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GRADUAL DISAPPEARANCE OF VACUOLATED ENTEROCYTES IN THE SMALL INTESTINE OF NEONATAL PIGLETS

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The unique feature of enterocytes in newborn mammals is the presence of an apical canalicular system (ACS) leading to production of large vacuoles, important for colostral macromolecule uptake. The vacuolated fetal-type enterocytes (VFE) enable transfer of colostral and milk proteins from the intestinal lumen across the epithelium without loosing their biological activity. First VFE are observed in the pig and lamb fetuses in the second trimester of pregnancy, located at the upper part of villi in the proximal region of the fetal small intestine and subsequently in the middle and distal regions. After birth the VFE are replaced with enterocytes lacking ACS. The present study aimed to investigate the depletion of VFE in the small intestine in the sow reared pig neonates during the first postnatal weeks using scanning electron microscopy (SEM). The SEM analysis demonstrated the gradual disappearance of vacuolated enterocytes in time. VFE remained in the jejunum for a few days after birth, whereas in the duodenum single VFE were present only at birth. In the proximal jejunum, the VFE were localized in the upper part of the villi, and disappeared until the day 3 of life. VFE were present in the mid and distal jejunum, and diminished gradually until day 14 of life. By the day 21 of life, the vacuolated cells were not observed neither in the jejunum nor ileum. In conclusion, morphology analysis of pig small intestinal mucosa suggests that replacement of fetal type vacuolated enterocytes is resumed within 21 days after birth.

Key words: *apical canalicular system, development, scanning electron microscopy, colostrum*

INTRODUCTION

Vacuolated fetal-type enterocytes (VFE) are observed in pig and lamb fetuses from the second trimester of pregnancy (1, 2). VFE appeared firstly in the upper part of villi in the proximal small intestine, and expanded downward with time on the mid and distal jejunum as well as ileum. The unique feature of VFE is the occurrence of cytoplasmic vacuoles of various size which constitute the apical canalicular system, ACS (1). VFE precede the occurrence of adult-type enterocytes in mammals (including humans), and show a high level of similarity of structural appearance across the species (1, 3). The habitual features of VFE is the craft ability to transport intact proteins from the gut lumen across the epithelium into circulation (VFE producing transport vacuoles) or to digest the gut content inside the cell (VFE containing digestive vacuoles) (3). The VFE producing transport vacuoles appear after the first colostrum feeding and play crucial role in the uptake of colostral macromolecules. In pigs these enterocytes are observed only during the first 2-3 days of postnatal life. The VFE producing transport vacuoles account for about 2% of intestinal population of enterocytes. On the other hand, the VFE which produce digestive vacuoles are present in the lower part of the small intestine. Their role is to support the digestion of milk. These enterocytes disappear gradually from proximal, mid and distal jejunum to the ileum. In porcine ileum, VFE with digestive vacuoles are present up to 3-4 w of life. Disappearance of vacuolated enterocytes in the small intestine is considered a good marker of intestinal maturation (4). Mammalian adult type enterocytes have neither the capacity to transport whole proteins to any significant degree nor to digest the intestinal content macromolecules inside the cell. The only mature epithelial cells having the capacity to sample gut content, like transport VFE, are the M cells overlying the Peyer's patches (2).

The presence of endocytic network under the apical cell membrane allows to recognize the fetal type enterocytes. Transmission electron microscopy studies revealed that the large vacuoles origin from small endocytic vesicles that appear during invagination of the cell membrane into the cell interior (2). The small endocytic vesicles are part of the tubulo-vesicular network (ACS) closely associated with invagination of the luminal plasma membrane (2). However, Godlewski *et al*. (5, 6) have demonstrated that MAP I LC3, a protein associated with formation of autophagosome membranes in cells dying *via* autophagy (type II of programmed cell death), is abundantly expressed in the vesicle membranes constituting the ACS. On the other hand, MAP I LC3 was not expressed in the cell membranes suggesting that the ACS vesicle membranes may not originate from the cell membrane. Nonetheless, the ACS vesicles fuse together in small vacuoles, cluster in larger and effectively arisen large vacuoles, often filling the majority of enterocyte volume. The vacuole contents is of uneven density. The features of VFE retain the primitive enterocytes characterized by the presence of long microvilli covered with glycocalyx,

extensive terminal web, large number of mitochondria, prominent golgi apparatus, increase in GERL (Golgi Endoplasmatic Reticulum Lysosom) and coated vesicles in lateral and basal regions.

The aim of present study was to investigate the VFE in the small intestinal mucosa of pig neonates using 3-dimensional images acquired with the scanning electron microscopy.

MATERIAL AND METHODS

The experimental protocol was approved by the Local Ethical Committee. Studies were carried out on 10 piglets, born on time, housed in standard farming conditions. The piglets were kept with their sow from birth up to 21 days of life. During the suckling period piglets received small amounts of prestarter solid food. The animals were sacrificed just after birth (day 0, unsuckling neonates, n=2), at the day 3 (n=2), 7 (n=2), 14 (n=2) and 21 (n=2) after birth by pentobarbiturate overdose (50 mg/kg b wt. i.p.). The gastrointestinal tract tissue were immediately removed, and the samples of small intestine were collected. Whole-thickness samples of the duodenum, proximal (25%), middle (50%) and distal (75%) jejunum and ileum were harvested and fixed in Bouin solution for optical microscopy. Serial histological sections of 5-μm thickness were cut and stained with haematoxylin and eosin. For visualization of mucopolysaccharide content in the goblet cells the slides were stained with alcian blue (stains acid and neutral mucopolysaccharides on blue and red, respectively) and PAS (Periodic Acid Shiff - stains neutral mucopolysaccharides on red-violet).

For scanning electron microscopy (SEM) 2-3 cm long whole tissue segment were taken from the duodenum 5 cm distal from pylorus, jejunum (proximal - 25%, mid - 50% and distal - 75% of the jejunum length), and ileum 5 cm proximal to ileo-cecal valve. For SEM the samples were rinsed with ice cold saline (0.9% NaCl), and then cut into square-fragments (1.5x1.5 cm) which were placed on a metal grid and washed in cold physiological saline for 1 hour. The tissues were then fixed in 10% buffered formaldehyde. The fixation time depended on the thickness of the samples and varied between 24 and 48 hours. After fixation the apical parts of the villi were removed to disclose the interior of the enterocytes and samples were washed four times in saline solution and dehydrated in alcohol. After drying in a Critical Point Drier (Polaron Range, CPD 7501), the samples were sputter coated (Polaron Range S.C. 7620 Sputter Coater) with 30 nm layer of gold palladium (Au/Pd) and examined using LEO 1430 VP scanning electron microscope at an accelerating voltage of 15 kV (7).

Minimum twenty measurements were made for each specimen. Data were calculated as means ± SEM. A one-way variance analysis followed by a Tukey's post-test was performed (GraphPad Prism, Graph Pad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Fig. 1a shows SEM micrograph of a fractured epithelial cell lining with revealed cell interior. Comparison of the micrograph in *Fig. 1a* with optical microscopy (OM) micrographs (*Fig. 1b-d*) allows to differentiate the vacuolated enterocytes from goblet cells, and identify them as the fetal-type enterocytes. The apical part of the enterocytes contains microvilli (*Table 1*), and a dense network of canalicular structures and vesicles ($\phi \leq 1$ μm) which resembles the apical

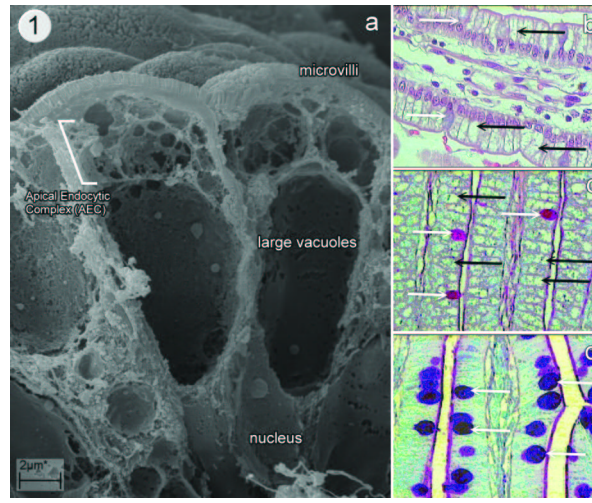

Fig. 1. a - SEM micrographs of the longitudal section of vacuolated foetal enterocytes from ileum at birth. Note the microvilli, AEC, large vacuoles and nucleus. The SEM micrograph shows only replicas contained cell's components. Horizontal bars depict scale. *b-d* - Representative photograph of the intestinal villi in the distal jejunum in 14 d old suckling piglets stained with HE (*b*). alcian blue, (*c*) and PAS (*d*) (obj. 60x). Abundant large size lysosomal vacuoles (*black arrows*) located between the cell apex and nucleus in the enterocytes can be distinguished from the goblet cells (*white arrows*) with their content stained with alcian blue (stains acid and neutral mucopolysaccharides on blue and red. respectively) and PAS (stains neutral mucopolysaccharides on red-violet).

canalicular system (ACS) described previously (1-3). Downwards, the size of vesicles increase, and the center of the cell is occupied by a single large size vacuole (supranuclear vacuole, SV) with a cross section area reaching up to 110 µm^2 in the distal jejunum (*Fig. 1*). The nucleus is pushed down to the basal cell membrane. The size of enterocytes changes with age and intestine segment as shown in *Table 1*, the smallest enterocytes were found in the unsuckling neonates, and the largest at day 14 in the duodenum, and at day 3 in the jejunum and ileum. The longest microvilli were found at day 3 of life (*Table 1*). A significant difference was found between the microvilli in VFE and in adult type enterocytes in the jejunum at the day 7 of life (0.75±0.02 *vs*. 1.09±0.04, respectively, $P<0.05$). The ACS and large vacuole membranes were thinner as compared to the cell membrane but it was impossible to precisely measure their thickness using SEM.

At birth the vacuolated enterocytes are present at the tip of duodenal villi are large and highlighted by the swelling of the apical part of the cell (*Fig. 2b*). In the mid-jejunum at birth all the surface of the villi was lined by swollen fetal enterocytes. The vast majority of enterocytes was hexagonal in shape, but rounded and flattened cells appeared as well (*Fig. 2a, b*). In the cross section of the villi the empty spaces nearby the large vacuoles and the structure of the apical canalicular system are visible (*Fig. 2a*). In the ileum at birth the enterocytes

Table 1. Enterocytes in the duodenum (D), mid jejunum (J) and ileum (I) of neonatal piglets analyzed with scanning electron microscopy. The microvilli were measured in adult type enterocytes. Mean±SEM.

Measurement	Segment	At birth	Day 3	Day 7	Day 14	Day 21
Enterocyte height (µm)	D	14.6 ± 0.34^{a}	20.0 ± 0.36^{b}	25.7 ± 0.78^{c}	33.7 ± 1.30^{d}	17.7 ± 0.76^{e}
	J	19.6 ± 0.45^{a}	22.2 ± 0.89^{b}	21.7 ± 0.31^{b}	12.1 ± 0.14^{c}	18.0 ± 0.28^{d}
	I	19.3 ± 0.30^{a}	23.9 ± 0.49^{b}	21.0 ± 0.68^{a}	17.5 ± 0.48^{c}	21.6 ± 0.81^{a}
Microvilli length (µm)	D	1.25 ± 0.03^{a}	1.52 ± 0.07^{b}	1.02 ± 0.04^{c}	0.92 ± 0.05^{c}	1.07 ± 0.03^{c}
	J	0.81 ± 0.03^{a}	1.57 ± 0.06^{b}	1.09 ± 0.04^{c}	1.09 ± 0.04^{c}	0.95 ± 0.02^{d}
	I	0.76 ± 0.04^{a}	1.02 ± 0.03^{b}	0.95 ± 0.06^{b}	0.7 ± 0.04^{a}	1.09 ± 0.03^{b}
ACS abundance	D	+	-	-	-	-
	J	+++	++	+	-	-
	I	+++	+++	++	+/-	-

ACS - apical canalicular system. One-way ANOVA followed by Tukey test, different superscript letters red in row indicate statistical difference, $p<0.05$.

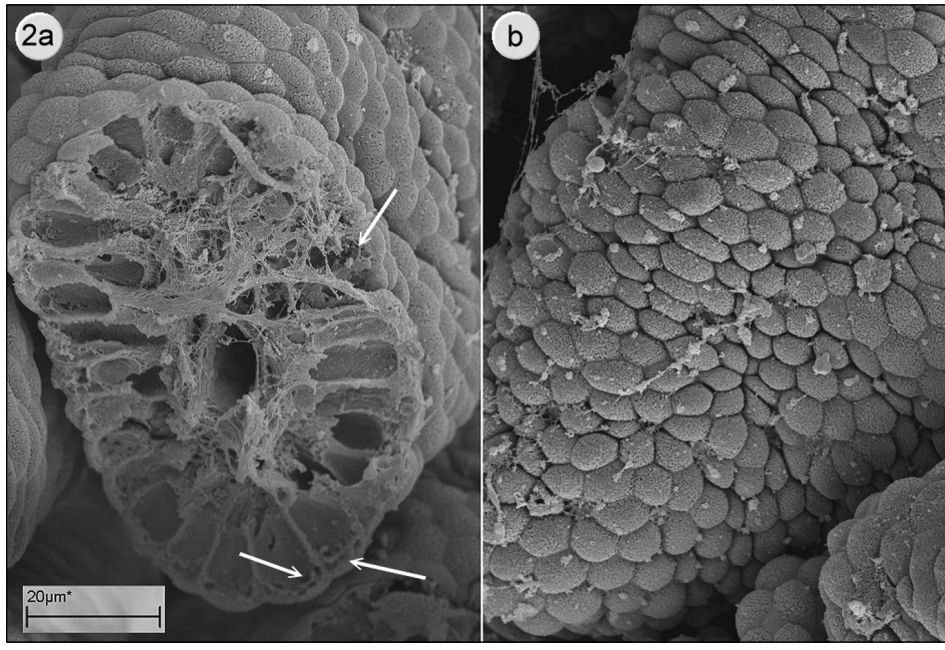

Fig. 2. SEM micrograph of the villi of middle jejunum at birth. The left SEM micrograph of cross section of middle jejunum villus at birth shows empty spaces in the cell body retain after a large lysosomal vacuole in the apical region of the enterocyte. White arrows indicate the remains after apical canalicular system. The right micrograph (b) shows the villus surface cover by fetal vacuolated enterocytes. Horizontal bars depict scale are the same on both micrographs.

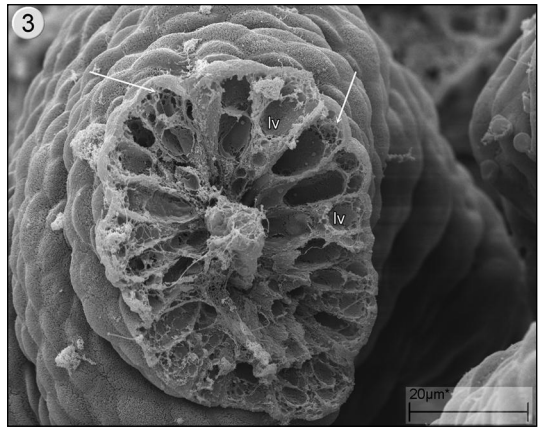

Fig. 3. SEM micrograph of cross section of villus from ileal mucosa at birth. Note the empty spaces in cell body after localization of the large lysosomal vacuoles in apical region of cell (LV). White arrows show the remnant of apical canalicular system.

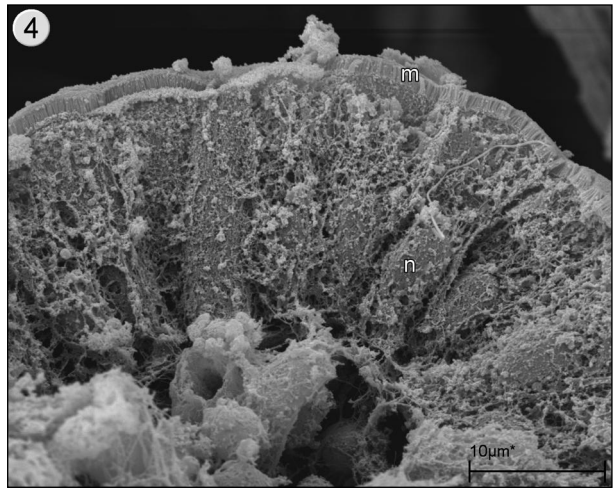

Fig. 4. SEM micrograph of enterocytes from duodenum at d 3 after birth. The microvilli (m) and nucleus (n) are visible. Note the lack of empty spaces after vacuoles and ACS . The apical surface of villi are flatten.

lining the villi are of fetal type and regular in shape (*Fig. 3*). The cross sections of the villi show regular arrangement of the enterocytes and well preserved remnants of one or two large vacuoles as well as the ACS (*Fig. 3*).

At the day 3 of postnatal life the cross sections of duodenal villi show already the adult-type enterocytes containing nuclei and lacking in ACS and large vacuoles (*Fig. 4*). In the mid-jejunum and in the ileum of 3 days old piglets the cross section of the villi revealed the presence of vacuole remnants and the ACS. At the day 7, 14 and 21 of postnatal life no major changes are observed in the duodenum, though in the jejunum the enterocytes still contained the ACS at day 7 (*Fig. 5*). In the cross section of ileal villi the remnants of large vacuoles and other cellular structures are still well distinguished at day 7 (*Fig. 6*), but not at postnatal day 21 (*Fig. 7*).

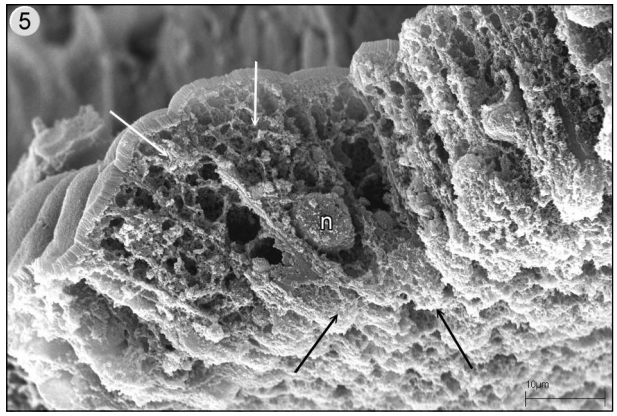

Fig. 5. SEM micrograph of villus part from middle jejunum at 7 day after birth. Enterocytes with ACS remains indicate white arrows. Nucleus is present (n). Black arrows indicate basal part of singular enterocytes.

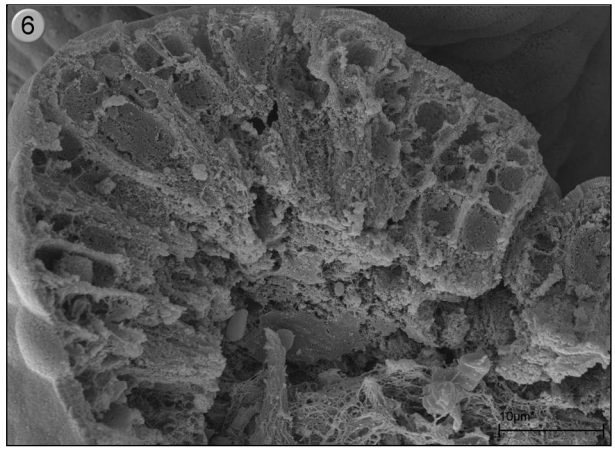

Fig. 6. SEM micrograph of villus cross section from ileum at d 7 after birth. The empty places, vestige of large vacoles, are still present at singular villus.

DISCUSSION

In the present study scanning electron microscopy technique was used to evaluate the presence of vacuolated enterocytes in the small intestine of pig neonates. Our studies using SEM technique corroborated with the results obtained with light microscopy (7). This three-dimensional technique also allowed analysis of microvilli, and the ACS and large vacuoles in the enterocytes. Interestingly, the microvilli length was found to differ in time in examined piglets. Previously, Markiewicz (8) reported the circadian fluctuations of microvilli length in humans. The circadian rhythm apparently did not contribute to our results since the animals were killed at the same time of the day. There was no coincidence with the ACS and large lysosomal vacuole size as well, though the microvilli in fetal-type enterocytes were shorter than in the

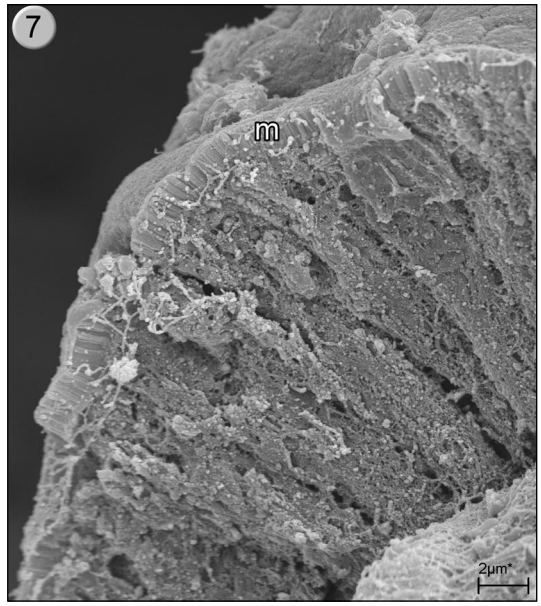

Fig. 7. SEM micrograph of ileal villi at d 21 after birth. brush border is visible (m). The mature population of enterocytes is present.

adult-type enterocytes. This observation needs further studies since the changes were quite large and contributed up to 40-50% of microvilli height and might be associated with the abundance of brush border enzyme and channel molecules. SEM analysis of the enterocytes demonstrated gradual disappearance of vacuolated enterocytes postnataly. The timing was different in the particular small intestine segments. Unfortunately, preparation of tissues for SEM washed out the vacuole content, so it was not possible to differentiate between the transport and lysosomal vacuoles. The present study demonstrated only the presence or lack of large-size vacuoles in pig small intestine. However, the results of our previous studies and literature data (1, 4, 7) allowed us to point out the empty areas present at the upper part of the enterocytes as the remnants of ACS and large vacuoles. Accordingly we used only one term, vacuolated fetal enterocytes, when any large vacuole traces were present.

The disappearance of VFE is a characteristic feature of mucosal remodeling and may be used as a marker of maturation of the small intestine. Vacuolated fetal-type enterocytes remained in the jejunum for few days after birth, whereas in the duodenum the VFE were present only at birth as reported previously (1, 3, 9). The large vacuoles seen in the duodenum and proximal jejunum are considered transport vacuoles, whereas in the lower parts of the gut - the digestive vacuoles. SEM microimages, however, showed no differences in the interior traces between the duodenal, jejunal and ileal VFE.

Concluding, morphology analysis of pig small intestinal mucosa suggests that replacement of fetal type vacuolated enterocytes is resumed within 21 days

after birth, and corroborate with the earlier findings (7, 10-12). SEM technique can be considered a complementary tool in studying the intestinal mucosa development since it gives three dimensional images allowing detailed analysis of the structure of epithelial cells.

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