The development of colon innervation in trisomy 16 mice and Hirschsprungs disease

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Subject headings: Hirschsprung’s disease; colon; Down syndrome; immunohistochemistry; nervous system; trisomy 16 mouse

Abstract

AIM To study the colon innervation of trisomy 16 mouse, an animal model for Down’s syndrome, and the expression of protein gene product 9.5 (PGP 9.5) in the stenosed segment of colon in Hirschsprung’s disease (HD).

METHODS Trisomy 16 mouse breeding; cytogenetic analysis of trisomy 16 mice; and PGP 9.5 immunohistochemistry of colons of trisomy 16 mice and HD were carried out.

RESULTS Compared with their normal littermates, the nervous system of colon in trisomy 16 mice was abnormally developed. There existed developmental delay of muscular plexuses of colon, no submucosal plexus was found in the colon, and there was 5 mm aganglionic bowel aparting from the anus in trisomy 16 mice. The mesentery nerve fibers were as well developed as shown in their normal littermates. Abundant proliferation of PGP 9.5 positive nerve fibers was revealed in the stenosed segment of HD colon.

CONCLUSION Trisomy 16 mice could serve as an animal model for Hirschsprung’s disease for aganglionic bowel in the distal part of colon. Abundant proliferation of PGP 9.5 positive fibers resulted from extrinsic nerve compensation, since no ganglionic cells were observed in the stenosed segment of the colon in HD. HD has a genetic tendency.

INTRODUCTION

The most significant pathophysiological change of Hirschsprung’s disease is its characteristic abnormal enteric nervous development of colon which resulted in the regional enterospasm and narrowed lumen, loss of intestinal peristalsis and relaxing reflex of the internal enteric sphincter[1]. The physiological peristalsis of the proximal gut pushes the intestinal contents forward, leading to the second megacolon in the proximal part of the narrowed gut. Since the cause of HD is still unknown and the experiment on humans is impossible, we used protein gene product 9.5 to study the enteric nerve development of colon in trisomy 16 mice, an animal model for Down’s syndrome[2,3], and the innervation of stenosed segment of the colon in HD so as to reveal the possibility of developing the congenital megacolon in trisomy 16 mice and the genetic tendency of HD in human[4-7].

MATERIALS AND METHODS

Animal stocks and breeding[8-10]

Trisomy 16 mouse embryos examined in this study were produced by crossing NMRI-female mice provided by the Experimental Center of Medical University of Lubeck, with male mice carrying the two Robertsonian (Rb) translocation chromosomes Rb (16,17) 8Lubw 8Lab3 perpendicularly and Rb (11,16)2H. The day on which a vaginal plug was observed was considered to be the first embryonic day (ED).

Cytogenetic analysis

The pregnant female mice were sacrificed one hour after intrauterine injection of colchicine (0.02 mg/25-35 mg body weight) by dislocation of the neck. The embryos were collected by laparatomy. Small samples of liver were taken from each embryo for direct preparation of karyotypes, by using the standard technique of hypotonic treatment with 0.563% KCl and methanol:acetic acid fixation (3:1). Air-dried slides were observed under light microscope. Accurate ascertainment of trisomy 16 was provided by the demonstration of two Rb metacentric chromosomes and a count of 41 chromosome arms. After the defrmy of the chromosomal status, the trisomic and the normal euploid embryos of each litter could be separated (Figure 1).

Immunohistochemical methods[11]

The guts were taken from trisomy 16 embryos and
normal littermates, and fixed in a solution containing 4% paraformaldehyde and 2% picric acid in phosphate-buffered saline (PBS) for approximately 2 hours at room temperature. Fifteen pieces of narrowed HD guts were obtained from the patients aged between 18 months and 9 years. Six segments of normal colons served as controls. The diagnosis of HD was based on the case history, clinical manifestations, examination of barium enema and pressure measurement of rectum and anal. All the cases were confirmed postoperatively by pathological examinations.

Whole mount preparations of the gut were dissected out under stereomicroscopic control[12-14], and freezing sections were made at a thickness of 12 µm, then washed in 0.01 M PBS and 50% ethanol for 2 hours at room temperature. For immunohistochemistry, peroxidase-antiperoxidase (PAP) was used in the present experiment.

The specimens were treated with 10% normal goat serum (Dakopatts × 907) for 30 min, then incubated in a primary antiserum protein gene product 9.5 (PGP 9.5) (Ultraclone RA 95101, UK) overnight at room temperature, diluted to 1 : 400 in a solution containing 10% normal goat serum, 0.01% sodium azide and 0.05% thimerosal in PBS. After three washes in PBS for 30 min, the specimens were incubated in goat anti-rabbit IgG (Dakopatts Z421) overnight at room temperature as the secondary antiserum, and diluted to 1 : 100 in the above solution. The specimens were then exposed in rabbit PAP (rabbit antibody to horseradish peroxidase and horseradish peroxidase) overnight at room temperature and diluted to 1 : 100 in a solution containing 0.1% bovine serum albumin and 0.05% thimerosal in PBS pH 7.4. 4 Cl1-naphtol or DAB was used as the chromogen for the peroxidase reaction. Control included the omission of the primary antiserum. PGP 9.5 is a cytoplasmic protein composed of 212 amino acids with molecular weight of 2.45 kb. This protein only exists in the neurons and neuroendocrine cells. PGP 9.5 is a specific protein with an excellent localizing function for the neurons[15-19].

RESULTS

Development of trisomy 16 mice and their normal littermates

Owing to the abnormality of their chromosomes, the trisomy 16 mice were characterized by their small size, generalized edema and a failure of closed eyelids after embryonic day 16 (ED 16). Both the small size and lid-gap defect were attributed to the generalized growth retardation and developmental delay, leading death at the time of birth in most mice. The trisomy 16 mice in the present study were chosen from the gestational age between ED13 and ED 18. The survival rate of the trisomy 16 mice was lower with the gestational age. The lowest survival rate of trisomy 16 mice was observed at the time of their birth (Figure 2).

Development of colon innervation in trisomy 16 mice and their normal littermates

The nerve development of colon in normal littermates is a continuous process. Based on the morphology and staining degree of the PGP 9.5 positive reactions, the nerve development of colon could be divided into three stages: primary stage (ED14), juvenile stage (ED15 and ED16) and mature stage (ED17 and ED18). At ED14, scantly-distributed PGP 9.5 positive neurons were observed with light staining and round nucleus in the center of the neurons. At ED15, the distribution density of neurons increased greatly with some nervous processes, through which the neurons were connected with each other (Figure 3A). At ED16, muscular plexuses appeared with gathered neurons to form the light-stained ganglion. But the number of neurons in the ganglion was less and sparsely arranged. At ED17 and ED18, the precursor cells of muscular plexuses migrated into the submucosal layer to form submucosal plexuses. The arrangement of nervous fibers in submucosal plexus was irregular, accompanied by some small-sized and sparsely-distributed ganglions, while muscular plexuses were composed of primary, secondary and tertiary strands displaying a nerve meshwork with regular wide meshes and numerous large ganglia. The muscular and submucosal plexuses were further developed with much stronger PGP 9.5 staining at ED18 (Figure 4A). The increasing number of large-sized ganglions was observed with stronger immunostaining and arranged along the primary nerve strands. Numerous fine nerve strands in the gut villi were observed at ED18 (Figure 5).

When compared with the normal littermates, the developmental delay of the colon-innervation was observed in trisomy 16 mice. Some neurons appeared with a scattered distribution and light staining at ED15 (Figure 3B) and the neurons were found with some nerve processes by which irregular nervous meshwork formed at ED16. In the colon of trisomy 16 mice at ED17 and ED 18, only one plexus was visible, i.e., the myenteric plexus with a few small-sized ganglions (Figure 4B). From ED14 to ED18 there was no submucosal layer in colon, and 5mm aganglionic bowel was found at the distal end of the colon in trisomy 16 mice (Figure 6), which was revealed as a characteristic morphological evidence of the development of congenital megacolon in trisomy 16 mice. This important finding was constantly verified in all the experimental trisomy 16 mice.

The exogenous innervation of colon in trisomy 16 mice and their littermates

As early as ED14, PGP 9.5 positive nerve fibers occurred in the mesentery of both trisomy 16 mice and their normal littermates, which gradually
increased with the embryonic development. At ED17 and ED18, rich mesenteric nerve fibers were detected. In all whole mounts of PGP 9.5 nerve fibers in mesentery, they were observed regularly running in parallel and close to the blood vessels and innervated the colon in both trisomy 16 mice and their normal littermates (Figure 7). Thus, the arcades of the mesenteric vessels were clearly delineated by their immunostained perivascular plexuses.

**The distribution of PGP 9.5 positive nerve fibers in the stenosed segment of HD and normal colons**

The ganglions of the muscular plexus and submucosal plexus from the normal colon all revealed highly positive reaction to PGP 9.5 (Figure 8). The number of ganglion in muscular plexus was greater than that in the submucosal ones. The neurons presented a dark brown color and gathered as a mass. The adjacent neurons extended processes, by which they were connected with each other. PGP 9.5 positive nerve fibers were distributed in all intestinal layers. The richest part was in the circular muscular layer and parallel to the muscular fibers. In the proper mucous membrane, nerve network was formed around the intestinal glands. In the submucosal layer, the nerve fibers were arranged around the vessels. But in the stenosed segment of HD colon, no ganglionic cell was observed in the muscular and submucosal plexuses. At the distal end of the stenosed segment of the colon, no nerve plexus was found. Occasionally, a few neurons existed, but with abnormal morphology, while PGP9.5 positive nerve fibers were great in number in all intestinal layers. Big, strong, disordered and even twisted nerve tracts could be observed (Figure 9). Such disorderly arranged nerve fibers could also be found in the submucosal layer and proper mucous membrane layer of the colon. Some of the proliferated fibers were twisted along the vessels.

Table 1  The development of the colon and mensenteric innervation of trisomy 16 mice and their normal littermates in chronological sequence

|        | ED13 | ED14 | ED15 | ED16 | ED17 | ED18 |
|--------|------|------|------|------|------|------|
| N      | T    | T    | N    | T    | N    | T    |
| PM     | -    | -    | +    | -    | +    | -    |
| PS     | -    | -    | -    | -    | -    | +    |
| MF     | -    | +    | +    | +    | +++  | +++  |

N: normal littermates; T: trisomy 16 mice.
PM: myenteric plexus; PS: submucosal plexus; MF: mesentery nerve fiber
-: no neurons and immunoreactive nerve fibers detected;
+: a few scattering neurons and no enteric plexus or sparse immunoreactive fibers detected;
++: lowly developed enteric plexus or moderate immunoreactive fibers detected;
+++: moderately developed enteric plexus or abundant immunoreactive fibers detected;
++++: fully developed enteric plexus or very abundant immunoreactive fibers detected.

Figure 1  a. The chromosome of normal mice.  b. The chromosome of trisomy 16 mice: two Rb metacentric chromosomes (†) and 41 chromosome arms. × 1000

Figure 2  The survival rate of trisomy 16 mice with different embryonic days (ED).

Figure 3  a. Myenteric plexus of normal littermates at ED 15. The nerve meshworks had regular meshes with many neurons (†). × 400  b. The colon of trisomy 16 mice contained some neurons (arrow) with different staining and size at ED15. × 400
Figure 4 At ED 18, the myenteric plexuses of trisomy 16 mice and their normal littermates in whole mount preparation. × 250
A. The developed myenteric plexus with primary (long arrow), secondary (arrow) and tertiary (arrowhead) strands were displayed in the colon of normal littermates. The primary strands were composed of broad fiber bundles and numerous ganglia with longitudinally arranged meshes. B. The myenteric plexus of trisomy 16 mice was poor with less ganglia containing some neurons (arrow) at different staining.

Figure 5 At ED 18, the nerve fibers (long arrow) stretching from the enteric plexuses reached the gut villi (Vi). The myenteric (arrowhead) and submucosal (arrow) plexuses were clear. × 160

Figure 6 Aganglionic segment of trisomy 16 mouse colon. × 160 B. The colon of normal littermate with ganglia (arrow). × 100

Figure 7 The myenteric nervous fibers (arrow) were detected in the colon (C) mesentery of trisomy 16 mice. × 160

Figure 8 The submucosal ganglia (arrow) of normal colon of human. S: submucosal muscular layer. × 100

Figure 9 The aganglionic colon of Hirschsprung’s disease. Abundant proliferation of nerve fibers (large arrow) was found in the stenosed segment of colon. A wave-like nerve fiber (arrow). × 100
DISCUSSION

The incidence of HD is 1/5000 due to the developmental defect of enteric nervous system [20-23]. Recently, HD incidence is increasing, especially the genetic HD and Down’s syndrome with congenital megacolon [24-29], which greatly aroused researchers’ interest in investigating the causes. The present study was based on PGP 9.5 as primary antibody to reveal the innervation of the narrowed gut in HD and the nerve development of the colon in trisomy 16 mice.

Enough evidences have been obtained from this study that trisomy 16 mice had a significant anomaly and developmental delay in colon innervation as well as 5 mm aganglionic colon at the distal end, which was the result of the chromosome abnormality. Our present experiment, found that there was a chronological sequence in the nerve development and a clear link between the gestational age and the morphological development in colon innervation of trisomy 16 mice and their normal littermates (Table 1). At ED 14, the precursor cells of ectoderm migrated into the gut along the course of the vagus nerves; at ED 15, nerve processes were observed in the neuron migrated gut; at ED 16, the muscular plexuses and ganglions appeared in the gut; at ED 17, the precursor cells in muscular layer migrated into the submucosal layer and developed into submucosal nervous plexuses; at ED 18, the relatively matured muscular and submucosal plexuses occurred, the former controls the intestinal peristalsis, and the latter manages the intestinal secretions and absorption [30, 31]. The lack of submucosal nerve plexuses of trisomy 16 mice accounted for the lower material absorption and poor intestinal secretions while the distal aganglionic colon strongly interfered with normal migration of precursor cells [47-51]. Payette and associates thought that the excessive accumulation of components of basal lamina material and muscularis mucosa interfered with normal migration of precursor cells [42-43].

The excessive accumulation of basal lamina material and muscularis mucosa interfered with normal migration of precursor cells [42-43]. Payette and associates [52] thought that the excessive accumulation of components of basal lamina material in the aganglionic region of the lethal spotted mutant mouse stopped precursor cells from migrating into the terminal bowel of lethal spotted mutant mice. Our study revealed that 5mm aganglionic bowel in length aparting from anus in trisomy 16 mice, might be caused by the change of regional micro-circumstance due to the abnormal chromosome, finally resulting in the nerve defect.

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