Ontogeny of Interstitial Cells of Cajal in the Human Intestine
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Background/Purpose: Interstitial cells of Cajal (ICC) recently have been identified as intestinal pacemaker cells. Abnormalities in ICC are increasingly recognized in a number of neonatal disorders such as infantile hypertrophic pyloric stenosis, Hirschsprung's disease, and transient intestinal pseudo-obstruction. The aim of this study was to determine the fetal and postnatal differentiation and development of ICC in the human gastrointestinal tract to aid interpretation of pathological specimens.

Methods: Specimens of human gastrointestinal tract from (1) fetuses (9 to 17 weeks' gestation; n = 12), (2) premature and full-term neonates with non-gut motility-related disorders, (age 26 to 59 weeks' gestation; n = 13), and (3) children (age 4 months to 13 years; n = 7) were immunohistochemically stained with antibodies to c-kit (a marker for ICC) and protein gene product 9.5 (PGP9.5, a marker for neural tissue).

Results: (1) C-kit-positive ICC were present throughout the gut in all specimens including those from the earliest gestational ages. C-kit and PGP9.5 immunoreactivities were present in different cell populations. (2) The distribution of ICC varied with gestational age and with region of the gut. (3) Maturation of ICC networks continues postnatally in a region-specific manner.

Conclusions: ICC are present from an early stage in human gut development. Interpretation of apparent abnormalities in ICC distribution as being of pathological significance should be tempered by the knowledge that ICC networks continue to develop postnatally and that ICC development varies throughout the gut.

Although interstitial cells of Cajal (ICC) have been recognized for over a century their roles as pacemakers and mediators of neurotransmission in the gut have only been demonstrated recently. The discoveries that ICC express the tyrosine kinase receptor c-kit and that disruption of the c-kit signalling pathway inhibits differentiation of subpopulations of ICC, has allowed rapid progress to be made.

In mice, mutations in the genes coding for the c-kit receptor or its ligand, stem cell factor (SCF), are associated with impaired differentiation of ICC at the level of the myenteric plexus (ICC MP) and loss of slow wave activity in the small intestine. In contrast, ICC-MP and slow wave activity are not affected in the stomach, whereas ICC in the muscle layers (ICC-IM) fail to develop, and inhibitory (nitric oxide-dependent) neuroregulation is impaired.

The "normality" of an ICC distribution even in adults currently is difficult to define because there are conflicting data on the distribution of ICC in the human colon and very little data on inter-individual variations in subjects with normal gastrointestinal function. Furthermore, the density of ICC may vary within regions of the gastrointestinal tract making comparisons between diseased groups and controls prone to error. In human neonates, abnormalities in the distribution of c-kit-positive ICC have been reported in a number of congenital gut motor disorders including Hirschsprung's disease and transient neonatal intestinal pseudo-obstruction. For interpretation of such data it is important to know the normal distribution of ICC in premature and full-term human neonates. Examples from children with anorectal malformations have been included with control material in some studies, whereas an abnormal distribution of ICC has been demonstrated in a proportion of such cases. A recent study on normal control colon included a full-term neonate, which clearly showed regional differences in ICC distribution in the colon. No data on postnatal differentiation were included; particularly important because in mice, subpopulations of c-kit-positive ICC only differentiate postnatally.

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The aim of this study was to determine the ontogeny of c-kit-positive ICC in the developing human gut and to explore the possibility of postnatal changes in the human intestine.

**MATERIALS AND METHODS**

**Specimen Collection and Processing**

Specimens were classified into 3 groups according to age: (1) Fetal (<17 weeks’ gestational age), (2) Premature and full-term infants (26 to 59 weeks’ gestational age), and (3) infants or children (>60 weeks’ gestational age). Gestational age was defined as postmenstrual age of the mother (time from onset of last menstrual period). Fetal samples were obtained after surgical termination of pregnancy for nonmedical indications. The study was performed with local research ethics committee approval in full accordance with recommendations of the Human Fertilization and Embryology Authority. Specimens were collected into 100 mL of a chilled solution of 0.1 mol/L phosphate-buffered saline (PBS) containing 25,000 IU of sodium heparin. Each fetus was aged according to heel-toe length using anthropometric data. 

**Specimen Processing**

Samples were fixed in 4% (wt/vol) paraformaldehyde in PBS for 3 hours then processed for wax embedding or cryopreserved in 15% (w/v) sucrose in PBS at 38°C for 3 hours, and the gelatine was then set by cooling to 4°C overnight. The gelatine blocks were trimmed to give the required orientation of the gut samples before snap-freezing in isopentane cooled with liquid nitrogen. Frozen samples and cryostat sections were stored at −80°C before use. Sections were routinely stained with H&E for morphological assessment.

**Table 1. Summary of Underlying Diagnoses of Patients From Whom Bowel Specimens Were Obtained**

<table>
<thead>
<tr>
<th>Site</th>
<th>Diagnosis</th>
<th>No.</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>Caustic esophageal stricture</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Segmental ileal atresia</td>
<td>requiring gastric tube</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Meckel’s diverticulum</td>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Colon</td>
<td>Necrotizing enterocolitis</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Gastrochisis with colonic atresia</td>
<td>(at stoma closure)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Penetrating pelvic trauma</td>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Pelvic tumour</td>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Previous necrotising tascitis</td>
<td></td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

*Group 2, premature and full-term neonates (gestational ages 26 to 59 weeks); group 3, children (>60 weeks)*

**Immunohistochemical Methods**

Three antisera against c-kit were used, 2 rabbit polyclonal antisera (c-kit [Ab-1, Cat # pc-34, Calbiochem, CA]: and c-kit [C-19, cat # sc-168, Santa-Cruz Biotechnology, CA] and mouse monoclonal antibodies (NCL-cKit, 57ASD8, Novocastra Laboratories, Newcastle upon Tyne, England). Although similar staining patterns were observed with all antibodies, the clearest labelling was obtained with monoclonal antibodies, so these were used for the rest of the study. Neurons were labelled using the rabbit antiserum against the neuronal marker Protein Gene Product 9.5 (PGP9.5).  

Immunoperoxidase staining was performed by incubating 10 μm cryostat sections with primary antibodies against c-kit (dilution 1:50) or PGP9.5 (dilution 1:2000) in 0.1 mol/L (w/vol PBS) for 18 hours followed by biotinylated antirabbit IgG or antimouse IgG (dilution 1:50 in 0.1 mol/L (w/vol) PBS), then streptavidin-peroxidase complex (dilution 1:50 in 0.1 mol/L (w/vol) PBS, ABC Duett kit, DAKO, Glostrup, England). Sites of antibody binding were marked by a brown deposit on incubation with Dabfast substrate in 0.1 mol/L PBS (DAKO). Sections were counterstained with eosin. No immunoperoxidase staining was observed when the primary antibodies were omitted.

Dual labelling was used to study the localization of c-kit immunoreactivity in relation to neural cells. Sections were incubated for 18 hours in a mixture of mouse anti-c-kit and rabbit anti-PGP9.5. Sites of antibody binding were detected by incubation for 1 hour with Texas Red-labelled antimouse IgG (Vector Laboratories, Peterborough, England), and biotinylated antirabbit IgG followed by streptavidin-FITC (Sigma Aldrich Co, Poole, England), all at a concentration of 1 in 50.

**RESULTS**

**Study Population**

The distribution of c-kit immunoreactivity was studied in the gastrointestinal tract of (1) fetuses (9 to 17 weeks’ gestation; n = 12); (2) premature and full-term infants with non-gut motility-related disorders (26 to 59 weeks gestational age; n = 13); and (3) children (age 4 months to 13 years; n = 7). Specimens of fetal stomach, small intestine, and colon were available for examination. In older patients, only specimens of ileum and colon were obtained with the exception of a specimen of fundal stomach from a 2½-year-old child. The underlying diagnoses in neonates and children are summarized in Table 1.

**C-kit immunoreactivity in speciments (Figs 1-3).** However, the morphology and distribution of c-kit-positive cells varied both during development and in the different regions of the gut. In addition to the cell staining described below, c-kit antibodies also stained ovoid connective tissue cells with a spherical nucleus resembling mast cells, which also express the c-kit receptor (Figs 2C, 3C, and 3E). These cells were distributed throughout the specimens with the greatest density being in the submucosal region and lamina propria.

In all specimens, PGP9.5 staining showed the presence of neurons in the myenteric plexus (Fig 1C).
ONTOGENY OF INTERSTITIAL CELLS

Stomach

In fetal stomach at 9.5 weeks' gestation, c-kit staining was extensive throughout both presumptive muscle layers and surrounding the myenteric plexus (Fig 1). Filamentous staining (which probably represents cytoplasmic processes) was prominent in the inner (circular) muscle layer. In addition, there was considerable generalized staining in the external muscle layer (Figs 1A and B). At 12 weeks' gestation, the staining pattern had altered: the generalized staining seen in the external muscle layer in the 9.5-week fetus had disappeared. The filamentous staining around the myenteric plexus and inner muscle layer remained and had increased in intensity (Fig 1C and D). In the fundal specimen from a 2½ year old child, c-kit-positive cells were distributed sparsely within the myenteric plexus and inner circular muscle layers only (Figs 1E and F). Stained cells were spindle shaped and had two to four long branching processes giving a characteristic spindle shape.

Small Bowel

In embryonic small bowel at 12 weeks, c-kit-positive cells with two to three branching processes were present surrounding neurones in the myenteric plexus (Figs 2A and B). No c-kit immunoreactivity was present in the muscle layers including the inner edge of the circular muscle (deep muscular plexus). By 26 weeks' gestation, a similar distribution of ICC was seen, but both the number of cytoplasmic processes and the density of c-kit staining of ICC had increased (Figs 2C and D). Only c-kit immunoreactive mast cells were present within the smooth muscle layers (Fig 2C). The same distribution was also seen in full-term neonates, although some immunoreactivity was present where the deep muscular plexus is described in adults (data not shown). By the age of 2 years, recognizable ICC were seen in a distribution similar to that described in adults within and around the myenteric plexus, in the deep muscular plexus, and in circular muscle (Figs 2E and F).
Colon

At 10 to 12 weeks' gestation, c-kit-positive cells with two to three processes and a nonimmunoreactive nucleus with sparse perinuclear cytoplasm surrounded the neurons in the myenteric plexus (Figs 3A and B). This pattern was very similar to that seen in the small bowel at a similar gestational age (Figs 2A and B). However, by 29 weeks, additional c-kit-positive cells could be seen in both muscle layers, although only very sparse immunoreactivity was seen at the inner aspect of circular muscle—the ICC SM layer (Figure 3C and D). This contrasts markedly with the pattern seen in the small intestine at the same gestational age (Fig 2C). A similar histological picture was seen in full-term neonates, although the ICC-SM layer was present (data not shown). With increasing age, an increase in cell size and in the number of cytoplasmic processes of individual ICC (from two to three to four to five), plus an overall increase in the complexity of inter-ICC networks was seen (Fig 3E and F, infant at 15 months).

Dual-Labelled Immunofluorescence

No colocalization of c-kit staining with the neural marker PGP 9.5 was seen in any of the specimens examined (Fig 1C).

DISCUSSION

This study has shown region-specific differences in the distribution of c kit positive ICC during development. Moreover, the morphological appearance of individual ICC and ICC networks become more complex with increasing age. The finding that these changes continue into postnatal life has implications when interpreting the pathological significance of abnormalities in distribution or density of ICC in neonates and young children.

Little variation in ICC distribution was observed
between cases with different diagnoses such as necrotizing enterocolitis and biliary atresia, although a lack of close age matching made quantitative comparisons invalid. This lack of variation and the heterogeneous nature of the underlying diagnoses reinforces the validity of using clinical material.

The embryonic origin of ICC in the human gut is unknown. Support for a mesenchymal origin comes from chick-quail chimeric and aneural mouse explant studies.\textsuperscript{21,22} Furthermore, γ enteric actin immunoreactivity has been found in developing murine ICC suggesting that they may be a specialized form of smooth muscle cell.\textsuperscript{23} The SCF ligand for c-kit is expressed in neural crest-derived cells\textsuperscript{24} and interaction between c-kit and SCF is essential for normal development of subpopulations of ICC in mice.\textsuperscript{4,6} The finding that c-kit-positive ICC are present from 9.5 weeks when neural crest colonization of the gut is approaching completion,\textsuperscript{25} therefore, is consistent with a modulating effect of the fetal enteric nervous system on ICC development.

Immunohistochemical studies in mice have shown c-kit expressing cells lacking the phenotypic characteristics of either ICC or smooth muscle present in the outer mesenchymal layer at E12.5.\textsuperscript{16} In our study, the diffuse staining seen in fetal stomach at 9.5 weeks’ gestation may reflect a similar developmental stage in humans.\textsuperscript{26} A similar distribution of c-kit–positive ICC was seen in early fetal small bowel and colon, although these similarities were lost by the third trimester suggesting the presence of extrinsic region-specific factors affecting the subsequent development of ICC networks. Hox genes exhibit regional specificity in individual subdomains of the developing gut and are candidate genes for such a role.\textsuperscript{27}

The key role played by these cells in generation of pacemaker activity makes it likely that a proportion of hitherto unexplained gut motility disorders are caused by functional or structural abnormalities in ICC. Defects in ICC populations have been described in neonates with infantile hypertrophic pyloric stenosis,\textsuperscript{28,30} Hirschsprung’s disease,\textsuperscript{11,12} anorectal malformations,\textsuperscript{14} and cases of neonatal transient intestinal pseudo-obstruction.\textsuperscript{13} In this
study, we have found that maturation of ICC and the development of adult-type ICC networks continues well into postnatal life (Fig 4). This has implications in the interpretation of apparent abnormalities of ICC in neonates. We previously have described an initial absence of colonic ICC immunoreactivity in two neonates with intestinal pseudo-obstruction: one premature neonate with complete absence and one neonate with absent ICC-SM. Normal ICC development coincided with the appearance of normal gut motility. The data presented in this paper confirm that these previous findings represent true deviations from normal development.

The finding that ICC morphology and network structure is age dependent during early life adds a further degree of complexity to the study of the possible pathological role that these cells may play. Immunohistochemical studies on ICC in neonates need to be controlled carefully and preferably supported by electrophysiological or molecular biological data.

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REFERENCES