Immunohistochemical Study on Regrowth of Myenteric Nerves Following Transection of the Muscle Coat in the Rat Small Intestine, with Special Reference to Glial Cell Line-Derived Neurotrophic Factor (GDNF) and its Receptor (Ret)

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ABSTRACT

The present immunohistochemical study was designed to investigate the manner of the myenteric nerve regeneration and expression of glial cell line-derived neurotrophic factor (GDNF) and its signaling receptor (Ret) in nerves after transection of the muscle coat (myectomy) in the rat small intestine. The enteric neurons and enteroendial cells were immunohistochemically determined using antibodies against protein gene product 9.5 and S-100 protein, respectively. The neuronal sprouts issued from the severed nerve stumps 12 h after the operation, and thereafter extended into the lesion. The proximal portions of outgrowing neuronal fibers were gradually enveloped by regenerating enteroendial cells to become thick nerve bundles. The regrowing neurons and their associated enteroendial cells developed into an irregular network in the myectomized area on postoperative day 5. The elongation of the regrowing nerves was conspicuously accelerated from postoperative day 3 in accordance with the regrowing neurons started to associate with the enteroendial cells. Under normal conditions, faint immunoreactivity for GDNF and Ret was selectively localized in the enteroendial cells and neurons within the myenteric ganglia, respectively. Following myectomy, the both immunoreactivities were significantly intensified in the nerve stumps and ganglia proximal to the lesion. The regenerating enteroendial cells in the lesion, being in close association with the regrowing neurons, exhibited a dense immunoreaction to GDNF, whereas the neuronal fibers were richly supplied with reaction products of Ret in their entire course. The present findings suggest that the enteroendial cells, contacting with regrowing neurons, may promote the myenteric nerve regeneration in the rat small intestine, via the GDNF-Ret signaling system.

Several studies have described the regeneration of enteric nerves in various experimental models including the guinea pig (6, 7, 10, 11, 13, 22), rat (5, 9) and mouse (16). The recovery of gastrointestinal motor function after transection of the gut may coincide with the regrowth of enteric nerves and other tissue elements (8, 17, 18). The recovery may be integrally controlled by the enteric nervous system. However, the precise mechanism of enteric nerve regeneration in the repairing process from tissues injury remains to be clarified. Therefore, an investigation of enteric nerve regeneration, involving the molecules promoting neural development, may give morphological basis for the functional recovery of the intestine following surgery.

In the present study, we applied double immunostaining for protein gene product (PGP) 9.5, a neuron-specific protein, and S-100 protein, a reliable marker of Schwann cells (enteric glial cells; see, Kobayashi et al. [12]), to whole-mount preparations, and we demonstrated the process of myenteric nerve regeneration.
regrowth following transection of the muscle coat in the rat small intestine. To obtain functional insight into the interaction between neurons and enteroglial cells in regeneration of the myenteric nerves, we further pursued the expression of immunoreactivity for glial cell line-derived neurotrophic factor (GDNF), a recently-identified promoter for the development of the enteric nervous system (2, 14, 15, 19, 21), and the expression of signaling receptor (Ret) (3, 4, 20, 23), in the regrowing nerves.

MATERIALS AND METHODS

Wistar rats of both sexes weighing 200–300 g were used in this study. They were maintained under standard laboratory conditions and allowed standard laboratory animal chow and water ad libitum. All experiments were carried out according to the Guidelines for Animal Experimentation, Oita Medical University.

Surgical procedure. The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) 20 min prior to surgery, then an incision was made along the abdominal midline. A segment of the jejunum was placed on sterilized gauze moistened with a physiological saline solution. The intestinal muscle coat was removed (5 mm in length) from 16 rats. The removed tissue included longitudinal and circular muscle layers, with adherent myenteric nerve plexus to sever the nerve pathways emanating from the ganglia (this procedure is provisionally called myectomy in this paper). The submucosal nerve plexus remained. Eight control rats underwent laparotomy, but not myectomy. The operated areas were conserved using a synthetic bioresorbable membrane (Seprafilm; Genzyme Co., Kimberlee Witkop, USA) to prevent the lesion from undergoing adhesional disorders. The abdominal walls were closed by suturing after surgery.

Tissue preparation. After the rats were sacrificed by exsanguination under deep anesthesia with ether, the operated intestines were removed from three animals (2 animals in the myectomy and one control) at 12 h, 1–7 consecutive days after surgery.

For light and fluorescence microscopy, the control and myectomy intestines were immersed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4) for 12 h at 4°C and then immersed in the buffer containing 30% sucrose. Some tissues were rapidly frozen in liquid nitrogen to prepare tissue sections of 15 μm thickness, whereas tissue strips (whole-mount preparations) were made from others, including the myectomized region of the operated rats or the myenteric layer of the controls. Both types of specimens were processed for immunohistochemical analysis.

Immunohistochemistry of tissue sections. Some tissue sections were incubated for 1 h in 1/15 M phosphate buffered saline (PBS) containing 0.3% Triton X-100 for the enhanced penetration of antibodies, followed by immersion in 0.3% H2O2 in methanol for 20 min to block the endogenous peroxidase activity. After being rinsed in PBS, the sections were incubated in 10% normal goat serum for 15 min and then in polyclonal antibody to GDNF or Ret (Table 1) at 4°C overnight. Following a rinse in PBS, they were treated with peroxidase-conjugated goat anti-rabbit IgG (ENVISION², DAKO, Carpinteria, CA, USA) for 1 h at room temperature. The antigen-antibody reaction was visualized by the diaminobenzidine (DAB) reaction, and examined under a light microscope (Olympus, BX-60, Tokyo).

The other tissue sections were preincubated in PBS containing 0.3% Triton X-100, and in 10% normal goat serum. They were then treated with one of the several mixtures of monoclonal antibody to PGP 9.5

Table 1 Characterization of the primary antibodies

<table>
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<th>Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Code</th>
<th>Source</th>
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<tr>
<td>PGP9.5</td>
<td>Rabbit</td>
<td>1:4000</td>
<td>RA95101</td>
<td>UltraClone, UK</td>
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<tr>
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<td>1:20</td>
<td>sc-328</td>
<td>Santa Cruz Biotech., USA</td>
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<tr>
<td>Ret</td>
<td>Rabbit</td>
<td>1:20</td>
<td>R787</td>
<td>IBL, Japan</td>
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</tbody>
</table>
or S-100 protein (Table 1), together with polyclonal antisera to GDNF or Ret (Table 1) at 4°C overnight. After washing with PBS, the sections were treated with a mixture of indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgG (diluted 1:400; Jackson Immuno Research, West Grove, USA) and fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (diluted 1:100; Jackson Immuno Research) for 1 h at room temperature. They were then rinsed in PBS, mounted in fluorescent mounting medium (DAKO), and examined with a fluorescence microscope (Olympus, BX-60, Tokyo).

**Immunohistochemistry of whole-mount preparations.** Some whole-mount preparations obtained from the control rat intestine were processed for immunostaining for Ret. After being rinsed in PBS containing 0.3% Triton X-100, the tissues were immersed in 0.3% H2O2 in methanol at room temperature for 30 min. They were incubated in 10% normal goat serum for 1 h, and then in a rabbit anti-Ret serum (Table 1) for 72 h at 4°C. After rinsing in PBS, the tissues were treated with a biotin-bound goat antisera immunostaining for rabbit IgG for 4 h, followed by peroxidase-bound streptavidin for 2 h (Histofine, Nichirei, Tokyo, Japan). The site of the immunoreaction was visualized by DAB reaction.

The remaining tissue strips from the control and myectomized intestines were processed for double immunostaining for PGP 9.5 and S-100 protein. The tissues, being preincubated in PBS containing 0.3% Triton X-100, were treated with 10% normal goat serum for 1 h, and then with a mixture of monoclonal anti-PGP 9.5 and polyclonal anti-S-100 protein antibodies (Table 1) for 72 h at 4°C. Following a rinse in PBS, they were incubated in a mixture of the fluorescence-bound secondary antibodies mentioned above for 4 h at room temperature. The stained tissues were mounted on glass slides with a fluorescent mounting medium (DAKO), and viewed with a fluorescent microscope (Olympus).

Control stainings were performed using the same procedures, except for utilization of a non-immunized serum, or an antisera preabsorbed with an excess amount of antigens (50–100 μg/mL diluted antisera), instead of the corresponding antibodies, and no nonspecific reaction products were seen.

**Length measurement of regenerating nerves.** To examine the rate of regenerating nerve elongation, the lengths of the regrowing nerves were measured from the severed stump to the terminal tips of the regrowing nerves in approximately twenty PGP 9.5-immunopositive nerves in every ten frames (each frame was 700 × 450 μm in area) on the whole-mount preparations immunostained for PGP 9.5 and S-100 protein by tracing their contours.

**RESULTS**

Following transection of the intestinal muscle coat, none of the rats developed any clinical disorders, such as ileus, throughout the experimental period. Since the regrowing profiles of myenteric nerves at the oral and anal sides of the operated region were essentially identical, we mainly describe the process of myenteric nerve regrowth from the oral stump.

**Immunohistochemistry for PGP 9.5 and S-100 protein on whole-mount preparations**

Double immunostaining for PGP 9.5 and S-100 protein on whole-mount preparations of the control animals clearly demonstrated that the myenteric nerve plexus was composed of PGP 9.5-immunopositive neurons and entericglial cells with S-100 protein-immunoreactivity (Fig. 1).

At 12 h after myectomy, the transected nerve stumps lost a considerable number of S-100 protein-immunopositive entericglial cells, and revealed many

![Fig. 1 Fluorescence micrograph of double immunostaining for PGP 9.5 (green) and S-100 protein (red) on a whole-mount preparation from a shamed-operated rat small intestine. A meshwork of the myenteric nerve plexus is seen to be composed of PGP 9.5-positive neurons and S-100-positive entericglial cells (EG). The double-headed arrow indicates the longitudinal direction of the intestine. NC, nerve cell. Bar, 100 μm](image-url)
PGP 9.5-immunopositive neuronal fibers expanding their terminals (Fig. 2a). A few neuronal microprojections emanated from the nerve stumps (Fig. 2a).

On postoperative day 1, the enteroglial cells immunoreactive for S-100 protein were renewed in the nerve stumps, whereas several fine varicose neuronal fibers with PGP 9.5-immunoreactivity lacking the enteroglial sheaths, extended into the injured area (Fig. 2b).

On postoperative day 3, the individual outgrowing neuronal fibers were assembled with regenerating enteroglial cells with S-100 protein-immunoreactivity (Fig. 2c).

By five days after myectomy, the regrowing neuronal fibers formed thick bundles covered with the enteroglial cells to elongate in a circular or longitudinal course, branching and anastomosing with each other, and developed into an irregular neural network (Fig. 2d). While proximal portions of the regrowing nerves were mostly equipped with the enteroglial sheath (Fig. 2d), the distal portions of which, including terminal tips, were not associated with the enteroglial cells (Fig. 2e).

**Elongation rate of regrowing nerves**
The length of nerves was measured during the regrowing process (Fig. 3). The growth rate of the nerve regeneration was 84 μm/day up to day 3, followed by a faster phase (170.3 μm/day) from days 3 to 7, and consequently displayed two phases during the experimental period (Fig. 3).

**Immunohistochemistry for GDNF**
Faint GDNF-immunoreactivity was preferentially located in the satellite cell region surrounding the nerve cell bodies within the myenteric ganglia in the

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**Fig. 2**  Fluorescence micrographs of double immunostaining for PGP 9.5 (green) and S-100 protein (red) on whole-mount preparations from rat small intestines at 12 h (a), on days 1 (b), 3 (c), and 5 (d, e) following myectomy. a: A transected PGP 9.5-positive nerve fascicle exhibits terminal expansion and small sprout (arrowhead). S-100-positive enteroglial cells (small arrow) are scant. b: Several PGP 9.5-positive varicose nerve fibers (arrowheads) extend into the lesion. Some S-100-positive enteroglial cells (small arrows) are seen in the severed nerve stump. c: Several PGP 9.5-positive regrowing nerves are ensheathed by S-100-positive enteroglial cells (small arrows). d: Many PGP 9.5-positive regrowing nerves are assembled with S-100-positive enteroglial cells to form an irregular network. e: The proximal portions of PGP 9.5-positive regrowing nerves are associated with S-100-positive enteroglial cells (small arrows), but the distal portions (arrowheads) are free from them. The large arrow, broken line and star in each figure indicate the direction of nerve regeneration, cut line of the muscle coat and myectomized lesion, respectively. Bars, 100 μm.
small intestine of control rats (Fig. 4a). Little was in the proper muscular layer. Double immunostaining for GDNF and PGP 9.5 or S-100 protein, showed that the immunoreaction products for GDNF were exclusively distributed in enterogial cells with immunopositivity for S-100 protein (Fig. 6a–c), but not within neurons with PGP 9.5-immunoreactivity (Fig. 5a–c).

Following myectomy, the GDNF-immunoreactivity was significantly more intense in the satellite cell regions of the myenteric ganglia proximal to the cut end (Fig. 4b, e). Irregularly-shaped cells in the nerve stumps also displayed strong immunoreaction to GDNF (Fig. 4c). The reaction products for GDNF were selectively localized in the S-100 protein-immunopositive enterogial cells (Fig. 6d–f), although they were often seen along the PGP 9.5-immunoreactive neurons (Fig. 5d–f).

Fig. 4  Light micrographs of immunostaining for GDNF on tissue sections from rat small intestines following sham operation (a), or on days 1 (b), 3 (c), and 5 (d–f) following myectomy. a: The satellite cell region in the myenteric ganglion shows faint immunoreaction, surrounding immunonegative nerve cells (asterisks). b: The satellite cell region (arrowhead) in the myenteric ganglion proximal to the lesion shows an accumulation of the immunoreactivity, surrounding immunonegative nerve cells (asterisks). c: The cells (small stars) with spindle processes at the cut end show extremely-intense immunoreaction. d: Many cells both in the stump and myectomized region (large star) are intensely immunostained. CM, circular muscle; LM, longitudinal muscle. e: Higher magnification of the ganglion indicated by asterisk in d. The satellite cell region in the ganglion shows a conspicuous accumulation of the immunoreaction, surrounding an immunonegative nerve cell (asterisk). f: Higher magnification of the area indicated by large star in d. Irregularly-shaped cells (small stars) show extremely-intense immunoreaction. The arrow in b–d and broken line in e, d indicate the direction of nerve regeneration and cut line of the muscle coat, respectively. Bars, 50 μm.
Fig 5  Fluorescence micrographs of double immunostaining for GDNF (a, d, g, red) and PGP 9.5 (b, e, h, green) on tissue sections from rat small intestines following sham operation (a–c), or on days 3 (d–f), and 5 (g–i) following myectomy. Each figure e, f, and i shows a merged image. a–c: The GDNF-immunoreaction products are sparsely seen in the satellite cell region, surrounding PGP 9.5-positive nerve cells (asterisks), in the myenteric ganglion. Neuronal somata are immunonegative for GDNF. d–f: The GDNF-immunoreaction products are densely seen along PGP 9.5-positive nerves both in the nerve stump (arrowhead in e, f) and in the satellite cell region, surrounding PGP 9.5-positive nerve cells (asterisks), in the myenteric ganglion proximal to the lesion. Neuronal somata are immunonegative for GDNF. g–i: The GDNF-immunoreaction products are densely seen along the proximal portions of PGP 9.5-positive regrowing nerves in the myectomized region, but absent from the distal portions (arrowheads in h, i). The arrow in f, i indicates the direction of the nerve regeneration. Bars, 50 μm

Fig. 6  Fluorescence micrographs of double immunostaining for GDNF (a, d, g, red) and S-100 protein (b, e, h, green) on tissue sections from rat small intestines following sham operation (a–c), or on day 3 (d–f), and 5 (g–i) following myectomy. Each figure e, f, and i shows a merged image. a–c: The GDNF-immunoreaction products are sparsely seen in S-100-positive enteroglial cells in the myenteric ganglion. Nerve cells (asterisks) are immunonegative for both antigens. d–f: The GDNF-immunoreaction products are densely seen in S-100-positive enteroglial cells in the myenteric ganglion proximal to the lesion. Nerve cells (asterisks) are immunonegative for both antigens. g–i: The GDNF-immunoreaction products are densely seen in S-100-positive regenerating enteroglial cells in the myectomized region. The arrow in f, i indicates the direction of nerve regeneration. Bars, 50 μm
In the regenerating region from postoperative day 3, intense GDNF-immunoreaction products were densely distributed in the cells being spindle or irregular in shape (Fig. 4, c, d, f). Our double immunostaining showed intense GDNF-immunoreactivity preferentially in the S-100 protein-immunopositive cells with tapered cytoplasm that were regarded as regenerating enteroglial cells surrounding the regrowing nerves (Fig. 6a-i). The reaction products for GDNF were often found along the proximal portions of the regrowing neuronal fibers with PGP 9.5-immunoreactivity, but absent from the distal portions including the terminal tips (Fig. 5g-i).

**Immunohistochemistry for Ret**

In the control rat intestine, granular immunoreaction deposits for Ret were sparsely distributed in neurons including some perikarya within the myenteric ganglia (Fig. 7a), but not in the proper muscular layer. The whole-mount preparations immunostained for Ret demonstrated the reaction products composing a few beaded fibrous structure within the ganglia (Fig. 7b), and some nerve cells showing a weak immunoreaction in their cytoplasm (Fig. 7b). Double immunostaining for Ret and PGP 9.5 or S-100 protein, showed that the Ret-immunoreactivity was selectively localized in PGP 9.5-immunopositive neurons (Fig. 8a-c), but often separated from the S-100 protein-immunopositive enteroglial cells (Fig. 9a-c).

Following myectomy, the myenteric ganglia proximal to the lesion revealed a conspicuous increase in intensity of the Ret-immunoreactivity in their neuronal elements (Fig. 7c). The Ret-immunoreactivity also accumulated in the severed nerve stumps (Fig. 7d). The condensed reaction products for Ret were

![Fig 7](image-url)
exclusively distributed in the PGP 9.5-immunoreactive neuronal elements including neuronal somata (Fig. 8d–i). In contrast, some reaction products for Ret were closely associated with the S-100 protein-immunopositive enteroglial (Fig. 9d–i), whereas others were segregated from those cells (Fig. 9d–i).

In the myectomized region after postoperative day 3, accumulated reaction products for Ret appeared as many varicose fibers to be assembled into thick bundles in their proximal portions (Fig. 7e–g). The PGP 9.5-immunopositive regrowing nerves in the lesion were richly supplied with immunoreaction products for Ret over their entire course including the terminal tips (Fig. 8g, h, i), although the reaction products were often seen to be free of the S-100 protein-immunopositive enteroglial cells in the distal portions of the regrowing nerves (Fig. 9g–i).

DISCUSSION

The present study using antibodies against PGP 9.5 and S-100 protein demonstrated the precise manner of myenteric nerve regrowth following transection of the muscle coat in the rat jejunum. Neuronal projections were sprouted from expanded nerve stumps soon after myectomy, and developed into fine varicose neurites spreading into the lesion. These nerves are referred to as regrowing neuronal fibers. Both the time lapeses and the time-dependent manner of the nerve regeneration observed in this study seem to correspond to those determined in a previous study using laser photocoagulation in the guinea pig small intestine (11). In contrast, Ekblad et al. (5) reported that reinnervation of neurons containing gastrin-releasing peptide, a peptidergic neurotransmitter, started 20 weeks after 10 mm wide myectomy in the rat small intestine. This temporal discrepancy might be due to differences in the surgical procedures (myectomy by Ekblad et al. [5] was wider than that in our study) and/or the neuronal markers (PGP 9.5 used in this study is a general neuronal marker).

Our immunohistochemical examination demonstrated that the transected nerve stumps contained few S-100 protein-immunoreactive cells immediately after the surgery. This may have resulted from either degeneration of the enteroglial cells in the stumps or a marked decline in the immunoreactivity of S-100 protein within the cells. The former view appears to be possible, since our preliminary TEM

![Fig 8](image-url) Fluorescence micrographs of double immunostainig for Ret (a, d, g, red) and PGP 9.5 (b, e, h, green) on tissue sections from rat small intestines following sham operation (a–c), or on days 3 (d–f), and 5 (g–i) following myectomy. Each figure e, f and i shows a merged image. a–c: The Ret-immunoreaction products are sparsely seen in PGP 9.5-immunopositive neuronal somata (asterisks) and neuropils in the myenteric ganglion. d–f: The Ret-immunoreaction products are densely seen in PGP 9.5-immunopositive neuronal somata (asterisks) and neuropils in the myenteric ganglion proximal to the lesion. g–i: The Ret-immunoreaction products are densely seen in PGP 9.5-positive regrowing nerves in their entire length both in the stump and myectomized region (star). The arrow in f, i and broken line in g–i indicate the direction of nerve regeneration and cut line of the muscle coat. G, myenteric ganglion. Bars, 50 µm.
examination demonstrated many degenerating cells, presumably enteroglial cells, at the cut end of the small intestine 12 h after surgery. The enteroglial cells, thereafter, came to ensheath the regrowing neurons from the severed nerve stumps with their tapered cytoplasm to form thick bundles. This may be due to migration of the renewed enteroglial cells from the stumps into the myectomized region.

It is noteworthy that the growth rate of the nerve regeneration showed two distinctly different phases during the present experimental period: regrowing nerve fibers lacking enteroigial investment, displayed a slow elongation rate in the early stage, while those equipped with enteroglial cells grew faster in the latter stage from postoperative day 3 onwards. This finding suggests that enteroglial cells play a crucial role in promoting the regrowth rate of the myenteric nerves in concert with the growing processes of individual neurons.

Since little if any information has been available as to the intercellular functions regulating nerve growth between enteroglial cells and neurons during the regeneration of enteric nerves, we further investigated the expression of immunoreactivity for GDNF, a potent promoter of vagal crest-derived enteric neuron development (2, 14, 15, 21), and its signaling receptor, Ret (3, 4, 19, 20, 23), during regeneration of the myenteric nerves. Both GDNF- and Ret-immunoreactivities were preferentially distributed within myenteric ganglia in the muscle coat of the control rat intestines, which corresponded to the findings in previous studies (1, 19). Bär et al. (1) described that reaction products for GDNF in the human intestine showed a similar distribution pattern to that of enteroglial cells, but they failed to define cellular immunolocalization of GDNF. Double immunostaining in our study clearly demonstrated that GDNF were selectively immunolocalized in the S-100 protein-immunopositive enteroglial cells both under normal and regenerating conditions. The distribution of the immunoreactive GDNF along the PGP 9.5-immunopositive nerve strands may indicate their localization in the enteroglial cells covering the neurons.

On the other hand, the present immunohistochemical findings, in the control and myectomized rat intestines, showed that immunoreaction deposits for Ret were exclusively distributed in the PGP 9.5-im-

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Fig 9  Fluorescence micrographs of double immunostaining for Ret (a, d, g, red) and S-100 protein (b, e, h, green) on tissue sections following sham operation (a-c), or on days 3 (d-f), and 5 (g-i) following myectomy. Each figure c, f and i shows a merged image. a-c: The Ret-immunoreaction products are sparsely seen in the myenteric ganglion. Some of them are found around S-100-positive enteroglial cells, but others (arrowheads) are free of the cells. d-f: The Ret-immunoreaction products are densely seen both in the stump and myectomized region (star). Some of them are found in close association with S-100-positive enteroglial cells, but others (arrowheads) are free of the cells. G, myenteric ganglion. g-i: The Ret-immunoreaction products are densely seen both in the stump and myectomized region (star). Some of them are found in close association with S-100 protein-immunopositive enteroglial cells, but others (arrowheads) are free of the cells. G, myenteric ganglion. The arrow in f, i and broken line in d-i indicate the direction of nerve regeneration and cut line of the muscle coat, respectively. Bars, 50 μm
munoactive neuronal processes, as well as in perikaryons, within the myenteric ganglia. Our data in the control state conform to the notion in the avian intestine (19). The present study demonstrated that the Ret-immuoneactivity was in the vicinity of the S-100 protein-immunoreactive enteroendelial cells. This may correspond to close relation between the immune deposits in the nerves and the enteroendelial cells. However, the Ret-immunoreactivity in the human intestine has been reported to be confined to neuronal cell bodies in the myenteric plexus (1). This discrepancy may be attributable to differences among species and/or in the antibodies used in the studies, because our antiserum was the same as that used in the avian study (19), but different from that used in the human study (1). These findings suggest that the GDNF and Ret may be involved in modulating neuronal functions in the ganglion including synaptic transmission, via their neurotrophic action.

The present study showed obvious increase in immunoreactivity for GDNF in enteroendelial cells and Ret in neurons for the ganglia at the cut ends during the myenteric nerve regeneration. This may indicate that a large amount of these molecules accumulates in respective cells. An in vitro study of rat neural crest-derived cells by Chalazonitis et al. (2) described that GDNF acts as a mitogen of enteric neural crest-derived neuronal precursors in the early developmental period, and, later, as a growth-differentiation factor, supporting enteroendelial development by its paracrine action. The two types of cells, therefore, may be required at high activity levels to survive from neuronal damage and to regenerate.

The dense supply of the reaction products for Ret in the outgrowing neuronal fibers, being demonstrated in the lesion, may indicate that the Ret is transported from the intraganglionic neurons by their axonal flow to accumulate over the entire course of the fibers. The regenerating enteroendial cells with extremely-intense GDNF-immunoreactivity was confirmed along the regrowing nerves in the myenterized area. The development of an investment of the regrowing nerves with such enteroendial cells seems to play a role in efficient transmission of neurotrophic signal by the molecules at their contact areas to facilitate the nerve regeneration. This GDNF-Ret signaling device is presumed to contribute to the notably-increased rate of the nerve elongation during the later stage of the experimental period. These possibilities remain to be explored in future studies.

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