Electron Microscopic Studies of Hirschsprung's Disease

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16.1 Introduction

The histopathology of Hirschsprung's disease is defined by a complete absence of intramural nerve cells and a concomitant hypertrophy of nerve fascicles in caudal regions of the gastrointestinal tract. These pathomorphological criteria are readily recognized by standard enzyme and immunohistochemical techniques in establishing the diagnosis of Hirschsprung's disease. In contrast, electron microscopic studies are more time-consuming and require several procedures prior to the assessment of the specimens (e.g. fixation, postfixations, semi- and ultrathin sectioning, and specific stainings with heavy metal compounds). Therefore, in clinical routine, electron microscopy is not the tool of first choice to confirm the histopathology of Hirschsprung's disease.

Nevertheless, electron microscopic examination allows a subtle assessment of the ultrastructural peculiarities (e.g. nerve fiber and glial cell morphology, subcellular and extracellular components) not discernible in detail at light microscopic level. Whereas the first description of the histopathology of Hirschsprung's disease [1] gave rise to a variety of light microscopic studies, reports on the ultrastructure are comparatively rare. In 1970, Howard and Garrett [2] documented the electron microscopic findings in human Hirschsprung's disease describing several of the ultrastructural features of the aganglionic bowel wall. Baumgarten et al. [3] ultrastructurally analyzed the nervous elements in human Hirschsprung's disease and offered comparative remarks on the normal colon and internal anal sphincter. Later on, electron microscopic studies were combined with immunohistochemical techniques to investigate distinct neurochemically defined subpopulations of enteric nerves [4].

In this chapter, the typical ultrastructural features of human Hirschsprung's disease are outlined, discussed in relation to the morphology of the unaffected intestine and compared to the findings obtained from animal models of this intestinal innervation disorder.

16.2 Ultrastructural Features of Intestinal Aganglionosis

16.2.1 Hypertrophic Nerve Fascicles

In contrast to the normally configured enteric nerve plexus composed of ganglia and interconnecting nerve fascicles, in Hirschsprung's disease the bowel wall is characterized by a complete lack of intramural nerve cells and, thus, the absence of ganglia. Conversely, the remaining nerve fascicles passing within the intermuscular zone, the submucosal and mucosal layer are considerably thickened. This striking nerve trunk hypertrophy has also been reported in light microscopic studies [5] using either conventional hematoxylin-eosin or immunostaining of the glial marker protein S-100. As 90% of rectal suction biopsies contain nerve fascicles greater than 40 µm in diameter, this feature is considered to be highly predictive of aganglionosis and represents an important additional parameter in the diagnosis of Hirschsprung's

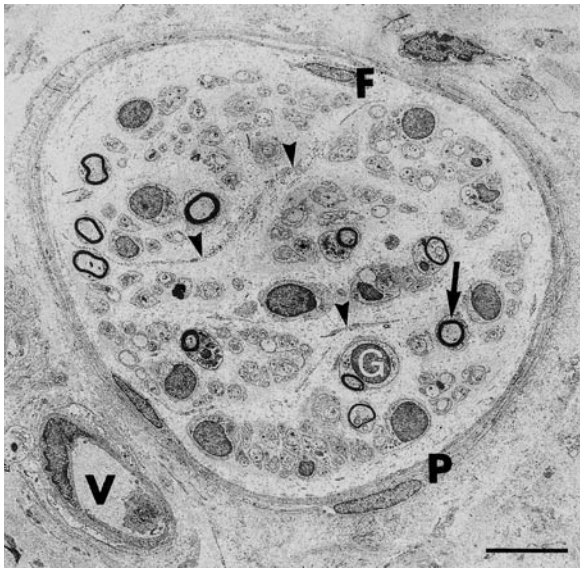

Fig. 16.1 Hypertrophic intermuscular nerve fascicle within the aganglionic segment. The nerve fascicle is composed of a prominent perineural sheath (*P*), numerous glial cells (*G*), naked and myelinated (*arrow*) nerve fibers. Abundant endoneural connective tissue composed of collagen and fibroblasts (*F*) with elongated processes (*arrowheads*) subdivide the nerve fascicle into different compartments (*V* adjacent blood vessel; *bar* 10 μm)

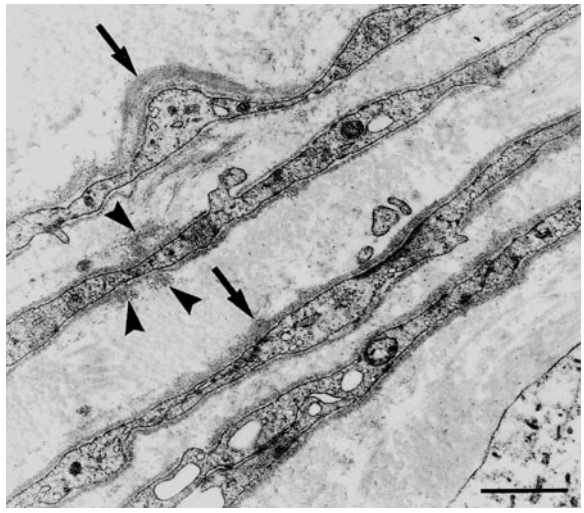

Fig. 16.2 Perineurium of a hypertrophic nerve fascicle within the aganglionic segment. Perineural cells exhibit a discontinuous thickening of basal laminae (*arrows*) and flocculent accumulations of basal lamina material (*arrowheads*) (*bar* 1 μm)

disease. The hypertrophic nerve fascicles are composed of a surrounding perineurium, abundant nerve fibers, distinctly shaped glial cells and a well-developed endoneurium (Fig. 16.1).

16.2.2 Perineurium

The prominent perineural sheath consists of multiple layers of flattened perineural cells connected by close intercellular contacts. Their cell borders are covered by a basal lamina delimiting the collagen-filled extracellular space between adjacent perineural cell layers (Fig. 16.2). Smaller nerve fascicles gradually lose their thick perineural sheath and possess a single-layered (Fig. 16.9), in some instances, discontinuous perineural envelope. When ramifying within the smooth muscle layers, the nerve fiber bundles are bare of a surrounding perineurium. The perineurium of hypertrophic nerve fascicles is richly supplied with blood vessels located adjacent to or intercalated within the perineural cell layers resembling typical features of vasa nervorum encountered in conventional peripheral nerves (Fig. 16.1). Large numbers of collagen fiber bundles and fibroblasts surround the outer border of the perineurium. The thin and remarkably elongated fibroblast processes frequently form loop-like, almost completely closed extensions ("collagen pockets") engulfing bundles of collagen fibers (Fig. 16.3).

16.2.3 Endoneurium

The nerve fibers within the hypertrophic nerve fascicles are not directly apposed but separated by endoneural connective tissue. The widened endoneural space is filled with densely packed collagen fibers and elongated fibroblasts subdividing the entire nerve fascicle into different endoneural compartments (Fig. 16.1). Whereas the majority of endoneural connective tissue fibers exhibit a typical periodical cross-banding (collagen fibers), a minor portion forms a hairy web of interdigitating reticular fibers (Fig. 16.4).

16.2.4 Glial Cells

The glial cell population within the hypertrophic nerve fascicles shows a remarkably homogeneous ultrastructure characterized by an oval nucleus lacking chromatinic condensations, a translucent cytoplasm poorly equipped with organelles and gliofilaments, and an individual basal lamina envelope (Fig. 16.5). In hypertrophic nerve fasci-

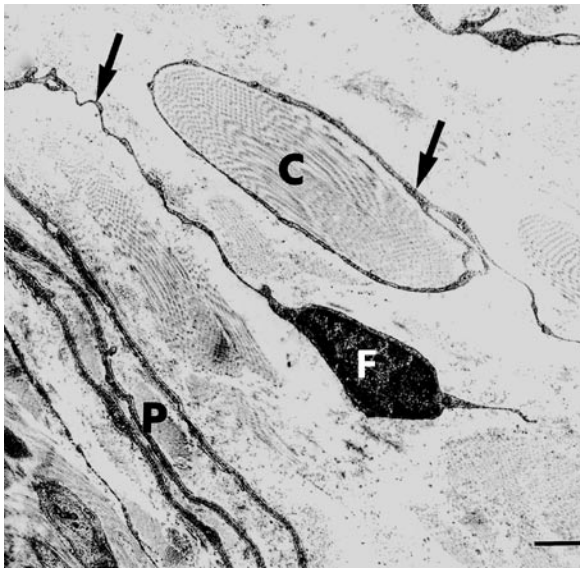

Fig. 16.3 Fibroblast (*F*) in close proximity to the perineurium (*P*) of a hypertrophic nerve fascicle within the aganglionic segment. Elongated fibroblast processes (*arrows*) engulf bundles of collagen fibers (*C*) forming "collagen pockets" (*bar* 2 μm)

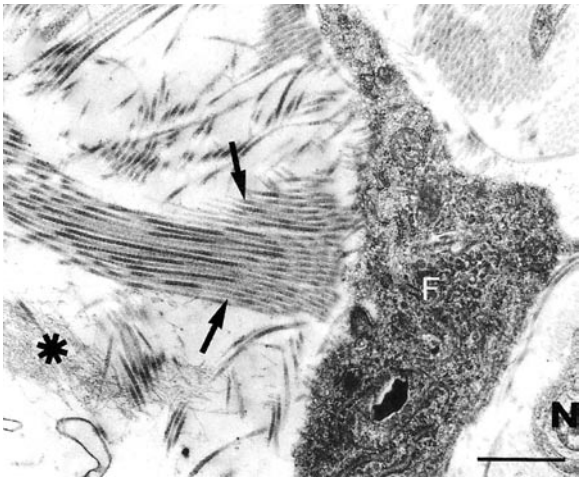

Fig. 16.4 Endoneural fibroblast (*F*) located within a hypertrophic nerve fascicle of the aganglionic segment. Collagen fibers extend from the cellular border into the endoneural space and aggregate to bundles (*arrows*). Reticular fibers form a hairy web of thin interdigitating filaments (*asterisk*) (*N* adjacent nerve fiber; *bar* 1 μm)

cles encountered in the aganglionic segment the glial cell processes generally ensheath a reduced number of nerve fibers forming mono- or oligoaxonal units (Fig. 16.6). Multiaxonal units are confined to smaller nerve fiber strands and their intramuscular ramifications. The observed ultrastructural characteristics correspond to the morphology typical of Schwann cells rather than of normal enteric glial cells: glial cells of the unaffected colonic wall possess a heterochromatinic nucleus with multiple indentations and a rich supply of cytoplasmic organelles and gliofilaments. Numerous nerve fibers are enveloped by extensively dividing glial cell processes resembling multiaxonal units (Fig. 16.7).

16.2.5 Nerve Fibers

The diameter of nerve fibers varies widely from 0.2 μm up to 8 μm. Their axoplasm is electrolucent and contains a reduced number of neurofilaments, microtubules and mitochondria (Figs. 16.5 and 16.6) in comparison to nerve fibers of normal interganglionic nerve fascicles (Fig. 16.7). While the axonal extensions of hypertrophic nerve fascicles within the intermuscular zone and the submucosal layer are almost completely bare of synaptic vesicles, the axoplasm of thinner nerve fibers enter-

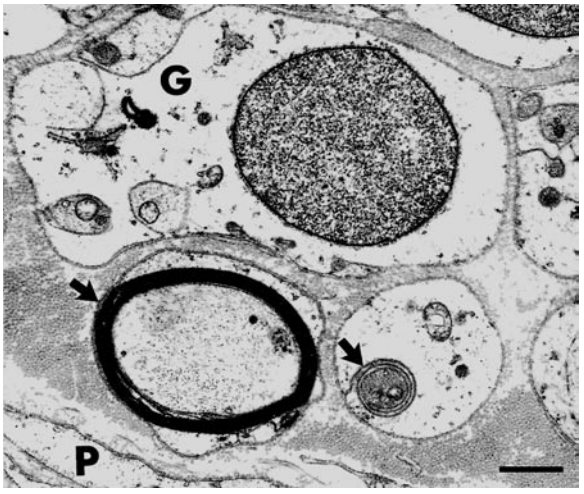

Fig. 16.5 Hypertrophic intermuscular nerve fascicle within the aganglionic segment. The glial cell (*G*) is characterized by a round euchromatinic nucleus and an electrolucent cytoplasm with a reduced number of organelles and gliofilaments. Two monoaxonal units display different stages of myelination (*arrows*) (*P* perineurium; *bar* 1 μm)

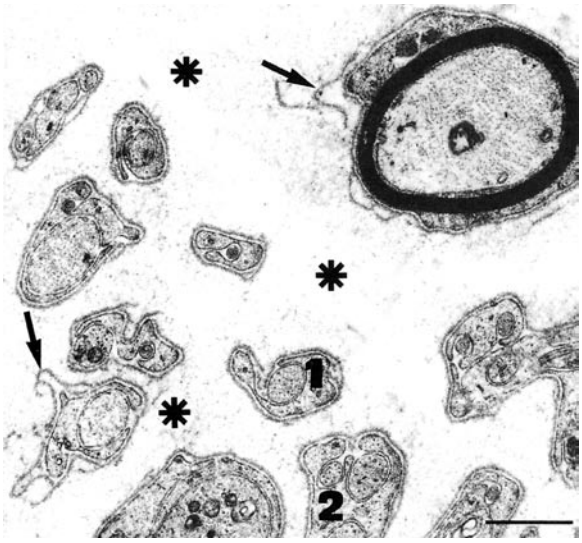

Fig. 16.6 Hypertrophic submucosal nerve fascicle within the aganglionic segment. Nerve fibers are separated by the widened collagen filled endoneural space (*asterisks*) and arranged in monoaxonal (*1*) and oligoaxonal (*2*) units. The basal lamina of glial cells enveloping naked or myelinated nerve fibers is frequently multilayered (*arrows*) (*bar* 1 μm)

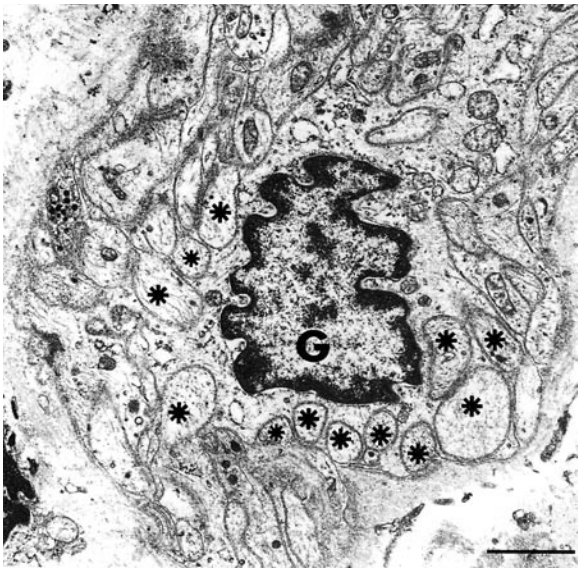

Fig. 16.7 Nerve fascicle of the myenteric plexus of a control specimen. Numerous nerve fibers (*asterisks*) are enclosed by one glial cell resembling a multiaxonal unit. The glial cell (*G*) is characterized by an indented heterochromatinic nucleus and a cytoplasm richly equipped with organelles and gliofilaments. The nerve fascicle lacks a prominent perineural sheath (*bar* 1 μm)

ing the smooth muscle layers exhibit varicose swellings predominantly filled with small empty, numerous small electrolucent and medium to large-sized electrodense vesicles. It has been shown that this distinct subpopulation of synaptic vesicles contrasts with the highly heterogeneous synaptic vesicle population of the unaffected bowel wall [3]. Immunohistochemical studies in human Hirschsprung's disease have confirmed a decreased number of synaptophysin-positive nerve fiber endings [6, 7]. Ultrastructural studies [8] have shown that the remaining neurotransmitters mainly correspond to acetylcholine (reaction deposits of acetylcholinesterase) observed between nerve terminals and smooth muscle cells, suggesting a direct innervation by extrinsic nerve fibers.

16.2.6 Myelination

An additional feature of hypertrophic nerve fascicles is the presence of myelinated nerve fibers (Fig. 16.1). Myelination of intramural nerve fibers is not confined to the intermuscular zone but also extends up to the inner submucosal layer with the ratio of myelinated nerve fibers to nonmyelinated nerve fibers ranging from 1:20 to 1:40. Whereas some nerve fibers are surrounded by a few lamellae probably indicating the initiation of myelination (Fig. 16.5), the majority of myelinated nerve fibers possess a myelin sheath composed of multiple apposed lamellae (Figs. 16.6 and 16.8).

16.2.7 Basal Laminae

Within the aganglionic bowel wall distinct basal lamina abnormalities can be observed. The basal lamina of perineural cells surrounding large and medium-sized nerve fascicles shows a discontinuous but marked thickening (Fig. 16.2). The width, measured from the perineural plasmalemma to the collagen-filled intercellular space, ranges from 50 nm to 200 nm. Additionally, flocculent accumulations of amorphous material exhibiting an electron density similar to the thickened perineural basal lamina are frequently disseminated along the perineural cell layers and protrude from the basal lamina into the extracellular space. Moreover, multilamination of basal laminae is found covering the glial cell plasmalemma of small monoaxonal units (Figs. 16.6 and 16.9) and myelinated nerve fibers of small- to medium-sized diameters including the nodes of Ranvier (Figs. 16.6 and 16.8). The basal lamina layers are irregularly apposed and show wriggling ramifications either connecting two adjacent basal laminae or blindly ending between endoneural

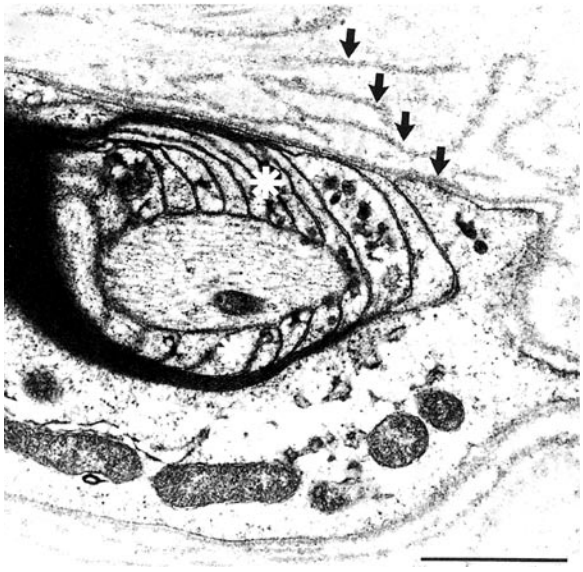

Fig. 16.8 Hypertrophic submucosal nerve fascicle within the aganglionic segment. A myelinated nerve fiber displays a node of Ranvier characterized by terminal loops (*asterisks*) of the glial corona and is surrounded by a multilayered basal lamina (*arrows*) (*bar* 1 μm)

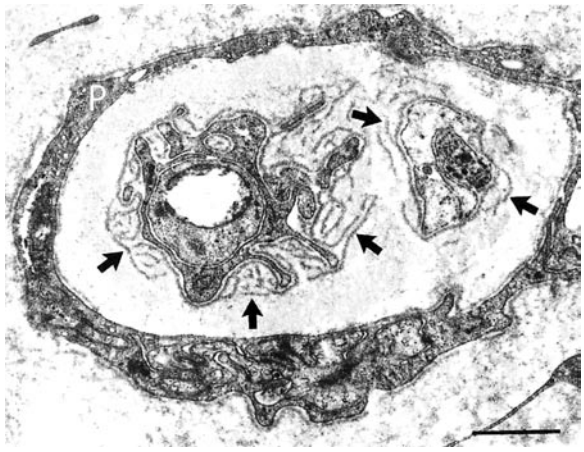

Fig. 16.9 Small submucosal nerve fascicle within the aganglionic segment. Glial cell processes of two monoaxonal units are concentrically surrounded by highly multilayered basal laminae (*arrows*) ramifying throughout the collagen-filled endoneurium (*P* single-layered perineurium; *bar* 1 μm)

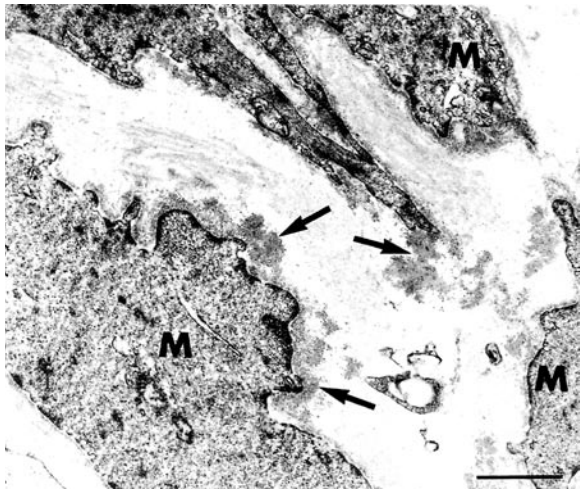

Fig. 16.10 Irregularly contoured smooth muscle cells (*M*) of the lamina muscularis mucosae of the aganglionic segment. Flocculent accumulations of basal lamina material (*arrows*) protrude into the interstitial space (*bar* 1 μm)

collagen fibers. Similar morphological abnormalities as observed in the perineural basal lamina are also discernible in the basal lamina surrounding smooth muscle cells, in particular those of the lamina muscularis mucosae. Prominent flocculent protrusions of amorphous basal lamina material are discontinuously distributed along the borders of the irregularly contoured smooth muscle cells (Fig. 16.10).

16.2.8 Subserosal Nerve Fascicles

Subserosal nerve fascicles approaching the aganglionic bowel wall via the mesentery exhibit ultrastructural characteristics very similar to those observed in intramural nerve fascicles located in the intermuscular and submucosal layer. Although the perineural sheath is more prominent, the amount of endoneural tissue is larger and

myelination occurs more frequently, both glial cells and nerve fibers virtually show the same ultrastructural arrangement.

16.2.9 Transitional Zone

Between the aganglionic and normoganglionic segment extends a transitional zone of varying length. This hypoganglionic region is characterized by small oligoneuronal ganglia and an irregular network of interganglionic nerve fiber connections. Although the hypertrophic nerve fascicles of the aganglionic segment enter the transitional zone, their number and diameter gradually decreases in the oral direction. The perineural sheath is thinner, the amount of endoneural tissue is diminished, and myelination is only rarely discernible. However, their ultrastructure still differs from the normal nerve plexus morphology by the predominance of mono- and oligoaxonal units and the Schwann cell-like appearance of glial cells.

16.3 Pathogenetic Implications

16.3.1 Extrinsic Origin of Hypertrophic Nerve Fascicles

The hypertrophic nerve fascicles encountered within the aganglionic bowel wall exhibit histological and ultrastructural features resembling those of extrinsic rather than intrinsic nerves. The presence of a thickened perineurium, wide endoneural spaces, vasa nervorum, Schwann cell-like glia, mono-/oligoaxonal units, myelination, and additionally the similarity of subserosal and intramural nerve fascicles suggest an extraenteric origin.

Indeed, whole-mount studies on the aganglionic colon from patients with Hirschsprung's disease [9] and of aganglionic spotted lethal rats [10] have confirmed the extrinsic origin of both intermuscular and submucosal nerve fascicles. Moreover, retrograde tracing experiments in aganglionic lethal spotted mice have revealed that the majority of nerve fibers originate from the inferior mesenteric ganglion and dorsal root ganglia, whereas only a minor proportion of intrinsic fibers seem to penetrate into the aganglionic segment [11].

Since Stach's [12] description of the "ascending nerves of the pelvic plexus" in various mammals, it is now well established that the distal colon is penetrated by large nerve bundles passing through the intermuscular zone and giving off branches to the myenteric plexus. It has been suggested that the over-abundance of thickened nerve fascicles found in the aganglionic segment results from a hypertrophy of these ascending pelvic nerves [10].

Experiments on extrinsically denervated cat colon have shown that the entire population of myelinated nerve fibers enter the colon from the pelvic plexus and, in part, from pudendal nerves [13]. As the stimulation of pelvic nerves induces contraction in the distal colon [14], it is suspected that the over-abundance of myelinated intramural nerve fibers originating from the pelvic plexus contributes to the functional colonic obstruction besides other intrinsic pathophysiological mechanisms. Constrictive influences mediated by hypertrophic nerves on nerve cell-deprived colonic segments have also been claimed by Baumgarten et al. [3] and Howard and Garrett [2], as these nerve fibers do not resemble blind endings, but form axonal varicosities richly supplied by synaptic vesicles in close proximity to smooth muscle cells.

16.3.2 Basal Lamina Abnormalities

As outlined above, ultrastructural peculiarities encountered in the aganglionic bowel wall include distinct morphological basal lamina abnormalities of perineural, glial and smooth muscle cells. These observations are in accordance with the light microscopic demonstration of an abnormal distribution of basal lamina-specific components such as collagen type IV, laminin and fibronectin in the aganglionic bowel wall [15, 16]. In particular, the extensive production of basal lamina material within the hypertrophic nerve fascicles provides an ultrastructural correlate of the findings reported by Parikh et al. [16] who have demonstrated intense immunoreactivity of the basal lamina constituent fibronectin in thickened nerve fascicles within the aganglionic segment.

However, morphological alterations of the basal lamina are not specifically related to Hirschsprung's disease, as they have also been documented in other neurological diseases such as diabetic autonomic [17] and hereditary peripheral neuropathies [18, 19]. In infantile hypertrophic and hereditary motor and sensory neuropathy type III the reduplication of the glial basal lamina has been attributed to reactivated Schwann cells from which the basal lamina material originates [20]. Thus, in Hirschsprung's disease the over-production of basal lamina material in hypertrophic nerve fascicles may reflect an increased activity of proliferating glial cells. In diabetic enteroneuropathy both a thickening and a reduplication of the glial cell basal lamina has been observed and is considered to represent a diffusion barrier for neurotransmitters impairing their release to neuroeffector sites [21]. However, this assumption may not apply to patients with Hirschsprung's disease, as in the aganglionic segment most of the axonal swellings located adjacent to smooth muscle cells are bare of a basal lamina thickening. Furthermore, it cannot be excluded that basal lamina abnormalities observed in Hirschsprung's disease may represent secondary defects resulting from the chronic mechanical distension resting upon the dilated bowel wall. In fact, intraluminal tension forces

are capable of provoking both basal lamina thickening and reduplication as demonstrated in the endothelium of blood vessels exposed to hypertension [22].

Abnormalities of the basal lamina in human Hirschsprung's disease remain of special interest, as they have also been found in mouse strains developing congenital megacolon [23, 24]. In particular, the thickening and reduplication of basal laminae surrounding smooth muscle cells of the lamina muscularis mucosae have been considered to reflect microenvironmental abnormalities during embryogenesis persisting into adult life. It has been proposed that the increased production of extracellular matrix components within the presumptive aganglionic segment may provide a good substrate for the ingrowth of extrinsic nerves, but seems to impair the colonization by nerve cell precursors [25–27]. These findings suggest that the pathogenetic mechanisms leading to aganglionosis in murine models of Hirschsprung's disease are not entirely related to the neural crest, but include microenvironmental abnormalities intrinsic to the colonic wall.

In summary, morphological alterations of basal laminae in human Hirschsprung's disease involve both neuronal and non-neuronal elements and provide further evidence that extracellular matrix components are abnormally distributed within the affected bowel wall. Moreover, the increased amount of collagen observed in the endoneurium, the perineurium and in areas adjacent to hypertrophic nerve fascicles indicates that the histopathology of the aganglionic intestine is not exclusively confined to nervous tissue alterations, but also includes an impressive over-production of connective tissue components.

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