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Morphometric Evaluation of PGP9.5 and NCAM Expressing Nerve Fibers in Colonic Muscle of Patients with Hirschsprung's Disease

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A quantitative assessment of the density of the protein gene product 9.5 (PGP9.5), the neural cell adhesion molecule (NCAM), and the low-affinity nerve growth factor receptor (NGFR) expressing nerve fibers in the circular muscle layer in the colon was carried out by morphometric analyses from 13 patients with Hirschsprung's disease (HD). The difference in the nerve fiber density between the ganglionic and aganglionic segments was compared by calculating the ratio of the sum of the areas occupied by positively stained nerve fibers per unit area of the muscle after immunohistochemical staining on paraffin embedded tissue sections using computer software. There was an obvious difference in the density of the PGP9.5 stained nerve fibers between the ganglionic (0.0380 ± 0.0171) and aganglionic segments (0.0143 ± 0.01661). The NCAM-positive nerve fibers were fewer in number than those of both the PGP9.5-positive fibers and NCAM-positive fibers, which were also markedly lower in number in the aganglionic segment (0.0066 ± 0.0076) than in the ganglionic segment (0.0230 ± 0.0195). Immunostaining for low-affinity NGFR revealed much fainter staining in the ganglionic and aganglionic segment without a statistically significant difference in their density. Considering the fact that PGP9.5 is a very sensitive marker for nerve fibers, the results of this study reaffirm the innervation failure of the proper muscle in HD. The decreased NCAM expression level in the aganglionic segment appears to be caused not by the selective down-regulation of NCAM expression among the nerve fibers but by a markedly reduced number of nerve fibers.

Key Words: Hirschsprung's disease, enteric nervous system, immunohistochemistry, morphometry, PGP9.5, NCAM, NGFR

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INTRODUCTION

The pathophysiology of Hirschsprung's disease (HD) has been extensively studied since Harald Hirschsprung presented his classic description of the disease in 1886. However, the exact etiology of this disease is not fully understood.[1] The key feature of HD is the absence of ganglion cells in the colonic nerve plexuses as a result of either migration failure of the neural crest cells or a failure of the cells to survive after proper migration.[2,3] Although the underlying mechanism hindering normal neural crest cell migration and survival has not been verified, the extracellular matrix, cell adhesion molecules, and neurotrophic factors have been suggested to play important roles in providing a migration pathway for neural crest-derived cells in a developing gut and in promoting their maturation.[3]

In addition to the absence of the ganglion cells in the submucosal and myenteric nerve plexuses, HD exhibits more generalized abnormalities in the enteric nervous system. It has been understood that the distal aganglionic segment in HD is innervated by an excessive number of nerve fibers[4,5] and a whole mount immunohistochemical study demonstrated innervation abnormalities, both quantitatively and qualitatively.[6] Abnormalities in the enteric nervous system were initially studied using acetylcholinesterase (AchE) histochemical staining[5] followed by immunohistochemical staining using various neural markers. Among the various markers used, the neural cell adhesion molecule (NCAM),[7-10] the protein gene product 9.5 (PGP9.5)[11] and the nerve growth factor receptor

(NGFR)[12-14] were suggested as possible factors that might play important roles in pathogenesis. However, the results of those reports varied and most of them focused on the abnormalities of the mucosal and submucosal enteric nerve system. However, there have been few studies focusing on the nerve fiber distribution in the proper muscle layer.[6-18] Furthermore, most of the reports adopted a semi-quantitative analysis, which lacked objectivity.

This study was designed to determine the abnormalities of innervation in the proper muscle of patients with HD using immunohistochemical staining on paraffin-embedded tissue sections with the aid of morphometric analysis methods.

MATERIALS AND METHODS

The entire resected specimens of the colon were obtained from 13 patients with HD undergoing pull-through operations. A diagnosis was established by clinical and conventional microscopic findings. All patients had a transitional zone on the sigmoid or below the sigmoid colon and they underwent a loop enterostomy before the pull-through operations. All the specimens was fixed in formalin, routinely-processed, and paraffin-embedded. After reviewing the hematoxylin-eosin stained tissue sections, representative blocks for immunohistochemical staining were selected. Immunohistochemical staining was performed using a labeled streptavidin-biotin immunoperoxidase technique (LSAB2 kit, Dako, Glostrup, Denmark) with 3,3'- diaminobenzidine used as the chromogen. For NCAM and low-affinity NGFR, the sections were subjected to heat-induced epitope retrieval in a 0.01 M citrate buffer (pH 6.0) for 15 minutes using a pressure cooker and a microwave oven (720W) prior to incubating them with the primary antibodies. The negative controls consisted of substitutions of mouse ascites fluid and rabbit serum for monoclonal and polyclonal antibodies. The sections were incubated with anti-NCAM 123C3 monoclonal antibodies (1:50, Monosan, PB Uden, the Netherlands), anti-PGP9.5 polyclonal rabbit antibodies (1:50, Dako) and anti-NGF-receptor ($p75^{NGFR}$) monoclonal antibodies (1:50, Neo Markers, Fremont, CA, USA) overnight at 4℃.

After immunostaining, morphometric analysis was performed by a digital camera (Polaroid DMG Ie) attached to the microscope (Olympus BX50) with an image analysis program, Image-Pro Plus® (Media Cybernectics, Silver Spring, Maryland, U.S.A). After locating the areas for analyses by comparing the hematoxylin-eosin stained sections, the positively stained nerve fiber density in the circular muscle layer was evaluated at X200 magnification by calculating the sum of the areas occupied by the positively stained nerve fibers per unit area of the circular muscle layer (Fig. 1). Morphometric analyses were done 3 times from the aganglionic and the adjacent ganglionic segment in each case and confirmed by two other examiners. The data obtained by morphometric analyses were statistically analyzed using the Student t-test.

RESULTS

PGP9.5, NCAM and low-affinity NGFR immunostaining demonstrated a similar pattern of positivity. All showed a positive reaction of the myenteric nerve plexuses, and both nerve fibers and ganglion cells within the plexuses showed a positive reaction. There was no definite difference in the staining intensity between ganglionic and aganglionic colon. The density and staining intensity of the nerve fibers in the circular muscle layer of ganglionic colon were most prominent after immunostaining for PGP9.5 followed by NCAM. Although most of the nerve fibers were transected thereby showing punctate positivity, some of them demonstrated a parallel linear positive reaction. The immunostaining results for low-affinity NGF were not distinct and difficult to interpret. Even with the scanning power examinations, some cases demonstrated a marked difference in the density of positively stained nerve fibers for PGP9.5 and NCAM between the ganglionic and aganglionic colon due to markedly decreased PGP9.5 and NCAM immunoreactivity in the circular muscle layer of aganglionic colon (Fig. 2 and 3).

By quantitative morphometric analysis, the mean density of the PGP9.5-positive nerve fibers

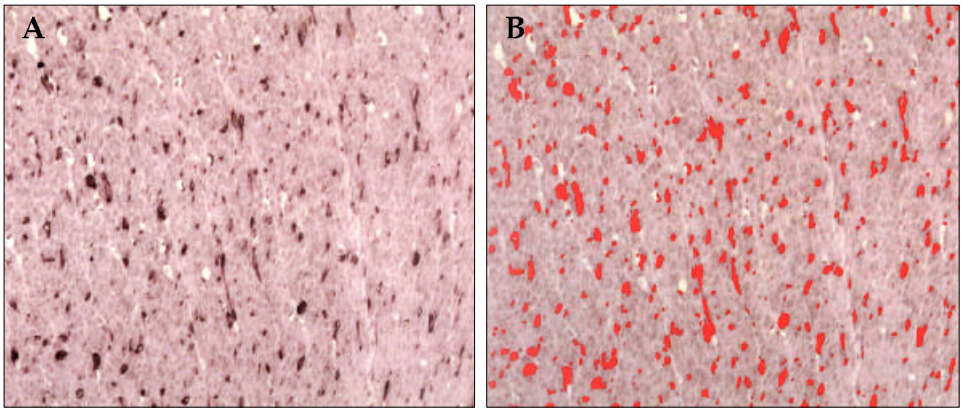

Fig. 1. A representative case showing that the selection of PGP9.5-positive nerve fibers for counting by the Image-Pro Plus®. Areas marked red for analyses in image B were selected from image A which shows a brown immunoreactivity.

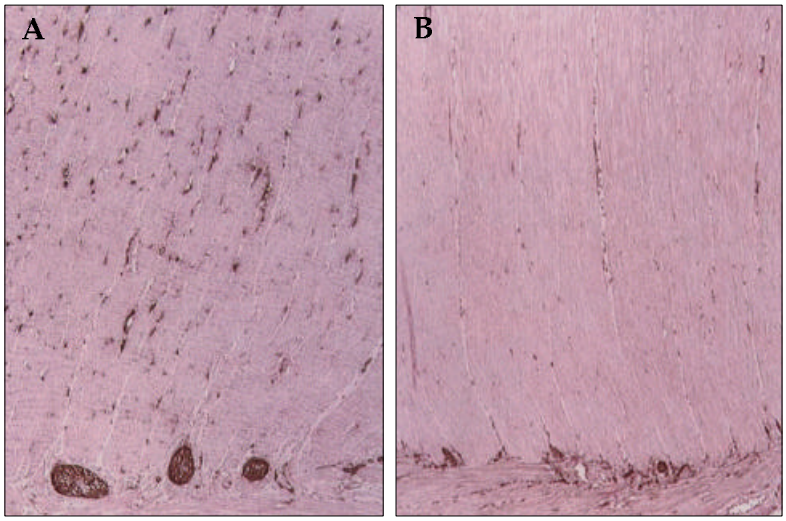

Fig. 2. A medium power microscopic view of a representative case showing marked difference in PGP 9.5-positive nerve fibers in the ganglionic (A) and aganglionic segment (B).

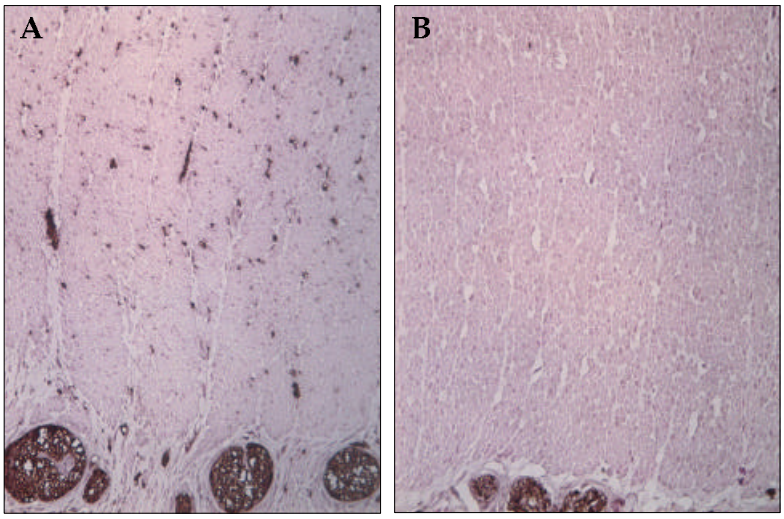

Fig. 3. A medium power microscopic view of a representative case showing marked difference in the NCAM-positive nerve fibers in the ganglionic (A) and aganglionic segment (B).

in the circular muscle layer showed a statistically significant difference between the ganglionic (0.0380 ± 0.0171) and aganglionic segment (0.0143 ± 0.0166) ($p < 0.001$). The NCAM-positive nerve fibers were also significantly reduced in the aganglionic segment (0.0066 ± 0.0076) compared

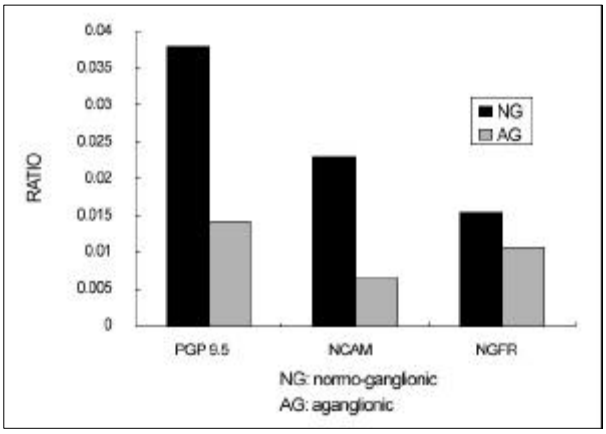

Fig. 4. Quantitative analyses of PGP9.5-, NCAM-, and NGFR-immunoreactive nerve fibers showing a significant difference in the NCAM- and PGP9.5-positive nerve fibers between the ganglionic and aganglionic segment

with the ganglionic (0.0230 ± 0.0195) segment ($p < 0.001$). However, the faintly stained low-affinity NGFR demonstrated no difference in the density of the positively stained nerve fibers (Fig. 4).

DISCUSSION

Immunohistochemical staining of the colon from patients with HD showed an obvious difference between the ganglionic and aganglionic segment in terms of the density of PGP9.5- and NCAM-positive nerve fibers in the circular muscle layer. There were significantly reduced numbers of PGP9.5- and NCAM-positive nerve fibers in the aganglionic segment and these results reaffirm previously results of absent NCAM and NADPH-diaphorase immunoreactive nerve fibers in the smooth muscle of HD reported by Kobayashi et al.[7] Although the absence of ganglion cells in the nerve plexuses is a key finding of HD, other abnormalities of innervation were also demonstrated with the aid of various neural markers.[5-18] Initially applied AchE histochemical staining demonstrated hypertrophic submucosal plexuses and nerve fibers, which has been used as an useful ancillary diagnostic tool for the interpretation of suction biopsies.[5,15] By developing sensitive immunohistochemical detection methods and specific neural markers, numerous studies have been able to evaluate the enteric nervous system. In contrast to the abnormalities of the mucosal and submucosal enteric nervous system, which have extensively been evaluated, there are few reports on the innervation abnormalities of the proper muscle layer and in these reports, there is some discrepancies.[6-18] Therefore, the main focus was on evaluating the innervation abnormalities of the proper muscle layer in patients with HD.

PGP9.5 has been used as a marker for neural crest-derived enteric neurons and neuronal precursor cells in the central and peripheral nervous system.[19,20] PGP9.5 is expressed in both the ganglion cells and nerve fibers of enteric nervous system and is understood to be one of the most sensitive markers for identifying intramuscular nerve fibers.[11,15] Evaluating PGP9.5-positive intramuscular nerve fibers using conventional tissue sections has some limitations compared with a whole-mount immunohistochemistry study[6,16] because the nerve fibers are transected. However, a morphometric evaluation of the density of positively stained fibers and paraffin section immunohistochemistry, which shows superior morphologic details compared with immunofluorescent staining or frozen section immunohistochemistry, can overcome these limitations. Therefore, the results in this study compare well with the findings reported by Fujimoto et al.[17] and reaffirm the markedly reduced innervation of the proper muscle layer in HD although there is some variation from case to case.

NCAM is a cell-surface glycoprotein involved in the adhesion between several types of neural cells, their processes, and in the initial formation of neuromuscular synapses.[21,22] Kobayashi et al.[7] demonstrated a lack of NCAM immunoreactivity within the muscles of HD patients and interpreted that down regulation as a characteristic finding related to the pathogenesis of HD. In contrast, the study done by Romanska et al.[9] using a polyclonal antibody demonstrated an abnormally high NCAM immunoreactivity in the muscularis mucosae in HD but they did not emphasize the difference in the distribution of NCAM-positive intramuscular nerve fibers. In this study, a commercialized monoclonal antibody for NCAM (Clone 123C3 from Monosan) and the muscularis mucosae in the aganglionic segment did not demonstrate any immunoreactivity. The immunohistochemical staining method used for detecting

NCAM is known to be sensitive enough to detect NCAM expression on the surface of NK cells.[23] Therefore, this data compares well with those reported by Kobayashi et al.[7] However, considering the results of PGP9.5 immunostaining that confirmed markedly decreased nerve fiber density in the aganglionic segments, the decreased NCAM immunoreactivity in the circular muscle of the aganglionic segment layer was interpreted as being caused not by the selective down regulation of NCAM expression within nerve fibers but by a markedly reduced number of nerve fibers.

NGFR is known to act as a trophic and chemotactic agent for developing nerves. It promotes the outgrowth of axons and the establishment of synapses.[24] Kuroda et al.[13] suggested that an abnormal NGF function might be a potential etiologic factor of HD, because the immaturity of synaptic connection in the enteric nervous system. This was suggested to be due to a reduction of NGF in the aganglionic bowel, which disturb the maturation or lead to the destruction of ganglion cells. Although a recently developed anti-NGF-receptor (p75NGFR) monoclonal antibody that works on paraffin-embedded tissue sections was used, the immunoreactivity was not distinct and we could not observe a significant difference in the expression between the ganglionic and aganglionic colon.

In summary, the results in this study reaffirm a generalized innervation failure of the proper muscle in HD. Although abnormal NCAM expression might be involved in the pathogenesis of HD, the decreased NCAM expression in the aganglionic segment appears to be caused not by the selective down-regulation of NCAM expression among the nerve fibers but by markedly reduced number of nerve fibers.

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