protozoan infection including *Entamoeba histolytica* [30]. Some papers stated that fecal calprotectin level is also increased in tuberculous colitis and associated with granuloma formation [31]. It can be used to predict intestinal involvement in cases of pulmonary tuberculosis and to evaluate patient's response to treatment [32], [33].

In both IBD and non-IBD colitis, intramucosal calprotectin expression is higher in active inflammation. The more severe the inflammatory reaction is, the more neutrophils are recruited to the mucosa. Neutrophils carry calprotectin in their cytoplasm; therefore, the number of neutrophils in most cases is positively correlated with degree of inflammatory activity. However, not all neutrophils have detectable calprotectin in their cytoplasm, probably due to calprotectin being secreted to extracellular matrix following neutrophil activation and phagocytosis [28]. Consequently, it is important to remember that the number of neutrophil is not always proportional to intramucosal calprotectin expression.

Some cases of inactive colitis, especially the ones presenting with dense lymphoplasmacytic and eosinophilic infiltrates, express more calprotectin than other cases. This finding indicates that neutrophil might not be the sole marker of inflammatory activity. It is supported by the development of several activity scoring indexes. Although most scoring indexes use the presence of neutrophil as activity parameter, some systems also include the presence and density of eosinophilic infiltrate [34], [35], [36]. Other literatures stated that evaluating intramucosal calprotectin expression is beneficial especially in cases with no notable inflammatory activity in histopathology examination, for calprotectin can be used as a parameter of subclinical and subtle inflammatory activity [5], [11]. Evaluating this minimal activity and eosinophilic infiltrate become important particularly in IBD cases, because it is associated with the presence of mucosal inflammation and probability of relapse [37], [38], [39]. Although the role of eosinophil in IBD pathogenesis is still unclear, it is an important player in immune response and immunoregulation. It releases cytoplasmic granules containing cytotoxic protein, cytokines, lipid mediator, and reactive oxygen metabolites that induce fibrosis and coagulation cascades in IBD [39], [40], [41]. This result is supported by a study showing that IBD cases with or without dense inflammatory infiltrates have more intramucosal eosinophils compared to control group [39].

Conclusion

Higher expression of intramucosal calprotectin is observed in IBD and non-IBD colitis compared to control group, indicating its role in inflammation. Higher expression was associated with active inflammation and activity grade. Non-IBD colitis had the highest intramucosal calprotectin expression. However, the utility of intramucosal calprotectin for distinguishing IBD from non-IBD still has to be further evaluated.

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