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Computer-aided morphometric analysis of the developing concentric structure of the human fetal intestinal tube

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Summary. The Image-Pro Plus 3.0 morphometric program was used to study the region-specific organization of the human fetal intestine across the radial axis of the gut at weeks 12 and 18 of gestation. The thicknesses of the epithelium, the submucosa, the muscular layers and the myenteric ganglia were measured in resin-embedded semithin sections. Statistical analysis of the collected data was performed by using the two-way ANOVA, the SNK test and the Pearson correlation. The structural changes relating to the gut morphogenesis within this developmental period were followed both light and electron microscopically. The various tissues forming the radial axis of the intestinal tube exhibited different trends concerning their individual development. The thickness of the epithelium did not change in the fetal period investigated, although the epithelial surface displayed characteristic ultrastructural changes. The thickness of the submucosal layer increased significantly, but with different dynamics along the longitudinal axis, whereas the increases in size of the muscular layers and the myenteric ganglia did not differ significantly along the longitudinal axis of the embryonic intestine. The Pearson correlation revealed a significant correlation between the development of the circular muscle layer and that of the myenteric plexus along the whole length of the intestinal tube. The epithelium, the submucosa and the longitudinal muscle layers developed independently between weeks 12 and 18 of gestation.

Key words: Region-specific organization, Statistical analysis, Light microscopy, Electron microscopy

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Introduction

There are two major steps in the development of the gastrointestinal tract: organ formation, which in the human fetus is complete by week 12 of gestation (Grand et al., 1976; Montgomery et al., 1999), and functional maturation, which starts with swallowing, first detectable at around week 17 of gestation (Pritchard, 1966; Abramovich, 1973). During gut organogenesis, the mesenchyme differentiates into distinct concentric layers around the endodermal epithelium, forming the thin layer of the lamina propria, the muscularis mucosae, the submucosa and the muscle layers. How this topographical organization of the gut mesenchyme is established is largely unknown, though a small number of studies have recently been made about the regulatory mechanism underlying this topographically organized differentiation of the gut (Fukada et al., 1998; Sukegawa et al., 2000). It has been established that the patterning across the radial axis in mice is basically organized by the epithelium (Sukegawa et al., 2000). Only sporadic data exist concerning the morphogenetic events relating to the functional maturation of the human fetal intestine. A changing pattern of myenteric plexus (MP) muscle contact has been revealed (Fekete et al., 1996). Up to week 18 of gestation, the circular muscle layer (CM) offers the mechanical surface for the developing MP, while after this time the longitudinal muscle (LM) is formed and the mechanical points of attachment have shifted from the CM to the LM. Evidence that the number of neurons, the density of the MP and the average neuronal size are greater in areas where the smooth muscle layers are thicker (Gabella, 1994) suggests a close neuron-target cell relationship for mutual survival and function. More recent evidence indicates that neurons are effective regulators of intestinal smooth muscle growth and for the maintenance of their differentiated state (Blennerhassett and Louressen, 2000).

There are as yet no data on the age- and region-specific differences in the final patterning across the radial axis in the developing human gastrointestinal tract. Accordingly, to elucidate the mechanism involved in the morphogenesis of the concentric structure of the human gut, we have investigated the changes in thickness of the tissue layers forming the final structure around the intestinal lumen in the different segments of the human fetal intestine at weeks 12 and 18 of gestation. Quantitative observations of the developing intestine may provide data of value to assess the individual tissue components, the modification of their relationships and the developmental correlations between tissue layers of different segments of the embryonic intestine. A knowledge of the exact timing and the quantitative features of the morphogenetic events might promote an understanding of the mechanisms underlying functional maturation. In the present work we have considered weeks 12 and 18 of fetal development because particularly striking morphogenetic events occur in this period (Grand et al., 1976; Moxey and Trier, 1979; Montgomery et al., 1999).

Materials and methods

Tissue preparation

Human fetuses at weeks 12 and 18 of gestation were obtained immediately after legally approved or spontaneous abortions. The crown-heel length was used to determine the gestation age (England, 1983; Moore, 1989). Three fetuses of each age were used for each examination. All the experiments were performed in accordance with the declaration of the Medical World Federation proclaimed in Helsinki in 1964. The whole intestine was flushed with phosphate buffer (0.1 M, pH 7.4) and distended by filling with the fixative (2% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer). The distended intestine was immersed in the same fixative overnight at 4 °C. After fixation, two measured segments of the small intestine and one measured segment of the colon were selected and processed for electron microscopy (Fekete et al., 1996). The proximal and distal samples of the small intestine were located at 1 cm distal to the pylorus and at 7 cm oral to the ileo-cecal junction, respectively. The colon samples were located at 0.5 cm distal to the ileo-cecal junction.

Measurements

Toluidine blue-stained semithin cross-sections prepared from the selected intestinal segments were subjected to morphometric analysis, and ultrathin cross-sections from the same sectional level were examined with a Philips CM 10 conventional electron microscope equipped with a MEGAVIEW II camera. Semithin cross-sections were examined with a Zeiss Axioskope light microscope equipped with a Hitachi (HV-C20 3-CCD) color camera. The Image-Pro Plus 3.0 morphometric program was used for measurements. The thicknesses of the epithelium, the submucosa, the circular and the longitudinal muscle layer and the diameter of the myentric ganglia were measured. 50 measurements per intestinal segment per age group were made at 50- or 100-fold magnification, depending on the age.

Data analysis

After logarithmic transformation of the collected data, statistical analysis was performed by using the two-way ANOVA and the SNK test on SPSS 9.0 software. A probability of $P<0.05$ was set as the level of significance in all analyses. Diagrams and tables were made with the Microsoft Excel 7.0 program. Data were expressed as means ± SE. In order to evaluate the interaction between the tissue layers around the radial axis of the developing intestine, the Pearson correlation was used with SPSS 9.0 software. The correlation coefficient (r) and the level of significance (P) were evaluated.

Results

Morphological observations

A gradual increase in the thickness of the intestinal wall, with a clear proximo-distal gradient, was observed between weeks 12 and 18 of gestation. Age-related changes were noted light microscopically in the submucosa, the muscle layers and also the MP. The

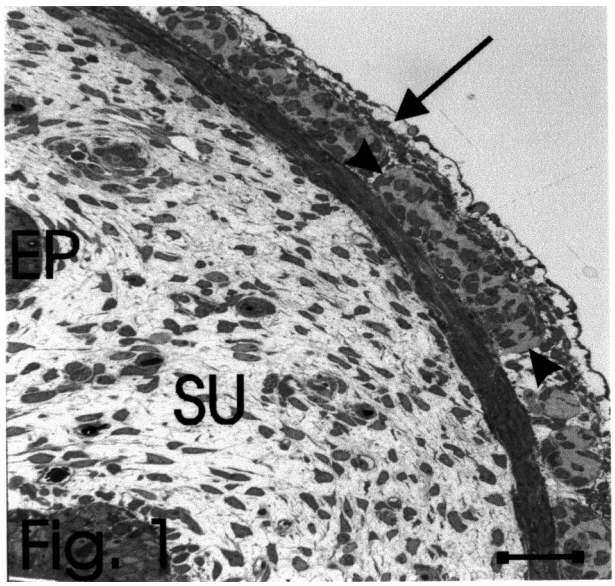

Fig. 1. Toluidine blue-stained semithin cross-section of resin-embedded human fetal small intestine at week 12 of gestation. The mesenchymal cells in the submucosal layer (SU) are loosely packed. The myenteric ganglia (arrowheads) appear as compact cell masses with no internodal segments. The longitudinal muscle layer (arrow) is identifiable only with difficulty. EP: epithelium. Bar: 35 μm.

mesenchymal cells in the submucosal layer became more closely packed during the developmental period examined and dense arrays of large blood vessels appeared (Figs. 1, 2). Whereas the LM layer was very difficult to identify at week 12 (Fig. 1), by week 18 it had formed a continuous layer with closely-packed smooth muscle cells (Fig. 2). The myenteric ganglia appeared as compact cell masses with no internodal segments at week 12 (Fig. 1) while the characteristic picture of ganglionated plexus was revealed at week 18 (Fig. 2), when the ganglia were separated by long internodal segments. At the same time, age-related morphological changes in the epithelium were observed only ultrastructurally. Zones of bilayered epithelium were frequently seen in the intervillar epithelium at week 12 of gestation (Fig. 3). Coherent cells in these areas

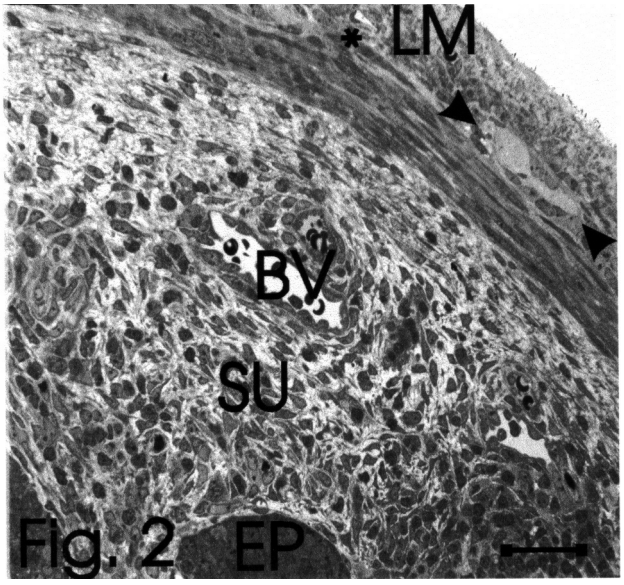

Fig. 2. Toluidine blue-stained semithin cross-section of resin-embedded human fetal small intestine at week 18 of gestation. The mesenchymal cells in the submucosal layer (SU) are more closely packed and dense arrays of large blood vessels have appeared (BV). The myenteric ganglia (arrowheads) are separated by long internodal segments (asterisk). The longitudinal muscle layer (LM) forms a continuous layer with closely packed smooth muscle cells. EP: epithelium. Bar: 35 μm.

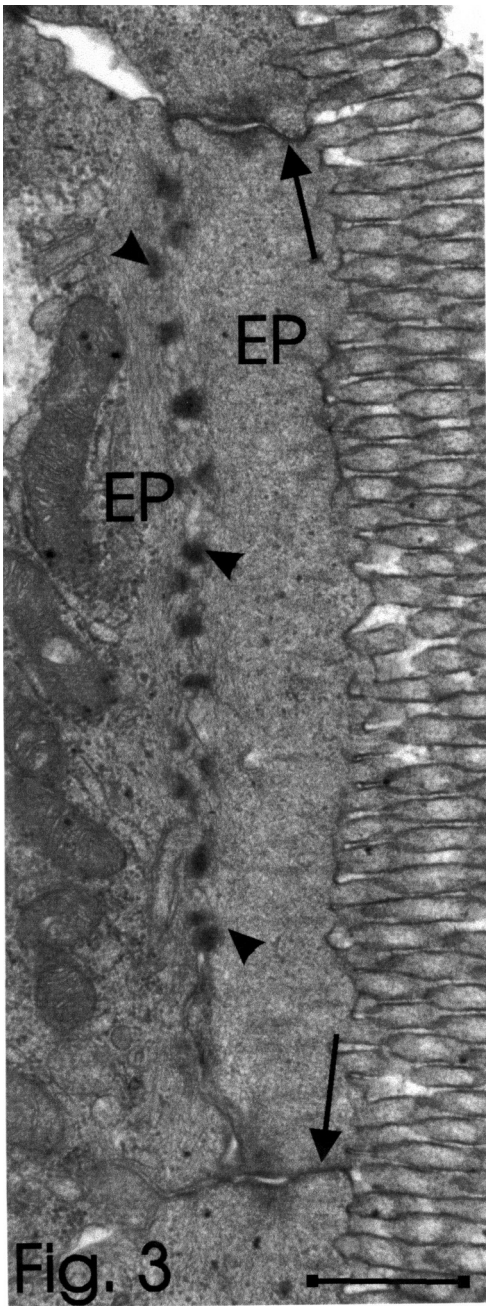

Fig. 3. Bilayered zone of the epithelium (EP) in the intervillar region of the human fetal small intestine at week 12 of gestation. Coherent cells are coupled with regularly-ordered desmosomes (arrowheads). Matured junctional complexes with zonula occludens (arrows) have also appeared. Bar : 1 μm.

Table 1. Thickness of the tissue layers of the human fetal intesinal tube in micrometer at weeks 12 and 18 of gestation. Data are expressed as means ± SE. In case of the older animals, when in a given tissue layer of an intestinal segment significant differences were measured, the value of significance ($p<0.05$) was indicated with an asterisk.

	PROXIMAL PART OF THE SMALL INTESTINE (μm)		DISTAL PART OF THE SMALL INTESTINE(μm)		COLON (μm)	
	12 weeks	18 weeks	12 weeks	18 weeks	12 weeks	18 weeks
Epithelium	28.590±10.54	29.396±2.48	28.851±5.76	31.456±4.61	35.265±5.91	35.427±5.61
Submucosa	238.357±30.81	291.270±88.02*	156.827±13.33	163.912±13.97*	118.130±18.9	160.85±33.81*
Circular muscle layer	15.470±4.26	30.890±6.88*	15.258±0.58	27.104±8.04*	14.610±1.82	36.753±10.07*
Myenteric ganglia	19.470±2.1	22.994±5.00*	15.425±2.45	19.849±2.48*	15.339±1.17	21.806±3.92*
Longitudinal muscle layer	unmeasurable	16.734±1.56	unmeasurable	20.505±1.46	unmeasurable	22.427±2.58

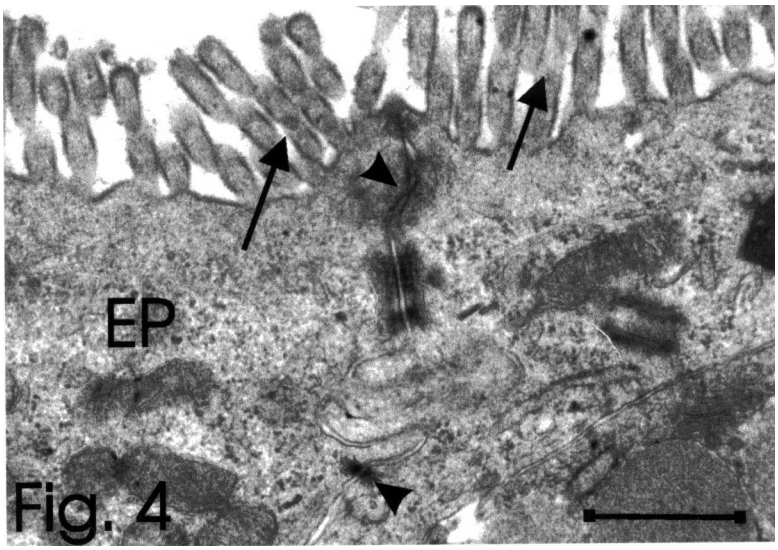

Fig. 4. Luminal surface of the epithelium of the human fetal small intestine at week 12 of gestation. The absorptive cells display an array of short, sparse and irregularly oriented microvilli (arrows). Immature junctional complexes are characteristic with an array of desmosomes (arrowheads). Bar: 1 μm.

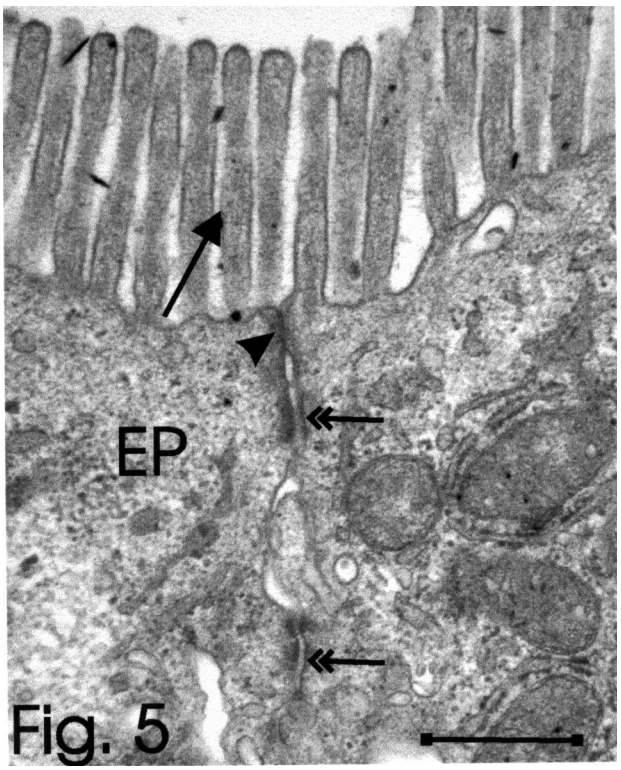

Fig. 5. Absorptive cells of the human fetal small intestine at week 18 of gestation. Simple columnar epithelium (EP) with the apical brush border is composed of regular microvilli (arrow). Matured junctional complexes with zonula adherens (double arrows) and zonula occludens (arrowhead) are present. Bar: 1 μm.

were coupled with regularly-ordered desmosomes (Fig. 3). Absorptive cells on the luminal surface already displayed an array of microvilli, although they were short, sparse and irregularly oriented (Fig. 4). By week 18, the luminal intestinal surfaces were covered by a simple columnar epithelium, and the apical brush borders were composed of regular microvilli similar to those found in the adult intestine (Fig. 5).

Statistical analysis

The different tissue layers forming the radial axis of the intestinal tube exhibited different quantitative trends in their individual development. The thickness of the epithelium did not change significantly ($P>0.05$) between weeks 12 and 18 of gestation and no significant difference in epithelial thickness was noted in the different intestinal segments (Figs. 6, 7 and Table 1). The thickness of the submucosal layer increased significantly ($P<0.05$) within this developmental period and the thicknesses of this tissue layer were significantly different in the proximal and distal parts of the small intestine and in the colon ($P<0.001$) (Figs. 6, 7 and Table 1). A significant thickening of the CM was noticed ($P<0.001$) within this period, though this thickening was uniform along the proximo-distal axis of the intestine (Figs 6, 7 and Table 1). At week 12, the LM layer was too thin to measure, whereas at week 18 it had formed a continuous thick tissue layer, with uniform thickness throughout the whole length of the intestine (Figs. 6, 7 and Table 1). A significant increase in the diameter ($P<0.05$) of the myenteric ganglia was found between weeks 12 and 18 in each segment investigated. At week 12, the diameter of the myenteric ganglia differed significantly ($P<0.001$) in each of the intestinal segments investigated (Figs. 6, 7 and Table 1), but at week 18 the regional differences in the diameter of the ganglia were no longer observed ($P>0.05$). The Pearson correlation parameters were $r=0.511$ and $p=0.01$ for the CM and the myenteric ganglia, and $r<0.5$ and $p>0.05$ for the remainder of the tissue layers.

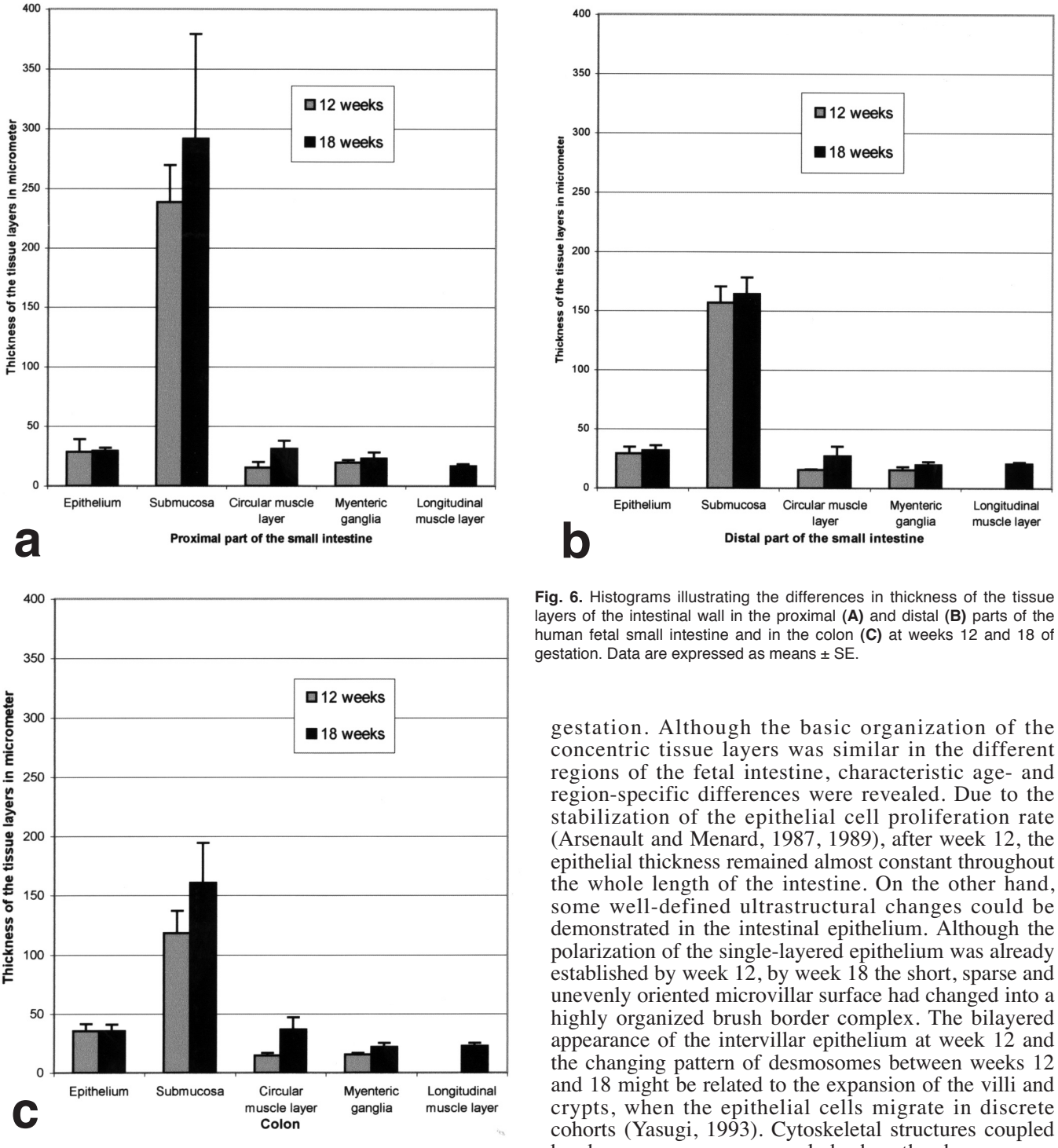

Fig. 6. Histograms illustrating the differences in thickness of the tissue layers of the intestinal wall in the proximal **(A)** and distal **(B)** parts of the human fetal small intestine and in the colon **(C)** at weeks 12 and 18 of gestation. Data are expressed as means ± SE.

Discussion

In the present work, the qualitative and quantitative changes in the tissue layers around the intestinal lumen were investigated in the different segments of the developing human fetal gut at weeks 12 and 18 of gestation. Although the basic organization of the concentric tissue layers was similar in the different regions of the fetal intestine, characteristic age- and region-specific differences were revealed. Due to the stabilization of the epithelial cell proliferation rate (Arsenault and Menard, 1987, 1989), after week 12, the epithelial thickness remained almost constant throughout the whole length of the intestine. On the other hand, some well-defined ultrastructural changes could be demonstrated in the intestinal epithelium. Although the polarization of the single-layered epithelium was already established by week 12, by week 18 the short, sparse and unevenly oriented microvillar surface had changed into a highly organized brush border complex. The bilayered appearance of the intervillar epithelium at week 12 and the changing pattern of desmosomes between weeks 12 and 18 might be related to the expansion of the villi and crypts, when the epithelial cells migrate in discrete cohorts (Yasugi, 1993). Cytoskeletal structures coupled by desmosomes, or uncoupled when the desmosomes disintegrate during maturation, might play an important role in the regulation of cell migration leading to the formation of matured villi. The significant region-specific thickening of the submucosal layer between weeks 12 and 18 was accompanied by an intensive vascularization, suggesting an enhanced oxidative metabolism in this fetal period. The present data

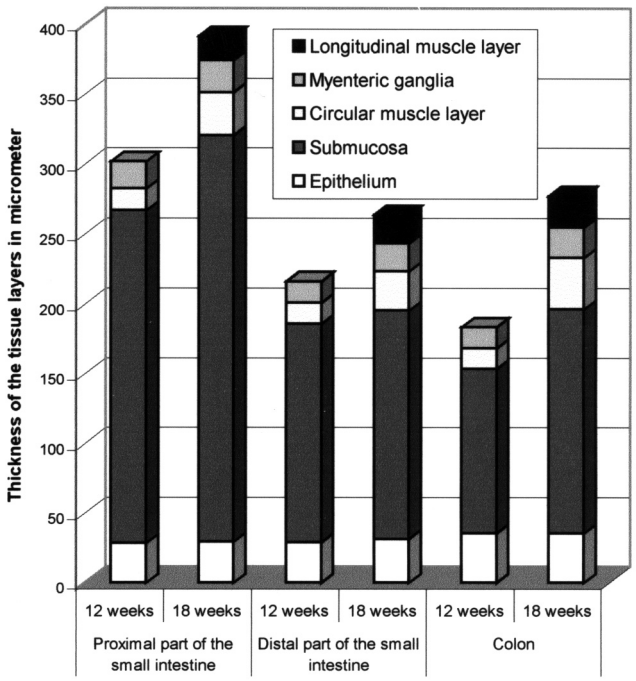

Fig. 7. Histogram showing the thicknesses of the concentric tissue layers of the human fetal intestinal wall at weeks 12 and 18 of gestation.

revealed significant increases in thickness of the smooth muscle layers, with no clear regional differences. Although the LM was not evident at week 12, by week 18 it appeared as a thick tissue layer with closely-packed smooth muscle cells. A significant increase in the diameter of the myenteric ganglia was also revealed, though the morphogenetic changes in the plexus were even more impressive. The compact cell masses on the outer side of the CM at week 12 had changed into the regular pattern of the ganglionated plexus by week 18. Long internodal segments were formed and the regional differences observed in the thickness of the plexus at week 12 had disappeared by week 18. The Pearson correlation data demonstrated a correlative development between the CM and MP, but not between the other tissue layers within this period. The interaction between nerve and muscle is directed partly through physical contacts (Fekete et al., 1996) and partly through trophic factors (Gittes et al., 1996; Blennerhassett and Louressen, 2000). Neurally-mediated inhibition or stimulation of smooth muscle growth might help to maintain the target organ growth within narrow parameters, such that the proper size is reached, but not exceeded. The tissue-specific variety of regional differences suggests that the regional identity is imprinted in the cells forming the concentric tissue layers around the intestinal lumen, although the imprinting rules differ in tissues with different embryonic origins. In the gut mesenchyme, the region-specific expression of regulatory genes such as Hox (Parr and McMahon, 1995; Wolpert, 1998) can organize the regional identity. In the nerve cells, on the other hand, region-specific expression of regulatory signal molecules responsible for the development of the enteric nervous system has been identified (Roberts et al., 1995; Montgomery, 1999; Kapur, 2000; Roberts, 2000). The locally-determined neuronal identity might well be influenced by the mesenchymal cells through which they migrate during colonization. This could create the final region-specific pattern of the concentric structures around the intestinal lumen which best suits the functional demands of the particular intestinal segment. The Pearson correlation data provided in present investigation suggest that the nerve-muscle interaction might have a crucial role in the final refinement of this patterning.

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