Smooth Muscle Differentiation During Human Intestinal Development

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Abstract

In this study we have examined the differentiation of smooth muscle cells, of the human gut, using antibodies against contractile (α-smooth muscle actin, smooth muscle myosin), cytoskeletal (cytokeratin, vimentin, desmin), regulatory (h-caldesmon, calponin) and membrane proteins (dystrophin). We show that the appearance of differentiation markers correlates with the gradual maturation of the intestinal wall.

At 7 weeks gestation, mucosa as well as prospective muscularis propria are reactive with antibodies to cytokeratin and all mesenchymal cells are vimentin positive. At the same time, α-smooth muscle actin is the first muscle specific protein to be expressed in the muscularis propria, immediately followed by calponin. By week 11 of gestation, the circular and longitudinal smooth muscle layers are established and their components express, besides α-smooth muscle actin and calponin, also smooth muscle myosin, h-caldesmon, desmin and dystrophin. At around 15 weeks the muscularis mucosae is formed and cytokeratin expression in the outer muscular layer is down-regulated. The intermediate filament switch from vimentin to desmin occurs between 18 weeks and early infancy (2-3 months).

The results show that gut smooth muscle displays a differentiation program which contrasts with smooth muscle cells of vascular origin in the temporal appearance of smooth muscle-associated proteins.

Key words: visceral smooth muscle, differentiation, human embryogenesis.

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Smooth muscle development and differentiation have been monitored closely during vertebrate embryogenesis. However, most investigations have centered around the de novo formation of blood vessels and comparatively little attention has been paid to the embryogenesis of visceral smooth muscle. During early human embryogenesis the intestinal wall is composed of an epithelial tube, surrounded by undifferentiated mesenchyme. Cellular differentiation of both layers is triggered by epithelial-mesenchymal interaction [10]. Between 8 and 11 weeks gestation in humans, the muscularis propria is subdivided into a thick circular and a sparsely formed longitudinal layer, separated by developing nerve plexus [16]. While this muscular coat serves to regulate overall intestinal peristalsis, a further thin layer of smooth muscle cells inserted between mucosa and submucosa is held responsible for the fine movements of the villi [22]. In contrast to the muscularis propria, the cells forming the muscularis mucosae acquire smooth muscle characteristics only around midterm of human gestation [16] and well after birth in rats [10].

Using antibodies associated with different stages of smooth muscle maturation, we have followed the development of human intestinal smooth muscle during a gestational period from 7 to 18 weeks and in the young infant (2-3 months) and compared the successive appearance of smooth muscle marker proteins in different muscular layers with the pattern described in vascular smooth muscle cells.

Material and Methods

Tissue preparation

Specimens of small and large intestine were obtained from 15 aborted human fetuses ranging from 7-18 weeks gestation. Ethical approval for this work was granted in accordance with the Polkinghorne report. The developmental age of fetal specimens was determined by foot length. Specimen of large bowel were obtained fresh at surgery from boys aged 2-3 months undergoing revision of colostomy for anorectal anomaly. Specimens were immersed in 4% paraformaldehyde for 4 hours at 4°C and rinsed twice in 15% (wt/vol) sucrose in 0.1 mol/L phosphate-buffered saline (PBS; pH 7.2) with 0.01% (wt/vol) sodium azide. Sections of 6 µm thickness were cut perpendicular to the long axis of gut from snap-frozen blocks in a cryostat at -25°C. These were mounted on gelatin-coated slides and air dried for 30 min. The sections were immersed for 20 min in PBS containing 0.2% (vol/vol) Triton X-100 (Sigma, Munich Germany) and fresh 0.3% (vol/vol) hydrogen peroxide, then rinsed in buffer for 10 min. Non-specific binding of antibodies was blocked by application of 5% normal goat serum in 1% bovine serum albumine (BSA) in PBS for 15 min. Sections were then incubated with the primary antibodies overnight in a humid chamber at 4°C. All antibodies were diluted in PBS containing 1% BSA. After rinsing in PBS, a secondary biotinylated antibody (goat anti-mouse: Dako, Hamburg, Germany; goat anti-rabbit: Vector Laboratories, Peterborough, England) was applied for 40 min at room temperature, followed by a brief wash and incubation with an avidin-biotin horseradish peroxidase complex (Vector Laboratories) for 15 min. After a rinse in PBS, followed by 0.1 mol/L acetate buffer, the peroxidase reaction was developed according to Hsu et al. [9] with nickel intensification of the reaction product [19]. Sections were counterstained with haematoxylin and examined under a transmitted light microscope. Photographs were taken using Agfa Pan 400 film.

Antibodies

Monoclonal antibodies against desmin (DE-U-10, [3]) and vimentin (V9, [15]) were a gift of Prof. M. Osborn (Göttingen), the monoclonal antibodies against α-smooth muscle actin [20], smooth muscle myosin (clone hSM, [5]), h-caldesmon (clone hHCD, [5]), calponin (clone hCP, [5]) and broad-spectrum cytokeratin (clone PCK-26, [14]) were obtained from Sigma (Munich, Germany), the monoclonal antibody against cytokeratins 8, 18, 19 (CAM 5.2, [13]), from Becton Dickinson. The polyclonal antibody against dystrophin was a gift from Dr. T. Sherratt (London) and was used as described previously [18] Negative controls which were performed by omitting first antibodies remained unstained (not shown).

Results

Smooth muscle has been distinguished from striated muscle or non-muscle cells by the expression of a number of characteristic proteins. Although all considered specific for this tissue, the majority is either permanently or transiently expressed also in other cell types [2, 7, 12, 21, 24]. We have therefore used antibodies against contractile (α-smooth muscle actin, smooth muscle myosin), cytoskeletal (cytokeratin, vimentin, desmin), regulatory (h-caldesmon, calponin) and membrane proteins (dystrophin) to monitor individual steps of intestinal maturation. A summary of our results is given in Table 1.

Embryonic development

At seven weeks gestation, the human intestine consists of a thin-walled tube with a simple epithelial lining. Differentiation of mesenchymal cells into smooth muscle can be followed by a ring of α-smooth muscle actin positive cells surrounding mucosa and submucosa (Fig. 1a). The corresponding area in a serial section shows the same set of cells faintly outlined with an antibody to calponin (Fig. 1b). Intermediate filament proteins expressed at this early developmental stage are vimentin (not shown) and traces of cytokeratins (Fig. 1c) which can be observed in a basal location in cells of the mucosa as well as in mesenchymal cells (arrows). One week later, the circular smooth muscle cells express in addition desmin, h-caldesmon (not shown) and smooth muscle myosin (Fig. 1d) besides calponin (Fig. 1e) and α-smooth muscle actin. The muscular layer is outlined with a monoclonal antibody reactive to cytokeratins 8, 18 and 19, although these proteins are now more predominant in the mucosa (Fig. 1f).

Foetal development

At twelve weeks gestation smooth muscle cells of the now divided circular and longitudinal layers also express dystrophin (not shown). Further differentiation of the intestinal wall can be observed at the age of 15 weeks with the first appearance of the muscularis mucosae (mm in Fig. 2d), well demarcated by α-smooth muscle actin positive cells beneath the mucosa. In addition, this early marker protein of smooth muscle clearly outlines the vascular wall of submucosal blood vessels (bv in Fig. 2d) while other smooth muscle-associated proteins such as dystrophin (Fig. 2b), desmin (Fig. 2c), h-caldesmon (Fig. 2e) and calponin (Fig. 2f) are not yet expressed in these structures. All mesenchymal cells express vimentin (Fig. 2a), regardless whether they are differentiating into smooth muscle cells or not. However, at this point only traces of immunoreactivity with an antibody to cytokeratin are still present in smooth muscle of the circular layer (not shown).

The end of the embryonic and the beginning of the foetal period is characterised by a positive reaction to all smooth muscle markers investigated and a clear separation between circular and longitudinal layers by the developing myenteric plexus.

Due to the difficulty in obtaining fetal material between 18 weeks gestation and delivery, we have no information on the temporal appearance of other smooth muscle-associated proteins in muscularis mucosae or blood vessels. However, at early infancy (2-3 months), all smooth muscle

Table 1. Temporal pattern of smooth muscle differentiation in the human intestine.

	α-smooth muscle actin	smooth muscle myosin	h-caldesmon	calponin	dystrophin	desmin	vimentin	cytokeratin
7 weeks	musc. propria	-	-	musc. propria	-	-	all cells except mucosa	musc propria, mucosa
8 weeks	"	musc. propria	musc. propria	"	musc. propria	musc. propria	"	"
12 weeks	"	"	"	"	"	"	"	"
15 weeks	musc. propria musc. mucos., blood vessels	"	"	"	"	"	"	mucosa
18 weeks	"	"	"	"	"	musc. propria musc. mucos., blood vessels	"	"
infant	"	musc. propria musc. mucos. blood vessels	musc. propria musc. mucos. blood vessels	musc. propria musc. mucos. blood vessels	musc. propria musc. mucos. blood vessels	" "	sub mucosa nerve plexus	" "

cells within the intestinal wall express the full range of muscle-associated proteins investigated.

This maturation is further reflected in the distribution of intermediate filaments: Cytokeratin expression is restricted to cells of the mucosa (not shown), whereas desmin (Fig. 3b) has completely replaced vimentin (Fig. 3a) in the muscularis propria and muscularis mucosae. The vascular wall of submucosal blood vessels contains vimentin and desmin-positive cells.

Discussion

Smooth muscle differentiation

During embryogenesis the sequential appearance of smooth muscle-associated proteins indicates a gradual differentiation of mesenchymal cells into a smooth muscle phenotype. In general, specific isoforms of actin and myosin are considered to be early markers of differentiation [5]. At seven weeks gestation, α-smooth muscle actin positive cells surround the submucosa in human intestine. These cells are still morphologically indistinguishable from other mesenchymal cells in the intestinal coat and can only be visualized by the antibody reaction. Unlike cells of vascular origin which synthesize regulatory components such as h-caldesmon and calponin only in late gestation [4, 5], the first cells to become α-smooth muscle actin-positive in the intestinal population were also faintly reactive with an antibody to calponin.

It is interesting to note that the structural maturation of the intestinal wall lags behind the differentiation of the individual smooth muscle cell: at eight weeks gestation when both contractile and regulatory proteins are expressed, the muscularis propria was still undivided.

At the gestational age of 15 weeks, α-smooth muscle actin was again the earliest indicator of the maturing muscularis mucosae and the vascular wall of submucosal blood vessels, whereas smooth muscle myosin, h-calde-

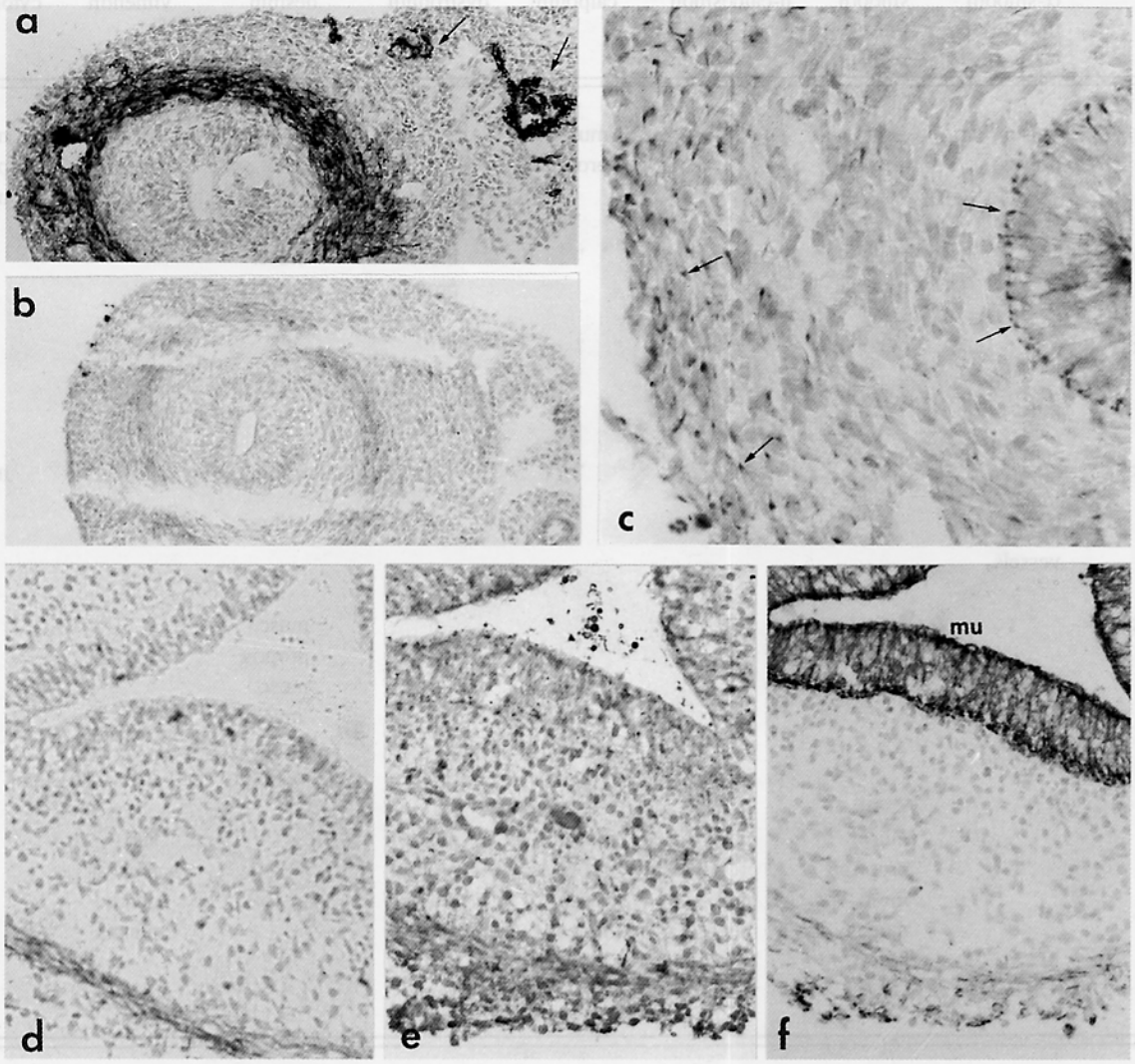

Figure 1. Intestinal smooth muscle differentiation during embryogenesis. Cross sections of human intestine at 7 (a-c) and 8 weeks gestation (d-f). The circular muscle layer is outlined with antibodies to α-smooth muscle actin (a) and calponin (b). α-smooth muscle actin is also present in mesenterial blood vessels (arrows). (c) Cytokeratin is expressed in a punctate pattern in mucosa and circular smooth muscle (arrows). One week later, smooth muscle myosin (d), calponin (e) and traces of cytokeratin can be observed in the circular smooth muscle layer, while in the mucosa (mu) the expression of cytokeratin is upregulated. Magnification a,b x 150, c x 280, d-f x 220.

smon, calponin, dystrophin and desmin are still negative at this stage.

In gut smooth muscle, the presumptive actin regulatory protein calponin [23] precedes the expression of the specific myosin isoform whereas in vascular muscle cells α-actin and myosin are expressed simultaneously [5].

Intermediate filaments

We have investigated intermediate filament expression on tissue sections of human gut, using highly specific antibodies for the different classes of intermediate filaments.

The detection of epithelial markers in smooth muscle cells has been regarded as a sign of immaturity [1, 8]. Between 7 and 15 weeks gestation, we have found cytokeratin co-expressed with vimentin in the muscularis propria of human intestine and with the onset of desmin expression at around 8 weeks [16, this study] the outer muscular layer contained three types of intermediate filaments simultaneously, as has been observed during human myocardial development [11, 17]. The fact that we were unable to detect cytokeratin in the developing muscularis mucosae or in the wall of submucosal blood vessels, suggests that

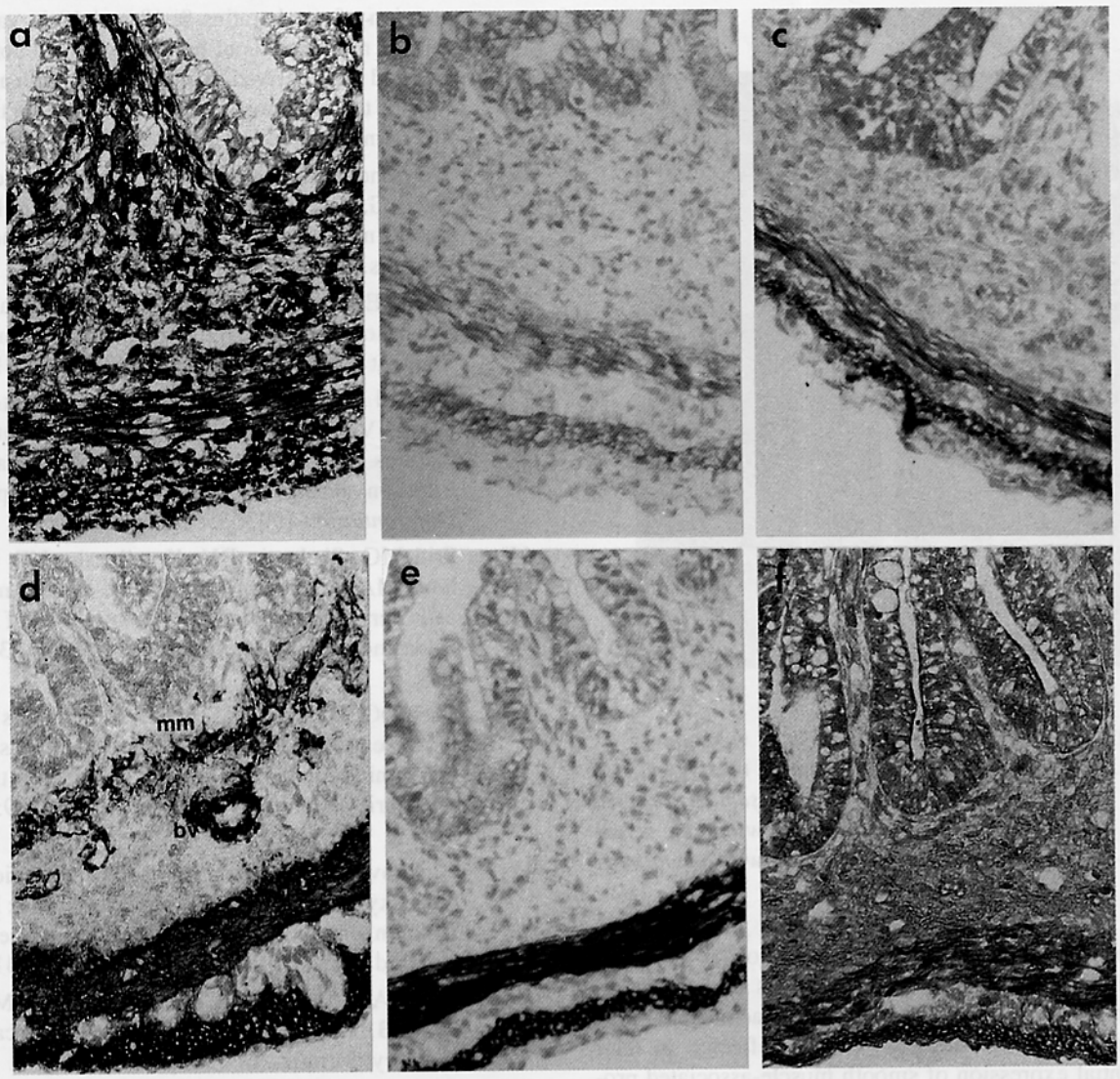

Figure 2. Expression of contractile and cytoskeletal proteins in human intestine of 15 weeks gestation. Vimentin is present in all cells with exception of the mucosa (a), Dystrophin (b), desmin (c), α-smooth muscle actin (d), h-caldesmon (e) and calponin (f) are expressed in smooth muscle cells only. Muscularis mucosae (mm) and submucosal blood vessels (bv) are just starting to differentiate as indicated by their reactivity with an antibody against α-smooth muscle actin (d). Magnification x220.

differentiation into smooth muscle does not necessarily require the transient expression of this intermediate filament type.

In particular in the early stages of gestation, a punctate intracellular pattern of cytokeratin expression can be observed in mucosa and smooth muscle. This distribution is reminiscent of the onset of desmin and titin synthesis during mouse embryogenesis [6,17]. However, only mucosa cells upregulate the expression of cytokeratin (Fig. 1f) whereas in smooth muscle the expression pattern does not change. It is therefore possible that this intermediate filament is never functionally integrated into the smooth muscle cytoskeleton.

Limited tissue availability made it impossible to investigate the gestational interval between 18 and 40 weeks. Consequently, we are unable to comment on the exact time of the switch from vimentin to desmin expression which occurs during this period. The functional significance for this replacement which takes place predominantly in visceral smooth muscle has not yet been established.

We assume that vascular and visceral smooth muscle cells arise from different lineages and do not share com-

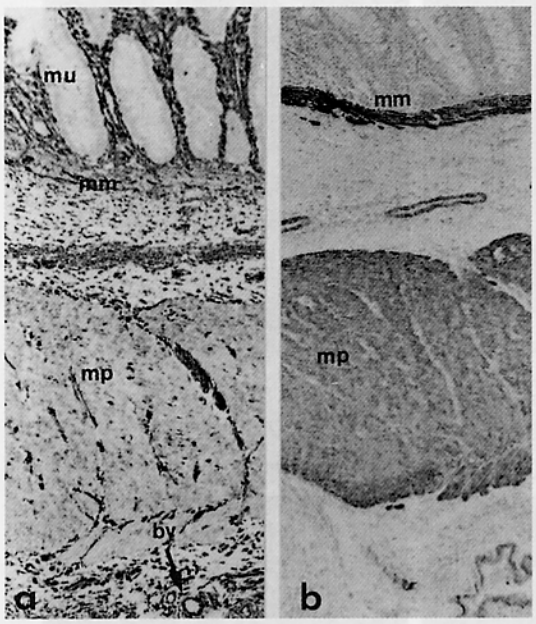

Figure 3. In the mature intestinal wall, vimentin (a) is replaced by desmin (b) in smooth muscle cells of muscularis mucosae (mm) and muscularis propria (mp). The vascular wall of vessels within the myenteric plexus (bv, arrow) is reactive with an antibody to vimentin. Magnification x 150.

mon precursors. Furthermore, the function of a smooth muscle cell is always dependent on its environmental surrounding and - unlike skeletal or cardiac muscle - may show considerable variation. This is reflected in their differential expression of smooth muscle-associated proteins as well as by a distinct pattern of cellular maturation.

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