Main topic

Abnormal peptidergic innervation in internal sphincter achalasia

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Abstract. The internal anal sphincter is morphologically derived from the circular muscle of the rectum, but marked differences have been observed in the motor activities of these two morphologically continuous structures. Immunocytochemical studies using anti-neurotransmitter antibodies (vasoactive intestinal peptide, substance P, met-enkephalin, neuropeptide Y), enzyme histochemistry for acetylcholinesterase, and electron microscopy were carried out on internal sphincter specimens from 14 patients with internal sphincter achalasia, 5 normal controls, and on rectum from 4 patients with Hirschsprung’s disease (HD). The various peptide-containing nerves were increased in internal sphincter achalasia compared to normal controls and patients with HD. The pathophysiology of internal sphincter achalasia appears to differ from that of HD. It is a distinct clinical entity and should be considered separate from the latter.

Key words: Internal anal sphincter – Achalasia – Peptidergic nerves – Enzyme histochemistry – Hirschsprung’s disease

Introduction

Histochemical staining for the detection of acetylcholinesterase (AchE) in rectal mucosal biopsy specimens has been established as a reliable and simple method for the diagnosis of Hirschsprung’s disease (HD). Ultrashort-segment HD, on the other hand, is diagnosed by anorectal manometry and treated by internal sphincter myectomy. There is confusion regarding the terminology and underlying pathology of ultrashort-segment HD. Lake et al. [12], in a detailed histochemical study of HD, suggested that ultrashort-segment HD is not related to HD proper and should be regarded as a disorder of the internal sphincter.

They proposed that to avoid confusion the term “ultrashort segment” and its association with HD pathology should be discarded and a more suitable term, “achalasia of the internal sphincter”, should be considered for this condition. Others have stated that internal sphincter achalasia more accurately reflects failure of relaxation of the internal sphincter, the causative factor in this condition [18]. It is now well-recognized that many neurons in the enteric nervous system are peptidergic and that many gut peptides are synthesized and secreted by the neurons that regulate motility and various other functions of the gut. In recent years several authors have reported abnormal peptidergic innervation in the aganglionic segment of bowel in HD, but there have been no detailed immunohistochemical studies of the internal sphincter in patients with internal sphincter achalasia. We have attempted to characterize the nature of the innervation of resected strips of internal sphincter muscle from patients with internal sphincter achalasia using anti-neuropeptide antibodies, AchE enzyme histochemistry, and electron microscopy.

Materials and methods

Strips of internal sphincter muscle from 14 patients aged 2 to 22 months with internal sphincter achalasia were obtained at the time of internal sphincter myectomy. For controls, internal sphincter muscle was obtained at the time of autopsy from 5 patients aged 4 months to 3 years with no evidence of gastrointestinal disease or tissue autolysis. Rectal tissue was also obtained from 4 patients at the time of pull-through operation for rectosigmoid HD. All 14 patients with internal sphincter achalasia presented with chronic constipation with or without abdominal distension. The diagnosis of internal sphincter achalasia was established by anorectal manometry, which showed the absence of a rectosphincteric reflex on rectal balloon inflation and the presence of marked rhythmic activity of the internal sphincter. HD was excluded in these cases by the presence of normal AchE activity on suction rectal biopsy examination.

Strips of specimens were processed in three ways for three different staining procedures. Immediately after it was obtained, the tissue was divided into three strips sagittally. One was fixed in Bouin’s solution for immunocytochemistry, one was snap-frozen for enzyme histochemistry, and the other was fixed with modified Karnovsky solution [11] for electron microscopic studies.
Routine H&E staining was performed on Bouin-fixed and fresh frozen sections. For enzyme histochemistry, fresh-frozen sections were cut at 10 μm and briefly fixed with 4% formaldehyde solution in 0.1M calcium acetate. AchE activity was visualized using a modified Karnovsky and Roots method with Hanks' enhancement [12]. Immunocytochemical studies were carried out on Bouin-fixed tissue sections using the avidin-biotin-peroxidase complex (ABC) method [9]. The following antisera were used in appropriate dilutions: anti-vasoactive intestinal polypeptide (VIP) antisera (Yanaihara, Japan), anti-substance-P (Sub-P) antisera (Yanaihara, Japan), anti-neuropeptide-Y (NPY) antisera (Amersham, England) and anti-met-enkephalin (Met-Enk) antisera (Amersham, England). Bouin-fixed 4-μm paraffin-embedded sections were mounted on glass slides, deparaffinized in xylene, and passed from absolute alcohol into water and then incubated with antisera for 24 h at 4 °C. The second- and third-layer antibodies were incubated for 30 min each at room temperature. Visualization of the peroxidase was achieved by the addition of 3,3'-diaminobenzidine as substrate. Resected sphincter tissue was fixed first with Karnovsky solution [11], then with 1% osmium tetroxide buffered with cacodylate. After dehydration, tissues were embedded in Epon 812 and ultra-thin sections were double-stained with lead uranyl acetate and lead citrate. A JEM-100-U electron microscope was used. The distribution and relative frequency of immunoreactive nerve fibers in the internal anal sphincter were described as follows: (+) very few; + moderate numbers; ++ fairly numerous; +++ numerous.

Results

H&E staining

All 14 cases of internal sphincter achalasia demonstrated no apparent inflammatory or fibrotic changes or necrosis in the muscle. Scanty populations of ganglion cells were noted in the myenteric plexus in both internal sphincter achalasia and controls. Thick nerve bundles were noted between smooth-muscle bands in both groups.

Acetylcholinesterase enzyme histochemistry

Normally innervated control internal anal sphincters had few AchE positive nerve fibers with low enzyme activity, and these were located only in the intermuscular space (Fig. 1). Internal sphincter achalasia specimens demonstrated increased AchE positive nerve fibers in both the intermuscular space and between the smooth-muscle cells, which was not seen in normal controls.

Immunocytochemistry

NPY-immunoreactive fibers were abundant in cases of internal sphincter achalasia. These fibers penetrated the adventitia of the anal canal and formed a dense network throughout the internal sphincter, running parallel to the smooth-muscle cells (Fig. 2). In the controls these tissues were located in the intermuscular space.

In the anal sphincter achalasia cases, VIP-containing nerve fibers were observed throughout the entire thickness of the sphincter muscle and most of these fibers were varicose and thin and ran in the direction of the smooth-muscle cells. Thick nerve bundles located in the intermuscular space also showed intense VIP immunoreactivity (Fig. 3). In the control sphincter muscles, only scanty VIP-positive nerve fibers were observed between the muscle bundles.

The distribution of Sub-P containing nerves in achalasia patients was significantly higher than that of VIP nerves. Numerous diffuse Sub-P-immunoreactive fibers were seen running parallel to the smooth-muscle cells. These fibers were the predominant type of peptide-containing fibers present in the internal sphincter achalasia specimens (Fig. 4). In contrast, very few Sub-P-containing fibers were observed in the normal controls. Their immunoreactivity was noted only in the large nerve bundles in the intermuscular septa. The immunoreactivity of Met-Enk in sphincter muscles of both groups was similar to that of VIP.

Electron microscopic findings

Individual nervous elements were supported by Schwann-cell processes and occasional myelinated fibers were seen between the unmyelinated fibers. These multi-axonal Schwann-cell units approached smooth-muscle cells and exposed varicose axonal swellings filled with heterogeneous vesicle populations. Adrenergic, cholinergic, and peptidergic nerves were present throughout the internal sphincter in patients with internal sphincter achalasia (Fig. 5).

Classical Hirschsprung's disease

The distribution of cholinergic and NPY-containing nerves was similar to that in internal sphincter achalasia. The most striking finding was the greatly reduced number of VIP- and Sub-P-containing nerves in the circular muscle coat of aganglionic segments in HD. This phenomenon was observed in the hypertrophied extrinsic nerve bundles that were located in the intermuscular sulcus (Fig. 6). The distribution of immunoreactive or enzyme histochemical-positive nerve fibers in the internal sphincter in both achalasia and the controls as well as in HD is summarized in Fig. 7.

Discussion

Normal intestinal motility is dependent on structural and functional integrity of the musculature and nerves. The muscle coat is a prominent component of the intestinal wall and extends through its entire length as a continuous structure. The internal anal sphincter is the last segment of the circular muscle coat and has a complex neural apparatus. It is influenced by five neural mechanisms: (1) adrenergic excitatory nerves, which travel in the hypogastric nerves and maintain sphincter tone via alpha-excitatory receptors [7]; (2) beta-adrenergic receptors whose pharmacologic stimulation leads to relaxation of the muscles [4, 6, 15, 16]; (3) cholinergic neurons whose influence is not yet well known but could have a biphasic action [8]; (4) purinergic neurons, which are responsible for both the peristaltic relaxation phase and relaxation of the internal sphincter [5]; and (5) peptidergic nerves, which have been postulated...