A quantitative study of collagen production by human smooth muscle cells during intestinal morphogenesis

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Summary

Differentiating mesenchymal cells and the extracellular matrix that these cells produce constitute the structural basis for developing organs. The splanchnopleuric mesenchyme surrounding the developing gut and respiratory tubes provides connective tissue cells to the lamina propria/submucosa and smooth muscle cells to the muscularis musosae/muscularis externa.

In human fetal intestine, the identity of the matrix-producing cell or cells has begun to be elucidated. The smooth muscle cell is one of the sources of collagen fibers in the extracellular matrix in the developing human fetal intestine and collagen production is a significant function of smooth muscle cells during intestinal organogenesis.

The aim of the current study was the quantitative investigation of collagen production by human fetal intestinal smooth muscle cells in various stages of development (10 to 23 weeks of gestational age).

Identification of the mesenchymal cells/extracellular matrix was confirmed by immunohistochemical techniques using the following monoclonal antibodies: actin, desmin, vimentin, collagen IV and fibronectin. Histochemical stains for the presence of extracellular matrix components were also performed.

Immunohistochemical analysis and the results of the histochemistry of the fetal human intestine in various stages of development revealed that the muscle cells of the muscularis externa contribute to the production of collagen in collaboration with the mesenchymal cells. This is more evident between 10 to 14 weeks of gestational age.

Key words: Smooth muscle cells; Collagen type IV; Intestine; Embryology.

Introduction

Embryology

As a result of the cephalocaudal and lateral foldings of the embryo, the endoderm-lined cavity is partially incorporated into the embryo to form the primitive gut. The endoderm of the primitive gut gives rise to most of the epithelium and glands of the digestive tract. The epithelium at the cranial and caudal extremities of the tract is derived from ectoderm of the stomodeum (primordium of the mouth) and proctdeum (anal pit), respectively. The muscular, connective tissue, and other layers of the digestive tract are derived from the splanchnic mesenchyme surrounding the endoderm of the primitive gut. The primordial gut is divided into three parts: foregut, midgut, and hindgut.

Mesenchyme

This is a term first introduced over a century ago by Hertwig [1] as an alternative to mesoblast. The first mesoblast population of the trilaminar disc (termed primary mesenchyme by Hay) [2] derives from epiblast cell ingression at the primitive node and streak. At the end of the fourth week, the sclerotome cells become polymorphous and form a loosely woven tissue known as mesenchyme or embryonic tissue. It is characteristic for the mesenchymal cells to migrate and to differentiate into a variety of stromal cells including muscle and fibroblasts [3].

In humans, collagen is the major constituent of the adult intestinal wall. Graham *et al.* [4] demonstated that in the adult human intestine, collagen production is a major function of smooth muscle cells. Furthermore, it has been hypothesized that the quantity and type of collagen produced by smooth muscle cells are responsible for the compliance of the bowel wall [5].

Recent work has demonstrated, in comparison with the production of collagen by fibroblasts in the skin, that smooth muscle cells produce collagen, which plays an important and critical role in the development of intestinal structure [6, 7].

Intermediate filaments:

1) Actin

Actin is an important component of the cytoskeleton and accounts for about 5% of the total protein load in most cell types. It is a globular protein (G - actin), which polymerizes to form filaments (F - actin) with all the subunits facing in one direction (polar filaments). There are six molecular variants (isoforms) of actin, which have specific distributions in different cell types, for example isoforms restricted to smooth muscle or skeletal muscle.

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2) Desmin (skeletin)

Desmin is found in smooth muscle and in the Z disks of skeletal and cardiac muscle (MW 53,000 - 55,000).

3) Vimentin

Vimentin filaments are characteristic of cells of mesenchymal origin and of embryonic or undifferentiated cells. Vimentin is a single protein (MW 56.000 - 58.000) and may copolymerize with desmin or glial fibrillary acidic protein.

Fibrillar proteins of the extracellular matrix:

1) Collagen IV type

Collagen IV type, which make up a very large proportion (about 30%) of all the proteins of the body, was formerly thought to be a single protein with an amino-acid composition that had been highly conserved in the course of evolution, but improved methods of analysis have led to the discovery of differences in the collagen extracted from various tissues in the body. Collagen IV type is now regarded as a family of closely related, but genetically distinct proteins that share certain features of molecular organization but have a-chains that differ in their amino-acid composition and sequence. Collagens are classified using roman numerals to reflect the chronological order of their discovery. At least 14 types are now genetically characterized and others are being investigated. Collagen IV type is a specialized form largely restricted to the basal lamina of epithelia. Together with laminin and heparan sulfate proteoglycan, it forms a close meshwork of fine filaments that is the physical support of epithelia and a selective filtration barrier for macromolecules.

2) Fibronectin

Fibronectin is a multi-functional glycoprotein and exists in three main forms. These are: a) a circulating plasma protein, b) a protein that transiently attaches to the surface of many cells, c) insoluble fibrils forming part of the extracellular matrix, when fibronectin dimers cross-link to each other by disulphide bonds. The functional importance of fibronectin stems from its ability to adhere to several different tissue components because it possesses sites binding collagen and heparin, as well as cell adhesion molecules. Fibronectin is recognized by fibronectin receptor proteins in cell membranes (integrins), allowing cell adhesion to extracellular matrix.

Materials and Methods

Source of tissues

Specimens of small (jejunum and ileum) and large intestine were obtained from autopsies after legal abortion.The embryos were between 10 and 23 weeks of gestational age. Autopsies were performed a few days after the intrauterine death. Samples of small and large intestine were fixed in 10% formaldehyde and processed routinely in paraffin wax. Tissue blocks were cut in serial sections 5 - 7μm thick and stained with :

1) Haematoxylin-eosin (routine examination) in order to demonstrate the general histologic architecture of the concentric layers of the intestine (mucosa, submucosa, muscularis and serosa).

2) Periodic acid-Schiff (PAS) to demonstrate carbohydrates and carbohydrate-rich macromolecules in the connective tissue.

3) Masson's trichrome to demonstrate the architecture of the muscle layers and collagenous matrix.

4) Verhoeff-van Giesson to demonstrate the architecture of the elastic fibers

5) Immunohistochemical stains to demonstrate:

a) The intermediate filamentous cytoskeletal proteins of the muscle cells, i.e. actin and desmin.

b) The interme diate filamentous cytoskeletal protein of the fibroblast, i.e. vimentin.

c) The fibrillar proteins of the extracellular matrix, i.e. collagen type IV and fibronectin.

Results

Immunohistochemical staining of cells for cytoskeletal proteins (actin, desmin and vimentin)

The immunohistochemical study for the presence of muscle-specific filament actin in the cells of the layers of the intestines, mesentery and mesocolon showed at 10 to 14 weeks gestational age, a strong positivity in: 1) Spindle mesenchymal cells and adipocytes of the mesentery and mesocolon and muscle cells of the middle layers of the vessels. No staining was seen in mesothelial and

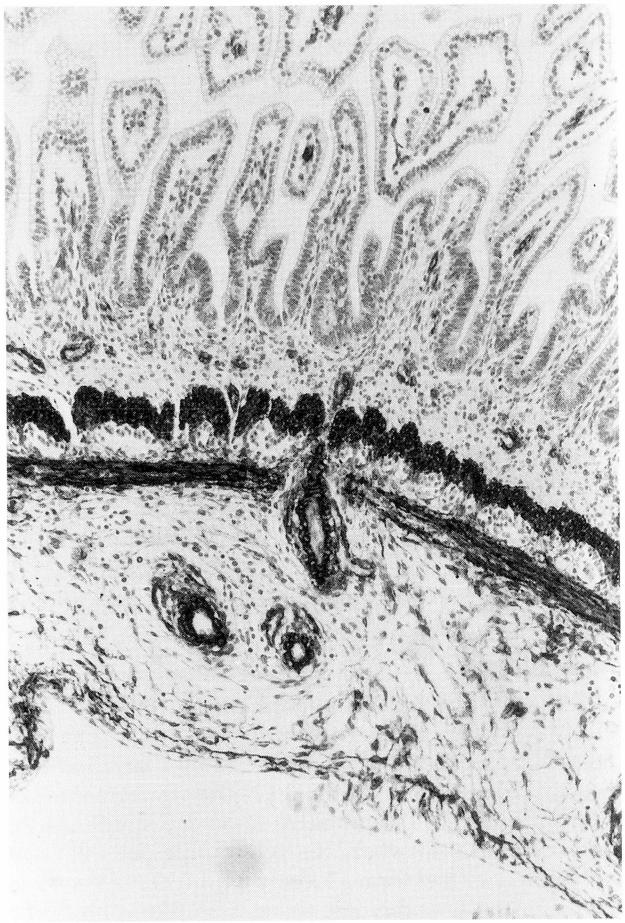

Figure 1. — Immunostaining for actin in the muscle cells of the muscularis externa of the intestine.

endothelial lining cells. 2) Spindle mesenchymal cells of the serosa. 3) Muscle cells of the muscularis externa (inner circular and outer longitudinal layers) (Figure 1). A weakly positive staining was seen in: 1) Spindle cells of submucosa adjacent to the inner circular layer of the muscularis externa. 2) Muscle cells of the mucosa muscularis. 3) Muscle cells of the middle layer of the vessels in the submucosa and lamina propria of the mucosa. At 14 weeks of gestation no positive reaction with actin in the cells of the mesentery and serosa was noted. At 18 to 23 weeks gestational age a weakly positive reaction with actin in the cells of the mesentery and a positive reaction with actin in the other layers of the intestine was noted similar to the one observed at the tenth week of gestation.

The immunohistochemical control for the presence of desmin was negative except in two cases (19 and 23 weeks gestational age respectively) where a positive staining was identified in the epithelial lining cells of the villi.

The immunohistochemical analysis for the protein of mesenchymal origin tissues, vimentin, showed a positive staining in the basement membranes of the blood vessels in the submucosa of the intestine and mesentery, at the tenth week of gestation. No staining was seen in the other layers of the intestine. Vimentin was also negative in the intestines in the subsequent periods.

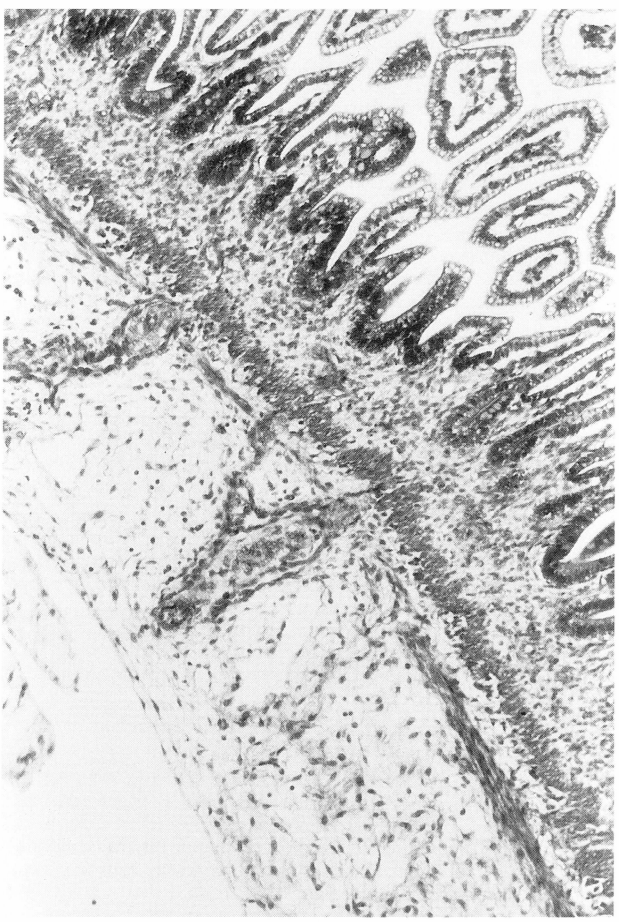

Figure 2. — Immunostaining for fibronectin in the spindle cells and adipocytes of the mesentery and mesocolon, serosa, submucosa and lamina propria of the mucosa of the intestine.

Immunohistochemical staining for extracellular matrix components (fibronectin and collagen IV type)

In all our cases the immunohistochemical analysis for fibronectin showed a strong positive staining in the spindle cells and adipocytes of mesentery and mesocolon, serosa, submucosa and lamina propria of the mucosa of the intestines (Figure 2). A weak staining for fibronectin was also seen in the muscle cells of the inner circular layer of the muscularis externa at the fetal intestine of ten weeks of gestational age.

Immunohistochemical control for collagen IV type revealed a weakly positive staining in the epithelial lining cells of the villi, in one case (19th week of gestational age).

Histochemical stainings

Sections of fetal human intestine, in various stages of development, were stained with haematoxylin-eosin, PAS, Masson's trichrome and Verhoeff-van Giesson, in order to demonstrate the complete architecture of the concentric layers of the organ and mainly the muscle layers (muscularis externa, muscularis mucosae), and the extracellular matrix components between them.

At ten weeks of gestation the muscularis externa showed a well-defined circular layer and a longitudinal layer containing only a few, well-oriented cells. The muscularis mucosae contained only a few cells. Staining for the extracellular matrix components showed a weak positive reaction in the mesentery, serosa adjacent to the longitudinal layer, submucosa and lamina propria of the mucosa.

At 12 weeks' gestation all the muscle layers were evident and well-formed, and staining for collagen revealed a strong reaction: 1) In the mesentery, in the spindle (mesenchymal) cells and especially those around the blood vessels. 2) In the serosa adjacent to the longitudinal muscle cells. 3) In the submucosa adjacent to the circular muscle layer. A weak positive staining for collagen was seen in the lamina propria of the mucosa.

Discussion

The fetal intestinal wall develops from a primitive mesenchyme into discrete layers of differentiated smooth muscle and collagenous matrix. Models have been developed by several investigators, in order to demonstrate that fetal human intestinal smooth muscle cells synthesize collagen [8, 9].

Human fetal intestinal smooth muscle cells are important in intestinal morphogenesis because of the multiple influence of the matrix on cell behavior. The matrix is well recognized as a scaffold upon which an organ develops. However, the components of this matrix, such as collagen, influence cell behavior and thus, morphogenesis, by serving as a reservoir for growth factors [10, 11], and by affecting cell attachment, cell migration [12, 13,

14, 15], cell proliferation [16], and cell differentiation [12, 16, 17]. These effects on cell differentiation have been observed in gastrointestinal epithelial cells. Coculture of human fetal mesenchyme derived from gastric or intestinal tissue with chicken or rat endoderm, induced endodermal differentiation to the organ of human mesenchymal origin [18, 19, 20, 21, 22]. The effect of intestinal matrix on the myogenesis of intestinal smooth muscle is not clear, but a relationship between collagen production and myogenesis in skeletal muscle has been shown. Studies of fetal rat and embryonic chicks demonstrated the differentiation of skeletal myoblasts into myotubules in response to collagen are associated with an increase in endogenous collagens and their associated mRNA's [23, 24]. Therefore, a relationship exists between changes in collagen synthesis and skeletal muscle cell differentiation in development. This relationship may similarly occur in intestinal smooth muscle cell differentiation.

These studies demonstrate that human smooth muscle cells isolated from fetal intestine synthesize collagen in culture, suggesting a significant role of collagen production by these cells during intestinal morphogenesis. The amount of collagen produced is influenced by fetal age, cell density and contact during growth, and the mesenchymal cell type. Collagen production by fetal intestinal smooth muscles is important in morphogenesis not only because collagen is a major structural constituent of the developing bowel wall, but because of the potential influence of collagen on the ultimate differentiation of both the epithelial and muscle layers. An understanding of the cellular events regulating human intestinal smooth muscle cell collagen production and cell differentiation would contribute to an understanding of normal and abnormal development of intestinal structure and motility.

The immunohistochemical analysis for the presence of actin at ten weeks of gestation reveals a strong positivity in the muscle cells of the muscularis externa and middle layers of the vessels, as well as in the spindle mesenchymal cells of the mesentery and mesocolon, and cells around the vessels. Furthermore, a positive reaction with actin is seen in spindle mesenchymal cells of the serosa and submucosa adjacent to the inner circular layer of the muscularis externa. A weak positive immunoreaction is seen at the subsequent periods of development (18 to 23 weeks' gestational age). No staining reaction with desmin is seen in the muscle cells and adjacent tissues. The immunostaining for vimentin shows a weak positive reaction in the basement membranes of the vessels at the mesentery and submucosa. Fibronectin, at ten weeks of gestational age is strongly expressed by the mesenchymal cells in mesentery, mesocolon, lamina propria of the mucosa and submucosa, but it is weakly expressed by the muscle cells of the muscularis externa.

These immunohistochemical results, in relation with the histochemical stainings especially with Masson's trichrome, reveal that collagen is a major component of the human fetal intestinal wall and that the submucosal, serosal, mesenterial and mesocolon distribution of collagen varies as the intestinal muscle layers develop. The immunohistochemical expression of actin and fibronectin especially adjacent to the externa muscle layer, suggests that the muscle cells could be a source of collagen in collaboration with the mesenchymal cells. This is more evident between 10 to 14 weeks of gestational age.

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