Surgery for inflammatory bowel disease, crossing borders

Gardenbroek, T.J.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Immunological and histological characteristics of the appendix in ulcerative colitis

Tjibbe J. Gardenbroek¹, Saloomeh Sahami¹, Jan P. van Straalen³, Hugo M. Horlings⁵, Marc J. van de Vijver¹, Cyriel Y. Ponsioen⁶, Gijs R. van den Brink⁴, Geert R. A. M. D’Haens⁶, Willem A. Bemelman¹ and CJ Buskens¹

Affiliations
¹Department of Surgery, ²Department of Clinical Chemistry, ³Department of Pathology, ⁴Department of Gastroenterology and Tytgat Institute for Liver and Intestinal Research, Academic Medical Centre, Amsterdam, The Netherlands

In progress
ABSTRACT

Background

Mucosal inflammation and cytokine production within the appendix has been suggested to play a causative role in the development and relapses of ulcerative colitis (UC). Objective of this study was to analyse if T-cell phenotype in appendiceal lavage fluid is associated with histological and immunohistochemical characteristics of UC appendices. Results were compared to the appendices of patients with Crohn's disease (CD), acute appendicitis (AA) and non-inflammatory controls, and correlated to clinical disease activity.

Methods

The appendix was removed from the surgical resection specimen and flushed with 2cc of phosphate buffered saline. Presence of CD4+ and CD8+ T-cells in the lavage fluid was determined by FACS analysis and the CD4/CD8 ratio was calculated. Histological analyses of the appendices assessed the degree of inflammation and mucosal ulceration. Furthermore, slides were immunohistochemically stained for CD4+ and CD8+ (grading 1-3), and T-cell infiltration grades were correlated to lavage results and clinical activity indices.

Results

Thirty-three patients were included; 13 with UC, 7 with CD, 5 with AA and 8 controls. In UC, an increased number of CD4+ cells was demonstrated in the lavage fluid, comparable to CD and AA. A relatively low number of CD8+ was found in UC, resulting in a significantly higher median CD4/CD8 ratio 7.1 (iqr 4.2-8.1) when compared to the median ratio of 3.2 (iqr 2.5-6.2) in the control group. At immunohistochemical evaluation the majority of UC (8/13 = 62%), CD (6/7 = 85.7%) and AA (3/5 = 60%) patients had increased levels of CD4+ lymphocytes (grade 2 and 3). Immunohistochemical results correlated to CD4+ percentages in lavage fluid in 80% of UC patients, and was able to predict clinical disease activity in 90% of UC, 100% of AA, and 80% of CD patients.

Conclusions

Despite a macroscopically normal appearance, appendices of UC patients with active disease show immunological activation with inflammatory characteristics and increased numbers of CD4+ lymphocytes in lavage fluid and immunohistochemical analysis.
INTRODUCTION

The triggering factor for the development of ulcerative colitis (UC) is still unknown\(^\text{1-2}\). In the pathogenesis of UC, cytokine imbalance and production of inflammatory mediators by activated CD4+ T cells are regarded to play an important role\(^\text{3-5}\). Extensive infiltration of lymphocytes, especially CD4+ T cells, has been observed in the inflamed mucosa of UC patients\(^\text{6}\). Activated CD4+ T cells exhibit increased cytotoxic activity and produce cytokines, enhancing the inflammatory state which results in tissue injury\(^\text{7-9}\).

Until recently the appendix was mostly seen as a rudimentary part of the human intestine, but nowadays it has been demonstrated that the appendix has distinct immunological functions. Reports are emerging linking this vermiform organ to the development of UC and a systematic review suggests that an appendectomy could modulate the course of the disease\(^\text{10-11}\). Although characteristic transmural histological changes in appendectomy specimens are hardly ever seen in UC, a quantitative and qualitative change of the lymphocyte phenotype in the appendix of UC patients has been described. Studies on T-cell subsets in the appendix have shown increased numbers of CD4\(^+\) lymphocytes. Because it is predominantly the early activation antigen CD4\(^+\)CD69\(^+\) and the activation marker CD25\(^+\) that are increased, this could indicate that the appendix acts as a priming site for this particular disease\(^\text{9,12-13}\). Furthermore, UC mouse models suggest that the CD4/CD8 ratio in the mucosa of the appendix represents and influences the inflammation degree in the colonic mucosa\(^\text{12-13}\).

In contrast, for patients with Crohn’s disease (CD) higher incidence rates after appendectomy have been described, being highest within the first six months after resection. However, these data are difficult to interpret since the appendix is frequently involved as part of the terminal ileitis which could result in overestimated incidence rates. The appendix as sole primary manifestation of the disease is rare\(^\text{14-17}\). Comparable to the affected terminal ileum, most specimens show macroscopically and microscopically affected appendices with transmural inflammation.

Acute appendicitis (AA) represents a different form of transmural inflammation. This non-autoimmune coordinated inflammation has been linked to bacterial invasion, diet, familial aggregation and an obstructing appendiceal faecolith possibly play a role in the etiology of the disease\(^\text{18-20}\). To gain insight in the distinct role the appendix plays in the development in UC, it would be interesting to compare immunological changes to appendices from patients with CD, AA, and healthy controls. So far, lymphocyte phenotyping has been done predominantly in murine colitis models. The scarce literature on human UC appendices only discusses inflammatory characteristics in resection material. However, if T-cell infiltration and characterization could be clinically determined in the appendix, it might be possible to utilize this as a measurement for the inflammatory process, and guide clinical decision making.
The objective of this study was to analyse if T-cell phenotype in appendiceal lavage fluid was associated with histological and immunohistochemical characteristics of UC appendices. Results were compared to the appendices of patients with Crohn's disease (CD), acute appendicitis (AA) and non-inflammatory controls, and correlated to clinical disease activity.

METHODS

This prospective cohort study was performed in a tertiary IBD center (Academic Medical Center, Amsterdam) in The Netherlands. Patients over 18 years of age with therapy refractory UC scheduled for colectomy in an elective setting, or UC patients in remission participating in a study analysing the role of appendectomy in this disease, were eligible. The control groups consisted of patients with Crohn's disease (CD) undergoing ileocolic resection, patients with acute appendicitis (AA) undergoing laparoscopic appendectomy, and patients undergoing (partial) colectomy for colonic carcinoma or familial adenomatous polyposis (FAP). Patients were pre-operatively included in the MIC study ('In depth characterization of the mucosal microbiota in patients with IBD using novel, potent high-throughput approaches and their interaction with the immune system') in which the mucosal microbiota in the resection specimens of abovementioned patients was analysed. The study protocol was approved by the institutional review board of the Academic Medical Center and the trial was registered at Netherlands Trial Register (NTR2908). For the current study, we analysed the appendix of the included patients. Patients were included between August 2011 and January 2013.

Procedure

Surgical procedures were performed laparoscopically by two gastrointestinal surgeons. Care was taken not to touch the appendix during dissection. After resection, the specimen was extracted from the abdominal cavity and the appendix was removed from the resection specimen under sterile conditions. The mesentery of the appendix was removed and the appendicular tissue was cleaned of peri-appendicular fat. The distal tip of the appendix was cut off to enable flushing the appendix with fluid. The appendix was inserted in a transparent tube to provide circular pressure during flushing. Subsequently, the appendix was flushed with 2cc of phosphate buffered saline (PBS). In case of faecal contamination of the fluid, a second flush with 2cc of PBS was performed. The lavage fluid was collected in a container with a protein medium and analysed in the clinical chemical laboratory of the AMC. Subsequently, the appendix was transported to the department of pathology for histological evaluation and immunohistochemical staining according to a standardized protocol.
Appendix lavage fluid analysis

The mononuclear cells in the lavage fluid were stained with fluorescein isothiocyanate (FITC) and phycoerythrin-conjugated (PE) monoclonal antibodies (anti-CD45, anti-CD3, anti-CD4, and anti-CD8). First the phenotyped cells were analysed by colour flow cytometry (FACS analysis). Cell suspensions were visualised in the forward scatter/side scatter profile, subsequently lymphocytes were gated. Then, the proportion of CD4+ and CD8+ T cells and relative ratio of CD4+ and CD8+ T-cells in CD3+ cells were calculated. Figure 1 shows an example of a result after FACS analysis of the lavage fluid. An ROC curve was created to determine the optimal cut-off value for increased CD4+ percentages.

Figure 1 Image of a colour flow cytometry of appendix lavage fluid. Cell suspensions were visualised in the forward scatter/side scatter profile, subsequently lymphocytes were gated. The proportion of CD4+ and CD8+ T cells and the CD4/CD8 ratio in the total lymphocyte populations were calculated.

Histology and immunohistochemistry

The appendix was cut transversely and longitudinally, and serial sections were cut for staining and analysis. The hematoxylin and eosin stain sections of the appendix were evaluated, assessing the architecture and inflammatory features. Architectural features included crypt atrophy, crypt distortion and surface irregularities. Inflammatory features included increased cellularity of the lamina propria, plasmacytosis, crypt abscesses, granulomas and lymphoid aggregates, assessing the degree of inflammation (mucosal, submucosal or transmural) and mucosal ulceration.

Paraffin embedded slides were also immunohistochemically stained for CD4 and CD8, by routine staining with primary antibodies.

Results were assessed by three reviewers (SS, HH and CB) independently, blinded for the patients’ clinical records and disease diagnosis, and scored according to the number of positive cells per high power field. A representative mucosal area was chosen which
was not directly covering a lymphoid follicle in the submucosa or lamina propria of the appendiceal wall. Scores were adapted from Stumpf et al. (grade 1 representing < 5 positive cells per HPF, grade 2 represents 6-19 cells per HPF and grade 3 >20 cells per HPF). 22

Statistical analysis
Continuous data are presented as mean ± standard deviation (SD) or as median and interquartile range (IQR) according to distribution. Categorical data are presented as frequencies and percentages. Independent t-test was used to compare means. Mann-Whitney-U test was used for continuous, not normally distributed data. To compare dichotomous data the χ²-test or Fisher’s exact test were used. All tests were two-sided and a P-value of <0.05 was deemed significant. Statistical analysis was done with IBM SPSS Statistics for Windows®, Version 19.0 (IBM Corp., Armonk, NY, United States).

RESULTS

Demographics
A total of 33 patients were included; 13 UC patients, 7 patients with CD, 5 patients with appendicitis and 8 patients with colonic carcinoma or FAP (non-inflammatory controls). The characteristics of all groups are summarized in Table 1.

Table 1 Baseline patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>UC (n = 13)</th>
<th>CD (n = 7)</th>
<th>AA (n = 5)</th>
<th>Controls (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender M/F</td>
<td>6/7</td>
<td>2/5</td>
<td>3/2</td>
<td>4/4</td>
</tr>
<tr>
<td>Age at surgery</td>
<td>43 [30.5-61]</td>
<td>26 [26-34]</td>
<td>40 [35-44.5]</td>
<td>60 [54-73.3]</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>63 [23.5-183]</td>
<td>45 [17-116]</td>
<td>-</td>
<td>0 [0-1]</td>
</tr>
<tr>
<td>Disease location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Left sided</td>
<td>8 (61.5)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>- Pancolitis</td>
<td>5 (38.5)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Medication at time of surgery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Steroids</td>
<td>7 (53.8)</td>
<td>2 (28.6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- AZA / 6MP / MTX</td>
<td>1 (7.7)</td>
<td>2 (28.6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- anti-TNF</td>
<td>-</td>
<td>1 (14.3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- anti-TNF + AZA / 6MP / MTX</td>
<td>-</td>
<td>1 (14.3)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Appendiceal lavage
In 26 of 33 patients, analysis of the lavage fluid could be performed. Since the amount of CD3+ T-lymphocytes per lavage was variable, only percentages of CD4+ and CD8+ cells were compared. An ROC curve demonstrated that the optimal cut-off for increased CD4+
was > 70% CD4+ lymphocytes per lavage (sensitivity 0.74 and specificity 0.65, data not shown). In UC patients, an increased proportion of CD4+ was seen in the lavage fluid when compared to the controls (75% versus 50%). This increase in CD4+ cells in the lavage fluid was also seen in CD and AA. Since the number of CD8+ cells remained relatively low in UC and CD, this resulted in high CD4/CD8 ratio’s in the lavage fluid for these patients groups (median 7.1 [iqr 4.2-8.1] and 5.2 [iqr 4.2-7.0], respectively) when compared to non-inflammatory controls (median 3.2 [iqr 2.5-6.2], P = 0.042). In AA patients, the associated increase in CD8+ cells resulted in a relatively low CD4/CD8 ratio. The results are shown in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>UC (n = 10)</th>
<th>CD (n = 5)</th>
<th>AA (n = 5)</th>
<th>Controls (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>75.7 [62.9-81.9]</td>
<td>75.0 [63.3-80.5]</td>
<td>73.9 [60.6-80.9]</td>
<td>64.6 [52.4-80.3]</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>7.1 [4.2-8.1]*</td>
<td>5.2 [4.2-7.0]</td>
<td>3.8 [3.1-6.0]</td>
<td>3.2 [2.5-6.2]*</td>
</tr>
</tbody>
</table>

* CD4/CD8 ratio UC patients versus controls P = 0.042, for all other comparisons P >0.05

**Histology and immunohistochemistry**

In all UC and control patients the appendices appeared macroscopically normal, but mucosal based inflammation with increased lymphocyte infiltration was seen in 9 of 13 UC patients (69%). CD and AA patients demonstrated macroscopically affected appendices with an increased diameter, thickened meso-appendix, and a fibrino-purulent exsudate covering the serosal surface. Histology confirmed the transmural inflammation with oedema and lymphocyte influx present in the macroscopically affected appendices. Immunohistochemistry demonstrated that the increased number of lymphocytes seen at histology were predominantly CD4+ cells.

The majority of UC (8/13 = 62%), CD (6/7 = 85.7%) and AA (3/5 = 60%) patients had increased levels of CD4+ lymphocytes (grade 2 and 3). In the control group, the majority of patients had no increased CD4+ cells (88% grade 1 CD4+). Extensive influx of CD4+ lymphocytes (grade 3) was seen in 4/13 UC patients (31%), in 5 CD patients (71%), 2 AA patients (40%) and was present in only 1 control patient (Figure 2).

Comparable to the lavage results, the amount of CD8+ lymphocytes in UC and CD appendices was found to be a relatively constant factor. Only 1 UC and 1 CD patient had increased CD8+ cells (grade 3). In the AA appendices with increased CD4+ cells, an accompanying increase in CD8+ cells (grade 3) was found.

Immunohistochemical results correlated to CD4+ percentages in lavage fluid in 80% of UC patients.
Correlation with clinical disease activity

Increased CD4+ was able to predict clinical disease activity in 90% of UC, 100% of AA, and 80% of CD patients. Two UC patients were in remission at time of surgery, both patients had low CD4+ cells in the lavage fluid and both scored grade 1 in the immunohistochemical analysis. In three UC patients, a peri-appendicular red patch was seen on preoperative colonoscopy. Unfortunately, this could not be correlated to disease activity or increased CD4+ levels or CD4/CD8 ratio because lavage was performed in only one of these patients. Of the CD patients, one patient had a CD4+ level grade 1, in this patient indication for surgery was a stenosis. One other CD patient operated upon for stenosis had grade 2.

DISCUSSION

This study aimed to analyse if T-cell phenotype in appendiceal lavage fluid of UC patients was associated with inflammatory activation. Despite a macroscopically normal appearance of the appendix, patients with active disease showed inflammatory characteristics and increased numbers of mucosal CD4+ lymphocytes in lavage fluid and in immunohistochemical analysis. The results suggest that appendiceal lavage may be a useful tool for monitoring disease activity and guiding clinical decision-making, particularly in cases where surgical intervention is contemplated.

Figure 2 Immunohistochemical analysis, CD4+ T cells are stained in this image (brown coloured cells). In image A, a normal appendix is shown. The appendix of patients with appendicitis showed extensive influx (grade 3 or 4) of CD4+ cells (B). Also in the appendix of UC patients, extensive CD4+ influx can be seen (C).
tochemical analysis, comparable to CD and AA. This increase in \( \text{CD}_4^+ \) lymphocytes was predictive of disease activity in the majority of patients. The \( \text{CD}_4/\text{CD}_8 \) ratio in lavage fluid was also significantly increased when compared to non-inflammatory controls. This elevated ratio in both UC and CD confirmed the difference in these auto-immune coordinated diseases when compared to AA, where a ratio comparable to non-inflammatory controls was found due to the associated increase in \( \text{CD}_8^+ \) cells.

In UC, the balance between T-helper cells and T-suppressor cells is shifted toward the T-helper cells. Infiltration of \( \text{CD}_4^+ \) T cells has been observed in the inflamed mucosa of UC patients, which results in enhancement of the inflammatory state by increased cytotoxic activity and cytokine production, eventually resulting in tissue injury. The appendix is known to be part of the gut-associated lymphoid tissue system. The mucosal lymphoid tissue of the appendix is predominantly composed of B cells and \( \text{CD}_4 \) T-helper cells. Here, T-lymphocytes are likely to get primed by various luminal antigens. A study by Matsushita et al. demonstrated an increased \( \text{CD}_4/\text{CD}_8 \) ratio in appendix biopsies of UC patients with active left sided colitis, when compared to non-inflammatory controls. Interestingly, as the \( \text{CD}_4/\text{CD}_8 \) ratio in the appendix increased, the ratio in the rectum tented to increase as well. Furthermore, the proportion of early activated T cells (\( \text{CD}_4^+\text{CD}_{69}^+ \) T cells) in the appendix of UC patients, irrespective of disease extension or activity, was increased compared to non-inflammatory controls. Another study reported that the histological inflammation grade in the entire colon (both in inflamed and non-inflamed parts) was higher in patients with left sided colitis with appendiceal involvement.

In a murine UC model with T-cell receptor (TCR)-\( \alpha \) deficient mice, \( \text{CD}_4^+ \) T-lymphocytes proliferate in the appendix, suggesting that the appendix is the priming site of cells involved in the disease process in these mice. The results of these studies have led to the suggestion that the \( \text{CD}_4/\text{CD}_8 \) ratio in the mucosa of the appendix represents the inflammation degree in the colonic mucosa and cytokine production within the appendix is proposed to trigger an immunological cascade in the colorectum. In this respect, the finding of an elevated \( \text{CD}_4/\text{CD}_8 \) ratio in UC patients in remission is intriguing. If indeed this appendiceal immunological disbalance would contribute to the development of this disease, this could explain why reduced relapsing rates have been described after appendectomy. The available literature suggests a more preferable clinical course with reduced need for immunosuppression therapy, reduced relapse rate and lower colectomy rates in appendectomised patients with UC, but strong evidence is lacking. Currently, an appendectomy is sometimes offered as an experimental treatment in UC patients, even though the efficacy has not been evaluated in a randomized setting.

Involvement of the appendix in UC is also observed clinically. Endoscopists have described a cecal patch or peri-appendicular red patch (PARP) in UC patients. This patch of focal activity surrounding the appendiceal orifice is seen in up to 86% of patients with
a left sided colitis and an otherwise normal right-sided colon. It has been hypothesized that this PARP could be used as a surrogate marker for ulcerative colitis.

In this study, we have prospectively identified patients with different inflammatory and non-inflammatory diseases and evaluated their appendical tissue. The appendices were evaluated on all levels; macro- and microscopically and by lavage of the appendical lumen. Assessment of the histological and immunohistochemical samples were blinded for diagnosis to avoid review bias. The intra- and inter-observer measurement error variability was restricted by independently repeating the sample scoring three times and by using three observers. This is a small exploratory study, that warrants further research. Currently, no objective measure of CD4+ cut off points in lavage fluid is available.

The results of this study indicate that despite a macroscopically normal appearance, appendices of UC patients with active disease show immunological activation with inflammatory characteristics and increased numbers of CD4+ lymphocytes in lavage fluid and immunohistochemical analysis. In contrast to AA there is no associated increase in the numbers of CD8+, resulting in a higher CD4/CD8 ratio. An increased immunohistochemical CD4+ level was associated with active inflammation in UC, CD and AA patients, with CD4+ proportions in patients without active inflammation comparable to healthy controls. These results support the notion that the appendix may play a pathogenic role in UC. Future research should focus on the role of the appendix in UC, the implications of (endoscopical) appendicular lavage as measurement for the inflammatory process in the appendix, and the effect of appendectomy in UC. Accordingly, we have initiated several studies including an international multicentre randomized trial on the effect of appendectomy on the clinical course of UC (the ACCURE trial, Netherlands Trial Register NTR2883).
OF THE APPENDIX IN ULCERATIVE COLITIS  CHAPTER 11

REFERENCES


19. Jones BA, Demetriades D, Segal I, Burkitt DP. The prevalence of appendiceal fecaliths in patients with and without appendicitis. A


