Increased smooth muscle contractility of intestine in the genetic null of the endothelin ETB receptor: a rat model for long segment Hirschsprung’s disease

K-J Won, S Torihashi, M Mitsui-Saito, M Hori, K Sato, T Suzuki, H Ozaki, H Karaki

Background and aims: The endothelin ETB receptor null rat (ETB(−/−)R) has an intestinal segment without ganglia, and this rat is characterised by intestinal obstruction similar to that observed in human Hirschsprung’s disease. In the present study, we have examined the myogenic mechanism responsible for obstruction in the ETB(−/−)R.

Results: The ETB(−/−)R had an enlarged belly and the average lifespan was 18.1 days. The bowel from the rectum to the lower part of the small ileum was constricted whereas the upper region was dilated with faecal stasis and thus presented as megaileum. The constricted muscle segments without ganglia had a greater increase in absolute force when stimulated by carbachol, high K+ and endothelin-1 compared with that of normal siblings. In contrast, in the dilated part with ganglia, the absolute contractile force due to these stimulants in the ETB(+/−)R was not different from that in the ETB(+/+)R. Such a functional hypertrophy of the musculature was observed in parts of the colon, caecum, and distal ileum without ganglia but not in the part of the proximal ileum and jejunum with ganglia. Morphological study demonstrated that the thickness of the circular and longitudinal muscle layers was greater in the constricted part of the intestine in the ETB(−/−)R, and these changes were associated with an increase in the number of smooth muscle cells.

Conclusions: Our findings suggest that both increased contractility of smooth muscle and increased thickness of the intestinal muscular wall may contribute to the intestinal obstruction in the ETB(−/−)R.

M motility disorders of the gastrointestinal tract are extremely important in the clinical field because they can lead to systemic diseases. Obstructive intestinal disorders caused by abnormalities of the enteric nerves are observed as an enlargement of the intestine or chronic constipation. It is known that Hirschsprung’s disease, which is usually detected during the neonatal period, is the most common developmental obstructive motility disorder of the colon, and the incidence of Hirschsprung’s disease is approximately 1 in 5000 live births. Thus, this disease is regarded as an important congenital obstructive disorder in paediatrics. Studies undertaken to further our understanding of the pathogenesis of Hirschsprung’s disease have focused on the development of the enteric nervous system (see discussion below).

Such pathological features of the inherited aganglionosis megacolon, present in Hirschsprung’s disease, are also commonly found in livestock, including horses and pigs, and in experimental animals, including rats and mice. A mutant spotted lethal rat with a congenital aganglionosis bowel (congenital aganglionosis rat) has been described as progeny of a Wistar-Imamichi female and a wild male rat. The aganglionosis rat has been found to be genetically lacking the endothelin ETB receptor. This rat is characterised by an aganglionosis intestine and white coat colour, with a small pigmented spot on the head caused by an autosomal recessive gene (sl). And in most cases showing symptoms of constriction in a longer region of the intestine. Heterozygotic rats (sl+/−), however, as well as those homozygous for the dominant gene (+/+) appear to be identical both histologically and anatomically. The ETB(−/−)R, pathophysiologically and histologically, resembles humans showing severe symptoms of long segment Hirschsprung’s disease, which led to its selection as an animal model of intestinal obstructive disease (including human Hirschsprung’s disease) in this study.

The pathophysiology of Hirschsprung’s disease is obscure even though several morphological and functional abnormalities have been identified. In particular, smooth muscle alterations have yet to be clarified. The experiments carried out in the present study were designed to functionally and morphologically examine the myogenic diversity of the ETB(−/−)R and to better characterise an animal model for human long segment Hirschsprung’s disease.

MATERIALS AND METHODS

Muscle preparations and measurement of contraction

At approximately 12–15 days of age, rats were killed by a sharp blow to the neck and exsanguination. The whole or some part of the intestine was dissected out and rinsed in physiological salt solution containing (mM): NaCl 136.9, KCl 5.4, CaCl, 1.5, MgCl, 1.0, NaHCO3, 23.8, EDTA 0.01, and glucose 5.5. This solution was saturated with a 95% O2 and 5% CO2 mixture at 37°C, pH 7.4. For measurement of contractility, the mucosa was removed from the intestinal segments. Segments were then cut into rectangular strips oriented towards the longitudinal axis of the circular muscle cell layer. The length of the strips was equal to the circumference of the intestine, and width was 2–3 mm. Muscle contraction was measured isometrically under a resting tension of 5 mN. At the end of the experiments the muscle strips were blotted with filter paper and weighed on an analytical balance. The contractile responses to the agonists are expressed as absolute force (mN per mg tissue wet weight).

Abbreviations: ETB (−/−)R, endothelin ETB receptor null rat; RT-PCR, reverse transcription-polymerase chain reaction; EFS, electrical field stimulation.
Histological examination
Segments of the intestine isolated from the ETB(+/+)R and ETB(−/−)R were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), post fixed with 1% OsO4, dehydrated through graded ethanol, and embedded in Epon 812. Semi-thin sections were stained with 0.1% toluidine blue in 0.1 M phosphate buffer.

For immunohistochemistry, whole mount preparations of the enteric nerve elements, terminal ileum, and colon from the ETB(+/+)R and ETB(−/−)R were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The muscle layers were separated and washed in phosphate buffered saline (pH 7.5). Samples were incubated with anti-PGP9.5 polyclonal antibody (Ultraclone, UK; 1:200). Immunohistochemistry was accomplished with an avidin-biotin complex system (Vectastaine ABC kit; Vector), and the reaction products were developed by 0.03% diaminobenzidine and 0.005% H2O2 in 0.05 M Tris HCl buffer (pH 7.6).

Semiquantitative RT-PCR
The oligonucleotide primers for the ETA receptor were designed based on data from the cDNA database (NM 012550) by selecting specific amino acid sequences from the cDNA database for the ETB receptor (X 57764). The forward and reverse primers for the ETA receptor were designed as 5′-ATG AGA TTA AGA ACC-3′, and 5′-CCA TTC ATG GGG ACC CAG G-3′, respectively. The suitable size of the reverse transcription-polymerase chain reaction (RT-PCR) product for the ETA receptor is 205 bp. The oligonucleotide primers for the ETB receptor were designed considering the deletion site of ETB(−/−)R, as described previously. The forward and reverse primers for the ETB receptor were designed as 5′-GGG TCG ACG CCA CCC ACT AAG ACC TCC-3′ and 5′-CAT CAA AAC CTA TGG CTT CAG GGA CAG-3′, respectively. The suitable size of the synthesised cDNA for ETB receptor is 586 bp. In the ETB(−/−)R however a suitable product cannot be obtained because of the deleted mutation corresponding to the first and second transmembrane domains of the ETB receptor.

The oligonucleotide primers for GAPDH were 5′-TCC TCT AAG ATT GTC AGC AA-3′ (forward primer) and 5′-AGA TCC ACA ACG GAT ACA TT-3′ (reverse primer), and the theoretical size of the RT-PCR product for GAPDH is 308 bp. The PCR products obtained from 35–50 cycles (five cycle interval) were electrophoresed onto 2% agarose gel containing 0.1% ethidium bromide and were detected using a UV transilluminator. The densitometric intensity at 45 cycles was quantified by NIH imaging. The quantitative results are expressed as the ratio of the densitometric intensity of GAPDH.

Statistics
The results of the experiments are expressed as mean (SEM). The unpaired Student’s t test was used for statistical analysis of the results; p values less than 0.05 were considered to be significant.

RESULTS
General biological features of ETB(−/−)R
The ETB(−/−)R had a unique appearance, with their almost white coat (caused by a reduced number of pigment cells in the coat) and abdominal distension. The ETB(+/+)R had a black coat limited to the head and part of the body. The physique of the ETB(−/−)R was poor with severe emaciation and growth retardation. The ETB(+/+)R hair was lustreless and rough. The death rate of the ETB(−/−)R was significantly greater than that of the ETB(+/+)R. The shortest life span of the ETB(−/−)R in the present study was three days and the longest was 34 days (mean 18.1 days).

Figure 1A shows the abdominal dissection and digestive tract from the stomach to the anus. The ETB(+/+)R had intestinal dilatation in the same location with abdominal distention. The ETB(+/+)R had a black coat limited to the head and part of the body. The physique of the ETB(−/−)R was poor with severe emaciation and growth retardation. The ETB(+/+)R hair was lustreless and rough. The death rate of the ETB(−/−)R was significantly greater than that of the ETB(+/+)R. The shortest life span of the ETB(−/−)R in the present study was three days and the longest was 34 days (mean 18.1 days).

Figure 1B shows the structure of the myenteric plexus in the colon and ileum of the ETB(+/+)R and ETB(−/−)R. Although there were some individual differences, the myenteric plexus in the colon and ileum of the ETB(+/+)R showed a similar shape and size of ganglia with a regular meshwork pattern (fig 1B). In the dilated (proximal) portion...
of the ETB(−/−) ileum however the myenteric plexus appeared to be decreased in density and stretched in the shape of meshwork compared with that in the ETB(+/+) ileum (fig 1B). In the constricted (distal) part of the ETB(−/−) ileum, the myenteric ganglia were completely absent, and only some extrinsic nerve fibres or bundles were observed (fig 1B).

**Contractionss**

Carbachol (0.001–100 µM), a muscarinic receptor agonist, produced concentration dependent contractions both in the constricted (distal) and dilated (proximal) portions of ileum from the ETB(+/+) and ETB(−/−)rat (ETB(+/+)R) compared with the homozygous wild type ETB(+/+)R ileum (fig 2). The contractions generated by carbachol in the constricted (distal) part of the ETB(−/−) ileum (100 µM carbachol: 5.88 (1.39) mN/mg ww; n=8) were significantly greater than those of the corresponding part of the ETB(+/+) ileum (1.99 (0.39) mN/mg ww; n = 8) (fig 2B). However, the carbachol responses induced by carbachol in the dilated (proximal) parts of the ETB(−/−) ileum (2.60 (0.64) mN/mg ww; n=8) were almost the same as those of the corresponding part of the ETB(+/+) ileum (2.25 (0.19) mN/mg ww; n=8) (fig 2A). The constricted (distal) ileum in the ETB(−/−)R showed greater contractility than the dilated (proximal) ileum.

Addition of KCl (5.4–72.7 mM) produced concentration dependent contractions in both the constricted (distal) and dilated (proximal) ileum from the ETB(+/+)R and ETB(−/−)R ileum (fig 3). The amplitude of the contractions generated by carbachol in the muscle strips of the constricted regions (colon, cecum, and distal ileum) from the ETB(−/−)R were significantly greater than those of the corresponding parts of the ETB(+/+)R ileum (1.75 (0.30) mN/mg ww; n=8) (fig 3B). However, the carbachol responses induced by KCl in the dilated (proximal) parts of the ileum in the ETB(−/−) ileum (1.86 (0.41) mN/mg ww; n=8) were almost the same as those of corresponding parts of the ETB(+/+) ileum (1.56 (0.22) mN/mg ww; n=8) (fig 3A). The contraction generated by KCl in the constricted (distal) part of the ETB(−/−) ileum was greater than that of the dilated (proximal) part.

Addition of endothelin-1 (0.1–3×100 nM), a non-selective agonist of endothelin ETA and ETB receptors, produced concentration dependent contractions in both the constricted (distal) and dilated (proximal) ileum from the ETB(+/+)R and ETB(−/−)R ileum (ETB(+/+)R) (fig 4). The amplitude of contractions generated by endothelin-1 of the corresponding parts of the ETB(+/+) ileum (1.21 (0.31) mN/mg ww; n=10) were also significantly higher than those of the corresponding parts of the ETB(+/+) ileum (1.11 (0.27) mN/mg ww; n=10) (fig 4B). In addition, the endothelin responses induced by endothelin-1 at higher concentrations (10–100 nM) in the dilated (proximal) parts of the ileum in the ETB(−/−) ileum (1.28 (0.75) mN/mg ww; n=9) were also significantly higher than those of the corresponding parts of the ETB(+/+) ileum (1.21 (0.31) mN/mg ww; n=10) (fig 4A). The constricted (distal) ileum of the ETB(+/+)R showed greater contractility than the dilated (proximal) ileum.

Contractionss induced by 10 µM carbachol in the muscle strips of the constricted regions (colon, cecum, and distal ileum) from the ETB(−/−)R were significantly greater than those of the corresponding parts of the ETB(+/+)R (8.04
non-competitive agonist of P2X and P2Y receptors, was unable to inhibit the contractility of the intestine in ETB(−/−)R. Possible involvement of inhibitory substances could inhibit the contractility of the intestine in ETB(+/+)R. RT-PCR analysis of RNA extracted from the muscle tissue of ETB(+/+)R and ETB(−/−)R was carried out. As shown in fig 6, expression of the RT-PCR product encoding the housekeeping gene GAPDH (308 bp) was identical in controls and ETB(−/−)R.

ETB receptor (586 bp) mRNAs were strongly expressed in the normal ETB(+/+)R. It has been reported that a 301 bp region intervening between direct repeat sequences was deleted in the ETB(−/−)R, and that the deletion produces various transcripts due to aberrant splicing. Consistent with this observation, we observed two bands in an area approximately 300 bp shorter than in normal rats. In contrast, ETA receptor (205 bp) mRNAs were expressed both in ETB(+/+)R and ETB(−/−)R, with no significant difference between the two. There was also no significant difference in ETA receptor mRNA between the contracted (distal) and dilated (proximal) ileum from the ETB(+/+)R compared with the homozygous wild type ETB(+/+)R. Contractile responses are expressed in mN per mg tissue wet weight (mN/mg ww). Each point represents the mean (SEM) of 9–11 experiments. Significantly different from the ETB(+/+)R: *p<0.05, **p<0.01.

Histology

A histological profile of thickness of the smooth muscle layer can be obtained by determining the number of piling smooth muscle cells of each muscle layer of the ETB(+/+)R and ETB(−/−)R. In general, both the longitudinal and circular smooth muscle layers were thicker in the ETB(−/−)R than in the ETB(+/+)R although the diameter of each smooth muscle cell around the nuclear region was not different, as shown in fig 7A.

To obtain more numerical data on the thickness of the smooth muscle layer, the smooth muscle cells in the circular or longitudinal muscle layers were counted in the ETB(+/+)R and ETB(−/−)R on the longitudinal or circular section, respectively. As shown in fig 7B, the number of cells piled in the longitudinal smooth muscle layers of both the caecum and colon of the ETB(−/−)R was significantly greater than that in the ETB(+/+)R although the diameter of each smooth muscle cell around the nuclear region was not different, as shown in fig 7A.

DISCUSSION

In Hirschsprung’s disease obstruction of the gut creates a distended oral gut followed by a constricted anal portion similar to a megacolon. The distended oral side is a ganglionic segment where the number of enteric neurones is gradually reduced and continues to the constricted aganglionic segment. This pattern was confirmed in the ETB(+/+)R. The constricted gut of the ETB(−/−)R was long and extended to the terminal ileum, resulting in megaileum (fig 1). Due to these severe gut conditions, the average lifespan was only 18.1 days.
This animal model therefore corresponds to long segment Hirschsprung's disease in humans.

Constriction of the aganglionosis segment has been explained as being characterised by a lack of an inhibitory nerve supply such as nitric oxide neurones and proliferation of extrinsic nerve fibres—that is, cholinergic neurones. Predominance of a contractile nerve supply and a scant number of inhibitory neurones seems to cause the characteristic constriction. However, the distribution of nerve terminals was significantly more sparse in the constricted segment than in the normal segment, suggesting poor innervation of the muscle layer in the constricted portion. Therefore, the neurogenic obstruction described above does not fully explain the abnormal constriction and pathophysiology of the disease. These results strongly suggest that a myogenic mechanism is involved in the constricted gut.

In the present study, we observed that the absolute contractile force in response to carbachol in the distal ileum of the ETB(−/−)R without ganglia (constricted) was larger than that of the ETB(+/+)R. In contrast, the proximal ileum of the ETB(−/−)R intestine with ganglia (dilated) showed an amplitude of force similar to that of the ETB(+/+)R. In addition, the constricted distal ileum had greater contractility than the dilated proximal ileum in the ETB(−/−)R. We also compared contractility to carbachol in the entire intestinal tract and found that the lower part of the intestines with no ganglia (distal ileum, caecum, and colon) induced greater contraction than the upper part of the intestine with ganglia (proximal ileum and jejunum). These results suggest that the increased contractility observed in the ETB(−/−)R intestine is closely associated with the absence of ganglia in the intestine.

Augmented contractility was demonstrated in the present study when the muscle was treated with a non-cholinergic agonist, endothelin-1, or with high K+ simply increasing the intracellular Ca2+ concentrations. These results suggest that the change in contractility is attributable to changes other than just membrane associated reactions. There are several possibilities for the increased contractility of the intestine in the ETB(−/−)R. One is that the absence of propulsive motor activity, mediated by ganglial dysfunction, upregulates the contractility of smooth muscle cells. It is also probable that inflammatory reactions may affect the contractility of smooth muscle.
muscle cells in the ETB(+/−)-R, as enterocolitis is frequently observed in this model animal.

In our study, Hillemeier and Biancani have reported that active force generated in vitro in the colonic smooth muscle in the mutant Hirschsprung mouse model is greater than that in wild type mice. However, Siegman and colleagues have failed to confirm these phenomena in which there were no differences in the force per cross sectional area of permeabilised muscles maximally activated in high concentrations of Ca²⁺. These findings suggest that the functional hypertrophy observed in the Hirschsprung mouse model is attributable to mechanisms other than activation of contractile elements, which is inconsistent with our findings in rats. Siegman and colleagues have further reported that there is an increase in the shortening velocity in the colon of obstructed mice, an increase that is associated with changes in the contractile elements such as isoforms of myosin and actin.

In our study of smooth muscle histoology, we found that the thickness of the smooth muscle layer in the portion of intestine with no ganglia in the ETB(+/−)-R was greater than that of corresponding parts in the ETB(+/+)R. Furthermore, the number of smooth muscle cells comprising both the circular and longitudinal muscle layers increased in the ETB(+/−)-R. These results were different from the observation in the Hirschsprung mouse model that the thickness of the circular smooth muscle layer in constricted intestine is the same as that in obstructed and control mice. This difference is probably related to the length of the aganglionic segment; the Hirschsprung mouse model has short constricted segments (only a terminal colon) that lack enteric ganglia.

The question is then raised as to how the muscle might be exposed to the agonists that were utilised to demonstrate hyperactivity of the gut in the aganglionic preparations. It has been reported that in normal mice, electrical field stimulation (EFS) induces an initial decrease followed by an increase in basal exposure whereas EFS induces only contraction in the mutant Hirschsprung mouse model, and that there is no significant difference in the amplitude of contractions in these preparations. An excitatory response to EFS has also been demonstrated in the aganglionic segment of intestine in ETB(+/−)-R. These contractile responses to EFS appear to be cholinergic as they are blocked by atropine. Similar atropine sensitive contractions have been demonstrated in Hirschsprung’s patients. Because extrinsic nerve innervation is intact in the aganglionic intestine, acetylcholine may be released and thus related to maintenance of an elevated muscle tone.

Both ETA and ETB receptors are responsible for activation of gastrointestinal motility. Although the contractility of the dilated (proximal) ileum to carbacol or high KCl does not differ between ETB(+/+/+)R and ETB(+/−)-R, contraction due to endothelin-1 in the ETB(+/−)-R was significantly greater than that in ETB(+/+)R, implying that the ETA receptor is upregulated in the ETB(+/−)-R. In the present study, we analysed the level of ETA receptor mRNA by RT-PCR. However, we observed no change in the expression of mRNA levels. These results suggest that the downstream signal-transduction pathway after ETA receptor stimulation may be upregulated by a deficiency of the ETB receptor.

It has been considered that the absence of periostalsis in response to deficiencies in the number of ganglion cells may cause the intestinal obstruction found in Hirschsprung’s disease. Our findings suggest that as an additional mechanism, increased contractility of smooth muscle and increased thickness of the intestinal muscular wall may contribute to the constriction of intestine in Hirschsprung’s disease. Thus the absence of periostalsis and the morphological and functional hypertrophy in the intestine may promote the intestinal obstruction in Hirschsprung’s disease. The present study also indicates that the results obtained in mutant rats (the short segment model) are different in terms of some of the abovementioned functional and histological features from those obtained in mutant mice (short segment model).

ACKNOWLEDGEMENTS
This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, and Science, Japan, Human Science Foundation, Yaktol Bio-Science Foundation, Takeda Science Foundation, and a Program for Promotion of Basic Research Activities for Innovative Biosciences (BRAIN).

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Gut 2002 50: 355-360
doi: 10.1136/gut.50.3.355

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