Enteric neuroglial apoptosis in inflammatory bowel diseases

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Apoptosis; Crohn's disease; Enteric glial cells; Enteric neurons; Ulcerative colitis

Abstract

Background: Enteric nervous system abnormalities have been described in patients with inflammatory bowel diseases. However, the mechanisms responsible for these abnormalities remain to date largely unknown.

Aims: We investigated the potential role of apoptotic phenomena in enteric neurons and enteroglial cells in patients with inflammatory bowel diseases.

Patients and methods: Full-thickness surgical specimens of 19 patients undergoing surgery for medically refractory disease (9 from the ileum of patients with Crohn's disease, 10 from the colon of patients with ulcerative colitis) were assessed for the presence of enteric neurons and enteroglial cells and for their apoptosis by two immunohistochemical methods, one also able to distinguish apoptosis from necrosis. The results were compared with those obtained in control specimens.

Results: Concerning Crohn's disease, the ileal segments displayed a significant increase of apoptotic enteric neurons and enteroglial cells in both the submucous and the myenteric plexus compared to controls. In patients with ulcerative colitis, compared to controls, apoptotic phenomena were significantly reduced in enteric neurons, whereas they were increased in the enteroglial cell population (submucous and myenteric plexus).

Conclusions: In patients with inflammatory bowel disease apoptotic phenomena involve both enteric neurons and enteroglial cells, and may play a role in the abnormalities of the enteric nervous system. The importance of these findings in the pathophysiology of these conditions remains to be determined.

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Enteric neugroglial apoptosis in IBD

1. Introduction

There is increasing evidence that the enteric nervous system (ENS) plays a pivotal role in the development of inflammatory bowel disease (IBD) and its associated symptoms, through the control and the modulation of the gut immune system. This role is mainly tuned by two cell populations, the enteric neurons and the enteric glial cells (ENS); the first control mucosal (through the submucosal plexus, MP) and motor (through the myenteric plexus, MP) functions, whereas the latter (by means of their modulation of neuronal functions) act directly or indirectly on the gut immune system and other functions.

Thus, it is not surprising that ENS abnormalities have been reported in IBD patients; besides, their occurrence in macroscopically uninvolved areas may actually suggest that they precede mucosal inflammation. Older studies carried out with standard pathological techniques have often yielded heterogeneous results, whereas more recent studies with immunohistochemical methods and electron microscopy have shown an increased expression of nitric oxide synthase in neuronal cell bodies, a variable increase of EGC, an increased number of mast cells and axonal necrosis of gut nerves, decrease and ultrastructural injury of ICC, and ganglioneuritis.

It is worth noting that most of the above studies only focused on one aspect of ENS in these patients. We have recently shown, in a study taking simultaneously into considerations various aspects of the ENS (neurons, EGC, interstitial cells of Cajal (ICC), cell types involved in the inflammatory response (T lymphocytes, mast cells)), that IBD patients with ileal Crohn’s disease (CD) and ulcerative colitis (UC) have discrete abnormalities in this area.

However, we had no clue on the origin of these abnormalities, apart from hypothesizing some influence of the inflammatory process. In previous studies we demonstrated that apoptotic phenomena may be involved in the death of at least some cell population (in particular, neurons) of the ENS in other pathologic conditions. Moreover, there is evidence in experimental animal models of colitis of ENS neuronal loss due to increased apoptotic phenomena. We tried to establish whether this mechanism could also be significantly present in the main ENS cell populations of IBD patients.

2. Patients and methods

Archival full thickness specimens from 19 IBD patients (9 from the ileum of CD patients, 5 men and 4 women, age range 37–49 years; 10 from the colon of UC patients, 5 men and 5 women, age range 43–57 years) were obtained from patients undergoing surgery for severe disease refractory to medical treatment in the period July 2007–January 2008. The diagnosis of CD or UC was based on clinical, radiologic, and endoscopic examination and histologic findings. All IBD patients had been treated with 5-amino-salicylic acid and immunosuppressive drugs.

The samples studied were taken from macroscopically involved areas. For each patient we chose the samples better oriented and more representative of disease.

Control specimens were obtained from patients undergoing colonic (n=10, 5 man and 5 women, age range 41–59 years) or ileocolonic (n=15, 10 men and 5 women, age range 35–50 years) resection for neoplastic disease. No control had received treatments likely to alter the ENS. The samples studied were taken at least 3 cm from the resection margin in tumour free areas. The same staining methods used for the patients were also used for the evaluation of these control samples. The time from tissue resection to fixation was similar for both patients and controls.

3. Methods

Full thickness sections were obtained from ileal or colonic samples. For conventional histology 5 μm paraffin sections were stained with hematoxylin–eosin, PAS and Trichrome stain.

3.1. Immunohistochemistry

At least 20 slides for each patient were processed for immunohistochemistry. We used monoclonal antibodies directed against neuron-specific enolase (NSE, NCL-NSE2, Novocastra laboratories, dilution 1:50) as a marker of neuronal cell bodies in the ganglia, and the glial marker protein S100 (S-100, Dako, dilution 1:50) for enteroagial cells, as previously described. Apoptosis was evaluated with two methods: by evaluating the expression the caspase-3 (a so-called executioner caspase), and by a monoclonal antibody to single-stranded DNA, using the formamide monoclonal antibody (formamide-MAb) method, as previously described.

NSE and S-100 immunostaining was carried out, as previously described, using a peroxidase-based visualization kit (Dako LSAB®), following the manufacturer’s recommendations. Diaminobenzidine tetrahydrochloride was used as chromogen. The slides were then counterstained with Mayer’s hematoxylin for 5 s, dehydrated and mounted in Clarion (Biomed). To account for non-specific staining, peptides that blocked polyclonal antibody bindings (passage cooling, endogenous peroxidase activity was suppressed by incubation with 3% solution of H2O2 for 5 min, then the sections were washed again in Tris, the latter carefully blotted, and incubated for 15 min with DAB Chromogen (Menarini), washed again in Tris and incubated for 15 min with Post Primary Block (Menarini). Thereafter, the sections were washed twice in Tris, incubated for 15 min with NovoLink Polymer (Menarini), washed again in Tris and incubated with DAB Chromogen (Menarini) for 5 min, washed with water and counterstained with hematoxylin for 1 min, dehydrated and mounted in Clarion (Biomed).

Concerning the formamide-MAb method, the sections were incubated for 5 min in PBS with the addition of 20% Tween 20, followed by a passage with proteinase K (Dako, Carpinteria, CA, USA) for 20 min. The sections were then rinsed with distilled water and heated in 50% formamide prewarmed to 60 °C for 20 min. The sections were then incubated with 5% formamide prewarmed to 60 °C for 20 min.
20 min. After cooling, endogenous peroxidase activity was suppressed by incubation with 3% solution of H2O2 for 5 min. Normal serum diluted 1:50 was applied for 10 min to room temperature, followed by anti-DNA MAb (Mab F7-26 BMS 156, Bender MedSystem, Vienna, Austria) for 30 min, according to the manufacturer’s recommendations. After that, the sections were incubated at room temperature with secondary polymeric antibody for 20 min and ABC (Kit super sensitive non biotin detection system, Menarini, Firenze, Italy) for 30 min. Finally, a 5 minute reaction in the dark with diaminobenzidine (DAB, BioOptica, Milano, Italy) was carried out, and the sections were then counterstained with Mayer’s hematoxylin for 5 s, dehydrated and mounted in Clarion (Biomeda, Foster City, CA, USA). Positivity was observed under the microscope as an intense brown reaction.

3.2. Data analysis

All slides were coded and analyzed blind by one pathologist (VV). Both the submucosal and the myenteric plexuses were taken into account by optical microscopy (Olympus BX 40, Tokyo, Japan) at ×20 magnification. For each patient, the number of immunopositive cells was calculated and expressed as the mean of cells on 10 well stained and well oriented microscopic fields for each region of interest (submucosal and myenteric plexus). Staining intensity was determined as 0=negative, 1=weak, 2=moderate, and 3=strong. To be considered as positive, the intensity of cell immunostaining in relation to possible background had to be from moderate to strong, as previously described.24

3.3. Statistical analysis

Data from controls and patients were compared by nonparametric methods, using the Wilcoxon test. Values of $p<0.05$ were chosen for rejection of the null hypothesis. Data are expressed as median (95% CI).

3.4. Ethical considerations

Since this was a retrospective study, no individual patient identification was involved and no study-driven clinical intervention was performed; therefore no ethical approval was necessary.

4. Results

4.1. Crohn’s disease

The evaluation with conventional and histochemical stains (H&E, PAS, Trichrome) was used only for the morphological characteristics of the disease, and to exclude neoplastic involvement in controls. Microscopy confirmed the presence of classical lesions, especially transmural lymphoid hyperplasia, in sections obtained from diseased areas and normal morphology in sections from non-involved tissue in all cases.

4.2. Immunohistochemistry

Results are shown in Table 1. In the affected ileal segments there were no differences between patients and controls in the number of enteric neurons, in both the submucous and the myenteric plexus. However, the two plexi displayed a significant increase of apoptosis (documented with both methods) of the enteric neurons compared to controls.

Concerning EGC, the number of these cells was significantly greater in patients compared to controls, in both the submucous ($p=0.04$) and the myenteric ($p=0.04$) plexus. Apoptotic phenomena in these cells were also significantly increased (with the two methods) in patients compared to controls, in both the submucous and the myenteric plexus (Table 1).

4.3. Ulcerative colitis

Again, the evaluation with conventional and histochemical stains (H&E, PAS, Trichrome) was used only to assess the morphological aspects of the disease, and to exclude neoplastic involvement in controls. Microscopy confirmed the presence of classical lesions in sections obtained from diseased areas and normal morphology in sections from non-involved tissue in all cases.

4.4. Immunohistochemistry

Results are summarized in Table 2. Compared to controls, the number of colonic enteric neurons was greater in the submucous plexus ($p=0.006$), whereas no differences between groups were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Immunohistochemical results in the ileum of CD patients and controls.</th>
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<tbody>
<tr>
<td></td>
<td>CD (59 (46–72))</td>
</tr>
<tr>
<td>NSE-MP</td>
<td>59 (46–72)</td>
</tr>
<tr>
<td>NSE-SP</td>
<td>35 (29–52)</td>
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<tr>
<td>S100-MP</td>
<td>259 (197–301)</td>
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<tr>
<td>S100-SP</td>
<td>115 (90–148)</td>
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<tr>
<td>Apoptosis-enteric neurons (formamide-Mab)-MP</td>
<td>17 (13–27)</td>
</tr>
<tr>
<td>Apoptosis-enteric neurons (formamide-Mab)-SP</td>
<td>19 (14–31)</td>
</tr>
<tr>
<td>Apoptosis-EGC (formamide-Mab)-MP</td>
<td>19 (16–31)</td>
</tr>
<tr>
<td>Apoptosis-EGC (formamide-Mab)-SP</td>
<td>21 (16–32)</td>
</tr>
<tr>
<td>Apoptosis-enteric neurons (caspase)-MP</td>
<td>10 (6–11)</td>
</tr>
<tr>
<td>Apoptosis-enteric neurons (caspase)-SP</td>
<td>8 (4–12)</td>
</tr>
<tr>
<td>Apoptosis-EGC (caspase)-MP</td>
<td>9 (4–12)</td>
</tr>
<tr>
<td>Apoptosis-EGC (caspase)-SP</td>
<td>10 (5–11)</td>
</tr>
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</table>
found in the myenteric plexus ($p=0.15$). When assessed by the formamide-Mab method, the number of apoptotic neurons was significantly decreased compared to controls, in both the submucous and the myenteric plexus, whereas no differences between groups were found for the caspase-3.

With respect to EGC, no significant increase was found compared to controls in the submucous plexus ($p=0.16$), whereas patients had a greater number of these cells in the myenteric plexus ($p=0.003$). Apoptotic phenomena of EGC were significantly increased in patients in both plexi with the formamide-Mab method, and only in the submucous plexus with the caspase-3.

Representative immunohistochemical images are shown in Figs. 1–3.

5. Discussion

A growing literature amount suggests that the ENS is more or less deranged in patients with IBD, with enteric neurons and

<table>
<thead>
<tr>
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<th>UC</th>
<th>Controls</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSE-MP</td>
<td>71 (63–87)</td>
<td>62 (52–76)</td>
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<tr>
<td>NSE-SP</td>
<td>32 (28–44)</td>
<td>50 (44–84)</td>
<td>0.006</td>
</tr>
<tr>
<td>S100-MP</td>
<td>267 (229–289)</td>
<td>204 (185–271)</td>
<td>0.03</td>
</tr>
<tr>
<td>S100-SP</td>
<td>104 (64–128)</td>
<td>115 (92–182)</td>
<td>0.16</td>
</tr>
<tr>
<td>Apoptosis-enteric neurons (formamide-Mab)-MP</td>
<td>1 (1–2)</td>
<td>9 (7–15)</td>
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</tr>
<tr>
<td>Apoptosis-enteric neurons (formamide-Mab)-SP</td>
<td>4 (4–5)</td>
<td>10 (8–18)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Apoptosis-EGC (formamide-Mab)-MP</td>
<td>1 (1–2)</td>
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<td>0.0001</td>
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<tr>
<td>Apoptosis-EGC (formamide-Mab)-SP</td>
<td>5 (3–6)</td>
<td>0 (0–0)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Apoptosis-enteric neurons (caspase)-MP</td>
<td>1 (1–2)</td>
<td>0.5 (0–1)</td>
<td>0.06</td>
</tr>
<tr>
<td>Apoptosis-enteric neurons (caspase)-SP</td>
<td>1 (0–1)</td>
<td>0 (0–0)</td>
<td>0.44</td>
</tr>
<tr>
<td>Apoptosis-EGC (caspase)-MP</td>
<td>1 (1–2)</td>
<td>1 (1–3)</td>
<td>0.66</td>
</tr>
<tr>
<td>Apoptosis-EGC (caspase)-SP</td>
<td>5 (3–6)</td>
<td>1 (0–2)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Figure 1  A. Representative image of enteric neurons in the submucous plexus of a CD patient (arrow). NSE, original magnification ×60. B. Representative image of enteric neurons in the myenteric plexus of a CD patient (arrow). NSE, original magnification ×100. C. Representative image of enteric glial cells in the submucous plexus of a UC patient (arrow). S100, original magnification ×100. D. Representative image of enteric glial cells in the myenteric plexus of a UC patient (arrow). S100, original magnification ×100.
EGC displaying several grades of abnormality in the various layers of the lower gut.\textsuperscript{10–16} However, to date little or no explanation is available for these abnormalities.\textsuperscript{16}

Apoptosis, also defined as programmed cell death, is one of the principal mechanisms by which cells are physiologically eliminated in metazoan organisms,\textsuperscript{25} and can be considered a

Figure 2  A. Representative image of an apoptotic enteric neuron (arrow) in the submucous plexus of a control. Formamide-MAb method, original magnification ×100. B. Representative image of apoptotic EGC (arrows) in the myenteric plexus of a CD patient. The long arrow shows a cell not interested by apoptotic phenomena. Formamide-MAb method, original magnification ×40.

Figure 3  A. Representative image of apoptotic enteric neurons (arrows) in the submucous plexus of a CD patient. Caspase-3, original magnification ×100. B. Representative image of apoptotic EGC (arrows) in the myenteric plexus of a UC patient. The thick arrow shows a cell not interested by apoptotic phenomena. Caspase-3, original magnification ×40.
cell-intrinsic mechanism for suicide that is regulated by a variety of cellular signalling pathways. Apoptosis is essential for development and adult tissue homeostasis, and its abnormal increase is observed in many pathologic processes. Some evidence in experimental animal models of colitis also suggests that the loss of myenteric neurons may be partly due to apoptotic phenomena.

For these reasons, we investigated whether the loss of these elements in IBD patients might be due to increased apoptotic phenomena. Thus, data were obtained from the main targets of specific IBD, i.e. the ileum in CD patients and the colon in UC patients.

To verify this hypothesis, we have adopted a conventional (the caspase-3, a so-called executioner caspase) and a non-conventional method to assess apoptosis, i.e. the monoclonal antibody to single-stranded DNA, using the formamide monoclonal antibody (formamide-MAb) method, already used with success in previous studies on the ENS. We adopted this method because: a) it is able to detect apoptotic cells in frozen sections and in sections of formalin-fixed, paraffin embedded tissues; b) it is unaffected by DNA breaks; and c) it specifically stains the apoptotic cells distinguishing them from the necrotic ones. In addition, we have previously demonstrated that the formamide-MAb method yields the highest number of apoptotic cells in the ENS compared to more traditional methods, such as the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) test and the caspase-3 expression.

In this study we provide evidence that apoptotic phenomena may be, at least in part, responsible for some of the abnormalities of enteric neurons and EGC described in IBD patients, with some differences between CD and UC.

In CD patients, the affected segments displayed a significant increase of apoptotic enteric neurons and EGC in both the submucous and the myenteric plexus compared to controls; in UC patients differences compared to controls were also found. In fact, apoptotic phenomena were significantly reduced in enteric neurons, whereas they were increased in the EGC population (submucous and myenteric plexus).

We are at present unable to explain the increased apoptotic phenomena in IBD patients. Several points merit to be discussed: a) although aging has been demonstrated to play a role in the loss of enteric neurons, we feel unlikely that this variable could have played a significant role in this setting, because our patients and controls were relatively young and, besides, were similarly aged; b) another factor to take into consideration is that these were all severely compromised patients requiring surgery, and had been treated with immunosuppressive drugs, including steroids, that are well-known pro-apoptotic agents; c) increased neuronal apoptosis could represent a mechanism to counteract the neurochemical remodeling of these cells observed in IBD patients, but it is unknown whether ENS repair is associated with neuronal renewal, even though progenitor-like cells have been documented in the ENS; d) the loss of EGC by apoptotic phenomena is interesting, since these cells control intestinal permeability, and their loss increases vascular and paracellular permeability before overt signs of inflammation appear. On the other hand, the higher levels of apoptosis we found in EGC were not mirrored by a parallel decrease of these cells in IBD patients. One possible explanation is that the increase in EGC, well documented in several studies in IBD patients, might represent the organism attempt to compensate for the loss of this cell type in IBD.

Finally, we have no explanation for the fact that apoptotic phenomena were reduced in enteric neurons of UC patients compared to controls. We feel unlikely that this might be attributed to the use of controls undergoing surgery for neoplastic disease, since the sections were obtained far from the tumor and were completely free from neoplastic cells. It could be hypothesized that in UC some still unknown mechanism somewhat acts as a protective factor toward this cell population, as shown by the different expression of inflammatory mediators in the enteric neurons of CD and UC.

In conclusion, we confirm that the ENS is involved in patients with IBD, and provide evidence that some of the abnormalities previously described may be due to increased apoptotic phenomena involving the neuro-glial network. Some differences between CD and UC are probably due to the more superficial involvement of the inflammatory phenomena in the latter condition. Further work is needed to establish the proper role of these findings, and to see whether these may be useful to provide some insight for alternative therapeutic approaches.

References

13. Dvorak AM, Monahan RA, Osage JE, Dickersin GR. Crohn’s disease: transmission electron microscopic studies. II. Immunologic