Histol Histopathol (1998) 13: 989-994
DOI: 10.14670/HH-13.989
http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

Myenteric plexus in the gastrointestinal tract of non-obese diabetic mice

A. Spångéus and M. El-Salhy
Section for Gastroenterology and Hepatology, Department of Medicine, University Hospital, Umeå, Sweden

Summary. The myenteric plexus was investigated in the gastrointestinal tract of pre-diabetic and diabetic non-obese diabetic (NOD) mice. The plexus was immunostained by the avidin-biotin complex method, using a general marker for nerve elements, namely protein gene-product 9.5. The nerve fibres were quantified by point-counting and the number of ganglia and their area were determined by image analysis. The relative volume density of the nerve fibres in duodenal muscularis propria was found to be significantly reduced in of both pre-diabetic and diabetic NOD mice. There was no statistical difference between controls and NOD mice regarding relative volume density of nerve fibres in antral and colonic muscularis propria. The number of myenteric ganglia/mm baseline was significantly decreased in the duodenum of diabetic NOD mice, and showed a non-statistically significant tendency to decrease in pre-diabetic mice. In the antrum and colon, there was no difference between the controls and NOD mice regarding the number of ganglia/mm baseline. Nor was there any significant difference between controls and NOD mice in the area of myenteric ganglia in either antrum, duodenum or colon. It is concluded that the changes in the duodenal myenteric plexus of NOD mice are prior to the onset of diabetes. It is suggested that the absence of changes in the antral and colonic myenteric plexus when using a general marker for neuroelements does not preclude a possible change in cholinergic, adrenergic or peptidergic innervation.

Key words: Enteric nervous system, Ganglia, Computerized image analysis, Immunocytochemistry, Nerve fibres

Introduction

The innervation of the gastrointestinal tract is of two types: one extrinsic (central) and the other enteric (intrinsic) (Ekblad et al., 1991). The extrinsic innervation is represented by parasympathetic, sympathetic and sensory nerve fibres. The intrinsic innervation represents the great majority of the nervous elements in the gastrointestinal tract with their cell bodies in the intramural ganglia (Ekblad et al., 1991). The enteric nervous system plays an important role in the regulation of gut motility and secretion (Allescher and Ahmed, 1991; Rangachari, 1991). Gastrointestinal dysmotility is common in patients with diabetes (Feldman and Schiller, 1983; Spengler et al ., 1989; Wegener, et al., 1990; Clark and Nowak, 1994; Locke III, 1995; Mearin and Malagelada, 1995). It is conceivable that these motility disturbances seen in patients with diabetes are associated with disorders of the enteric nervous system. It has not been possible to study the enteric innervation in patients with diabetes, as whole-wall biopsies could not be obtained, because the condition of these patients never calls for intestinal resection. The use of animal models may, therefore, be useful in this respect. Even so, few studies have been made on the morphology of the enteric nervous system of these models. The myenteric plexus has been studied in the stomach of rats with alloxan-induced diabetes (Nwolokolo et al., 1992), peptidergic innervation has been studied in the small intestine of diabetic BB Wistar rats (Buchan, 1990) and the enteric nervous system in the ileum and colon of rats with streptozotocin-induced diabetes (Belai and Burnstock, 1990; Belai et al., 1991).

In the present study the myenteric plexus in an animal model, the non-obese diabetic (NOD) mouse was used. This strain is genetically predisposed to develop a condition clinically similar to that of type I human diabetes (Makino et al., 1980; Kolb, 1987; Tochino, 1987). For this purpose, a general marker for nerve elements, namely protein gene product 9.5 (PGP 9.5) was used (Krammer et al., 1993, 1994).

Offprint requests to: Magdy El-Salhy, Section for Gastroenterology and Hepatology, Department of Medicine, University Hospital, S-901 85 Umeå, Sweden. Fax: 46-90-120923, E-mail: magdy.el-salhy@medicin.umu.se

Materials and methods

Animals

Female NOD/Bom mice, 22-24 weeks old (Bomholtgård Breeding and Research Centre, Denmark)

were studied. As controls, sex- and age-matched mice of the sister strain to the NOD mouse (Fujishima et al., 1989), namely BALB/cJ Bom (Bomholtgård) were used. The animals were housed, 5 in each cage, in a room with a 12/12 h light-dark cycle, and were fed on a standard pellet diet (Astra-Ewos AB, Södertälje, Sweden) with free access to water. The mice used in this investigation have been characterised in detail elsewhere (El-Salhy et al., 1998). The NOD mice were divided into two groups: pre-diabetics, and diabetic according to the following criteria: pre-diabetic mice were not glucosuric as established with Ecur-Test sticks (Boehringer Mannheim), did not have a significantly decreased volume density of pancreatic islet and insulin cells, and did not have a significantly decreased pancreatic insulin content as determined by RIA. They did have, however, grade 1-2 insulitis (islets with significantly peri-insular diffused to mild infiltration of lymphocytes) and reduced body weight. The diabetic mice had glucosuria in the range 2-4 (5.6-55 mmol/l), significantly decreased volume density of pancreatic islets and insulin cells and significantly reduced pancreatic insulin content. They also had grade 3-4 insulitis (islets with peri- and intra-insular infiltration of lymphocytes or islets entirely dominated by lymphocyte infiltration), and significantly reduced body weight.

Eight pre-diabetic and 8 diabetic NOD/Bom mice as well as 8 BALB/cJ Bom mice were used in this investigation. Their food was withheld 24 h before the animals were sacrificed in a CO_2 chamber. The abdomen was opened with a median incision and the antrum, proximal duodenum and distal colon were excised. The tissue specimens were fixed overnight in 4% buffered formaldehyde, embedded in paraffin wax and cut into 10 μm-thick sections. The investigation was approved by the Local Committee on Animal Ethics, Umeå University.

Microwave antigen retrieval

The sections were hydrated, and placed in plastic Coplin jars filled with 0.01M citrate buffer, pH 6. They were then incubated in a microwave oven at 350W for 15 min. The slides were allowed to cool to room temperature for 20 min, rinsed in Tris-buffer and were then immunostained. In a preliminary study the sections were immunostained with or without antigen retrieval. When antigen retrieval was applied, the number of the nerve fibres increased and small branches of the nerve fibres could be detected.

Immunocytochemistry

The avidin-biotin complex (ABC) method (Dako-patts, Glostrup, Denmark) was applied as described in detail earlier (El-Salhy et al., 1993). Briefly, following microwave antigen retrieval, the sections were incubated first with 1% bovine serum albumin for 10 min to block the non-specific binding sites, then with rabbit anti-PGP 9.5 (diluted 1:600, code no. RA 95101, Ultra Clone Ltd, Isle of Wight, England) overnight at room temperature. The sections were incubated with biotinylated swine anti-rabbit IgG, diluted 1:100 for 60 min at room temperature, and finally with the avidin-biotin-peroxidase complex, diluted 1:50 for 30 min at room temperature. The sections were developed in 50 ml Tris buffer containing 10 μl of 30% H_2O_2 and 25 mg diaminobenzidine tetrahydrochloride (DAB) and counterstained lightly with Mayer's haemotoxylin. Negative controls were performed by substituting the anti-PGP 9.5 by non-immune rabbit serum. Positive controls were obtained by staining sections from rat brain and human colon. In the control experiments an identical immunostaining procedure was used.

Computerized image analysis

To quantify the myenteric plexus, the video image analysis Quantimet 500MC image processing and analysis system (Leica, Cambridge, England) linked to an Olympus microscope (type BX50) was used. The image analysis software was "QWIN" (version 1.02), a Windows-based image analysis program from Leica. Leica's interactive program, QUIPS (version 1.02), was also included in the system. Quantification was performed using x4, x20 and x40 objectives. At these magnifications each pixel of the image corresponded to 2.12, 0.414 and 0.21 μm, respectively and each field in the monitor represented a tissue area of 1.3, 0.04 and 0.009 mm^2, respectively.

In order to determine the relative volume density of the nerve fibres in muscularis propria, the classical stereological point-counting method (Weibel and Elias 1967; Weibel et al., 1969), as adapted for computerized image analysis, was used (El-Salhy et al., 1997b). Briefly, an automated standard sequence analysis operation was applied, in which a regular 400-point lattice was superimposed on the frame containing the tissue. Points covering tissue other than muscularis propria were erased and the points covering the immunoreactive nerve fibres were pointed out with the computer "mouse" and by clicking the mouse a series of blue highlight points appeared. A step introduced at the end of the sequence was to use the editor menu to add or erase errors. The ratio of points lying on nerve fibres vs. those lying on muscularis propria in each field was tabulated. The sum of all fields in the specimen was computed and statistically analysed automatically. Twenty fields (from 2-4 different sections, separated from each other by 50 μm) were randomly chosen from each individual and analysed by a x40 objective.

The number of ganglia in the myenteric plexus/mm baseline was determined in three sections from each mouse, with the x4 objective and by using interactive measurements in the manual menu. This was done by drawing a line parallel to the muscle fibres and counting the number of ganglia. The area of each ganglion was measured with the x20 objective and by using interactive

measurements in the manual menu. This was carried out by drawing a line around fifteen randomly-chosen ganglia from each mouse.

The thickness of muscularis propria in the antrum, duodenum, and colon was determined in four randomly-chosen fields from 2-4 perpendicularly-cut sections, 50 μm apart, taken from each mouse. Fifteen measurements in each field were performed with the x20 objective and by using the interactive measurements in the manual menu.

Statistical analysis

Comparisons between two groups were made with the non-parametric Wilcoxon (Mann-Whitney) test. P-values below 0.05 were regarded as significant.

Results

Immunocytochemistry

PGP 9.5-immunoreactive nerve fibres were found in muscularis propria, in submucosa and lamina propria as well as neurones of both the myenteric- (Fig. 1) and submucosal plexus. Specificity controls disclosed that replacement of the primary antiserum by non-immune rabbit serum did not produce immunostaining. The antiserum used immunostained nerve elements in the rat brain and the enteric nervous system of human colon.

Image analysis

The results of the morphometric measurements are reported in Figs. 2-4. The relative volume density of the nerve fibres in duodenal muscularis propria was significantly reduced in both pre-diabetic and diabetic NOD mice (P=0.015 and P=0.038, respectively). There was no statistical difference between controls and NOD mice, regarding the nerve-fibre relative volume density in antral and colonic muscularis propria. The number of myenteric ganglia/mm baseline was significantly reduced in the duodenum of the diabetic NOD mice (P=0.021). Although the number of these ganglia showed a tendency to decrease in the pre-diabetic mice, it was not statistically significant (P=0.06). In antrum and colon, there were no differences between the controls and NOD mice regarding the number of ganglia/mm baseline. Nor was there any significant difference between controls and NOD mice regarding the area of myenteric ganglia in either antrum, duodenum, or colon.

The values of antral muscularis propria thickness in

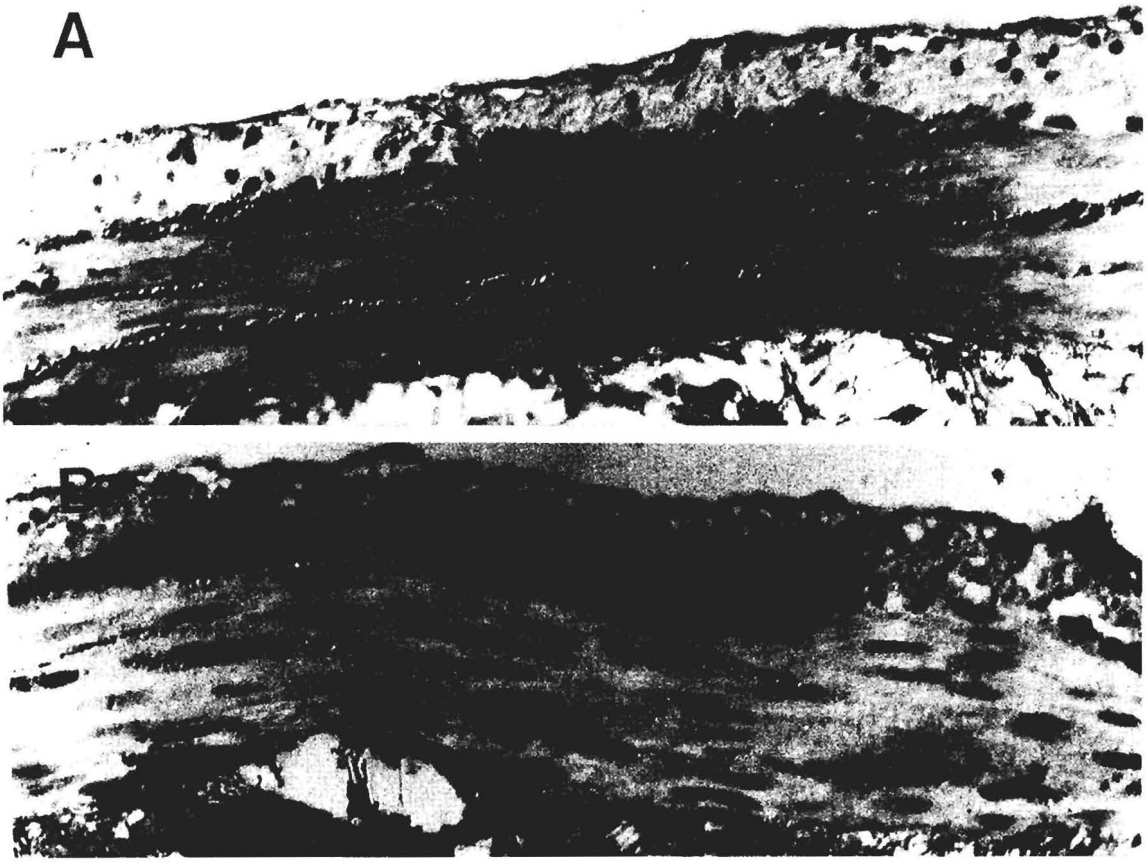

Fig. 1. Protein gene-product 9.5 (PGP 9.5)-immunoreactive nerve fibres in muscularis propria as well as myenteric ganglia (arrow) in the duodenal of a control **(A)** and of a diabetic NOD mouse **(B)**. x 200

the controls, pre-diabetic and diabetic NOD mice were 182±6.7, 173±10.6 and 156±12 μm, respectively. The corresponding figures for duodenum were 60±2.7, 57±3.6 and 61±8.3 μm, respectively, and for colon were 83.9±5.6, 97±6.2, 104±10.4 μm. All values expressed as mean ± SEM. There was no statistical difference between controls and NOD mice in the thickness of muscularis propria from antrum, duodenum and colon.

Discussion

In the present study a reduced relative volume

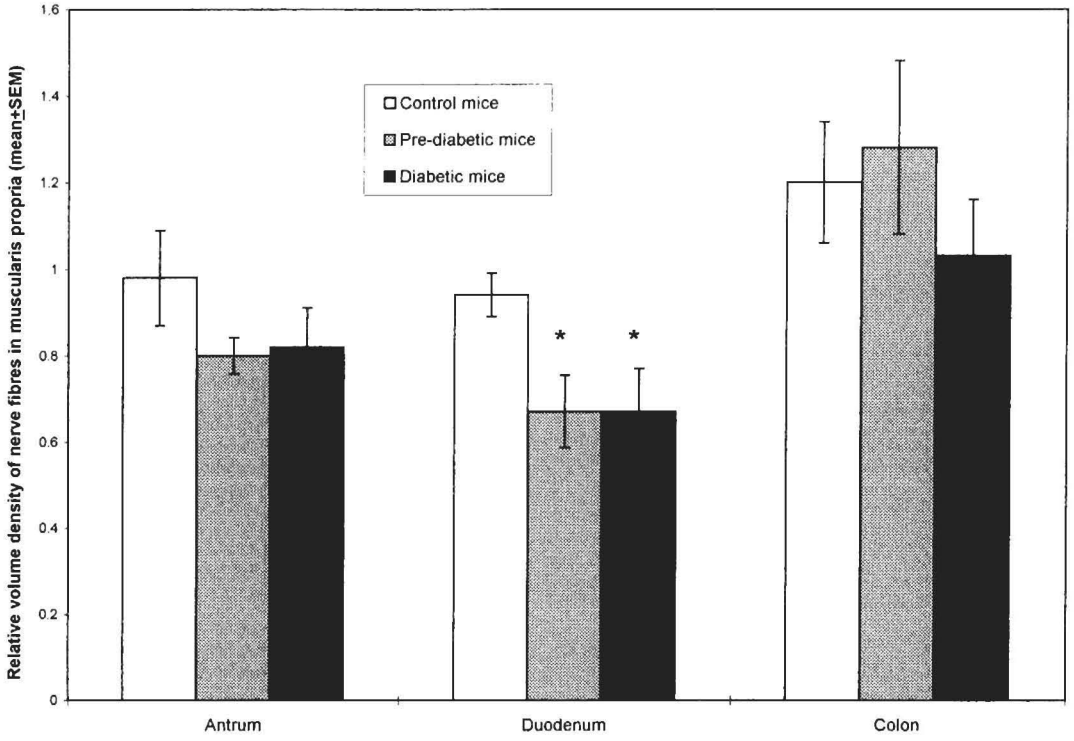

Fig. 2. The relative volume density of PGP 9.5-immunoreactive nerve fibres in muscularis propria of controls and NOD mice. *: P<0.05.

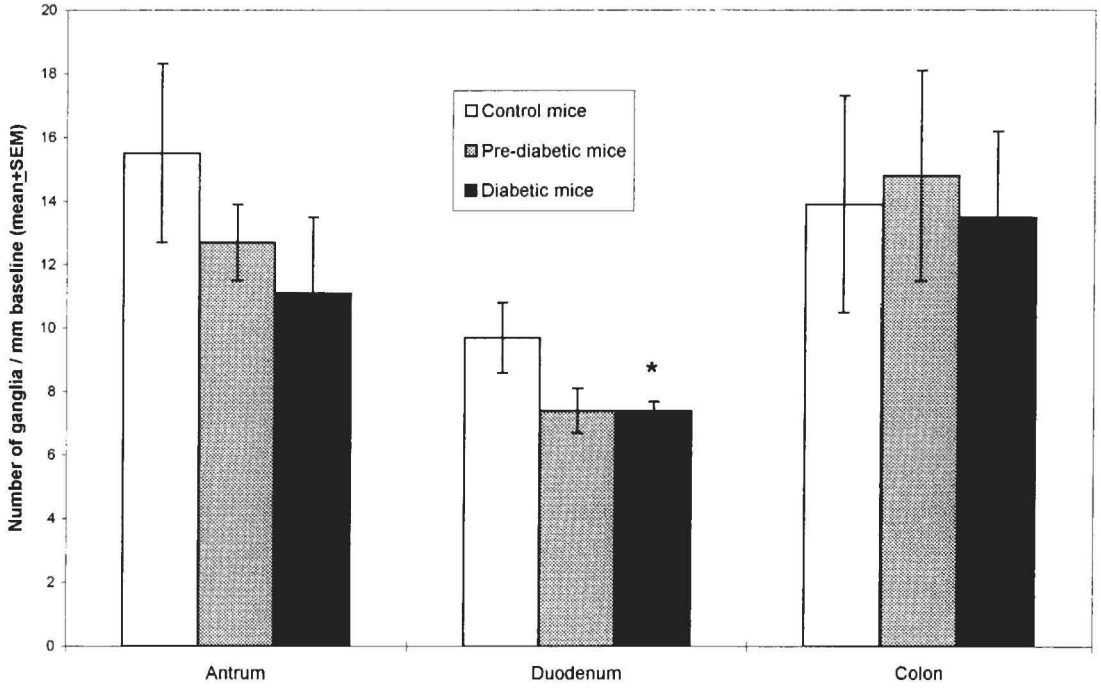

Fig. 3. Number of ganglia/mm baseline of controls and NOD mice. Symbols as in Fig. 2.

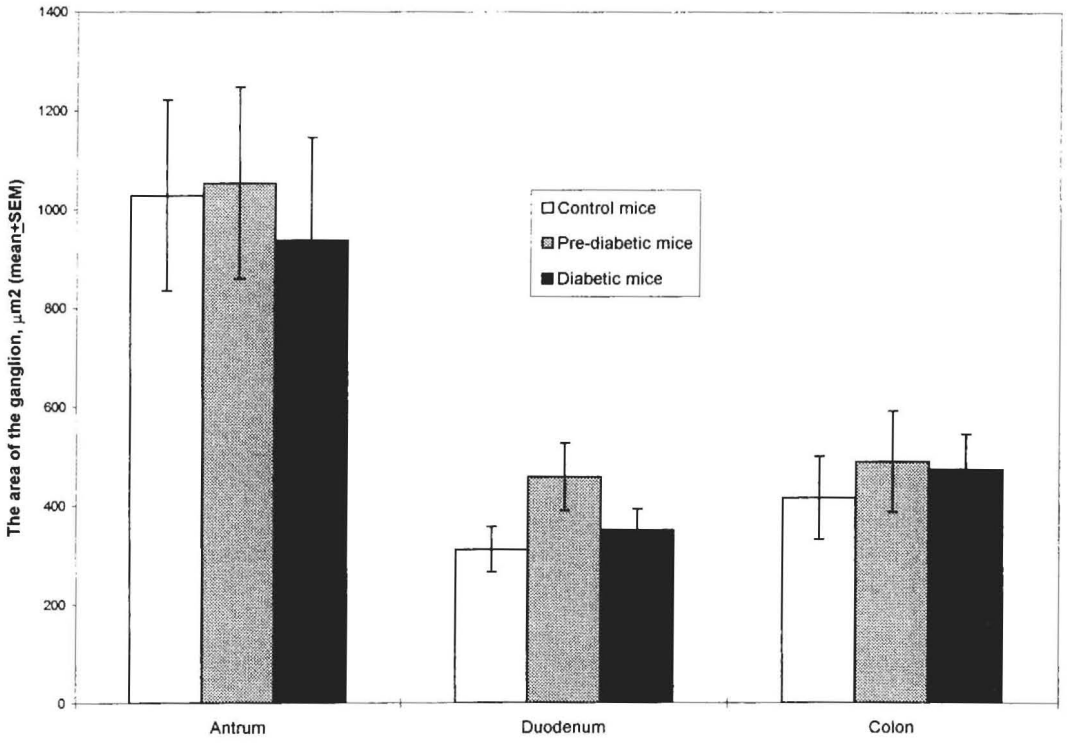

Fig. 4. The area of ganglia in the myenteric plexus of controls, and of NOD mice. Symbols as in Fig. 2.

density in the nerve fibres of NOD mice, as well as a reduced number of ganglia in the duodenal myenteric plexus were observed. This decrease in nerve fibres and ganglia occurred in both pre-diabetic and diabetic NOD mice, indicating that these abnormalities precede the onset of diabetes and are not secondary to the diabetic state. In an earlier report (Buchan, 1990), both the number and the intensity of staining of galanin-immunoreactive nerve fibres were increased in the small intestinal myenteric plexus of hyperglycaemic diabetic BB Wistar rats. Radioimmunoassays (RIA) of duodenal extracts from whole-wall biopsies from NOD mice have shown high levels of substance P, vasoactive intestinal polypeptide (VIP) and enkephalin (El-Salhy and Spångéus, 1998a,b). Moreover, an increased concentration of VIP, norepinephrin and dopamine and a decreased level of substance P and met-enkephalin have been reported in the duodenum of alloxan-induced diabetes in rats (Di Giulio et al., 1989). These RIA results of whole-wall biopsies are difficult to correlate with the present observations on the myenteric plexus.

There were no differences in antral and colonic myenteric plexus between controls and NOD mice regarding the relative volume density of nerve fibres, or the number and area of ganglia. Similarly, in an earlier study of rats with streptozotocin-induced diabetes, the antral nerve density in muscularis propria was found to be unchanged (Nwokolo et al., 1992). It has been reported that in rats with streptozotocin-induced diabetes, there were no evident changes in colonic myenteric plexus regarding dopamine-beta-hydroxylase-, substance P-, calcitonin gene-related peptide-and VIP-immunoreactive nerve fibres (Belai et al., 1991). An increased concentration of substance P has been shown by RIA from whole-wall biopsies of the colon of NOD mice (El-Salhy and Spångéus, 1998a).

The present study shows that the myenteric plexus in the small intestine of NOD mice, an animal model for type I human diabetes, is affected. On the other hand, this plexus was unchanged in the stomach and large intestine. It should be remembered, however, that these results were obtained by using a common marker for nerve elements, namely PGP 9.5, and should be interpreted as a summary of possible changes in cholinergic, adrenergic and peptidergic nerve fibres.

Acknowledgements. This study was supported by grants from Sahlberg's Foundation, and the Bengt Ihre's Foundation.

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Accepted March 26, 1998