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Dynamics Of Differentiation Of Chromogranin A-Immunoreactive Endocrine Cells And Myenteric Plexus Of The Human Fetal Duodenum In The Third And The Fifth Month Of Development

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SUMMARY

The aim of the paper was to examine the neuroendocrine differentiation of endocrine cells and neurons of myenteric plexus of fetal duodenum.

The material consisted of duodenums of 14 fetuses of different gender, in the third and fifth month of gestational age. Fetal duodenal tissue was fixed in the Bouin solution, routinely processed and embedded in paraffin blocks, from which were made the sections stained with HE and PAP immunostaining method for identification of chromogranin A (Cg A), marker of neuroendocrine differentiation. The number of endocrine cells was quantified by determining the numerical areal density, while the morphological development of myenteric plexus was determined by measuring its area.

There is a large number of Cg A-immunoreactive endocrine cells in the mucosis of duodenum in the third and fifth month of development. These cells show a high degree of polymorphism in shape, size and immunoreactivity. No significant difference was observed in the number of cells in the epithelium of duodenum in the third and the fifth month of development. At the same time, myenteric plexus was significantly more developed in the duodenum in the fifth month of fetal development compared to the third month.

The results indicate that the differentiation of Cg A-immunoreactive cells occurs earlier in the epithelium of the duodenum than in neurons of myenteric plexus.

Key words: **fetus, duodenum, chromogranin A, endocrine cells, myenteric plexus**

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INTRODUCTION

Numerous factors that include hormones and neurotransmitters, secreted by the endocrine cells and neurons of enteric nervous system (ENS) regulate secretion, blood flow, motility and differentiation of the digestive tract. The products of secretion of endocrine cells and neurons are peptides, amino acids, biogenic amines, purines or gases. The proteins found in the secretory granules and cytosol, common to several types of epithelial endocrine cells or neurons, are used as universal markers of their neuroendocrine differentiation. One of them is chromogranin A (acid soluble glycoprotein, vesicular marker) (1-3). Several types of endocrine cells (P/D1, EC, D, L, G, CCK) are identified in the surface and glandular epithelium of duodenum (4-6).

ENS consists of the neurons and glial cells that are present in the wall of the digestive tube. ENS is a complex network of different morphological and functional types of neurons which, although under the control of the central nervous system (CNS), can function independently. Neurons of ENS are classified into seven types based on their morphological characteristics, while there are much more types of these cells based on their function.

The myenteric plexus (MP), localized between the inner circular and outer longitudinal layers of the muscularis externa, and submucosal plexus, localized in submucosis of the digestive tube are the main components of ENS. ENS, as the most complex part of the autonomic nervous system, is composed of a large number of ganglia in the form of nodules which are connected by internodal bundles that represent the grouped extensions of the nerve cells (7). Ganglia of ENS are spaced evenly in the myenteric and submucosal plexus. While myenteric ganglia are located in all parts of gastrointestinal tract (GIT), submucosal ganglia are not present in the esophagus and stomach.

Duodenum develops in the fourth week from the anterior gut. Epithelial and glandular structures arise from endodermal epithelium of the anterior gut while connective tissue and muscular structures originate from splanchnopleural mesoderm that surrounds the anterior gut. Submucosal and myenteric ganglia originate from the neural crest (8, 9).

Endocrine cells of duodenum occur as a result of differentiation of multipotent stem cells localized in the duodenal epithelium. The process of neuroendocrine differentiation in the duodenum is related to the period from the 8th to the 12th week of development. However, in the literature there are often wide variations in the time of occurrence of certain cell types (10).

ENS originates from the vagal portion of neural crest whose cells migrate in the cranial part of the primitive gut, extending to the caudal direction. The cells of the sacral segments of neural crest also participate in the formation of ENS of the posterior gut. Inductive factors that react with tyrosine - kinaze receptors play important role in the regulation of migration and differentiation of neuroblasts in the intestines (7, 11-14).

AIM

The aim of this paper was to determine and compare the dynamics of duodenal neuroendocrine differentiation in two periods of intense morphofunctional differentiation, in the 3rd and the 5th month of development. Morphological characteristics of fetal duodenums, the numerical areal density of Cg A-imuno-reactive endocrine cells in the surface epithelium and the area and the areal fraction of MP were analyzed, based on the presence of Cg A.

MATERIAL AND METHODS

The material consisted of 14 duodenums taken from fetuses of different sex and age (6 in the 3rd and 8 in the 5th month of development). Fetuses were obtained legally at the Clinic of Gynecology and Obstetrics of the Clinical Center Niš, after artificial abortions or spontaneous miscarriages. Age of the fetus was determined by taking the anamnestic data, ultrasound examination and by determination of crown - rump length. Local ethical committee permission was given for the study as well as patient/parent or guardian's consent.

The duodenums were fixed in the Bouin solution, routinely processed and embedded in paraffin blocks. The 5 μm thick tissue slices were stained by the hematoxilin - eosin method and immunohystochemically by using PAP method and Cg A monoclonal antibody (Anti-Human Chromogranin A Dako, M0869, 1:100). The immunohistochemical staining was performed on positive and negative control (pancreas of rat). Endocrine cells were quantified by determining the numerical areal density ie. number of cells per mm^2 of tissue. The areal fraction of MP was determined by measuring the area of MP and the area of the whole muscularis externa (per μm^2 of tissue), and by dividing these two values and multiplying the result with 100 in order to obtain the percentage share ratio of MP within the muscularis externa.

The digital pictures were taken on the Olympus BX50 microscope using a camera Olympus PM-C35 at 100x magnifications for the determination of numerical areal density of Cg A-immunoreactive cells and 200x for the analysis of area of MP. The whole mucosis and muscularis externa from the tissue sections were analyzed. Counting was done using the software "Image J", and by using the plugin "Point picker".

For statistical analysis, the Student's t-test for large independent samples was used.

RESULTS

Histological characteristics of the duodenum

Duodenal wall in the 3rd month of development is still not fully differentiated. Intestinal villi are developed and covered by simple columnar epithelium. Duodenal glands as well as intestinal glands are not differentiated. Muscularis mucosae is not present, and the connective tissue of the lamina propria and submucosis are unique. Muscularis externa is very thin but already differentiated into inner circular and outer longitudinal layer. Subserosis is extremely narrow and is covered by peritoneum.

In the 5th month of development, duodenal wall shows a greater degree of layer differentiation. The size of duodenum is slightly bigger, duodenal villi are elongated, wider in their apical portion. The folds of the submucosis are more pronounced, but the duodenal and intestinal glands as well as the muscularis mucosae cannot be observed. Muscularis externa is much thicker with differentiated inner circular and outer longitudinal layer, between which clearly evident MP is present.

Chromogranin A-immunoreactivity

Endocrine cells. In the 3rd month of development Cg A - immunoreactive endocrine cells are present in the duodenal epithelium. Endocrine cells are single or in small groups, primarily in the basal parts of intestinal villi (intervillous region), but also can be seen in the middle and apical segments of the duodenal villi epithelium (Figure 1). They show pronounced polymorphism in shape, size and degree of immunoreactivity (Figure 2). Most of these cells have elongated or triangular shape, with a wide base and thin supranuclear region whose apex reaches the surface of the epithelium (open type cells). Minority of these cells are small, located close to the basement membrane and do not reach the surface of the epithelium (closed type cells). Chromogranin A-immunoreactivity is relatively well defined and intense in the basal parts of cells.

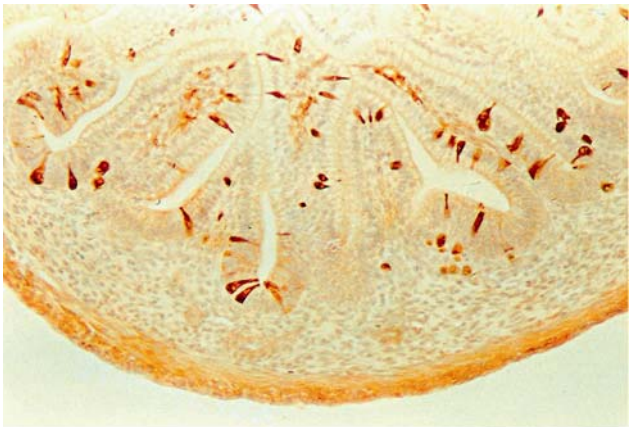

Figure 1. *Cg A-immunoreactive endocrine cells (brown colored) in the duodenum of the fetus in the 3rd month of development; PAP, X200*

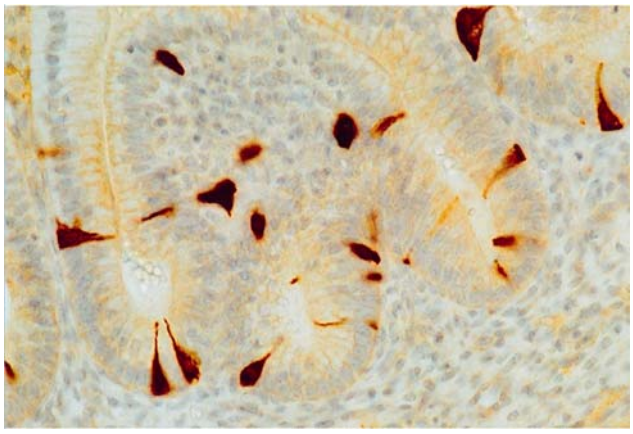

Figure 2. *Polymorhism of Cg A-immunoreactive endocrine cells in the basis of the intestinal villi of fetal duodenum in the 3rd month of development; PAP, X400*

In the 5th month of development Cg A-immunoreactive endocrine cells of duodenum are relatively numerous and show similar morphological features and immunostaining reaction (Figure 3).

Myenteric plexus. In the 3rd month of development Cg A-immunoreactivity is very weak and observed in limited, irregularly oval areas, between layers of muscularis externa. The immunoreactivity is present in rare gaglionic cells and nerve fibers that surround them. The cells and fibers showed weak immunoreactivity (Figure 4).

In the 5th month of development, Cg A-immunoreactivity is significantly more pronounced in the components of the MP, while sporadically present in submucosal plexus (Figure 5). Myenteric plexus is in the form of linear continuous fields (Figure 5), or clearly limited groups (Figure 6) that contain ganglionic cells and nerve fibers. In some cases, Cg A-immunoreactivity is more intense in ganglionic cells in peripheral parts of MP, while sometimes it is evenly distributed in all ganglionic cells of MP (Figure 6). Nerve fibers show a characteristic morphology, they are thin, convoluted, and show the presence of the thicknesses along their wall.

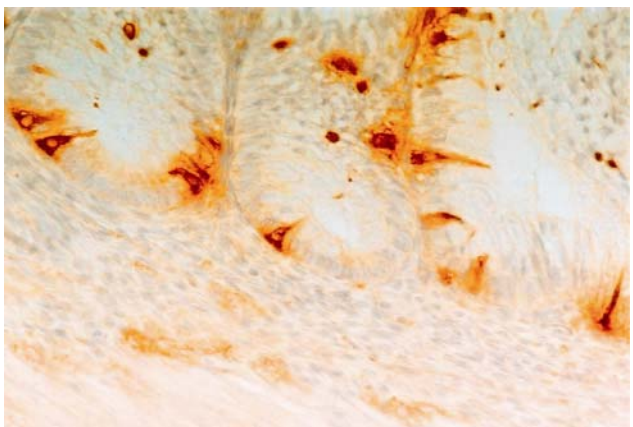

Figure 3. *Cg A-immunoreactivity in epithelial endocrine cells of fetal duodenum in the 5th month of development; PAP, X400*

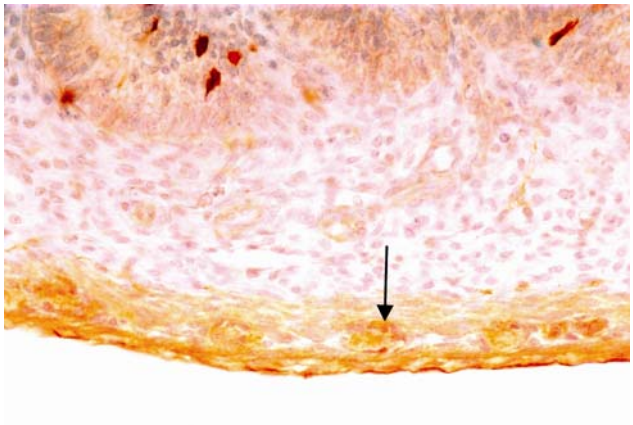

Figure 4. *Cg A-immunoreactivity in myenteric plexus (arrow) of the fetal duodenum in the 3rd month of development; PAP, X400*

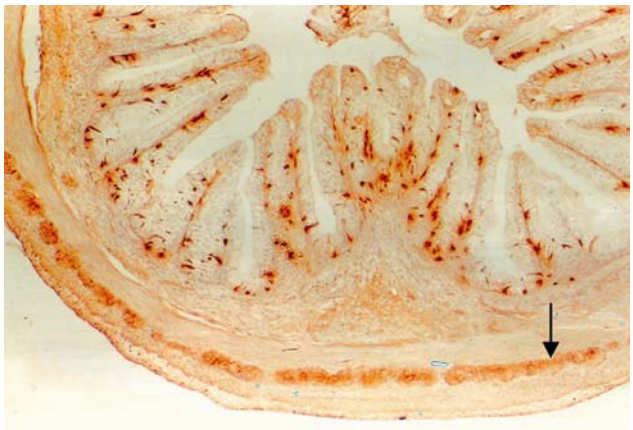

Figure 5. *Cg A-immunoreactivity in a well-pronounced myenteric plexus, in form of continuous fields (arrow), of the fetal duodenum in the 5th month of development; PAP, X100*

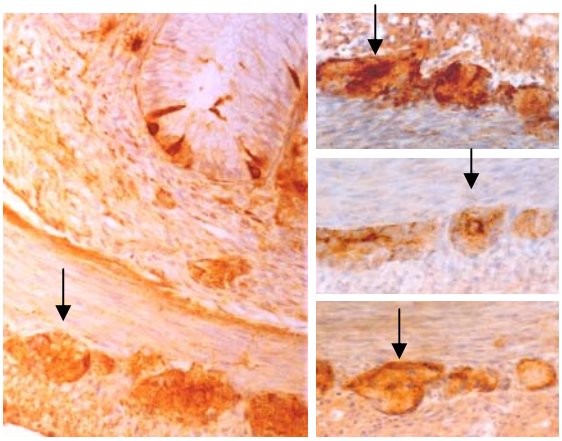

Figure 6. *Cg A-immunoreactivity of different localization and intensity in ganglionic cells of the myenteric plexus (arrow) in fetal duodenum in the 5th month of development; PAP, X400*

Results of quantification

In the 3rd month of development, the average value for numerical areal density of CgA-immunoreactive endocrine cells is 236.81 cells per mm². In the 5th month of development, the average value for numerical areal density of CgA-immunoreactive cells is 264.57 cells per mm². The comparison of the values for numerical areal density of Cg A-immunoreactive fetal endocrine cells in the mucosis of duodenum in the 3rd and 5th month of development did not show statistically significant difference (p = 0.85) (Figure 7).

The average area of MP in the third month of development is 8531.75 μm^2, and represents 15.24% of the total area of muscularis externa. In the fifth month, the average value for area of MP is 61367.12 μm^2, and represents 37.93% of the muscularis externa. The comparison of the values for areal fraction of MP in the third and the fifth month of development showed statistically significant difference (p=0.0001) (Figure 8).

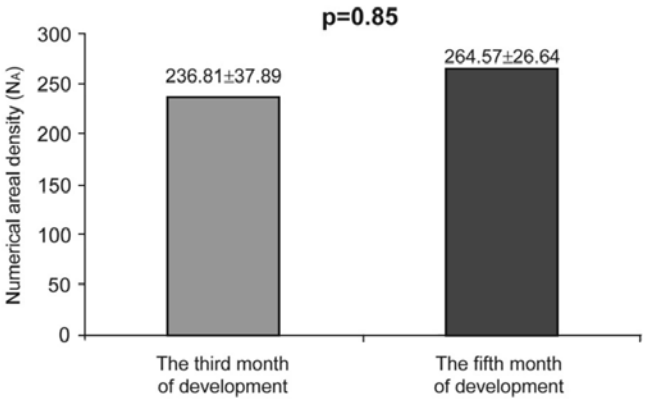

Figure 7. *Numerical areal density (N_A) Cg A-immunoreactive cells in the 3rd and the 5th month of development*

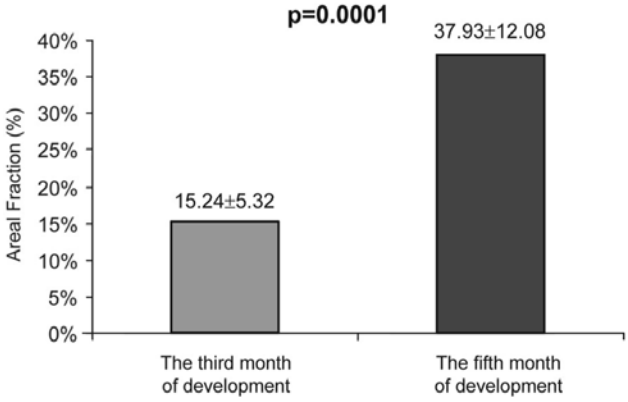

Figure 8. *Areal fraction of the myenteric plexus within the muscularis externa of the fetal duodenums in the 3rd and the 5th month of development*

DISCUSSION

Results of our research showed that the Cg A-immunoreactive endocrine cells are numerous in the epithelium of duodenum of human fetuses in the third and the fifth month of development. Facer et al. reported the Cg A-immunoreactivity in epithelial endocrine cells of the gastrointestinal tract (GIT) at the end of the 2nd month of development, with the significant increase in number of these cells from the 10th to the 22nd week of development (15). The same author showed that the number of Cg A-immuno-reactive cells increases proportionally with the growth of the gut (15). Our results, however, did not show statistically significant difference in the number of Cg A-immunoreactive endocrine cells in duodenal mucosis in the 3rd and the 5th month of development, regardless of the growth of duodenum. Even more, we obtained a low value of standard deviation inside the groups, which speaks in favor of homogeneity of the sample. Our results indicate that the majority of endocrine cells differentiate in the duodenum at the beginning of the 3rd month of the development.

Facer and al. have reported that Cg A-immunoreactive endocrine cells in duodenum start presenting themselves from the 8th to the 12th week of development (15). Such variations in time of occurrence of Cg A-immunoreactive cells could be explained by different approaches in determining the age of the fetuses.

Great presence of endocrine epithelial cells in the duodenum from the 3rd month of development coincides with the establishment of the functions in GIT; the formation of intestinal glands begins during the period from the 10th to 14th week, while the active transport of amino acids as well as glucose and absorption of fatty acids in enterocytes begins in the period from the 14th to the 24th week of gestation (10).

Our results demonstrated a high degree of polymorphism of Cg A-immunoreactive endocrine cells. Similar findings have been reported by Moxey et al (6). They have identified 13 different types of endocrine cells in the small intestine in the 12th week of development, using the electronic microscope. These authors have described the existence of precursors and transition cell forms, which are present only during neuroendocrine differentiation. Heterogeneity in the intensity of Cg A-immunoreactivity of endocrine cells of duodenum points out the existence of different morphofunctional types of cells, and to different degree of differentiation of the same cell types. Cetin et al. have reported that EC cells of the intestine show strong Cg A - immunoreactivity, while the D, CCK and S cells have weak positivity, and that Mo cells almost never show positive Cg A-immunoreactivity (3).

The differentiation of endocrine cells of GIT occurs at the end of the 2nd month of development. G cells differentiate from the 8th to the 14th week, I, D and EC cells from the 10th to the 11th week, and S and D1 cells from the 9th to the 10th week of development (1, 6, 15, 16). The active substances present in the secretory granules of endocrine cells (gastrin, somatostatin, cholecistokinin, motilin, etc.) are shown to have important role in the processes of growth and differentiation of gastrointestinal structures. Cg A is present in all duodenal endocrine cells and it is believed that Cg A could have positive effect on the growth and differentiation of gastrointestinal system (6).

A large number of ENS neurons, with its numerous neurotransmitters, have very important functions such as: regulation of motility in GIT, secretion of epithelial cells (including endocrine cells), regulation of vascular permeability, as well as in immune response. In addition, the crucial role of ENS in development of GIT lies in the fact that some diseases such as Hirsch-sprung's disease or neuronal intestinal dysplasia type B may occur as a result of inadequate ENS development (7).

Our research showed that the CgA-immunoreactive components of ENS are poorly developed in the duodenum in the 3rd month of development. On the other hand, in the 5th month of development, between the inner circular and outer longitudinal layer of muscularis externa, there is expansion of ENS components, with CgA-immunoreactive cells and nerve fibers. During this period, ganglia begin to have the characteristics of ganglia in the adult duodenum. They are distributed in the form of nodules that are connected by intermodal bundles of nerve fibers.

Fekete et al. gave detailed information on ENS development (7). They reported that the nerve cells from vagal and sacral region of the neural crest reach parts of the gut wall trough the process of anterioposterior migration. In this process interaction goes trough the signaling molecules of the immature neurons on the mesenchymal cells of the gut wall and vice versa. Functional differentiation of ENS is different for different functional types of neurons. Most neurons morphologically and functionally become mature in the period from the 10th to the 18th week of development. The intestinal peristaltic starts about the 12th week of development (7), which is in accordance with our results that show a remarkable increase in the area of myenteric plexus in relation to the area of muscularis externa. Our finding is partially in accordance with the results of Bagyánszkie who performed the morphometric analysis of intestine of human fetuses that were 12 to 18 weeks old (11). She found a correlation between the thickness of inner circular layer of the muscularis externa and myenteric plexus (11).

Future studies, by using specific antibodies and extensive stereological analysis, could give a better insight in ENS development.

CONCLUSION

The quantification of the number of endocrine cells and measuring the area of MP of human fetal duodenum in the 3rd and 5th month of development

showed that there is a difference in their neuroendocrine differentiation. There is statistically significant difference between the values for area and areal fraction of myenteric plexus between the groups. The area of MP is significantly higher in the duodenum in the 5th than in the 3rd month of development. There is not a statistically significant difference between the values for numerical areal density for CgA-immunoreactive endocrine cells among the examined groups.

Acknowledgements

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DINAMIKA DIFERENCIJACIJE HROMOGRANIN A-IMUNOREAKTIVNIH ENDOKRINIH ĆELIJA I MIJENTERIČNOG PLEKSUSA DUODENUMA HUMANOG FETUSA U TREĆEM I PETOM MESECU RAZVIĆA

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Sažetak

U radu je ispitivana neuroendokrina diferencijacija endokrinih ćelija i neurona mijenteričnog pleksusa u fetusnom duodenumu.

Materijal je činilo 14 duodenuma fetusa različitog pola, u 3. i 5. mesecu gestacione starosti. Duodenumi fetusa su fiksirani u Buenu i rutinski obrađeni do parafinskih kalupa. Od kalupa su pravljeni preseci, bojeni HE metodom i PAP imunohistohemijskom metodom za identifikaciju hromogranina A (Cg A), markera neuroendokrine diferencijacije. Broj endokrinih ćelija određivan je izračunavanjem numeričke arealne gustine, dok je morfološka razvijenost mijenteričnog pleksusa određivana merenjem njegovog areala.

Pokazano je da je veliki broj endokrinih ćelija prisutan u mukozi duodenuma u 3. i 5. mesecu razvića. Ove ćelije pokazuju visok stepen polimorfizma oblika, veličine i imunoreaktivnosti. Nije pronađena statistički značajna razlika u njihovoj zastupljenosti u epitelu duodenuma u 3. i 5. mesecu razvića. Istovremeno, mijenterični pleksus je znatno razvijeniji u duodenumu u 5. nego u 3. mesecu razvića.

Rezultati ukazuju da se diferencijacija CgA-imunoreaktivnih ćelija završava ranije u epitelu duodenuma, u odnosu na neurone mijenteričnog pleksusa.

Ključne reči: **fetus, duodenum, hromogranin A, endokrine ćelije, mijenterični pleksus**