BRIEF ARTICLE

Differential changes in intrinsic innervation and interstitial cells of Cajal in small bowel atresia in newborns

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Abstract

AIM: To investigate morphological changes of the enteric nervous system (ENS) and the interstitial cells of Cajal (ICCs) in small bowel atresia.

METHODS: Resected small bowel specimens from affected patients (*n* = 7) were divided into three parts (proximal, atretic, distal). Standard histology and enzyme immunohistochemistry anti-S100, anti-protein gene product (PGP) 9.5, anti-neurofilament (NF), anti-c-kit-receptor (CD117) was carried out on conventional paraffin sections of the proximal and distal part.

RESULTS: The neuronal and glial markers (PGP 9.5, NF, S-100) were expressed in hypertrophied ganglia and nerve fibres within the myenteric and submucosal plexuses. Furthermore, the submucous plexus contained typical giant ganglia. The innervation pattern of the proximal bowel resembled intestinal neuronal dysplasia. The density of myenteric ICCs was clearly reduced in the proximal bowel, whereas a moderate number of muscular ICCs were found. The anti-CD117 immunoreaction revealed additional numerous mast cells. The distal bowel demonstrated normal morphology and density of the ENS, the ICCs and the mast cells.

CONCLUSION: The proximal and distal bowel in small bowel atresia revealed clear changes in morphology and density of the ENS and ICCs.

INTRODUCTION

Small bowel atresia is a congenital disorder that carries a substantial morbidity[1,2]. The etiology of bowel atresia remains unclear. One of two accepted theories of its pathogenesis is the concept of a lack of recanalization of the solid organ cord during the late stage of intestinal development[3]. Another concept is the occurrence of a late intrauterine mesenteric vascular accident[4]. The lack of revacuolization is the probable cause for most cases of duodenal atresia. Further studies have demonstrated that

jejunoileal atresias occur as a result of intestinal volvulus, intussusception, internal hernia, or strangulation in a tight gastroschisis or omphalocele defect[5-11].

Newborns with small bowel atresia are operated on soon after birth. Owing to the severity of the dilatation of the proximal bowel and the hypoplasia of the distal bowel, various postoperative gastrointestinal motility problems may occur; such problems include prolonged adynamic ileus (11%) and the need for total parenteral nutrition (30%-70%)[12]. The underlying cause of this postoperative intestinal motility disorder is still unclear.

Basically, normal gastrointestinal motility depends on the coordinated function of the enteric nervous system (ENS), the intestinal smooth muscle and the interstitial cells of Cajal (ICCs). Previous studies revealed histological changes within the wall of the atretic and adjacent bowel in small bowel atresia. Hypertrophy of the bowel muscle proximal to the atresia was found in clinical and experimental studies on small bowel atresia. Various changes have been reported within the ENS in small bowel atresia[13]. Nevertheless, the relationship between the macroscopic and histological changes of the affected bowel and the postoperative motility disorder are still under investigation. Furthermore, the role of the ICCs in small bowel atresia needs to be elucidated further.

ICCs play a major role in gastrointestinal motility. ICCs express the tyrosine kinase receptor c-kit. Therefore specifically designed c-kit antibodies have been developed which stain ICCs but also other cell groups such as stem cells and mast cells. However, c-kit positive ICCs can be identified by clear morphological features.

The aim of this study was to investigate the morphology of the ENS and the ICCs in resection specimens of small bowel atresia.

MATERIALS AND METHODS

Patients and tissues
Resected small bowel specimens (ileum) from affected newborn patients ($n = 7$) were included in the investigation after parental consent. The resected ileal specimens were divided into three parts (proximal, atretic, distal).

Tissue processing
The specimens were fixed in 4% paraformaldehyde and processed into paraffin blocks. Paraffin-embedded tissues were sectioned at 2-4 μm (Leica SM 2000 R) followed by drying at 37℃ in an incubator overnight. Before immunohistochemical staining, the paraffin sections were dewaxed for 10 min in xylene, followed by 10 min in acetone and 10 min in acetone/Tris-buffered saline (TBS; 1:1). After this treatment, the slides were washed in TBS.

Antigen demasking
If heat antigen retrieval was required, dewaxed paraffin sections were placed in microwave-proof tubes containing target retrieval solution (Dako). The slides were treated in the tubes for 5 min at 600 W in a microwave (SS 566H;

Table 1 Primary antibodies

Antibody	Clone	Company	Dilution
S-100	Polyclonal (Z0311)	Dako, Glostrup, Denmark	1:500
PGP-9.5	Polyclonal (Z5116)	Dako, Glostrup, Denmark	1:25
Neurofilament	2F11 (M0762)	Dako, Glostrup, Denmark	1:50
c-kit	Polyclonal (A4502)	Dako, Glostrup, Denmark	1:50

PGP: Protein gene product.

Bosch, Munich, Germany). The evaporated volume was replaced by distilled water, and the procedure was repeated twice. After microwave treatment, the slides were left to cool down, then were washed in TBS.

Histochemistry and immunohistochemistry
Standard histology [hematoxylin/eosin (HE)] was performed on the sections. For immunohistochemistry, an alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining kit (Dako Real™ Detection System, APAAP, Mouse) using anti-S100, anti- protein gene product (PGP) 9.5, anti-neurofilament (NF) and anti-c-kit-receptor (CD117) antibodies was employed. A non-sense mAb (clone: MR 12/53) served as negative control by omitting the primary antibody. The final concentrations are given in Table 1.

Evaluation
The evaluation of the immunohistochemical staining results was focused on the proximal and distal parts of the resected ileum.

The sections were evaluated by two independent investigators using light microscopy (magnification, × 40). HE staining was used to determine the overall histology of the investigated specimen. The distribution and density of immunoreactive ganglion cells, glial cells, nerve fibers and c-kit positive cells (ICCs, mast cells) were studied in each part of the resected bowel (proximal, atretic, distal). Since quantitative analysis of immunohistochemical staining is not possible, semi-quantitative scoring was performed as follows; - no expression (no staining), + low expression (few neuronal fibers and cells/few ICCs), ++ moderate expression (numerous neuronal fibers and cells/numerous ICCs), +++ high expression (dense networks of neuronal fibers and cells/dense network of ICCs).

RESULTS

Patients
The study included resected ileal segments from seven newborn patients (gestational age 38-40 wk). Six patients presented with type Ⅲa ileal atresia and one patient presented with multiple ileal atresia.

Neuronal markers (PGP 9.5, NF)
The neuronal markers PGP 9.5 and NF were strongly expressed within the ganglion cells and nerve fibers of the myenteric plexus (and submucosal plexus in the proxi-
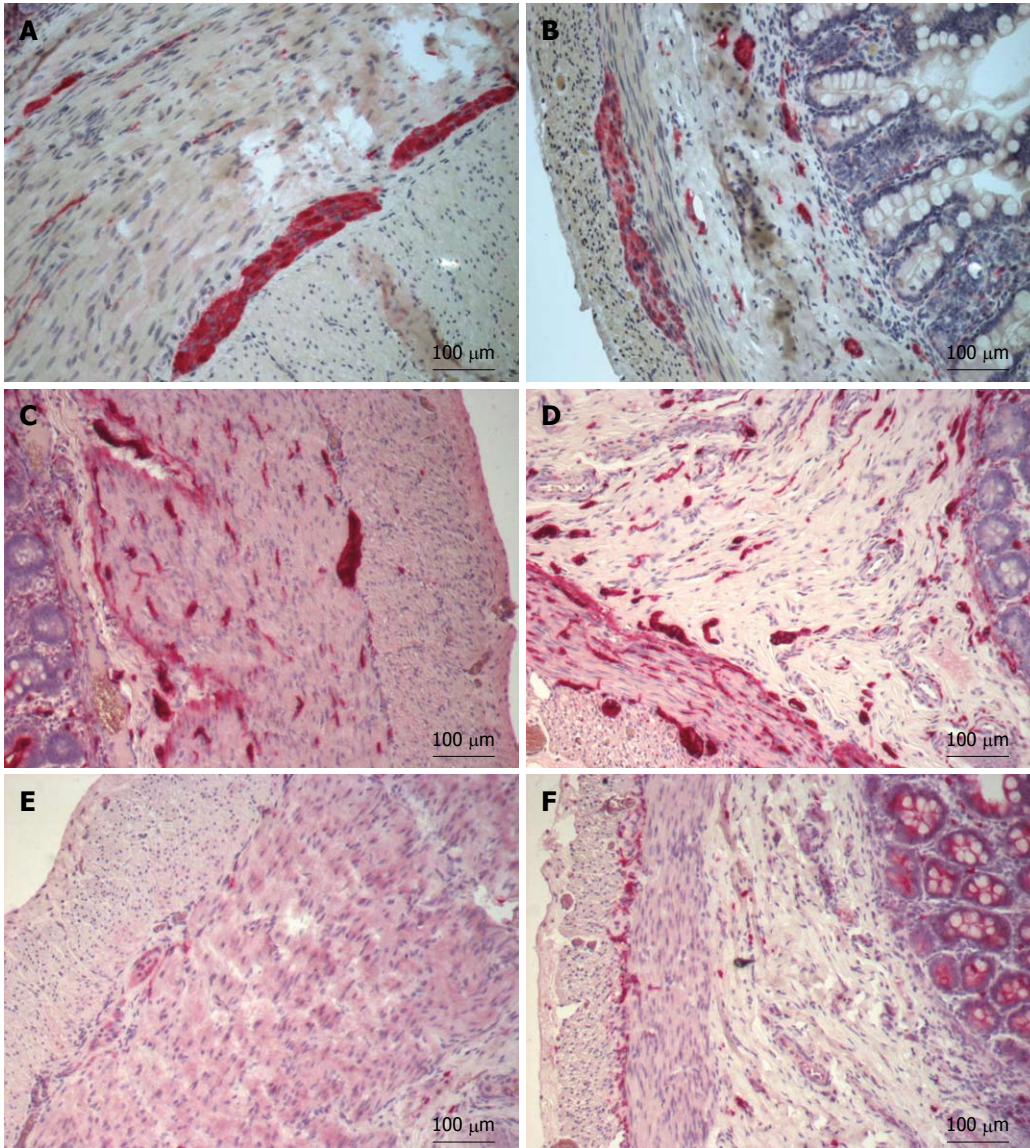
Figure 1 Immunohistochemistry of a full-thickness section of the ileum. A: Protein gene product (PGP) 9.5 in the ileum proximal to the atresia shows a dense innervation within the submucosal and myenteric plexus; B: PGP 9.5 in the ileum distal to the atresia shows a normal innervation pattern of the submucosal and myenteric plexuses; C: S-100 in the ileum proximal to the atresia shows numerous fibers and large ganglia (> 20 μm) within the submucosal plexus and normal innervation within the myenteric plexus; D: S-100 in the ileum distal to the atresia shows a normal innervation pattern within the submucosal and myenteric plexuses; E: c-kit in the ileum proximal to the atresia shows that myenteric and muscular interstitial cells of Cajal (ICCs) are almost absent; F: c-kit in the ileum distal to the atresia shows normal distribution of myenteric and muscular ICCs.

Table 2 Distribution and density of immunostaining (proximal *vs* distal)

	Proximal				Distal			
	SP	CM	MP	LM	SP	CM	MP	LM
PGP 9.5	++	+	++	+	++	+	++	+
S-100	+++	+	+++	+	++	+	++	+
NF	++	+	++	+	++	+	++	+
c-Kit (ICCs)	-	-/+	+/-	-	-	+	++	+

PGP: Protein gene product; ICCs: Interstitial cells of Cajal; SP: Submucosal plexus; CM: Circular muscle; MP: Myenteric plexus; LM: Longitudinal muscle; NF: Neurofilament; -: No expression (no staining); +: Low expression (few neuronal fibers and cells/few ICCs); ++: Moderate expression (numerous neuronal fibers and cells/numerous ICCs); +++: High expression (dense networks of neuronal fibers and cells/dense ICCs networks).

mal ileum (Figure 1A). The submucosal plexus contained some giant ganglia (*n* > 10 ganglion cells) and numerous thick nerve fibers. The overall numbers of ganglia were not increased within the myenteric and submucosal plexuses (Table 2). The immunohistochemical staining revealed features of intestinal neuronal dysplasia (giant ganglia, hypertrophied nerve fibers).

The distal bowel had a normally expressed ENS within the submucosal and myenteric plexuses (Figure 1B).

Glial marker (S-100)
Numerous S-100 immunoreactive glial cells were found in the two plexuses of the proximal ileum (Figure 1C). The expression of glial cells was increased within the proximal dilated bowel compared to the atretic and distal bowel

(Table 2). Additional giant ganglia (> 10 ganglion cells/ganglion) were stained by S-100 within the submucosal plexus of the proximal bowel. Regular distribution and morphology of S-100 immunoreactive cells and fibers was found within the myenteric and submucosal plexuses of the distal ileum (Figure 1D).

c-kit (CD-117) staining (ICCs, mast cells)

The density of ICCs within the myenteric plexus was clearly reduced in the proximal bowel (Figure 1E), whereas some ICCs were found in the circular and longitudinal muscle layer (Table 2). The anti-CD117 immunoreaction revealed numerous mast cells within the proximal bowel. The distal bowel had a normal distribution of ICCs within the myenteric plexus and muscular layers and a normal number of mast cells (Figure 1F).

DISCUSSION

This study revealed distinct changes in the morphology of the ENS and ICCs in parts of the resected bowel proximal to small bowel atresia. In general, our findings are not unique since previous studies described changes within the ENS in small bowel atresia. Ozguner *et al*[14] reported that the proximal segment of the atretic intestine showed structural deficits. Abnormal ganglia cells and defects in the intestinal musculature were prominent, but the intestinal mucosa remained intact. They found abnormalities in both the antimesenteric side and mesenteric side. Their interpretation supported a vascular accident as a causative factor.

Di Nardo *et al*[15] presented an interesting case report of small bowel atresia that was initially diagnosed as hyper-ganglionosis and later changed to an enteric degenerative neuropathy. Watanabe *et al*[16] contributed an elegant study investigating the myenteric plexus in small bowel atresia using whole-mount preparations. This study showed mild hypoplasia of the ENS plexuses in the proximal segments[16].

In our study the expression of nerve fibers and glial cells appeared to be slightly increased, and some submucosal giant ganglia were found, which resembled intestinal neuronal dysplasia within the proximal dilated bowel. The expression of c-kit positive ICCs was clearly decreased in the dilated proximal bowel. The reduction of ICCs within the proximal dilated bowel has also been described before. In contrast to our study, some previous investigators have shown a reduction of the innervation density within the proximal bowel segments[13]. The innervation pattern and the expression of ICCs within the distal small bowel were normal in our study, similar to comparable investigations[13].

The crucial problem of investigating the developing ENS or pathological changes within the ENS in order to define hyper-ganglionosis or hypoganglionosis is the uncertainty in the normal numbers and distribution of enteric ganglia. The normal neuron density of the human myenteric plexus was investigated in several studies and resulted in a huge variation of more than 200-fold[17-20].

Schuffler *et al*[17] found 27.8 nerve cells per 100 mm of smooth muscle, Ikeda *et al*[19] described more than 30 nerve cells per 10 mm of smooth muscle as normal, whereas Smith[18] estimated 7 nerve cells per mm of smooth muscle. Finally Meier-Ruge *et al*[20] found 756 ganglion cells per 10 mm of smooth muscle. All these investigations were performed on conventional paraffin sections[17-20], which usually display only a two-dimensional image of the investigated structures. Since these profound differences have already been found within normal bowel specimens, conflicting results on numerical changes of ganglia and nerve fibers within the changed ENS in bowel atresia must be expected. Another major aspect is the widely accepted concept of ongoing postnatal plasticity of the ENS, which has to be taken into consideration.

A previous animal study which created a partial obstruction in dog ileum revealed that the ganglion cells increased in size and the smooth muscle of the dilated bowel became thicker[21]. The number of ganglion cells related to the muscle mass was decreased above the constriction. Although some ganglion cells increased in size, there was no evidence of any increase in number. Experimentally, a similar increase in ganglion size was observed proximal to a stenosis in another study[22]. In a chicken embryonic model of bowel obstruction, a marked reduction of the submucous plexus ganglia with intact muscular plexus ganglia was noted in the dilatated gut[23].

Our study also showed an increase in size of ganglia (hypertrophy). These hypertrophic myenteric and submucosal ganglia in the proximal resected bowel could be considered the result of adaptive phenomena to the long-lasting prenatal bowel obstruction. Several experimental models have been instrumental in supporting the concept that adaptive changes to the ENS occur proximal to the obstructed gut[24-31]. These studies revealed an increased density and size in both myenteric[26,28,29,31] and submucosal neurons[29-31], along with neurochemical[28,30] and cytoskeletal abnormalities of the myenteric neurons[31].

Ekblad *et al*[28] showed that, in the hypertrophic ileum, several neuronal populations showed changes in the expression of neuro-messengers. Myenteric neurons expressing vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide, and galanin were notably increased in number. In submucosal ganglia, the number of VIP-immunoreactive neurons decreased, while those expressing VIP mRNA increased. NADPH diaphorase-positive submucosal neurons increased dramatically, while the number of neuronal-type nitric oxide synthase expressing neurons was unchanged. The number of ICCs decreased markedly in the hypertrophic ileum.

Another observation is the delayed maturation of ENS within the distal bowel during fetal development in cases with small bowel atresia[32]. Distortion of the polygonal architecture of the myenteric plexus has been shown postnatally in atretic parts of ileal type IIIa atresia[33]. It could only be speculated that after the restoration of bowel continuity does maturation of the ENS occur.

A previous animal study using a partial small obstruc-

tion model in mice revealed that 2 wk following the onset of a partial obstruction, the bowel increased in diameter, and hypertrophy of the tunica muscularis was observed oral to the obstruction site[27]. Networks of ICCs were disrupted oral to the obstruction, and this disruption was accompanied by the loss of electrical slow waves and responses to enteric nerve stimulation. These defects were not observed aboral to the obstruction. Furthermore, it was shown that removal of the obstruction led to the redevelopment of ICC networks and the recovery of slow wave activity within 30 d. Neural responses were partially restored in 30 d[27]. Similar repair mechanisms may occur after surgical correction of small bowel atresia. It seems obvious that decreases in ICCs in small bowel atresia and their restoration after removal of the obstruction contributes to the regulation of gastrointestinal motility.

Masumoto *et al*[34] showed muscular alterations in both segments of IA cases, which further contributes to the postoperative motility disorder. Another case report recently revealed long lasting chronological changes within the ENS, muscle components and ICCs in small bowel atresia[35].

The ENS and ICCs are altered in the proximal and dilated bowel in small bowel atresia. The innervation pattern of the proximal bowel resembles intestinal neuronal dysplasia. These changes might be the result of long-lasting bowel obstruction and stasis of bowel contents. The presented histological features do not contribute to the search for the pathogenesis of small bowel atresia. Nevertheless, the confirmed innervation abnormalities and defective expression of CD117-positive cells of the proximal bowel may have an influence on the postoperative gastrointestinal motility of the affected patients.

COMMENTS

Background
Small bowel atresia is a congenital anomaly of unknown cause. Despite early corrective surgery, patients carry a substantial morbidity because of postoperative gastrointestinal motility problems. Normal gastrointestinal motility is generated by the complex interaction of the enteric nervous system (ENS), the intestinal smooth muscle and the interstitial cells of Cajal (ICCs). Alterations in the ENS and ICCs may contribute to the motility problems in patients with small bowel atresia after surgery.

Research frontiers
Previous studies revealed histological changes within the wall of the atretic and adjacent bowel in small bowel atresia. Nevertheless the relationship between the macroscopic and histological changes of the affected bowel and the postoperative motility disorder are still under investigation. Furthermore, the role of the ICCs in small bowel atresia needs to be elucidated further.

Innovations and breakthroughs
This study showed that the ENS and ICCs are altered in the proximal and dilated bowel in small bowel atresia. The innervation pattern of the proximal bowel resembles intestinal neuronal dysplasia.

Applications
The changes within the ENS and the ICCs may be the result of a long-lasting bowel obstruction and stasis of bowel contents. The presented histological features do not contribute to the search for the pathogenesis of small bowel atresia. However, the confirmed innervation abnormalities and defective expression of CD117-positive cells of the proximal bowel may have an influence on postoperative gastrointestinal motility in the affected patients.

Terminology
Bowel atresia is a congenital defect in the continuity of the bowel. The incidence of small bowel atresia is higher than that of large bowel atresia and varies between 1:300 to 1:3000.

Peer review
The authors studied the ileum of seven patients with ileal atresia. They reported that the proximal dilated intestine had an increased expression of nerve fibers and glial cells, with a decrease in ICCs, and an increase in ganglia size, whereas the distal intestine displayed normal features. They conclude that these alterations may influence on postoperative intestinal motility. The authors performed a well designed study in a very uncommon congenital disease, which is very interesting for the readers.

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