Immunohistochemical Study of the Colonic Muscle and Innervation in Idiopathic Chronic Constipation

H. J. Park, M.D.,*† M. A. Kamm, M.D.,* A. M. Abbasi, M.D.,* I. C. Talbot, M.D.*

*From *St. Mark's Hospital, London, United Kingdom, and the †Department of Internal Medicine, Yonsei University Medical College, Seoul, Korea*

PURPOSE: This study was designed to investigate neural and muscular features of the colonic wall in patients with severe idiopathic constipation. METHODS: By using quantitative immunohistochemistry, resected specimens from 14 patients with idiopathic chronic constipation and 17 nonobstructed cancer controls were studied. RESULTS: Routine histology revealed no significant histologic abnormality throughout the colon apart from four cases of melanosis coli. Ratio of the thickness of circular to longitudinal muscle was significantly lower in the left colon in constipated subjects. The myenteric plexus appeared morphologically normal in all subjects. S-100 protein, which stains neuronal supporting tissues, demonstrated an increase in the proportion of neural tissue in the myenteric plexus. There was an increased number of PGP-9.5 immunoreactive nerve fibers in the muscularis propria in constipated patients, and this was significantly higher in the ascending and descending colon. CONCLUSION: Intractably constipated patients have alterations in the neural composition of the colonic myenteric plexus and innervation of the circular muscle. [Key words: Colon; Innervation; Nerves; Muscle; Immunohistochemistry; Constipation]

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Severe constipation in women, in the absence of a recognized cause, may rarely necessitate a therapeutic colectomy.[1] Recent studies have reported morphologic abnormalities of the myenteric plexus in the colon of such patients.[2, 3]

Krishnamurthy *et al.*[2] studied 12 patients with constipation who underwent colectomy. Conventional light microscopy, using hematoxylin and eosin serial sections, showed melanosis coli in four patients, thinning of the circular muscle in one, and no apparent abnormalities of the myenteric plexus. However, silver stains of the myenteric plexus showed quantitatively reduced numbers of argyrophilic neurons, morphologically abnormal argyrophilic neurons, decreased numbers of axons, and increased numbers of variably sized nuclei within ganglia. They concluded that idiopathic chronic constipation is associated with a pathologically identifiable abnormality of the myenteric plexus and hypothesized that these abnormalities might be of developmental origin. However, neuropathologic changes of the colonic wall in chronic constipation require further definition and quantitative analysis.

To search for intrinsic neuronal abnormalities in the colonic wall in patients with severe constipation, we have used immunohistochemical staining for PGP-9.5 and S-100 protein. To make our results objective, we undertook quantitation using computer-assisted image analysis,[4] after manual validation.

MATERIALS AND METHODS

Specimens were obtained from 14 patients (1 man, 13 women; mean age, 45 (range, 18–81) years) undergoing colectomy for treatment of idiopathic chronic constipation. All patients had a colon of normal diameter, and secondary causes of constipation were excluded. All patients had a spontaneous bowel frequency of less than one bowel movement per week and had documented slow transit assessed by radio-opaque marker colonic transit studies.[5] Control specimens were obtained from 17 patients (4 women, 13 men; mean age, 61 (range, 41–80) years) undergoing colectomy for nonobstructive carcinoma of the colon or rectum. All control patients had a normal bowel frequency and no history of other colonic disease. Tissue was taken from a site distant from the cancer that appeared macroscopically and histologically normal. From the constipated patients, sections were examined from the ascending colon, transverse colon, descending colon, sigmoid colon, and rectum.

Corresponding tissues were taken from control resections, depending on the site of cancer resections. Data on sites examined are shown in Table 1.

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Table 1.
Ratio of Circular to Longitudinal Muscle Thickness

	Control	Patient	
Ascending colon	3.0 ± 1.2 (n = 8)	2.6 ± 1.3 (n = 12)	NS
Transverse colon	3.3 ± 1.6 (n = 10)	3.1 ± 1.8 (n = 8)	NS
Descending colon	3.2 ± 0.7 (n = 4)	2.4 ± 0.9 (n = 6)	$P < 0.05$
Sigmoid colon	3.1 ± 1.9 (n = 8)	2.0 ± 1.2 (n = 13)	$P < 0.05$
Rectum	4.2 ± 2.9 (n = 8)	3.6 ± 1.6 (n = 5)	NS

Data are presented as mean ± SD. NS = Not significant ($P > 0.05$).

Immunohistochemistry

Tissues were fixed overnight in 10 percent unbuffered formal-saline and processed into paraffin wax. Tissue sections (5 μm) were cut for both hematoxylin and eosin staining and for immunohistochemistry. In all cases the latter used the avidin-biotin complex for S-100 protein and PGP-9.5.[6–8]

The process of immunohistochemical staining was as follows. The deparaffinized sections were immersed in methanol containing 0.3 percent hydrogen peroxide for 30 minutes at room temperature to eliminate endogenous peroxidase activity. Trypsin (Sigma, Poole, UK) pretreatment was performed for S-100 protein stain to reveal over-fixed antigenic sites according to previously described methods.[9]

After washing in phosphate-buffered saline and incubating with normal swine serum (1:20, DAKO, Copenhagen, Denmark) for ten minutes, PGP-9.5 (Ultraclone, Isle of Wight, UK) and S-100 (DAKO, Copenhagen, Denmark) antisera were applied to sections at 1:200 dilutions, and preparations were incubated at room temperature in a moist chamber for 60 minutes, followed by biotinylated antirabbit antiserum (1:200) (DAKO, Copenhagen, Denmark) for 45 minutes as a link antibody. After washing, the avidin-biotin complex (ABC) (Amersham, Amersham, UK) was applied for 45 minutes. Finally, sections were soaked in 3,3′-diaminobenzidine solution (Vector, Peterborough, UK) with 0.03 percent hydrogen peroxide for five minutes and counterstained with Meyer's hematoxylin.

Image Analysis

Sections were viewed on a microscope (Nikon, Tokyo, Japan) and linked to a Sonata computer-assisted image analysis system (Seescan, Cambridge, UK) with 512-pixel resolution, 256 shades of gray, and digital signal processor. By using a graded graticule, the mean measurement from six fields for longitudinal muscle and circular muscle thickness at ×4 magnification was used to determine the ratio of longitudinal to circular muscle thickness. This was performed using sections stained by both S-100 protein and PGP-9.5. As one measurement of neuronal content, the "myenteric fraction" was then determined as the percentage area that neural tissue (fibers plus cells) occupied as a proportion of the whole myenteric plexus within the plane of the plexus in a whole-mount preparation.[10] In the present study, the myenteric fraction was obtained from three fields in longitudinal sections. This was performed using both neural stains.

To assess changes in the density of innervation of the circular muscle coat, the immunoreactive nerve count per square mm in this layer was determined. Three different fields in each section from one patient were measured at a magnification of ×20. Images of immunoreactive nerve fibers were enhanced to increase the signal to noise contrast, which is important for fine fibers. This was performed using sections stained with PGP-9.5. We did not use the same analysis for the longitudinal muscle coat because a cross-section of the this layer was not obtained in the longitudinal mounts we used.

To ensure that computer-derived nerve counts of innervation of the circular muscle were correct, a manual analysis was also undertaken. This validation was performed using a microscope and grid, and nerve counts were recorded in three fields from each patient. These counts were then compared with computer-derived data to ensure reliability of the latter, although only the computer data are presented.

Statistical Analysis

Two-tailed Mann-Whitney *U* tests were carried out for statistical comparison between patients and controls for the same colonic sections. *P* values of <0.05 were considered significant.

RESULTS

On hematoxylin and eosin examination, there was no significant histologic abnormality in any specimen throughout the colon except for four cases of melanosis coli in constipated patients. Ratio of the thickness of circular to longitudinal muscle was lower in the constipated group than in controls throughout the

colon. This was statistically significant in the left colon only (Table 1).

The myenteric plexus appears morphologically normal in all subjects and did not appear to differ morphologically between patients and controls. The myenteric fraction with PGP-9.5 did not differ significantly between the two groups, whereas with S-100 protein immunostaining the myenteric fraction was higher in the constipated group compared with controls (Tables 2 and 3).

On subjective examination, distribution of PGP-9.5 immunoreactive nerve fibers in the muscularis propria of the constipated group did not appear to be different from those of the control group. But, image analysis revealed increased PGP-9.5 immunoreactive nerve fibers in circular muscle in the constipated group, and this increase was significantly different in the ascending and descending colon (Fig. 1).

DISCUSSION

We have used PGP-9.5 and S-100 protein antibodies immunohistochemically to quantify neural and muscular structures of the colonic wall from constipated patients and cancer controls, aided by computer-assisted image analysis. This study is quantitative rather than morphologic. PGP-9.5 is a neuron-specific cytoplasmic marker that stains all types of efferent and afferent nerve fibers.[11–13] Functionally, it is thought to be an ubiquitin carboxyl-terminal hydrolase.[14] S-100 protein was first described by Moore[15] as a protein specific for nervous tissue and that stains Schwann cells surrounding both myelinated and nonmyelinated nerve fibers and satellite cells around ganglion cells such as glial cells.[16, 17]

Colonic smooth muscle in idiopathic chronic constipation has been described as normal on conventional light microscopy, although occasionally there are variations in the colonic wall muscular thickness.

Table 2.
"Myenteric Fraction" Measurements Using PGP-9.5 (See Text)

	Control		Patient
Ascending colon	48.1 ± 14.5	45.0 ± 10.3	NS
Transverse colon	60.0 ± 10.4	53.7 ± 18.0	NS
Descending colon	47.6 ± 11.1	44.6 ± 19.4	NS
Sigmoid colon	46.5 ± 15.9	43.2 ± 12.9	NS
Rectum	46.5 ± 11.9	50.5 ± 10.4	NS

Data are presented as mean ± SD. NS = not significant.

Krishnamurthy *et al.*[2] found no abnormalities of the smooth muscle, on subjective examination, except for thinning of the circular muscle in 1 of 12 cases. By using a more detailed quantitative analysis, we found thinning of the circular muscle throughout the colon and rectum in the constipated group.

In addition, we found changes in density of innervation of the circular muscle coat. This increased PGP-9.5 immunoreactive nerve fiber count in the circular muscle may be a primary or secondary adaptive change. What evidence is there for a functional disturbance of the circular muscle in idiopathic chronic constipation, which may relate to these pathologic changes? There are normally two principal types of contractions in the large intestine, namely haustral and mass contractions.[18] Haustral contractions involve low pressure contractile activity, and their function is presumed to relate to colonic mixing. There are few data available about a possible disturbance of these contractions in severe constipation. Mass contractions involve much higher pressure colonic contractions, thought to be produced by the circular muscle in coordination with the longitudinal muscle and taenia. Bassotti *et al.*[19] reported that mass movements occur less frequently in patients with slow transit constipation. However, propulsive waves were of normal amplitude, suggesting an abnormality of neural initiation rather than of the colonic muscle itself. Our findings of altered innervation of the circular muscle layer may bear some relationship to this disorder of motility.

Neurotransmitter studies have also demonstrated abnormalities that may be of relevance to these morphologic and motility changes. Vasoactive intestinal polypeptide, an inhibitory neurotransmitter that produces relaxation of human colonic circular muscle *in vitro*, has been studied in resected colons from patients with severe constipation. In one study immunoreactive vasoactive intestinal polypeptide-containing nerve fibers were not identified in the circular muscle in three of four patients.[20] In another study the concentration of vasoactive intestinal polypeptide in the resected bowel wall was reduced in constipated patients compared with controls.[21]

We have not studied innervation of the longitudinal muscle layer. In view of known changes in motility, this may also be important.

Benson *et al.*[22] performed an immunohistochemical study for neurofilament, S-100 protein, and neuron-specific enolase in 12 patients with slow-transit constipation. They observed an increase in small

Table 3.
"Myenteric Fraction" Using S-100 Protein

	Control	Patient	
Ascending colon	34.9 ± 15.1	47.0 ± 17.4	$P < 0.05$
Transverse colon	40.3 ± 12.8	51.4 ± 17.8	NS
Descending colon	43.2 ± 8.2	50.2 ± 13.0	NS
Sigmoid colon	37.8 ± 11.2	43.0 ± 9.5	NS
Rectum	33.1 ± 11.7	50.3 ± 13.0	$P < 0.05$

Data are presented as mean ± SD. NS = not significant.

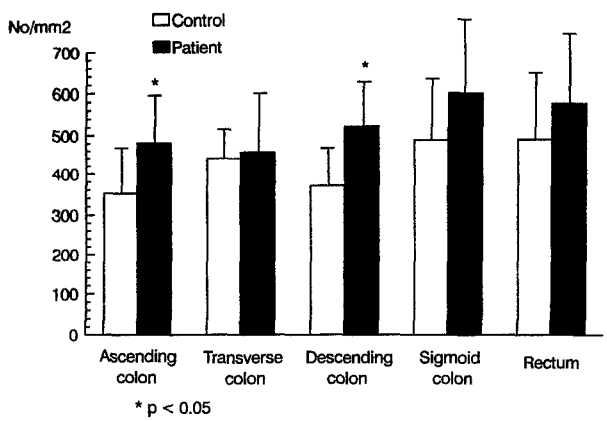

Figure 1. Immunoreactive nerve fiber count for PGP-9.5 in circular muscle. There was a significantly increased count in the constipation group in the ascending and descending colon (*).

nerve fibers of the muscularis propria, but no other neural or myocyte abnormalities were detected by light microscopy.

In addition to studying the circular muscle, however, we have also studied the density of neural tissue in the myenteric plexus. Qualitative morphologic changes in the myenteric plexus of the colon have been reported in chronic idiopathic constipation, but quantitative data on neuronal numbers are lacking. Determination of the myenteric fraction demonstrated a possible increase in the amount of neural support tissue relative to neuronal tissue in constipated patients. This provides some evidence for possible neural tissue loss in these patients. Alternatively, these changes may represent a Schwann cell proliferative reaction. Whether this change is of clinical significance and whether it is a primary abnormality or secondary to disturbed motility or laxative ingestion is unknown.

These changes in neural "density" may bear a relationship to changes in myenteric neural morphology, Schwann cell hyperplasia, and lack of axonal staining described in the myenteric plexus.[2, 23, 24]

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