Neuronal hypertrophy and mast cells in histologically negative, clinically diagnosed acute appendicitis: a quantitative immunophenotypical analysis

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Abstract

Introduction and Aim In about 20–25% of appendicectomies performed for clinically suspected acute appendicitis, definite morphological changes are lacking on histopathological examination. The present study was done to investigate whether any changes in neurons and mast cells could be detected in patients presenting with clinical acute appendicitis but found to have normal appendix at histopathology.

Methods A descriptive study was conducted on 50 appendix specimens which were categorized as histology-positive acute appendicitis (HPAA), clinically acute appendicitis but histologically negative (HNAA), appendices resected for other causes and appendices from forensic autopsy. A morphometric and quantitative evaluation of nerve fibers and ganglion plexus and its relation to mast cell density were studied. All sections were subjected to hematoxylin and eosin stain, toluidine blue stain, S 100 protein and neuron specific enolase (NSE) immunostaining and a quantitative image analysis system.

Results Mucosal and submucosal neuronal components highlighted by NSE and S100 immunostaining observed in cases of HNAA were comparable to cases of HPAA. With S100 immunostaining in HNAA cases, the increase in number and size of myentric neuronal plexus were mild in 40% (10/25) cases, moderate in 40% (10/25) and marked in 20% (5/25) cases as compared to 66.7% (10/15) cases of HPAA showing moderate and 33.3% (5/15) cases showing marked increase (p = 0.018). The mean mast cell count was highest in the HNAA cases (2.74) in all the four layers as compared to the HPAA (1.85) and control group (2.05). There was no difference in the relationship of the size of ganglion cells and the mast cell concentration.

Conclusions Neuronal hypertrophy and mast cells may play a role in the pathogenesis of appendicitis-like pain in patients with histologically normal appendices.

Keywords Acute appendicitis · image analysis

Introduction

Acute appendicitis (AA), a common surgical emergency continues to be a challenging disease. Nearly 25% of the appendices resected for AA histologically show no definite inflammatory changes by conventional histological examination. Since appendectomy relieves pain, an unknown pathology is likely to exist. The role of inflammatory reactions involving local endocrine cells and neoproliferation in causing repeated attacks of pain has been described and several staining techniques including immunohistochemistry have been tried in detecting the pro-inflammatory mediators. The present study combined routine histopathology, immunohistochemistry and quantitative image analysis to investigate the discrepancy between the clinical and histopathological diagnosis and to elucidate the underlying pathophysiology.

Methods

A prospective study was conducted on 50 appendix specimens of which 48 were surgically resected and two were obtained at forensic autopsy. Of the 48 appendices surgically resected, 40 cases were clinically diagnosed as acute appendicitis. Eight cases removed during surgery for some other cause like cholecystectomy (one case) and intestinal
resection (seven cases) and two appendices from forensic autopsy were taken as normal control appendices. The patients’ name, age, sex and clinical data were recorded.

Hematoxylin and eosin (H & E) was used as a routine stain to establish the histopathological diagnosis and for general study of the tissue. Toluidine blue stain was performed to study mast cells. Immunohistochemistry with polymer labelling two-step method using Super Sensitive™ Polymer-HRP IHC detection system (Biogenex) was used to assess the markers, S 100 protein and neuron specific enolase (NSE). Immunostained sections were subjected to quantitative microscopic measurement with an in-house developed image analysis system and integrated software. This technique was used to quantify ganglion plexus in the submucosa and muscularis externa. The immunohistochemical images were taken by an Olympus BX 41 microscope with multihead attachments, and attached to Olympus DP20-5E microscope (Scientific Equipment Group of Olympus America Inc.) and transferred to a personal computer. The investigated microscopic visual fields consisted of areas mostly occupied by the largest ganglia and more number of ganglia under high power field (hpf). These non-overlapping representative fields were captured on to the camera. The images were then analyzed using the in house developed software. The facility for choosing the particular color representing the maximum density of tissue as a reference color is provided in the software. Using this facility, the color setting was done for analysis. The same setting was used for analysis of all images. The software assigns scores to the various shades of the color represented by each pixel of the image, based on how close the shade is to the reference color. Using these values the total tissue present in the image is quantified. Only the stained tissue is chosen for quantification. From such image, only the ganglia were first segmented. Later, the size of the ganglion was calculated as the area of the segmented ganglion structure and expressed as arbitrary pixels (AP).

The 50 appendices were divided into four categories on microscopy – histology-positive acute appendicitis (HPAA), clinically acute appendicitis but histologically negative (HNAA), appendices resected for some other cause (others) and appendices from forensic autopsy. HPAA was diagnosed on the basis of signs of inflammation that included neutrophils infiltrating throughout the muscular layer, epithelial erosion, vasodilatation, edema, abscesses and fibrinous exudates over the serosa. Scattered inflammatory cells within the lumen and/or in the serosa was not considered sufficient for a diagnosis. Perivascular neutrophils were also excluded. The grading of inflammation was based on number of neutrophils in the muscularis externa: less than (<) 5/hpf = Grade 1+, 5-15/hpf = Grade 2+ and more than (> = Grade 3+. Cases of HNAA were categorized based on absence of inflammation and intact epithelium with or without lymphoid hyperplasia.

The number and size of ganglion plexus were assessed in the wall of the appendices. The ganglion plexus in the muscularis externa stained by H & E were graded as: <1/hpf = Grade 1+ (mild hyperplasia), 1/hpf = Grade 2+ (moderate hyperplasia) and >1/hpf = Grade 3+ (marked hyperplasia). At least 10 non-overlapping contiguous fields were examined under high power and an average per hpf was taken. The size of the ganglion plexus stained immunohistochemically by S100 was calculated in ap and graded as in Table 1. The increase in number and size of ganglia (ganglion density) stained by S 100 immunostain was considered mild with Grade 1+ to 2+ ganglion size, moderate with Grade 3+ to 5+ and marked with Grade 6+ to 7+ ganglion size. The nerve fibers were stained by NSE in the mucosa and submucosa and their presence or absence was also noted.

The mast cells were counted in 10 contiguous fields using 40× objective lens and 10× eye piece. The average number of mast cells per hpf was calculated and expressed as 0-2/hpf (Grade 1+), 2.1-4/hpf (Grade 2+), 4.1-6/hpf (Grade 3+) and more than 6/hpf (Grade 4+). In the mucosa, areas with lymphoid tissue were excluded and only area where the mucosa was continuous was selected.

Different parameters were compared using Pearson’s Chi square (χ2) test or Fisher’s exact test, as applicable. Correlation between neutrophils and size of ganglion plexus in the

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**Table 1**  Distribution of cases according to grading of ganglion plexus in submucosa by S100 immunostaining

<table>
<thead>
<tr>
<th>Grading of ganglion plexus *</th>
<th>Histologically negative acute appendicitis</th>
<th>Histologically positive acute appendicitis</th>
<th>Others</th>
<th>Autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+ (&lt;50,000)</td>
<td>19 (76)</td>
<td>10 (66.7)</td>
<td>5 (62.5)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>2+ (50,000–1,00,000)</td>
<td>6 (24)</td>
<td>1 (6.7)</td>
<td>3 (37.5)</td>
<td>0</td>
</tr>
<tr>
<td>3+ (1,00,000–2,00,000)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4+ (2,00,000–3,00,000)</td>
<td>0</td>
<td>2 (13.3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5+ (3,00,000–4,00,000)</td>
<td>0</td>
<td>1 (6.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6+ (4,00,000–5,00,000)</td>
<td>0</td>
<td>1 (6.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7+ (5,00,000–6,00,000)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values in parenthesis denote size of ganglion plexus (in arbitrary pixels). Data are as n (%)
muscularis externa in cases of HPAA was calculated using Spearman’s rank correlation coefficient, p value < 0.05 was considered significant.

Results

The 50 specimens of appendix analyzed in this study comprised four broad categories: 25 cases of HNAA (15 females, 10 males), 15 cases of HPAA (10 females, 5 males), eight cases of ‘others’ and two cases of autopsy. Of the 25 cases of HNAA category, 22 cases showed lymphoid hyperplasia. Twenty-two cases (44%) were in the age group of 21–30 years. Chronic pain (>2 weeks) was seen in 10 of 25 (40%) cases of the HNAA group as compared to 4 of 15 (26.7%) cases with HPAA. Acute pain (<2 weeks) was seen in 11 (73.3%) cases of HPAA and 15 (60%) cases of HNAA. The length of appendices varied from 3.5 cm to 12 cm (median 5.5 cm).

Fine nerve fibers identified by NSE immunostaining were positive in 7 of 25 (28%) cases of HNAA category, as compared to 5 of 15 (33.3%) cases of HPAA and one of 8 (12.5%) cases in the control group. The nerve fibers in the mucosa (M) were near the bottom and around the crypts. Nerve fibers in mucosa stained by S100 protein were seen in 18 (72%) cases of HNAA appendices as compared to 10 (66.6%) cases of HPAA and four (50%) cases in the control group. The nerve fibers were in contact with the marginal zone of the reactive lymphoid follicles. In the submucosa (SM), 19 cases of HNAA category showed grade 1+ ganglion plexus and 6 cases, grade 2+, while larger ganglion plexus (> grade 4) was seen in four (26.7%) cases of HPAA compared to none of the cases in other categories (p = 0.005; Table 1). S 100 protein immunostaining augmented the detection rate of ganglion plexus in muscularis externa (ME; Fig. 1). Moderate neuronal hyperplasia on H & E stained sections was seen in eight (32%) cases of HNAA category as compared to mild neuronal hyperplasia seen in all 15 cases of HPAA (p = 0.016). With S 100 immunostaining, the increase in number and size of ganglia in 25 HNAA cases was classified as mild in 10 (40%), moderate in 10 (40%) and marked in 5 (20%) cases when compared to 15 HPAA cases in which it was classified as moderate in 10 (66.7%) and marked in 5 (33.3%; p = 0.018). Seven of eight cases of the control group with H & E staining showed mild increase in number and size of ganglia, and a moderate increase were seen in three of 10 cases with S100 staining.

Of the 50 appendices, mucosal mast cells ≤4/hpf was seen in 46 (92%) cases and >4/hpf in four cases. In muscularis externa, mast cells ≤4/hpf were seen in 42 (84%) cases and >4/hpf in eight cases; such a pattern was not observed in the distribution of mast cells in submucosa. There were no cases with mast cells >4/hpf in the serosa (S). Distribution of cases according to presence of mast cells in all the four layers is given in Table 2. The mean mast cell count was highest in submucosa in cases of HPAA and HNAA (Fig. 2). Identification of mast cells in the mucosa was difficult due to the presence of large lymphoid follicles. In the submucosa, the mast cells were larger, ranging from spindle to polygonal in shape and they contained coarse and numerous granules as compared to mucosal mast cells.

In HNAA cases, there was no statistically significant relationship between ganglion cell size and mast cell concentration. Increased mast cell concentration (4.1–6/hpf) was seen in 12% (3/25) of HNAA cases showing nerve fibers in the mucosa.

In cases of HPAA, the grade of inflammation paralleled proportionately with the size of ganglion plexus (p = 0.027; Table 3). Seven of eight cases of HPAA with neutrophils >15/hpf had ganglion plexus of Grades 5, 6 and 7 and five cases with neutrophils <15/hpf had Grade 3 and 4 ganglion plexus. However, a decreased number of mast cells (0–2/hpf) was seen in 77.8% (7/9) cases of HPAA with increased neutrophils (>15/hpf) and 60% (9/15) cases with ganglion plexus >2,00,000 AP.

**Fig. 1**  S 100 protein immunostaining of ganglion cells in muscularis externa (original magnification × 400)

**Fig. 2**  Mast cells in submucosa in histologically negative cases of acute appendicitis (Toulidine blue × 400)
Table 2  Distribution of cases according to presence of mast cells in all the four layers of appendices

<table>
<thead>
<tr>
<th>MC/hpf</th>
<th>HNAA</th>
<th>HPAA</th>
<th>O</th>
<th>A</th>
<th>HNAA</th>
<th>HPAA</th>
<th>O</th>
<th>A</th>
<th>HNAA</th>
<th>HPAA</th>
<th>O</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–2</td>
<td>18</td>
<td>9</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>12</td>
<td>11</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>2.1–04</td>
<td>3</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4.1–6</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>&gt;6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>15</td>
<td>8</td>
<td>2</td>
<td>25</td>
<td>15</td>
<td>8</td>
<td>2</td>
<td>25</td>
<td>15</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

MC : mast cells, HNAA : histopathologically negative acute appendicitis, HPAA : histopathologically positive acute appendicitis, O : others, A : autopsy

Table 3  Relationship between neutrophils and size of ganglion plexus in the muscularis externa in cases of acute appendicitis

<table>
<thead>
<tr>
<th>Neutrophils</th>
<th>Grade of ganglion plexus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade 3</td>
</tr>
<tr>
<td>&lt;5/hpf</td>
<td>1</td>
</tr>
<tr>
<td>5–15/hpf</td>
<td>1</td>
</tr>
<tr>
<td>&gt;15/hpf</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are as number of cases. Spearman’s correlation coefficient, \( \rho = 0.568 \) (\( p = 0.027 \)).

Discussion

Appendicitis still continues to challenge the diagnostic skills of a surgeon. The exact etiopathogenesis is poorly understood in those appendices resected for suspected appendicitis and subsequently classified as normal by histopathology on conventional staining; some showing presence of enlarged lymphoid follicles.6

In the present study NSE positive fine nerve fibers were seen in the mucosa, especially near the base and around the crypts, whereas, Xiong et al observed fine nerve fibers near the epithelial surface and large nerve fibers near the bottom and in between the crypts in 40% of HNAA cases.7 Neural components seen in all four layers of appendices in HNAA cases were increased or comparable with cases of HPAA suggesting the possibility of right iliac fossa pain in the absence of inflammation. The S 100 positive nerve fibers were in contact with the marginal zone of the reactive lymphoid follicles as observed by Di Sebastiano et al, wherein they demonstrated increased growth associated protein-43 and substance P immunoreactive nerve fibers in HNAA cases and concluded that the close spatial arrangement of nerve fibers and lymphoid cells suggest neuroimmune interaction as a cause of pain in neuroimmune appendicitis; a distinct pathologic entity.3 Neural hyperplasia restricted to the mucosa was seen in 4% cases (6/150) of HN cases studied by Naik.8 Few authors have reported significant submucosal and myenteric neuronal hypertrophy in both HNAA and HPAA.4,7,8 Franke et al observed ‘neurogenic appendectomy’ (NA) in 3.8% of patients with HPAA and in 47% of those which are HNAA and found neither history nor clinical examination will enable preoperatively to differentiate HPAA and NA.9

On comparing H & E staining with S100 staining, 100% accuracy for H & E staining was found for the presence of ganglion plexus as compared to 93% accuracy observed by Franke et al in the diagnosis of ‘neurogenic appendicitis’ (NA).3 On H & E staining, 32% (8/25) cases in the HNAA group showed moderate neuronal hypertrophy in muscularis externa as compared to 0% cases of AA which was statistically significant (\( p = 0.0162 \)) and in concordance with that of Franke et al. Ganglia were found between the circular and longitudinal layers and also deep within the muscle layers. Only mild neural hyperplasia was seen in all cases of AA on H & E staining and may be explained by the mucosal destruction and dense inflammatory exudates that obscure the morphology of the tiny nerve twigs. Immunostaining by S 100 is mandatory in such cases to make an accurate estimation like in the present study and was in agreement with that of Xiong et al.7

Mast cells, seen in all the four layers of the appendices were in highest concentration in the submucosa in all the categories which was in contradiction to Xiong et al, Naik et al and Coşkun et al, wherein they found higher mucosal mean mast cells count as compared to the submucosa.7,10,11 This may be because identification in the mucosa was difficult due to the presence of large lymphoid follicles. In the submucosa, the mast cells were larger, spindle to polygonal in shape and contained coarse numerous granules as compared to mucosal mast cells. The mean mast cell count was highest in the HNAA category in all the four layers as compared to the HPAA and the control group and its distribution was similar to that observed by Naik et al.10 HPAA was associated with reduced mast cell count as compared to HNAA cases; perhaps because the initial stimulus is luminal and the reaction of mast cells with injury is either elimination through the mucosa or progressive degranulation with subsequent inability to detect them. Xiong et al got divergent findings with increased mast cells associated with increased ganglia and Schwann cells in cases of HPAA.7
Neuronal hypertrophy and mast cells in histologically negative

NSE positive mucosal nerve fibers, enlarged submucosal ganglion plexus (Grade 5 and 6) and marked myentric neuronal hypertrophy seen in HPAA paralleled with increased neutrophils (>15/hpf) and negatively correlated with the mast cell counts. Inflammation or injury can affect peripheral nerve remodeling and neural proliferation represents a form of inflammatory response.

To summarize, quantitative immunohistochemical analysis provide a supplementary technique to the conventional staining method to observe the changes in the pattern of innervation and morphology of nerve fibers in the appendiceal wall. Significant neuronal hypertrophy highlighted by NSE and S100 immunostaining was observed in cases of HNAA, similar to that of cases of HPAA. In all the four layers of the appendix, the concentration of mast cells was increased supporting the concept that repeated episodes of inflammatory process exist in its underlying pathogenesis. Hence, neurogenic appendicopathy is a distinct histopathological entity clinically presenting in the same way as acute appendicitis and is diagnosed on H & E staining with further confirmation by S100 and NSE immunostaining.

Acknowledgement

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References