Original Article

Immunohistochemical Study of Adhesion Molecules in Irritable Bowel Syndrome: A Comparison to Inflammatory Bowel Diseases

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Abstract

Background: The surface of endothelial cells is covered with cell adhesion molecules including E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) that mediate the adhesion and extravasation of leukocytes and play a pivotal role in inflammatory response. The aim of this study was to investigate the role of expression of adhesion molecules in inflammatory bowel disease (IBD) patients, irritable bowel syndrome (IBS) patients, and normal colonic mucosa. **Materials and Methods:** IBS and IBD patients along with normal colonic mucosa were recruited in the study. In all groups, two biopsies were taken from each of the three anatomical sites (terminal ileum, cecum, and rectum). Three monoclonal antibodies, E-selectin mAb, VCAM-1 mAb, and ICAM-1 mAb, were applied for immunohistochemical analysis. **Results:** In IBD patients, the expression of intensity of E-selectin, VCAM-1, and ICAM-1 was found decreased, at least in cecum and rectum, in comparison with IBS patients and controls ($P < 0.001$, $P < 0.005$, and $P < 0.007$, respectively). Comparison of the expression of intensity of the aforementioned molecules in IBS patients and controls revealed significant augmentation at the cecum and rectum of IBS patients. **Conclusions:** The expression of adhesion molecules appeared lower in IBD patients compared to IBS patients and controls. In addition, the expression of adhesion molecules appeared higher in IBS compared to the control group. Therefore, it could be hypothesized that the expression of adhesion molecules could be considered as an early event in the process of proinflammatory IBS group and may be other factors play a crucial role in the process of intestinal inflammation in IBD patients.

Keywords: *E-selectin, intercellular adhesion molecule-1, inflammatory bowel diseases, vascular cell adhesion molecule 1*

Introduction

Cell adhesion mediated by molecule families such as selectins, integrins, and immunoglobulins (intercellular adhesion molecule-1 [ICAM-1] and vascular cell adhesion molecule-1 [VCAM-1]) plays a central role in the function of the immune system by initially tethering leukocytes to the endothelium and then enabling their emigration from the vasculature and recruitment at sites of inflammation. An effective host response to pathogens, injury, or foreign antigen requires this focal accumulation of leukocytes, but excessive accumulation can lead to inflammatory disease and tissue pathology.[1]

The process of leukocyte recruitment to a site of inflammation encompasses the engagement and efficient arrest of leukocytes into the vascular endothelium and their subsequent transmigration. This sequence is composed of several major steps including capture, rolling mediated by selectins, activation mediated by chemokines, and firm adhesion.[2] Among the adhesion molecules, selectins are expressed on the surface of endothelial cells, leukocytes, and platelets, influencing the localization of circulating leukocytes on the endothelium at the site of inflammation. E-selectin is the most important vascular expressed in the first few hours of immune-inflammatory reactions and is responsible for leukocyte rolling, which is a transient stage of leukocyte adhesion. After rolling, firm adhesion of leukocytes to endothelium is accomplished by endothelial proteins with immunoregulatory domains such as ICAM-1 and VCAM-1. ICAM-1 helps to localize leukocytes to areas of tissue injury and VCAM-1 regulates the adhesion of

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monocytes, lymphocytes, basophils, and eosinophils to activated endothelial cells.[3]

Adhesion molecules have been shown augmented in mucosal biopsies of inflammatory bowel disease (IBD) patients,[4-6] whereas elevated levels of soluble forms of adhesion molecules have been documented in such patients.[7] Regarding IBS, a remanent unraveled mystery covers underlying pathology. The past few years an interesting supposition regarding inflammation as a potential pathophysiological mechanism of IBS has emerged;[8,9] nowadays, the low-grade multifactorial inflammation is believed to play a key role in IBS pathogenesis.[10]

The aim of this study was to evaluate the immunoexpression of E-selectin, VCAM-1, and ICAM-1 in inflamed mucosa (IBD patients), in noninflamed mucosa (normal controls), as well as in mucosa of patients with irritable bowel syndrome (IBS).

Materials and Methods

Ethical considerations

This study was conducted according to the Ethical Guidelines for Medical and Health Research Involving Human Subjects. The present study was approved by the Ethics Review Committee of University of Ioannina (approval No. 515α/11-11-2003). No additional permissions were required to review the patient records including the hospital from which the records were obtained.

A total of 86 patients were studied. Thirty-six (41.86%) were females and 50 (58.14%) males, median age of 46 years range from 17 to 72 years. All subjects underwent colonoscopy after cleansing of their whole colon with polyethylene glycol-electrolyte lavage solution. In all cases, two biopsies were taken at each of the three anatomical sites, namely, terminal ileum, cecum, and rectum, and were immediately placed in 10% neutral formalin and transferred at the Department of Pathology and after 6–8 h and embedded in paraffin for histological examination. None of IBD, IBS, or controls cases received any anti-inflammatory regimen before time of biopsy. The diagnosis of IBD and IBS was based on the clinical, endoscopic, and histological parameters using the hematoxylin-eosin (H-E) staining in sections obtained of uniform size (4 μm). Nineteen subjects' median age 46 years (range: 18–72), included as normal controls, had undergone colonoscopy for investigation of abdominal pain or for change of bowel habits, and no abnormality was found. Seventeen cases of IBS patients were included in this study. IBS patients fulfilled the Rome IV criteria.[11] Five cases were IBS-D, mean age of 44 years (range: 19–61); nine cases were IBS-C, mean age of 54 years (range: 22–66); and three cases were IBS-A, mean age of 43 years (range: 40–52). Fifty patients with IBD were included in this study. Twenty-nine patients were diagnosed with UC mean age of 38 years (range 24–48). In eight of them, the disease was active and 21 patients were inactive UC. Twenty-one patients were diagnosed with CD mean age of 35 years (range 17–61 years). In seven of them, the disease was active and 14 patients were inactive. During colonoscopy, biopsies were taken from the most inflamed site. Both UC and CD activities were examined on the basis of histopathological findings.

Immunohistochemistry was performed on paraffin blocks, from each case and tissue section placed on poly-L-lysine-coated glass slides. Antibodies directed against E-selectin (dilution 1/50, clone 16G4, Vision Biosystems), VCAM-1 (dilution 1/50, clone 1.4C3, Thermo Fisher Scientific Inc.), and ICAM-1 (dilution 1/50, clone 23G12, Vision Biosystems) were applied. Then, slides were washed in tap water, dehydrated, and mounted with glass coverslips. Positive controls were used to confirm the adequacy of the staining, and negative controls were included and consisted in the same immunohistochemical method with omission of the primary antibody. All slides were observed and examined using a light microscope (BX40; Olympus, Tokyo, Japan). The immunoexpression was assessed by a single pathologist (AM) to each slide using a ×40 objective, blinded to the clinical, endoscopic, and histological features of the patients. The following system was used for expression on inflammatory cells: 0, no immunoreaction was observed; 1, positive immunoreaction was observed in >10 cells/field.

Statistics

Superior Performance Software System (SPSS, IL, USA) software 16.0 for windows was used by the authors to compare morphological features and protein expression data. Significant differences between the expression of the target proteins with regard to clinicopathological parameters were compared by one-way ANOVA test and Kruskal–Wallis H-test for independent values. $P \leq 0.05$ was considered statistically significant.

Results

E-selectin

Epithelial elements were E-selectin negative. Eleven out the 29 UC specimens (37.9%) and 7 (33.3%) CD specimens were selectin-E positive. Immunoreactivity of selectin-E was absent at sites of active inflammation. In IBS patients, the immunoexpression of selectin-E was noted in 17 (89.4%) specimens. The expression of E-selectin in endothelium and crypts of terminal ileum showed no significant differences among IBS patients, IBD patients, and controls [Table 1]. On the other hand, the expression of E-selectin in the endothelium of cecum, rectum as well as crypts of IBD patients was significantly lower in comparison with IBS patients and controls (IBD vs. IBS/controls, $P < 0.001$), while no statistically significant differences among IBS patients and controls were found [Figure 1].

Vascular cell adhesion molecule 1

Immunoexpression of VCAM-1 was found in normal colonic mucosa in 48% of the cases. VCAM-1 immunoreactivity was observed in 14 cases (48.2%) of UC, in 12 cases (57.1%) of CD, and in 18 cases (94.7%) of IBS patients. No statistically significant difference was noted between IBD patients in quiescent or active disease ($P = 0.25$). No statistically significant differences were observed between IBS patients, IBD patients, and controls [Table 1]. A statistically significant difference was found in IBD patients of VCAM-1 in cecum endothelium and crypts ($P < 0.005$) as well as in rectum endothelium ($P < 0.01$) when compared to IBS patients and controls [Figure 2]. Comparison of IBS patients and controls showed a statistically significant increase of VCAM-1 expression in rectum endothelium ($P = 0.01$) and crypts ($P = 0.05$) in IBS patients.

Intercellular adhesion molecule-1

ICAM-1 immunopositivity was detected in 18 (94.7%) of IBS specimens; in 12 (41.3%) UC cases and 9 (42.8%) CD samples. In inflamed mucosa, ICAM-1 was positive in infiltrating chronic inflammatory cells within the lamina propria. In tissue specimens of inactive IBD patients, ICAM-1 expression was detectable on mononuclear cells. In active IBD, epithelial cells rarely expressed ICAM-1. No statistical differences were observed between activated and inactivated IBD. There were no statistically significant differences in the expression of ICAM-1 between UC and CD patients. The expression of ICAM-1 was significantly decreased in endothelium and crypts of terminal ileum and cecum as well as in rectum endothelium in IBD patients in comparison to IBS patients [Table 1]. Furthermore, the expression of ICAM-1 was significantly decreased in cecum and rectum endothelium in IBD patients compared to controls ($P < 0.007$) [Figure 3]. Comparison of the expression of the intensity of ICAM-1 among IBS patients and controls showed no significant differences except a statistically significant increase in crypts of terminal ileum in IBS patients ($P = 0.04$).

Discussion

In the present study, immunohistochemical evaluation of E-selectin, ICAM-1, and VCAM-1 was assessed in patients with IBD, IBS, and patients with normal colonic mucosa.

Selectins contribute to recruitment of lymphocytes to the place of inflammation. They take part in the initial phase of rolling of leukocytes to the blood vessel epithelium. The concentration of E-selectin increases in a process of inflammation in gastrointestinal tract and correlates with the activity of inflammatory process.[12] The influence of inflammatory mediators causes dislocation of selectins from the cytoplasm to the cell surface. This process marks the beginning of leukocytes rolling along the endothelial surface.[13] Selectins on the surface of moving leukocytes bind to ligands presented on the surface of endothelial cells.[13]

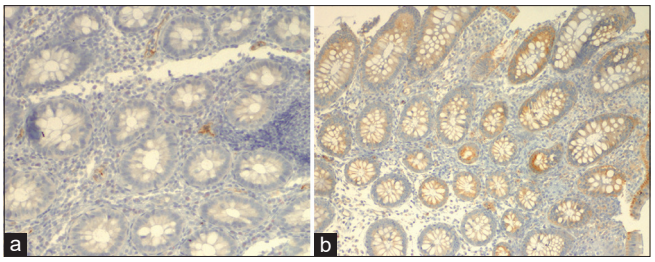

Figure 1: Immunohistochemical expression of E-selectin in inflammatory bowel disease patients (a) and irritable bowel syndrome patients (b) of cecum and rectum (×40)

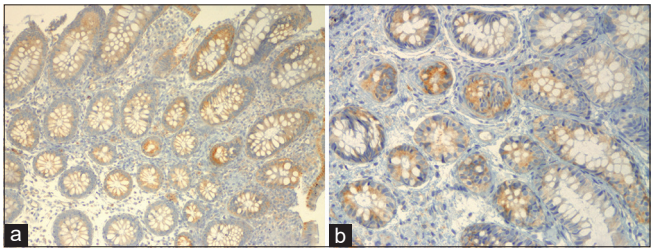

Figure 2: Immunohistochemical expression of vascular cell adhesion molecule/CD106 in irritable bowel syndrome patients (a) and inflammatory bowel disease patients (b) of cecum and rectum (×100)

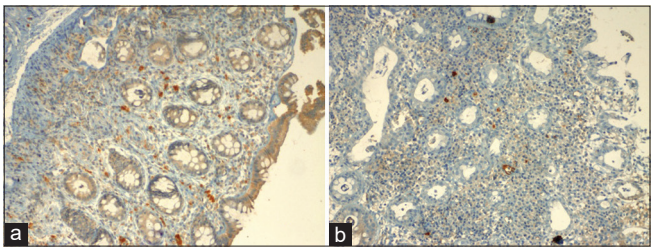

Figure 3: Immunohistochemical expression of intercellular adhesion molecule-1 in irritable bowel syndrome patients (a) and inflammatory bowel disease patients (b) of cecum and rectum (×40)

In the present study, we found an increased expression of intensity of E-selectin at the endothelium of rectum, cecum, and crypts of IBS patients compared to IBD patients. There is conflicting evidence and results in studies and reports examined the expression of E-selectin in IBD. Expression of E-selectin has been shown upregulated up to 5.5-fold over controls in IBD patients.[14] Lazaris *et al*. demonstrated in their study that increased expression of E-selectin on vascular endothelial cells of the colon is characteristic of active form of IBD.[15] Cellier *et al*. assessed both the expression of E-selectin in biopsies from the colonic mucosa and the concentration of the soluble E-selectin in serum in IBD patients. They showed a positive correlation between the expression of E-selectin in intestinal biopsies and the degree of activity of clinical, endoscopic, and histological IBD.[16] Selectin gene expression is normally not detected in resting endothelium but is strongly and rapidly induced in inflamed endothelial cells by the inflammatory cytokines.[17] E-selectin appears on the endothelial cell surface within

Table 1: E-selectin , VCAM-1 and ICAM-1 expression in terminal ileum, cecum and rectum of IBS patients, IBD patients and controls

	Intensity					
	IBD		IBS		CONTROLS	
	Endothelium	Crypts	Endothelium	Crypts	Endothelium	Crypts
E-selectin						
T. Ileum	1.77[a]	1.42[a]	1.89[a]	1.47[a]	1.84[a]	1.84[a]
Cecum	1.23[a]	1.14[a]	2.05[b]	1.47[b]	1.83[b]	1.59[b]
Rectum	1.28[a]	1.33[a]	1.90[b]	1.60[b]	2.00[b]	1.52[b]
VCAM-1						
T. Ileum	2.21[a]	2.17[a]	2.31[a]	2.38[a]	2.77[a]	2.37[a]
Cecum	1.75[a]	1.83[a]	3.09[b]	2.90[b]	2.83[c]	2.52[c]
Rectum	2.09[a]	2.44[a]	3.04[b]	2.86[b]	2.80[c]	2.35[c]
ICAM-1						
T. Ileum	2.50[a]	1.75[a]	3.21[b]	2.78[c]	2.94[b]	1.95[b]
Cecum	2.67[a]	1.75[a]	3.00[b]	2.43[b]	3.50[b]	2.35[b]
Rectum	2.55[a]	1.68[a]	3.50[b]	2.61[b]	3.45[b]	2.10[b]

Statistically significant. [a]P<0.05, [b]P<0.01, and [c]P<0.001

1–2 h, is expressed maximally at 4–6 h, and then rapidly declines even in the continuous presence of cytokines.[18]

VCAM-1 is expressed on activated human endothelial cells and is upregulated in response to tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and lipopolysaccharide. It is involved in the adhesion of lymphocytes, monocytes, and eosinophil to the vascular endothelium. VCAM-1 has been found in mucosa of both inflamed and noninflamed mucosa,[4] while in the study of Gulubova *et al*., VCAM-1 was found to be constitutively expressed in normal colonic mucosa and was not significantly enhanced or altered in distribution in mucosa of patients with IBD regardless of the activity of the inflammatory process.[19] Lazaris *et al*. demonstrated increased expression in vascular endothelium in areas of inflammation in patients with IBD.[15] Researches showed higher levels of ICAM-1 and lower VCAM-1 in Crohn's disease patients than in controls, but they did not show statistically significant values.[20] Our study showed no significantly higher values of VCAM-1 in IBD patients, but a statistically significant difference was found between VCAM-1 average values in IBS patients. The results of our study were in line with the findings of other laboratories who have failed to consistently demonstrate enhanced expression of VCAM-l in biopsies obtained from patients with active colitis.[5,20]

ICAM-1 is expressed by activated and nonactivated endothelial cells,[4] but several different reports have demonstrated enhanced staining for ICAM-l in biopsies obtained from patients with active UC and CD.[6,19] Vainer and Neles found higher expression of ICAM-1 in the inflamed fragment of the colonic mucosa in ulcerative colitis, but this relationship was not confirmed in patients with Crohn's disease.[21] On the other hand, other studies have failed to present an increased expression of ICAM-1 in IBD endothelium,[5,6] according to the results of our study. In this context, oligonucleotide (ASO)-based therapeutic strategies were developed. Anti-ICAM-1 oligonucleotide Alicaforsen had some positive randomized clinical trials,[22,23] but a *post hoc* meta-analysis of the results of four Phase 2 clinical studies showed only modest efficacy.[24]

IBS is a chronic, relapsing, and remitting functional disorder of the gastrointestinal tract characterized by abdominal pain, bloating, and changes in bowel habits that lack structural or anatomic explanation.[25] Until now, the exact cause of IBS is unknown, and various factors are implicated including mucosal inflammation, postinfectious low-grade inflammation, genetic and immunologic factors, alteration of the human microbiota, alterations of the intestinal permeability, and dietary and neuroendocrine factors. Usually, routine histologic examinations do not show significant abnormalities; however, recent quantitative histologic, immunohistochemical, and ultrastructural analysis have indicated subtle organic alterations in patients with IBS.[26]

Studies have been indicated that, although very distinct in pathology, IBD patients are three times more likely to have past history of IBS.[27] Moreover, there is accumulated evidence that IBS and IBD possibly share a common basis that is chronic, low-grade inflammation. Proinflammatory cytokines (TNF-alpha, IL-6, and IL 1beta) are prevalent in IBS as well as IBD,[28] and leukocyte infiltrations are found in histopathologic specimens of both IBS and IBD mucosa and epithelium.[29] In addition, it is thought that alterations in gut microbiota and dysbiosis play an important role in IBD pathogenesis, either directly, by disrupting epithelial barrier or indirectly by producing proinflammatory microbial metabolites. The consequent low-grade inflammation is a common early event in IBS and IBD course.[30]

Normal controls displayed augmented intensity of E-selectin, VCAM-1, and ICAM-1 in intestinal

endothelium. The later finding could be appointed to "physiological inflammation," which is a state that appears at the intestinal mucosa even under normal conditions, manifested by the presence of abundant leukocytes in the intraepithelial and subepithelial compartments.[31] Leukocyte transendothelial migration is a vital physiological process, and tight regulation of this process is critical for preventing inflammatory disorders.[32] Normal controls seem to achieve such a tight regulation of leukocyte migration preventing from chronic inflammatory manifestations.[33,34]

To the best of our knowledge, expression of adhesion molecules in mucosal biopsies from IBS patients has never been studied before. Our study in general showed an increased intensity of E-selectin, VCAM-1, and ICAM-1 in IBS patients in comparison to IBD patients and partially increased in comparison to normal controls. It could be that the intensity of expression of adhesion molecules at the intestinal mucosa is not that crucial into pointing out to certain phenotype of clinical disorders. The continuing and persistent expression of adhesion molecules leading to protracted leukocyte transendothelial migration and subsequently to inflammatory disease is probably of more importance.

Expression of adhesion molecules in our study was proved increased in IBS patients, but it does not solely explain potential underlying disorders of the disease; yet, it could possibly be considered as an early event in the process of the disease. On the other hand, the lower expression of adhesion molecules in IBD in comparison to IBS group, although we expected the opposite findings, may indicate that other factors implicate to continue the process of inflammation of this disease. Further studies are necessary to explain this phenomenon. The discrepancies found in the present study may be due to differences in the immunostaining techniques, quantification methods, and IBS-related recruitment criteria.

In a second stage, it would be interesting to assess the presented data in a future time point in the same IBS patients in order to examine whether those adhesion molecules are indeed an early event in IBS. Furthermore, the pathological process underlying IBD involves the dysregulated synthesis of pro-inflammatory cytokines.[35] Thus, it would be interesting to estimate expression of inflammatory mediators that promote leukocyte/endothelial cell adhesion such as TNF-alpha, IL-1, or IL-6 which correlates with neoplasia and autoimmune disorders[36-38] or signaling pathways such as nuclear factor-κB, which can be activated by TNF-alpha and IL-1beta in IBS patients.[39] Finally, expression of other adhesion molecules, namely, Syndecan-1, E-cadherin/β-catenin, and proteins related with neoangiogenesis which are known for their role in colon tumorigenesis[40] could be a field for further investigation in IBS and IBD pathogenesis.

Limitations of the present work

One serious limitation of the present study is the small number of histological specimens in some categories which may restrict generalization of the results. An additional limitation is that our analysis used the immunohistochemical method and no other techniques such as mRNA extraction and expression of the proteins studied or Western blotting extraction. We suggest further future investigation in the field of inflammation in IBS as any possible pathophysiologic relationship will lead to proper treatment.

Conclusions

The expression of adhesion molecules appeared lower in IBD patients compared to IBS patients and controls. In addition, the expression of adhesion molecules appeared higher in IBS compared to the control group. Therefore,it could be hypothesized that the expression of adhesion molecules could be considered as an early event in the process of proinflammatory IBS group.

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Conflicts of interest

There are no conflicts of interest.

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