J. Cell. Mol. Med. Vol 12, No 2, 2008 pp. 471-478

Delayed development of interstitial cells of Cajal in the ileum of a human case of gastroschisis

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Received: January 9, 2008; Accepted: February 5, 2008

Abstract

The Interstitial Cells of Cajal (ICC) are responsible for rhythmic electrical activity. A paralytic ileus is present in gastroschisis (GS), a malformation due to a defective closure of the abdominal wall through which part of the intestine herniates during pregnancy. In experimental GS, ICC morphological immaturity was shown in the rat foetus at-term but it could not be demonstrated whether differentiation is accomplished post-natally. For this purpose we morphologically investigated ICC, as well as enteric neurons and smooth muscle cells, in a case of human GS at birth and 1 month later when peristaltic activity had initiated. A 36 weeks gestation female was born by c/section with prenatal diagnosis of GS and possible volvulus of the herniated intestine. At birth, the necrotic intestine was resected and both ileostomy and colostomy were performed. The intestine continuity was restored after 4 weeks. Intestinal specimens, taken during both operations at the level of the proximal stoma, were immunostained with *c-kit*, neuron-specific-enolase and α-smooth-muscle-actin antibodies and some processed for electron microscopy. ICC were present at the myenteric plexus only. At birth, these cells were rare and ultrastructurally immature; 1 month later, when partial enteral feeding was tolerated, they formed rows or groups and many of them were ultrastructurally differentiated. Neurons and smooth muscle cells, immature at birth, had developed after 1 month. Therefore, ICC differentiation, as well as that of neurons and smooth muscle cells, is delayed at birth and this might explain the paralytic ileus in GS. One month later, differentiation quickly proceeded at all cellular levels paralleling the increasing tolerance of enteral nutrition.

Keywords: interstitial cells of Cajal • enteric neurons • smooth muscle cells • gastroschisis • human

Introduction

In the intestine, the interstitial cells of Cajal (ICC) are the cells responsible for generation and propagation of peristalsis [1–4].

Gastroschisis (GS) is a malformation whose prevalence in Europe is increased 4-fold (from 0.54 to 2.12 per 10,000 births) in the last 20 years, with the highest recorded rate in the UK [5]. This malformation is characterized by a defect of the abdominal wall through which part of the intestine herniates into the surrounding amniotic fluid since the first trimester of pregnancy. The damage of the herniated intestinal loops is quite variable among patients born with GS, from normal looking intestine to extremely damaged

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doi: 10.1111/j.1582-4934.2008.0277.x

loops. The main consequence is a characteristic paralytic ileus and the more affected the intestinal loops are, the more prolonged can the ileus be anticipated. Once the baby is born, one or more surgical operations, a central vein catheter and the parenteral nutrition are required. The period of admission may vary between few weeks and many months, depending on the time-course of enteral nutrition tolerance, *i.e.* on onset of the peristaltic activity. In addition, a minority of patients will require either parenteral or enteral supplementation for many months or years at home. Several articles regarding the pathogenesis of the intestinal damage in GS have been published [6–8] and the prolonged contact with the amniotic fluid, rich of gastrointestinal enzymes and waste products, seems to be the main cause of the damage [9]; however, the aetiology of the paralytic ileus is not completely understood. Experimental studies in different animal models have addressed this aspect [8, 10–13] and, recently, the possible role of ICC has been raised in a rat model of GS [8]. In this latter study, an immaturity of the ICC was observed with immunohistochemistry and transmission electron microscope (TEM) in the herniated loops of the rat at term foetuses with GS [8]. In the same rat model, immature neurons and smooth muscle cells were also seen [11–13]. Unfortunately, due to mortality of the animals, the possible maturation of these cells in parallel with a normal peristaltic activity could never be investigated after birth.

An immunohistochemical and electron microscope study has been presently performed in a baby born with GS on intestinal specimens collected at birth and 1 month later. The aim of the study was to follow the developmental steps of human ICC in parallel with the onset of a peristaltic activity.

Patient and methods

A 36 weeks gestation female was born by c/section with the prenatal diagnosis of GS and possible volvulus of the herniated intestine. At birth, some of the protruding loops were necrotic, probably due to twisting of the intestine, and were, therefore, removed. A terminal ileostomy and colostomy were performed at the level of the proximal and distal intestinal segments that were normal looking, not herniated, and not covered by the peel. A central vein catheter for total parenteral nutrition (TPN) was introduced as the intestinal volvulus had left a picture of typical short bowel syndrome. Indeed, the remaining small bowel was 50 cm in length and without ileocecal valve. During this first operation, specimens of viable normal looking and not ischemic small intestine at the level of the proximal stoma were taken. The TPN was started on the first post-operative day and the minimal enteral feeding was begun few days after the operation. The intestine continuity was restored at 1 month of age and intestinal specimens were again taken at the level of the proximal stoma. The enteral nutrition was gradually increased in the following months, while the composition and volume of the TPN was progressively decreased according to patientís growth. At 2 years of age, the patient is free from TPN, eating a full diet *per os*, and thriving within normal ranges for her age both of weight and height.

For light microscopy, the bowel specimens were fixed in 10% neutral formalin, dehydrated through an ascending series of alcohol (1×5 min each), cleared in Histoclear (2×5 min) and then left overnight in molten wax at 56°C prior to embedding. Some sections 4 μm thick were stained with haematoxylin & eosin and others processed for immunohistochemistry. Antisera anti *c-kit* (to label ICC), anti α-SMA (to label smooth muscle cells) and anti-NSE (to label enteric neurons) were used. Immunolabelling was obtained by using the detection Kit-Polymer (Novocastra, Newcastle upon-Tyne, UK) according to the manufacturer's instruction. The endogenous peroxidase was inhibited by Novocastra Peroxydase Block RE7101 Kit, followed by the protein block by Novocastra Protein Block RE7102. This was followed by incubation with the primary antibodies for 45 min at room temperature, rinsing in PBS, incubation with the post-primary block RE7111 for 20 min at room temperature and final incubation with NovoLink Polymer RE7112 for 20 min at room temperature. Immunoreactivity was detected at room temperature by the addition of 3,3′-diaminobenzidine (DAB, Novocastra) as a substrate. All the immunolabelled sections were counterstained by Harris haematoxylin. No staining was observed when the respective primary antibody was omitted. The primary antibodies used, and their respective dilutions, are listed in Table 1.

For transmission electron microscopy, full-thickness specimens were immersed in a fixative solution of 2% cacodylate-buffered glutaraldehyde (pH 7.4)

Table 1

Antigen	Clone	Supplier	Dilution
α-SMA	1-A4	Dako	1:100
NSE	Polyclonal	Dako	1:100
C-Kit	CD-117	Dako	1:300

for 6 hrs. They were then rinsed in cacodylate buffer supplemented with 15% sucrose, post-fixed with 1% phosphate-buffered OsO_4 (pH 7.4) for 2 hrs, dehydrated with graded alcohol, clarified in propylene oxide and embedded in Epon using flat moulds. Semi-thin sections were cut with a LKB NOVA ultramicrotome, stained with a solution of toluidine blue in 0.1 M borate buffer, and then observed under a light microscope. Ultra-thin sections of the selected areas were obtained with the same ultra-microtome using a diamond knife and stained with uranyl acetate, followed by a solution of concentrated bismuth subnitrate. The sections were examined under a JEOL 1010 electron microscope and photographed.

Results

Interstitial cells of Cajal

At birth, c-kit-immunoreactive (c-kit-IR) cells were present at the myenteric plexus (MP) level. However, unlike in normal pre- and term babies [14], these cells, named ICC-MP, were few, frequently solitary (Fig. 1A), and rarely grouped (Fig. 1B). All of them had a thin, fusiform body and two short processes. Moreover, myenteric regions completely devoid of these cells were often seen. At 1 month of life, there were many ICC-MP most of which forming rows (Fig. 1C) or groups (Fig. 1D), and the solitary ones were rare. Their body was provided with long and sometimes branched processes by means of which these cells interconnected to each other (Fig. 1D). No c-kit-IR cells, identifiable either as intramuscular ICC (ICC-IM) or ICC-DMP, *i.e.* those ICC related to the deep muscular plexus (DMP), were ever seen within both the circular and longitudinal muscle layers. Also under TEM, the cells identifiable as ICC were exclusively located at the MP level. At birth, these cells had a spindle-shaped body containing an oval nucleus surrounded by a thin ring of cytoplasm. Some of them had only free ribosomes (Fig. 2A) and some others fibroblast-like features, as several cisternae of the rough endoplasmic reticulum (RER) were detected (Fig. 2A and B). Moreover, those with a more extended RER also had a large Golgi apparatus (Fig. 2B). After 1 month, the ICC-MP had a larger body provided with several processes (Fig. 2C); some of them still had the fibroblast-like features (Fig. 2C) and others characteristically had cisternae of the smooth endoplasmic reticulum, filaments and caveolae (Fig. 2D). ICC processes were frequently seen close to both the neighbouring ICC bodies and processes (Fig. 2C and D).

Neurons

At birth, most of the myenteric neurons were faintly and few of them intensely neuronal specific enolase immunoreactive (NSE-IR) (Fig. 3A); all neurons were small-sized and provided with short processes. One month after birth, most of the neurons were intensely NSE-IR, large-sized and provided with longer and ramified processes (Fig. 3B). The intramuscular nerves, rare at birth (Fig. 3C), were numerous 1 month later (Fig. 3D). Some of them began to assemble in proximity to the innermost row of the circular smooth muscle, although not yet forming the nerve plexus named DMP, which in normal babies is present at birth [14].

Smooth muscle cells

Alfa–smooth muscle actin immunoreactivity (αSMA-IR) was faint at birth (Fig. 3E) but intense 1 month after, especially in those cells forming a thin monolayer at the submucosal border of the circular muscle layer (Fig. 3F). These modifications are suggestive for the further development in the so-called inner circular layer (ICL) that usually is well recognizable in normal term foetuses [14].

Discussion

During the last 10 years a series of articles regarding the possible role of the ICC in the gastrointestinal malformations, including GS, has been published [8, 15–18]. In the rat model of GS, a morphological immaturity of various cell types, including ICC,

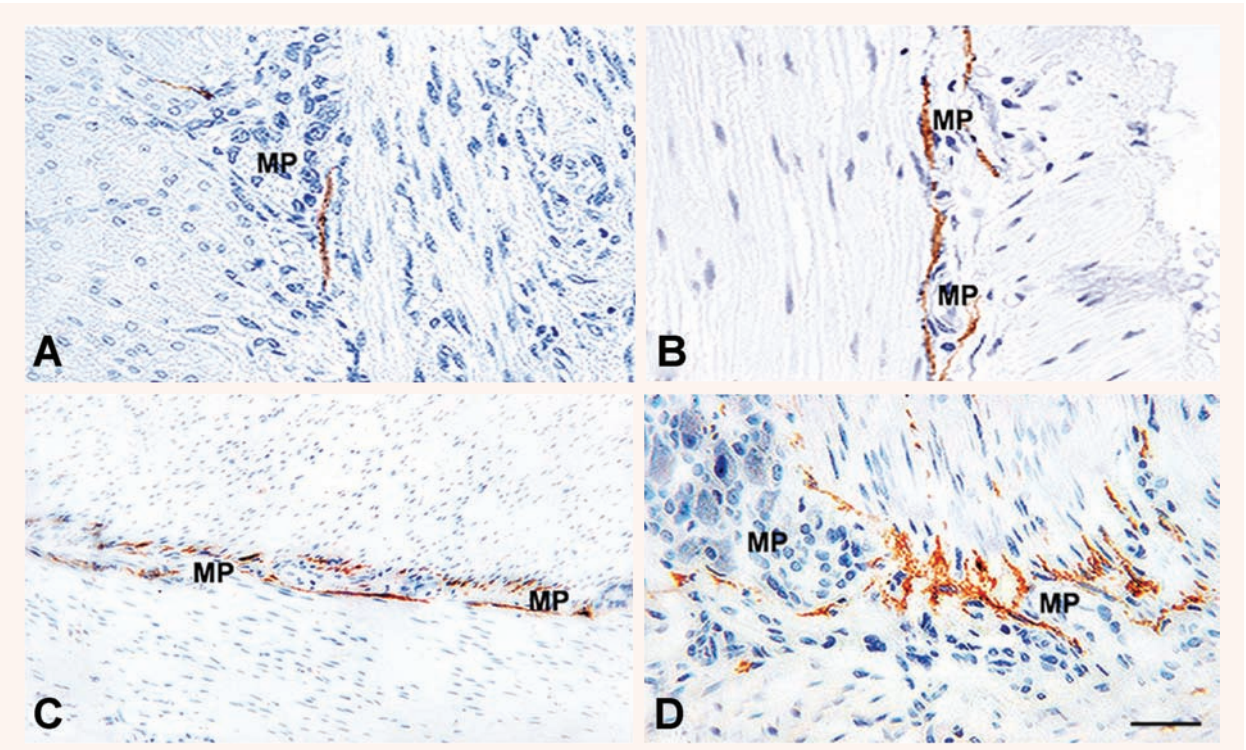

Fig. 1 Interstitial cells of Cajal, c-kit immunohistochemistry (c-kit-IR). **A** and **B**: GS ileum at birth. A solitary c-kit-IR cell in **A** and c-kit-IR cells lined up as rows of cells in **B**; in both cases, these cells are located in between the circular and longitudinal muscle layers and, therefore, these ICC can be considered ICC-MP. **C** and **D**: GS ileum 1 month after birth. Numerous c-kit-IR cells aligned in rows (**C**) or forming groups (**D**) in between the circular and longitudinal muscle layers. **MP**: myenteric plexus. Bar: **A, B, D** = 20 μm; **C** = 50 μm.

smooth muscle cells and enteric neurons, has been reported [8, 11–13] thus suggesting a possible explanation for the paralytic ileus characteristic of this malformation. In the present study, the immature features reported for the experimental GS were confirmed by examining samples of small intestine of a baby born with GS. Moreover, looking at samples collected 1 month later, it was possible for the first time to demonstrate the occurrence of a progressive maturation and, in particular, to ultrastructurally document the maturative steps of the human intestinal ICC.

According to studies performed with c-kit immunohistochemistry, the developmental steps of the intestinal ICC populations (ICC-MP, ICC-IM and ICC-DMP) have a different time course in humans. The ICC-MP are present by 7–9 weeks of foetal life [19], increase in number, and progressively differentiate up to birth and likely after birth [14, 20–22]. The ICC-IM begin to differentiate in the pre-term foetuses and the ICC-DMP, together with the related DMP and ICL, at birth [14]. Under light and electron microscope, we presently observed that, among these ICC populations, only the ICC-MP were present in GS, both at birth and 1 month later. The presence of the ICC-MP is important because, as intestinal pacemaker cells, they can ensure the peristaltic movements early in pregnancy, even without food intake [23–25]. Interestingly, the spatial organization and the morphology of these cells at birth were those typical of the first trimester of pregnancy, during which GS occurs. TEM confirmed immaturity of ICC-MP at birth, as some of them had the ultrastructural features of scarcely differentiated cells. Some other ICC-MP, the fibroblast-like ones, appeared similar to the immature ICC-MP described in the mouse foetuses [26] and to the newly differentiating ICC [27] and, therefore, be reasonably considered an intermediate step of the ICC differentiation process. From

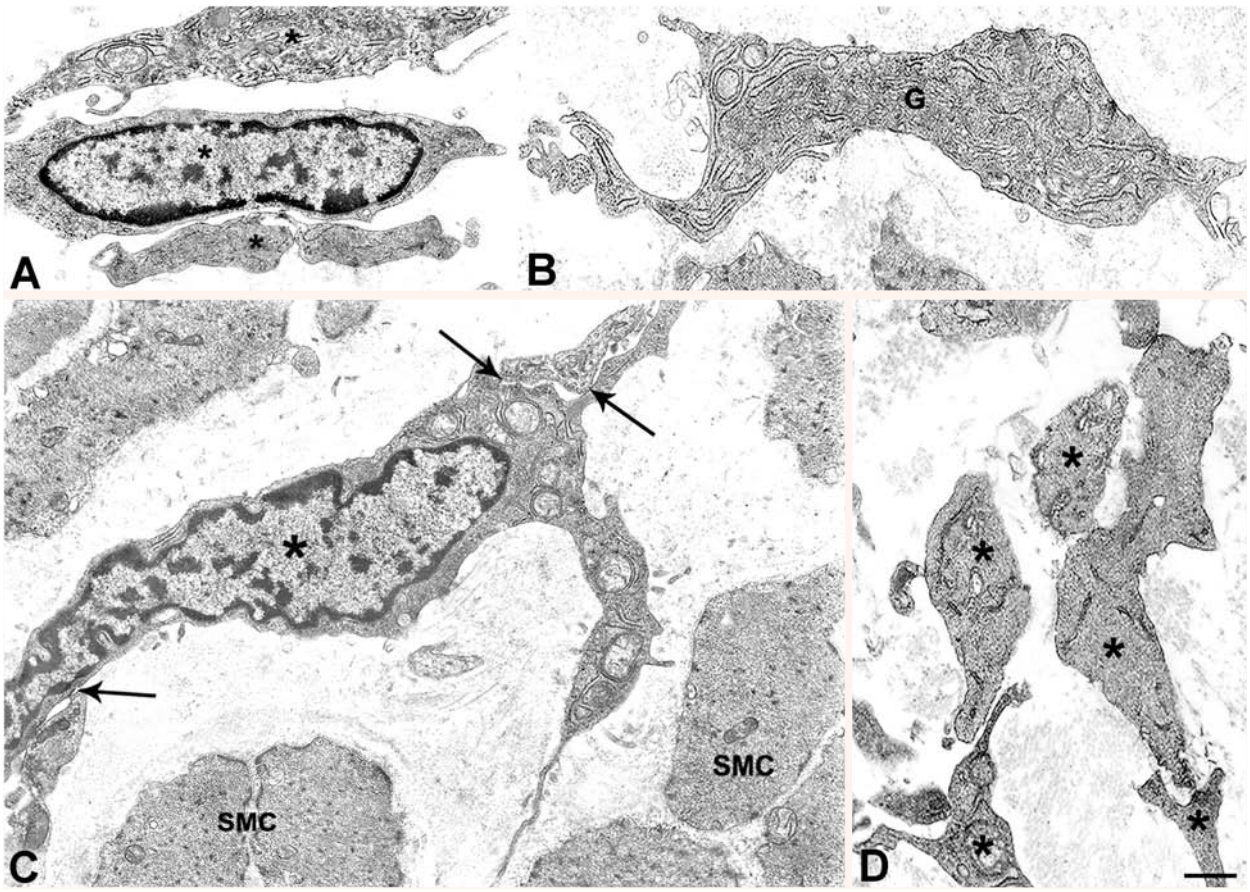

Fig. 2 Interstitial cells of Cajal, transmission electron microscope. **A** and **B**: GS ileum at birth. In **A**, three immature ICC-MP (*asterisks*). These cells are small-sized and have a spindle-shaped body and an ovoid nucleus. The cell in the upper side has some cisternae of the RER and the others free ribosomes. In **B**, a detail of an immature ICC with an extended RER and a large Golgi apparatus (**G**). **C** and **D**: GS ileum 1 month after birth. In **C**, an ICC (*asterisk*) with a large body and two processes. This cell is contacting processes (*arrows*) of neighbouring ICC. In the cytoplasm, several mitochondria and RER cisternae are present. In **D**, ICC processes (*asterisks*) close to each other and containing cisternae of the smooth endoplasmic reticulum and filaments. **SMC**: smooth muscle cells. Bar: **A** and **C** = 0.8 μm; **B** = 0.6 μm; **D** = 0.5 μm.

a functional point of view, this immaturity might explain the paralytic ileus of our baby; moreover, we may also hypothesize the fibroblast-like ICC-MP are not able to give raise to peristalsis.

Once the intestine was back into the abdomen in its proper environment, the process of differentiation had quickly continued at all cellular levels. In particular, 1 month after birth the ICC-MP pattern was similar to that of pre-term babies [14] and under TEM many ICC-MP had mature features. This maturative trend seemed to parallel the clinical evolution, as the patient was able to tolerate increasing amounts of food indicating the peristalsis was active at 1 month of age. Both the ICC-IM, responsible for neurotransmission, and the ICC-DMP, part of the intestinal stretch receptor [28, 29], were missing in GS, both at birth and 1 month later. Nerve structures and smooth muscle cells, scarcely differentiated at birth, showed mature features 1 month later; however, the DMP and the ICL were not yet so organized as in normal babies at this age [14]. Indeed, the clinical recovery was not completely accomplished 1 month after birth and the patient required partial parenteral nutrition until 2 years of age.

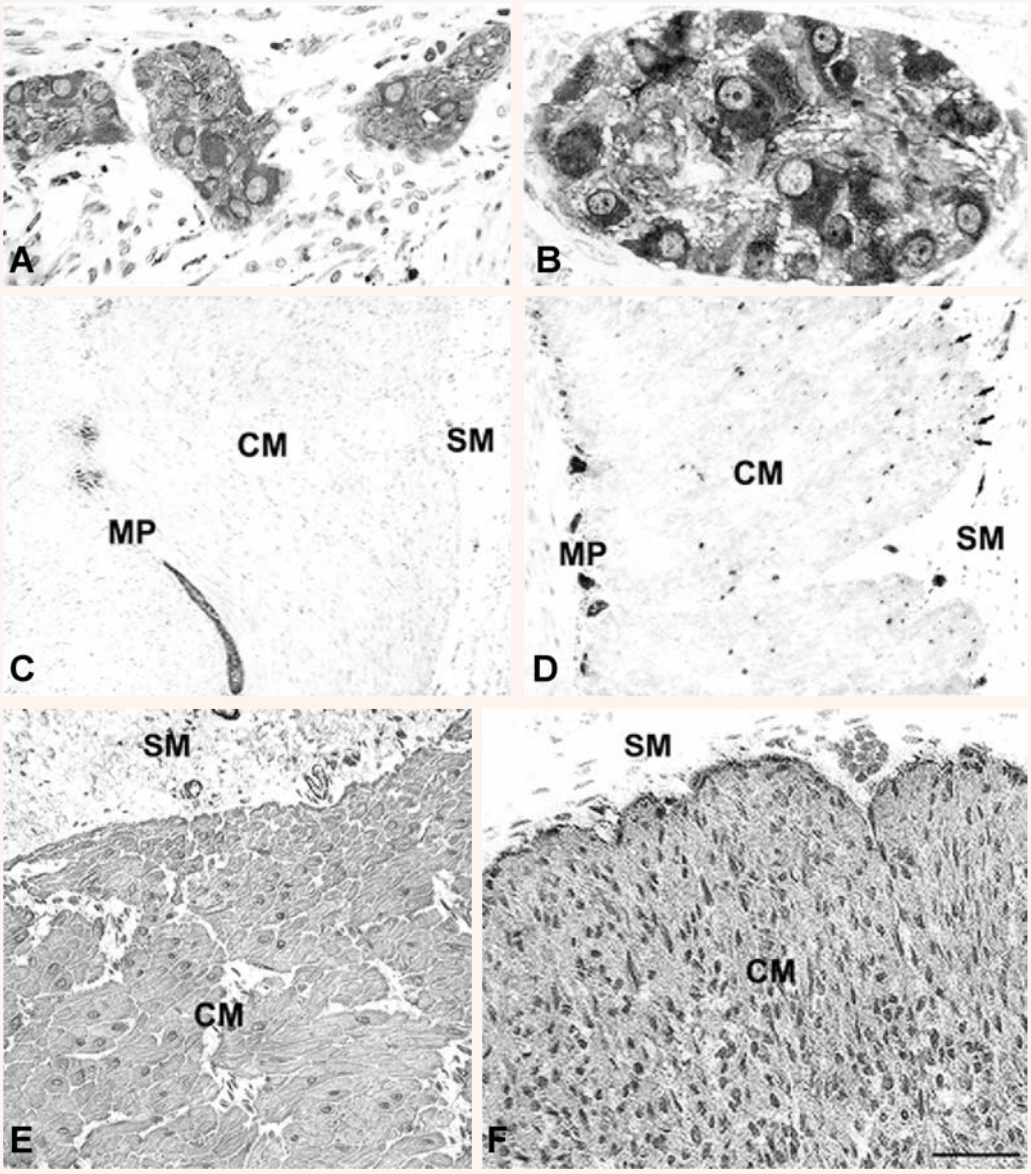

Fig. 3 A–D: Neurons, neuron-specific enolase immunohistochemistry (NSE-IR). **A**: GS ileum at birth. The neurons are small and mainly round in shape. Most of them are faintly NSE-IR. **B**: GS ileum 1 month after birth. Most of the neurons are intensely labelled, have a triangular or piriform body and long processes; a few neurons are faintly labelled. **C**: GS ileum at birth. The myenteric ganglia are few, the submucous plexus is not present and the intramuscular nerve fibres are scarce. **D**: GS ileum 1 month after birth. The myenteric ganglia are numerous and the submucous ganglia are present. The intramuscular nerve fibres are many and some are located at the level of the future DMP (*arrows*), although not yet organized to form a plexus. **E** and **F**: smooth muscle cells, α-smooth muscle actin immunohistochemistry (αSMA-IR). **E**: GS ileum at birth. αSMA-IR is faint and the circular muscle layer is not subdivided into two portions. **F**: GS ileum 1 month after birth. αSMA-IR is more intense than in **E** and the smooth muscle cells located at the submucosal border of the circular muscle layer are particularly intensely labelled. **MP**: myenteric plexus; **SM**: submucosa; **CM**: circular muscle layer. Bar: **A, B, E, F** = 20 μm; **C** and **D** = 50 μm.

The immaturity picture of the cells responsible for intestinal motor activities presently observed at birth in the baby with GS is very similar to that reported for animal models of GS [8, 11–13, 18]. Moreover, the absence of degenerative processes in all the specimens, together with the presence of a progressive maturative course, supports the hypothesis of a delayed differentiation, rather than an arrest. The aetiology of GS is still unknown but it is generally accepted the amniotic fluid has noxious effects on the herniated loops and, indeed, the damaged loops are those in contact with this fluid [9]. The delayed differentiation we observed might be due to the fact that in GS some of the herniated loops are tightly matted, shortened and covered by a peel. These conditions might halt the migration of the neural crest cells and, consequently, cause a delayed differentiation of both neurons and their target cells, such as ICC and smooth muscle cells.

In conclusion, in the intestine affected by GS we presently demonstrated: (*i*) at birth, in the presence of paralytic ileus, a morphological immaturity of all the structures responsible for the intestinal motor activities, (*ii*) after birth, the differentiation process has continued and its time-course paralleled the clinical recovery. This is the first report in which the differentiative steps of the human ICC are shown; this information may help in understanding the patho-physiology of affected peristalsis in newborns and, maybe, also in estimating the time-course of the disease.

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