Possible Role of E-Cadherin, CD86 and L-Selectin in Antiapoptotic Mechanisms of Ulcerative Colitis

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Abstract

The current study was designed to determine the pattern of E-cadherin, CD86 and L-selectin expression in different histopathological grades of activity of ulcerative colitis (UC), and to determine the degree of apoptosis in patients with UC and whether this apoptotic death could be correlated with cellular expression of E-cadherin, CD86 and L-selectin in different histopathological grades of activity of UC.

Forty five paraffin-embedded UC samples and 10 resection margins of colectomy specimens for diseases other than UC (control group) were enrolled in this study. The age range of the patients was 9-70 years and 18 were females while 27 were males. Hematoxylin and Eosin stained sections were used to define the disease activity.

Adequate, 4 µm thick sections of both UC and resection margins samples were prepared and used to detect E-cadherin, CD86 and L-selectin expression employing immunohistochemical analysis. Evaluation of apoptosis in mucosa with UC was performed using the TUNEL assay.

Based on current outcome, by immunohistochemical analysis, there was a significant downregulation in E-cadherin expression and over expression of CD86 in the inflamed mucosa compared to control group and the differences were statistically significant in both compared to control group (p < 0.05).

The expression of L-selectin was relatively higher in the inflamed mucosa compared to controls but the frequencies and percentages of L-selectin expression between patients and controls were statistically not significant (p > 0.05).

Using the TUNEL assays, thirty cases were investigated for evaluation of apoptosis. The results showed that the number of TUNEL positive cells was not significantly different from that in the control group and the frequencies and percentages of apoptosis between patients and controls in both lymphocytes and epithelial cells were statistically not significant (p > 0.05).

None of the four investigated parameters including E-cadherin, CD86, L-selectin and apoptosis demonstrated any significant correlations of their expressions with the grade of inflammation (p > 0.05).

Keywords: CD86, L-selectin, E-cadherin, apoptosis, UC.

Introduction

Ulcerative colitis (UC) is an inflammatory chronic disease primarily affecting the colonic mucosa; the extent of severity of colon involvement are variable, in its most limited form it may be restricted to the distal rectum, while in its most extended form the entire colon is involved [1].

E-cadherin is the predominant cell adhesion molecule in the intestinal epithelium. Experiments with a murine model indicate the important role of E-cadherin in the maintenance of the intestinal barrier, showing that dysregulation of this molecule leads to IBD [2].

CD86 (B7-2) Member of B7 family, which are immunoglobulin (Ig) superfamily molecules Pathways in the B7-CD28 family have key role in the regulating T cell activation and tolerance and are promising therapeutic targets. These costimulatory molecules could be implicated in the pathogenesis of autoimmune diseases and that blocking this interaction, could control the development and the activity of many immune disorders, included IBD [3].
UC was an inflammatory condition with infiltration of leukocytes into the mucosal lining of the colon and that these leukocytes were capable of causing inflammation and tissue damage [4]. The selectin family of adhesion molecules mediates the initial attachment of leukocytes to venular endothelial cells before their firm adhesion and diapedesis at sites of tissue injury and inflammation [5].

Apoptosis (programmed cell death) is a highly regulated process of cell death that is required for the development and homeostasis of multicellular organisms, in contrast to necrosis, apoptosis eliminates individual cells without inducing an inflammatory response. Activation or prevention of cell death could be a critical factor in the outcome of an infection. Apoptosis is one of the mechanisms that maintain unresponsiveness of the intestinal mucosal immune system. It is induced in a variety of GI cell lineages by either TNF or Fas-mediated pathways [6].

Materials and Methods
Collection of Samples
Forty five archived paraffin-embedded blocks of patients diagnosed at the GIT and Liver Disease Hospital-Baghdad to have ulcerative colitis were reviewed.

Resection margins of colectomy due to other causes rather than ulcerative colitis were utilized as control group.

Haematoxylin and Eosin stained sections were examined by the same senior pathologist to confirm the diagnosis and assign the grade of activity of inflammation based on simplification of the activity scale proposed by Geboes et al. [16]

Thin paraffin-embedded sections (4 µm thick) of UC and resection margin tissue section were prepared on positively charged slides for the immunohistochemistry (IHC) and TUNEL technique.

Materials
Labeled streptavidin-Biotin 2 system, horseradish peroxidase (L SAB2 system-HRP-Dakocytomation, Denmark):
- Peroxidase-block (3% hydrogen peroxide in water).

Biotinylated secondary antibody (biotin labeled affinity isolated goat anti-rabbit and goat anti-mouse immunoglobulin in phosphate buffered and 0.015 mol/L sodium azide).
- Streptavidin-HRP conjugates.
- DAB substrate buffer.
- DAB chromogen.

Anti-E-cadherin (Dako, Denmark): clone and isotype: NCH-38* mouse IgG1, kappa.
Anti-L-selectin (Dako, Denmark): clone and isotype: FMC 46 mouse IgG2b, kappa.
Anti-CD86 (Dako, Denmark): clone and isotype: BV63 mouse IgG1, kappa.

Preparation of Tissue Sections and Reagents:
Paraffin-embedded sections were cut 4 µm thick, placed on Fisherbrand superfrost/plus slides, left for 15 minutes at room temperature and then left overnight at 55 °C.

PBS-tween or PBS-0.1% tween 20 was prepared by adding 0.1 ml tween 20 to 100 ml 1XPBS.

Primary antibody was diluted in antibody diluents and as follows:
- 1:25 for anti-E-cadherin Ab.
- 1:10 for anti-L-selectin Ab.
- 1:10 for anti-CD86 Ab.

Absolute ethanol was diluted in distilled water to prepare 95% and 70% concentrations of alcohol.

Dilution/Blocking buffer was diluted ten folds in deionized distilled water.

Negative controls were included for each run of immunohistochemistry. The negative control was obtained by replacing the primary antibody with PBS buffer.
- E-cadherin expressed on epithelial cells, CD86 expressed on epithelial cells and lymphocytes, and L-selectin expressed on lymphocytes. The percent of (IHC) positive cells (brown color) was determined from the average evaluation in 5 randomly selected fields, calculated as the number of brown-stained cytoplasmic cells per 100 cells.

Quantitative IHC scoring was done according to the following table:
Quantitative immunohistochemistry scoring.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E-cadherin</th>
<th>L-selectin</th>
<th>CD86</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score</td>
<td>&lt; 5%</td>
<td>&lt; 2%</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5-25%</td>
<td>2-10%</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>25-75%</td>
<td>11-40%</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>&gt; 75%</td>
<td>&gt; 40%</td>
<td>4</td>
</tr>
</tbody>
</table>

Reference: [17] [18] [19]

Dead End™ Colorimetric TUNEL System for Detection of Apoptotic Cells:

Analysis of apoptosis in tissue sections:

Phosphate buffered saline (PBS), 0.3% hydrogen peroxide, Fixative (e.g., 10% buffered formalin), Xylene, Ethanol (100, 95, 85, 70 and 50%), 0.85% NaCl solution, Proteinase K buffer, DNase I, Poly-L-lysine-coated or silanized microscope slides, Coplin jars, Humidified chambers for microscope slides, 37°C incubator, Micropipettors, Glass coverslips, Clear nail polish or rubber cement, Microscope.

The Dead End Colorimetric TUNEL System end-labels the fragmented DNA of apoptotic cells using a modified TUNEL assay. Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown.

* The percent of TUNEL positive nuclei (brown color) was determined from the average evaluation in 5 randomly selected fields, expressed as the number of brown-stained nuclei per 100 cells.

Statistical Analysis:

The scores of the parameters used in this study were expressed as frequency and percentage for each score. The comparison between frequencies of the parameters in both groups of the study (patients and control) was done by chi square (X²) test using Kwikstat software program. The correlation among different parameters and with the grade of disease was done by Spearman correlation (r_s) using Statistica software program (version 5.0). The statistical significance level was defined as p < 0.05.

Results

Identification of E-cadherin, CD86 and L-selectin expression:

Histopathological grading of activity of 45 UC tissue samples (18 females and 27 males, age range 9-70 years) revealed that 9 samples were grade 6, 8 samples were grade 5, 18 samples were grade 4, 9 samples were grade 3 and only one sample was grade 2.

There was significant differences of the frequencies and percentages of E-cadherin and CD86 between patients and controls (p < 0.05) as shown in Tables (1) and (2) respectively.

Table (1)

Comparison of frequencies and percentages of E-cadherin expression between patients and controls.

<table>
<thead>
<tr>
<th>IHC Score (+)</th>
<th>Patients n =45</th>
<th>Control n =10</th>
<th>Chi-Square test (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1 (2.22)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>22 (48.89)</td>
<td>0 (0.0)</td>
<td>0.013</td>
</tr>
<tr>
<td>3</td>
<td>22 (48.89)</td>
<td>10 (100)</td>
<td></td>
</tr>
</tbody>
</table>
Table (2)
Comparison of frequencies and percentages of CD86 expression between patients and controls.

<table>
<thead>
<tr>
<th>IHC Score (+)</th>
<th>Patients (n = 45)</th>
<th>Control (n = 10)</th>
<th>Chi-Square test (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8 (17.78)</td>
<td>5 (50)</td>
<td>0.019</td>
</tr>
<tr>
<td>2</td>
<td>18 (40)</td>
<td>5 (50)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>19 (42.22)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
</tbody>
</table>

IHC: immunohistochemistry.

The expression of L-selectin relatively higher in the inflamed mucosa compared to control but statistically was not significant (p > 0.05) as shown in Table (3).

Table (3)
Comparison of frequencies and percentages of L-selectin expression between patients and controls.

<table>
<thead>
<tr>
<th>IHC Score (+)</th>
<th>Patients (n = 45)</th>
<th>Control (n = 10)</th>
<th>Chi-Square test (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>38 (84.44)</td>
<td>10 (100)</td>
<td>0.182</td>
</tr>
<tr>
<td>2</td>
<td>7 (15.56)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

Detection of apoptosis (TUNEL test):
Thirty cases were investigated for detection of apoptosis based on TUNEL assays. These included seven samples grade 6, 7 samples grade 5, 8 samples grade 4, 7 samples grade 3, and only one sample was grade 2. There was no significant differences of frequencies and percentages of apoptosis between patients and control in both lymphocytes and epithelial cells (p > 0.05), (Tables (4) and (5)).

Table (4)
Comparison of frequencies and percentages of apoptosis for lymphocytes between patients and controls.

<table>
<thead>
<tr>
<th>TUNEL Score (+)</th>
<th>Patients (n = 30)</th>
<th>Control (n = 10)</th>
<th>Chi-Square test (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30 (100)</td>
<td>10 (100)</td>
<td>0.999</td>
</tr>
</tbody>
</table>

TUNEL= Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay.

Table (5)
Comparison between frequencies and percentages of apoptosis for epithelial cells between patients and controls.

<table>
<thead>
<tr>
<th>TUNEL Score (+)</th>
<th>Patients (n = 30)</th>
<th>Control (n = 10)</th>
<th>Chi-Square test (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30 (100)</td>
<td>10 (100)</td>
<td>0.999</td>
</tr>
</tbody>
</table>

TUNEL= Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay.
Correlation between the investigated parameters and different histopathological grades:

Table (6) indicates that there was no significant correlation among the expressions of E-cadherin, CD86 and L-selectin, and apoptosis with the histopathological grades of activity in ulcerative colitis patients ($p > 0.05$) based on Spearman test of correlation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$r$ value</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD86(+)</td>
<td>0.210</td>
<td>0.166</td>
</tr>
<tr>
<td>L-selectin(+)</td>
<td>0.120</td>
<td>0.431</td>
</tr>
<tr>
<td>E-cadherin(+)</td>
<td>-0.226</td>
<td>0.136</td>
</tr>
<tr>
<td>TUNEL lymphocyte(+)</td>
<td>0.017</td>
<td>0.928</td>
</tr>
<tr>
<td>TUNEL epithelial cells(+)</td>
<td>0.099</td>
<td>0.601</td>
</tr>
</tbody>
</table>

Discussion

Immunohistochemical analysis for E-cadherin, CD86 L-selectin expression in UC: E-cadherin:

In the 45 patients studied here, alteration of E-cadherin expression was found to be not correlated with the grade of inflammation as shown in Table (6). In actively inflamed UC, expression of E-cadherin was reduced in the luminal surface epithelium. While in non inflamed (UC), the tissue expression of E-cadherin was not different from that seen in control tissue (Table (1)).

Disease activity in IBD is linked to an influx of transepithelial migration of neutrophils (PMN) into the mucosal epithelium (cryptitis) and subsequently into the intestinal lumen resulting in the formation of so-called crypt abscesses. Expression of E-cadherin was down-regulated only in epithelial cells immediately adjacent to transmigrating PMN [7].

Schmitz et al. [8] have shown a strong correlation between the deregulation of E-cadherin and the progression of human colitis. In actively inflamed IBD tissue, the expression of E-cadherin and α-catenin protein was highly reduced, as shown by both immunofluorescence and western blot analysis. This study shows that inappropriate intestinal inflammation could be one mechanism responsible for down-regulation of several classes of junction-associated molecules in IBD.

CD86:

This study showed that the immunohistochemical expression of B7-2 was observed in both normal and inflamed mucosa in UC. Although the expression was statistically more in inflamed mucosa but there were no significant differences in the expression of B7-2 with different histopathological grades of UC (Table (2) and (6)).

These results are in agreement with results of study done by [9] who reported increased expression of B7-2 in the inflamed mucosa of UC patients. They suggested that these results due to the small number of lymphocyte that infiltrating normal mucosa compared with inflamed mucosa. Furthermore, in the inflamed IBD specimens, the frequency of lymphoid structures was significantly increased and because such structures were generally larger and exhibited a density of B7-2 and B7-1 cells compared with normal control mucosa, the number of aggregated cells expressing these costimulatory molecules in IBD was evidently increased.

Polese et al. [3] analyzed by polymerase chain reaction (PCR) the expression of B7-2 and B7-1 in the large bowel mucosa of patients affected by IBD. They found B7-2 expression in the large bowel mucosa of patients affected by ulcerative colitis, higher during the onset of the disease.

Nakazawa et al. [10] by reverse-transcription of polymerase chain reaction, found that CD86 and CD80 messenger RNA was detected in isolated colonic epithelial cells from normal and inflamed mucosa of
patients with ulcerative colitis. By immunohistochemistry of colonic epithelial cells they confirmed that cell surface expression of CD86 protein was increased, demonstrating that colonic epithelial cells may act as antigen presenting cells and contribute to the activation of T cells through costimulatory molecule CD86 expression in inflamed colonic mucosa.

**L-selectin:**
In this study, the numbers of stained cells were relatively higher in the inflamed mucosa compared to normal tissue but the differences were statistically not significant (Table (3)). Also the correlation between the expression of L-selectin with histopathological grade was statistically not significant (Table (6)).

These results are in agreement with a study done by Munro et al., [11], who demonstrated that L-selectin positive leukocytes were rare in normal tissue and relatively few were seen in most inflammatory settings.

Griffin et al. [12] demonstrated that in most forms of inflammation studied, relatively few leukocytes within the inflamed tissue expressed L-selectin constituent with shedding of this molecule upon activation.

After activation by antigen, T cells show profoundly altered migration pathways characterized by loss of capacity to enter lymph nodes and acquired capacity to enter non lymphoid organs such as the lungs, skin, or gut and sites of inflammation. L-selectin is down-regulated from the T cell surface within minutes after antigen receptor engagement and most antigen-activated T cells found in non-lymphoid organs expressed L-selectin at low levels [13].

**Evaluation of apoptosis in ulcerative colitis (TUNEL test):**
In this study the TUNEL positive colonic epithelial cells were few in both patients and controls, In general the current study showed that the TUNEL positive cells were rare in colonic mucosa and the number of TUNEL positive cells was similar in controls and patients.

Statistically there was no significant differences in the frequencies and percentages of apoptosis between patients and controls in both lymphocytes and epithelial cells $(p > 0.05)$ as shown in Tables (4 and 5) respectively. Also there was no significant correlation between apoptosis in lymphocytes and epithelial cells with grade of inflammation as shown in Table (6).

These results are in accordance with other studies done by [14] that showed colonic epithelial cells of active UC to contain activated nuclear factor κB (NF-κB) which was an important regulator of proinflammatory cytokines and inhibitor of apoptosis also demonstrated low expression of Bax both at the protein and mRNA levels in inflamed colonic epithelium of UC.

Experiments carried out in humans showed that at least 15% of the LPT cells from normal bowel were undergoing apoptosis. In the case of IBD, there was a lower proportion of cells undergoing apoptosis [6].

In this study, the TUNEL positive lymphocytes were rare in both patients and controls. These results in agreement with the theory that T cells in inflamed tissue express increased levels of Bcl2. It appears that the reduced apoptosis in IBD is a consequence of inflammation rather than a cause of IBD, thus reduced apoptosis could be due to pro-inflammatory cytokines that are abundantly present in the inflamed mucosa. Many pro-inflammatory cytokines, such as IL-1, IL-6, IFNγ and IL-15, are expressed in IBD. In particular, IL-15 might have an important effect in counteracting apoptosis of LP cells, because it was shown that IL-15 was produced by macrophages in inflamed mucosa of patients with IBD. Bcl2 expression could be induced as a result of signaling via the common chain of the IL-2 receptor, which serves as a signaling component of the receptor for IL-2, IL-4, IL-7 and IL-15. Thus IL-15 might greatly enhance the expression of Bcl2, which further enhances resistance of LPT cells against induction of apoptosis [15].

**Conclusions**
From this study we conclude that ulcerative colitis is associated with:

- Down regulation expression of E-cadherin in the inflamed mucosa compared to controls.
- Over expression of CD86 in the inflamed mucosa compared to controls.
• No significant differences in the expression of L-selectin between patients and controls.
• Down regulation of apoptosis in colonic mucosa in both epithelial cells and lymphocytes.
• The expression of E-cadherin, CD86 and L-selectin and apoptosis does not correlated with the grade of inflammation.

References
[16] Geboes K., Riddell R., Ost A. et al.: A reproducible grading scale for histopatholgical assessment of


