INTO THE UNKNOWN - THE DEATH PATHWAYS IN THE NEONATAL GUT EPITHELIUM

Apoptosis is a fundamental process in the development of the fast growing intestinal mucosa. Apoptotic cells are present along the whole length of the villi and in the crypts. The mechanisms involved in the induction of apoptosis in the gut mucosa are still unknown. Cytokines are believed to play a role in auto- and paracrine models because the cells are dying in so-called "packets" containing neighboring cells. In the rapidly developing gut of neonates, the apoptosis rate is transiently reduced in the first days of life, enhancing the growth of mucosa. Afterwards, apoptosis plays a role in the exchange of the enterocyte population, facilitating maturation of the mucosa. The presence of autophagic cells has been confirmed for the first time in the developing gut. Deprivation of growth factors during feeding artificial milk formula led to an increased apoptosis rate. Supplementation with leptin reduced cell apoptosis and increased the mitosis-to-apoptosis ratio. Autophagy was also diminished. The key to healthy gut mucosa growth in early life, especially in fast-growing animals, is colostrum, which supplies nutritional and defensive components together with supplementary growth factors, cytokines and hormones essential for growth and maturation of gut mucosa.

Key words: apoptosis, autophagy, enterocyte, gut development, leptin, mitosis
The term apoptosis originates from the Greek word "falling", as in the falling of leaves or flower petals. It was proposed in 1972 by Kerr, Wyllie and Curie (1) to distinguish this genetically programmed, physiological and suicidal form of cell death from necrosis. Apoptosis is a fundamental process in cell biology, which contributes to the survival of every multicellular organism. This so-called physiological death facilitates removal of unnecessary (at a given point), old, defective and mutated cells during embryogenesis, development, remodeling and involution. It also underlies cancer therapy and prevention. The life of a multicellular organism is sustained by a dynamic equilibrium between mitosis and apoptosis, growth and involution. Maintenance of this equilibrium within certain boundaries allows physiological remodeling of a tissue. A shift in this equilibrium towards any extreme is considered pathological. Uncontrolled mitosis or impaired apoptosis results in neoplastic and autoaggressive diseases, whereas excessive apoptosis leads to degenerative disorders such as Alzheimer's and Parkinson's diseases or AIDS.

Apoptosis is the classical form of programmed cell death that coexists with autophagy, death associated with formation of autophagolysosomes, anoikis which results from the detachment from the lamina propria, amorphosis associated with distortion of the cytoskeleton, and the recently described mitotic catastrophe (2).

Programmed cell death is induced by a variety of factors that can be divided into four major groups (3). 1. Physical factors such as gamma and UV irradiation (4, 5), hyperthermia and hydrostatic pressure. 2. Trophic- and growth factor deficiency resulting from their prolonged deprivation or increased cell number (6). Removal of specific growth factors can also initiate apoptosis i.e. neuronal cells deprived of NGF. 3. Hormones and cytokines such as glucocorticoids, thyroid hormones, TNF-α (7), or TGF-β1 (8, 9). 4. Cytotoxic drugs and factors: granzyme A and B (10, 11), reactive oxygen species (12), inhibitors of enzymes like camptothecin (13), and antimetabolites (2-chloro-2’deoxyadenozine) (14). Various apoptosis-inducing factors work via different induction mechanisms that merge into one common pathway of promotion (reversible, regulated by the Bcl-2 family proteins), and degradation (irreversible, executed by caspases) in a later step of apoptosis.

There are two major pathways of apoptosis: receptor and mitochondrial (Fig. 1). Apoptotic signals that activate death receptors (CD95, TNF-R1, DR-5, TGFβ-R) localized on the cellular membrane facilitate their oligomerization, recruitment of an adapter molecule (like Fas-associated death domain - FADD) and procaspase 8. In this way the death-inducing signaling complex (DISC) is created. In DISC, procaspase 8 is autoproteolytically cleaved into two subunits. Subsequently, a heterotetramer of two large and two small subunits is formed to create an active form of caspase 8 (15, 16). Caspase 8 has the potential for
autoactivation and direct cleavage of procaspase 3, thereby propagating apoptosis.

The mitochondrial pathway of apoptosis originates in activation and heterooligomerization of proapoptotic proteins belonging to the Bcl-2 family, such as BAX and BID. BAX - BID complexes translocate to mitochondria where they induce the release of cytochrome c either directly or through complexes with membrane proteins (17-19). Cytochrome c together with Apaf-1 and procaspase 9 forms a 700 kDa complex called the apoptosome where activation of caspase 9 occurs (20-23). Together with cytochrome c, ROS, calcium ions (Ca\(^{2+}\)) and AIF are released from mitochondria. AIF translocates into the nucleus where it facilitates NUC-18 DNase activation and nuclear protein degradation (24, 25). During the apoptosis process, Ca\(^{2+}\) is also released from the endoplasmic reticulum. It is a necessary component for activation of calpain and DNases (i.e. DNase-I and NUC-1). Caspase 9 participates in activation of other caspases, i.e. caspase 3 and 7 responsible for cellular protein degradation (26). Caspase 8 and 9 are thus called regulator caspases, whereas caspase 3 and 7, executor caspases. The two pathways of apoptosis merge, as caspase 9 is capable of either directly or indirectly activating caspase 8 (26-29) and caspase 8 mediates the proteolytic activation of BID (18). One of the first targets of caspase 3 is PARP. PARP is an enzyme activated in the presence of DNA strand breaks (30, 31). It catalyzes modification of histones, topoisomerasers I and II, DNA polymerase α and other DNA-binding
proteins engaged in the DNA repair system (32, 33). The complexity of the apoptotic process and interactions between the different proteins involved are presented in Figure 2.

Apoptosis and mitosis within the gut endothelium were quantified as described by Biernat et al. (34) and Woliński et al. (35). For confocal analysis the cells were labeled with secondary Alexa Fluor 488 conjugated antibodies and 7-AAD to counterstain the DNA. The following primary antibodies were used: anti-caspase 3, anti-TGF-β1, anti-p53 (FITC-conjugated) and anti-MAP I LC-3. Next, the gut mucosa was visualized using the FV-500 (Olympus Poland) confocal laser scanning microscope system. The combinations of excitation / emission were: Argon 488 nm laser vs. 505-525 nm filter for Alexa Fluor 488 and FITC, and HeNe 543 nm laser vs. 610 nm filter for 7-AAD. For scanning electron microscopy the samples were fixed in 10% buffered formaldehde, dehydrated in a series of alcohols, chemically dried in HMDS (1,1,1,3,3,3 - hexamethyldisilazane), sputter-coated (Au/Pd 30 nm) and examined in a LEO 1430 VP (EHT 15kV).

POSTNATAL DEVELOPMENT OF THE SMALL INTESTINAL EPITHELIUM

Intensive growth of the small intestine starts several weeks before parturition and is characterized by a faster growth rate than the fetal body as a whole (36). Small intestine growth further accelerates after birth following the first

![Fig. 2. Interactions between proteins involved in apoptosis](image-url)
administration of colostrum. Thus, in the neonatal pig the small intestine doubles its weight and increases its length by 30% within the first 3 days of postnatal life (37). Accordingly, the intestinal crypt depth and villi length increase by 40 and 35 percent, respectively (34, 38). Growth is, on the one hand, associated with mucosa (in particular, enterocyte) incrustation by colostrum proteins (39). The size of a single enterocyte changes from $169 \pm 27 \mu m^2$ in unsuckling neonates to $229 \pm 26$ and $242 \pm 34 \mu m^2$ ($P<0.001$) in 24-hour-old and 7-day-old piglets, respectively. There was a 3 - 4-fold difference in intestinal protein synthesis described between piglets fed colostrums or milk formula and those fed water. The differences in intestinal protein mass seemed to be a result of IgG accumulation and retention as it was markedly higher in piglets fed colostrum while no significant difference was observed between water and milk formula fed ones (40). On the other hand, mucosa growth is tied to an increased cell proliferation rate, which can be deduced from the enhanced mucosa DNA content (41). In pig neonates we observed a 40 to 50% increase in the mitotic index in the crypt region of the jejunum within the first 1-2 days after birth (34). Cell proliferation increases, resulting in shortening the cell renewal cycle from 20 days in the fetal intestinal epithelium to 2-3 days in the neonatal tissue. Besides intensive growth, in the early postnatal period the small intestinal epithelium undergoes an essential maturation process. The enhanced proliferation in the crypt area is linked to major structural and functional remodeling (42). Morphologically, the so-called "fetal type" enterocytes containing large lysosomal vacuoles are replaced with "adult type" enterocytes that do not contain the vacuoles. In the "fetal type" enterocytes, two categories of lysosomal vacuoles, transport and digestive, have been described (43). The former, present on the entire length of the small intestine but only within the first two days after birth, are involved in transferring macromolecules in their intact form from the gut lumen into the circulation through the enterocyte. The transport vacuoles allow the biologically active colostral molecules, including immunoglobulins, to pass the gut epithelium without affecting their activity. Lysosomal vacuoles of the latter category are present in the lower half of the small intestine and seem to be responsible for intracellular digestion of nutrients and for controlling the pH of the gut lumen (44). The digestive vacuoles are observed for about 3-4 weeks in the pig ileum (42). Functionally, the activity of brush border lactase, aminopeptidases A and N, and dipeptidase IV is markedly reduced within a few days after birth (45). The high absorption rate of large molecules associated with the open "gut barrier" is abolished about two days after birth (43, 46, 47).

Stem cells, which constitute a replication center for the gut epithelium, occupy the lower third of the intestinal crypts (Fig. 3). The mitosis rate is high in the small intestine crypts. The highest percentage of dividing cells was observed in the mid-jejunum on the second postnatal day (Table 1). The fate of the newly constituted epithelial cells depends on the direction they "choose". Some cells migrate down to the crypt bottom and transform into Paneth cells.
Fig. 3. Scheme of intestinal villus and crypts, with stem-cell region marked in orange and Panneth-cell region in green. The fate of the gut epithelial cell depends on the direction of their movement. Cells that migrate to the villi differentiate into enterocytes, goblet cells (~5%) or endocrine cells (~1%) and are characterized by quick turnover, with a life-span seldom exceeding 48 hours. Cells directed downwards into the depth of the crypts differentiate into Paneth cells. These cells with life-span reaching 21 days produce lysozyme and defensins and are involved in protection of the gut.

Table 1. Mitotic and apoptotic indexes, and mitosis-to-apoptosis ratio in whole gut cross sections of newborn (unsuckling - 0 days), suckling (24 hours, 7 days) and weaned (12 weeks) piglets. $^\text{a}$According to Woliński et al. (35). $^\text{b}$According to Biernat et al. (34).

<table>
<thead>
<tr>
<th>Piglets</th>
<th>Newborn 0 days</th>
<th>Suckling 24 hours</th>
<th>Suckling 7 days</th>
<th>Weaned 12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotic index$^\text{a}$</td>
<td>4.50%</td>
<td>6.10%</td>
<td>4.45%</td>
<td>4.80%</td>
</tr>
<tr>
<td>Apototic index$^\text{b}$</td>
<td>21.8%</td>
<td>15.9%**</td>
<td>21.8%</td>
<td>25.1%</td>
</tr>
<tr>
<td>Mitosis/Apoptosis</td>
<td>0.20</td>
<td>0.38</td>
<td>0.20</td>
<td>0.19</td>
</tr>
</tbody>
</table>

$^\text{a}$ whole gut crosssections; ** P<0.01
With a life span exceeding 20 days, these cells produce lysozyme and defensins and are responsible for the protection of gut mucosa (48). The majority of epithelial cells migrate upward to the top of the villi and differentiate into goblet and endocrine cells and enterocytes. Enterocytes responsible for the secretory and absorptive functions of the gut epithelium are the most abundant. The population of mucus-producing goblet cells and endocrine cells make up approximately 5% and 1% of the epithelial cells covering the villi, respectively (Fig. 3). Their life span seldom exceeds 48 hours and the quick turnover is based on the dynamic equilibrium between cell mitosis and apoptosis. In neonatal piglets, enhanced mitosis was observed parallel with a significant decrease in apoptosis during the first two days after birth, resulting in transient elevation of the mitosis/apoptosis ratio (34). This phenomenon substantially contributes to the enlargement of the gut mucosa. The mitosis rate then stabilizes until the weaning period when another increase is observed, but this time it is associated with enhanced apoptosis (Tab. 1) (34). The mitosis-to-apoptosis ratio is a good marker of gut growth intensity. In particular, it clearly demonstrates that the first rapid phase of postnatal growth is not only due to passive incrustation of the mucosa with colostrum proteins but also due to an increased number of epithelial cells. This parameter allows differentiating between intensive growth of the gut mucosa occurring during the first 24 h of postnatal life from remodeling of the gut that occurs after the weaning of piglets, when the mitosis-to-apoptosis ratio remains unchanged while mitosis increases.

APOPTOSIS IN THE GUT EPITHELIUM

Until recently, apoptosis in the gut was the subject of only a few reports. The apoptotic cells were localized in the top of the villi of adult human subjects and rodents, and in the upper one third of the villi in monkeys (49, 50). These observations came from the gut of adult animals and supported the view of apoptosis as a common way of eliminating old, used epithelial cells from the villi. Species-specific differences are observed in the fate of eliminated epithelial cells. In adult mice and rats, the apoptotic cells are detached and desquamated into the intestinal lumen (49). In the guinea pig and monkey, apoptotic cells are not only desquamated but some of them are phagocytosed by macrophages present in the gut mucosa. In the end of last millennium, the first observations confirmed the presence of apoptotic cells in the intestinal crypt region as well (51). Our studies in newborn animals confirmed the localization of apoptotic cells in intestinal crypts (34, 52). In contrast to the observations made in adult animals, in neonatal piglets apoptotic cells were present along the entire length of the villi, including the lower half (Figs. 4 and 6). Similar pictures have also been found recently in chicken broilers (52). In the neonatal pig gut mucosa, dying cells are eliminated into the lumen as single cells or groups of cells (Fig. 4b), or shed with the entire
top of the villus (Fig. 4c and 6a). They can also be brought under the epithelium layer where they undergo phagocytosis (Fig. 4d). Interestingly, unlike the reports describing apoptosis in the gut of adults, the cells in neonatal piglets seem to die in packets (Fig. 5a and 6c-f), suggesting involvement of auto- or paracrine factor(s) in the induction phase of apoptosis (34, 52). Furthermore, the close proximity of the observed apoptotic cells in neighboring villi imply transmission of the apoptogenic signal via the luminal space (Fig. 5b). This peculiar pattern of apoptosis was observed in mucosa slides using confocal microscopy and, even more convincingly, with scanning electron microscopy (Fig. 6a) as groups of apoptotic cells observed along the entire length of the villi. In three-dimensional SEM images, the surface of the villi is flat with honeycomb-shaped outlines of

Fig. 4. Localization of apoptotic cells in the gut mucosa of neonatal piglets. The late apoptotic cells, characterized by high expression of active caspase-3 (green fluorescence), were localized in the crypts and at the basis of the villi (a), in the middle of the villi (b) and on the top of the villi (c and d) Cells from the top of the villi were either shed to the gut lumen (c) or internalized under the enterocyte layer where they underwent phagocytosis (d). Gut cross sections were stained with Alexa Fluor 488-conjugated secondary antibodies against caspase-3 (green fluorescence) and 7-AAD against the DNA (red fluorescence).
the top of the enterocytes. The groups of apoptotic cells are either sunken or protrude by 1-2 µm over the epithelial cell surface (Fig. 6 c-f).

The mechanisms of apoptosis induction in the gut epithelium remain elusive. A decrease in anti-apoptotic members of the Bcl-2 family as a result of total parenteral nutrition, so-called death from unuse, was suggested by Wildhaber et al. (53), increased free radical formation associated with passive smoking in rats was observed by Wang et al. (54), and infections of the gastrointestinal tract were associated with increased apoptosis (55). All of the above-mentioned mechanisms may apply to enterocyte apoptosis in the neonatal gut as well, but the most significant apoptosis-inducing mechanism during the physiological development of the gut epithelium seems to be local deprivation of growth factors and tissue hormones like EGF, IGF, IGF-BP, and leptin (35, 56). This induction pathway presumably is especially important during the first days of life as the mucosa of newborn animals grows extremely rapidly and, simultaneously, the gut has limited ability to produce its own growth factors. Thus the neonatal gut heavily depends on external regulators coming with colostrum and milk (57). Our recent data showing that the apoptosis rate in gut mucosa is higher in formula-fed newborns as compared with sow-reared newborns strongly support this hypothesis (Table 2).

Enhanced proliferation of crypt stem cells results in increased susceptibility and frequency of replication errors and mutations, which trigger the p53-
Fig. 6. Three-dimensional scanning electron microphotographs of gut mucosa of neonatal piglets. Apoptotic cells are manifested either as sunken or protruding by 1-2 mm over the epithelial cell surface: a) an overview of the intestinal mucosa showing the apoptotic cells along the entire length of the villi (magnification 300x); b) shedding of the entire top of the villi (2500x); c - e sequence of microphotographs showing packets of apoptotic cells localized in the middle section of the villus (magnification ranged from 3000x to 10000x); f) packet of the apoptotic cells localized at the base of the villus (15000x).

Table 2. Mitotic and apoptotic indexes, and mitosis-to-apoptosis ratio in isolated gut mucosa in 7-day-old piglets reared with sows, fed milk formula alone or milk formula supplemented with leptin (10 µg/kg bw every 8 h).

<table>
<thead>
<tr>
<th>Piglets</th>
<th>Kept with sows 7 days</th>
<th>Milk formula 7 days</th>
<th>Milk formula + leptin* 7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotic index</td>
<td>4.45%</td>
<td>3.45%</td>
<td>5.12%</td>
</tr>
<tr>
<td>Apoptotic index*</td>
<td>11.0%</td>
<td>14.5%</td>
<td>12.9%</td>
</tr>
<tr>
<td>Mitosis/Apoptosis</td>
<td>0.41</td>
<td>0.23</td>
<td>0.39</td>
</tr>
</tbody>
</table>

* +10 µg leptin/kg bw every 8 h; * isolated mucosa
dependent cell cycle arrest and, in case the error cannot be repaired, the p53- and Bcl-2 family-dependent death pathways (58). The epithelial cells expressing p53 were restricted solely to the crypt region and base of the villi.

TGF-β1-induced apoptosis is another pathway involved in cell death promotion that cannot be excluded. TGF-β1 has been recently proved to be a potent apoptosis-promoting factor in the epithelial cells of the mammary gland (9, 59, 60). This cytokine was observed to be secreted from macrophages upon the uptake of apoptotic bodies (49) and since it acts as paracrine factor, it may influence the neighboring gut epithelial cells forcing them into the receptor (extrinsic) pathway of apoptosis (Fig. 7). The involvement of TGF-β1 in inducing apoptosis in the gut mucosa could explain why the epithelial cells die in packets rather than alone, as shown in Figs. 5 and 6.

AUTOPHAGY IN THE GUT EPITHELIUM

Autophagy is a newly described form of programmed cell death set apart from apoptosis because of differences in molecular and morphological patterns. In cells that undergo autophagic death the prominent observed feature is the formation of autophagolysosomes where cell organelles are degraded (61) resulting in total destruction of the cytosol compartment of the cell with no changes observed

Fig. 7. Expression of TGF-β1 in the intestinal mucosa of neonatal piglets. The TGF-β1-positive cells were observed in the entire mucosa layer, but the most were localized at the lower third of the villi. Gut cross sections were stained with Alexa Fluor 488-conjugated secondary antibodies against TGF-β1 (green fluorescence) and 7-AAD against the DNA (red fluorescence).
within the nucleus (62-65). Cathepsins are enzymes catalyzing this pathway of cell death with cathepsin-B and -D suggested to be the most potent. There is a crosslink between the apoptosis and autophagic pathways (66) since cathepsin-B is capable of activating BID via N-terminal cleavage (67) and cathepsin-D, of activating BAX (68). Both cathepsins are capable of activating executor caspases 3 and 7 (69) (Fig. 8). The recent studies of Lamparska-Przybysz et al. on MCF-7, a mammary epithelial cell line, (70) suggest that autophagy is a complementary form of cell death to apoptosis, and is switched on in cells that are more resistant to typical apoptosis-inducing signals in the death-promoting environment. In mammalian cells, autophagy is regulated by homologues of the ATG and AUT family of yeast genes. Beclin 1, a homolog of the yeast autophagy protein Atg6, is required for vacuolar transport and can induce autophagy in human cells (71). The mammalian homologue of the yeast Atg8 gene codes microtubule-associated protein I (MAP I) light chain 3 (LC-3), which binds with autophagic membranes and is currently the only reliable marker of autophagosomes (72-75). Our studies confirmed the presence of MAP I LC-3-positive, autophagic cells within the gut in

**Fig. 8.** Autophagic pathway and its crosslink with the apoptosis pathways.
piglets fed milk formula and milk formula supplemented with leptin (Fig. 9). In the piglets fed milk formula alone, the MAP I LC-3-positive cells were grouped in packets, similar to cells dying via the apoptotic pathway (compare Fig. 9a with Fig. 5). When milk formula was supplemented with leptin, the amount of autophagic cells decreased and the packet pattern was no longer observed (Fig. 9b). In comparison with apoptosis, autophagy seems to play a less significant role in gut development as the number of MAP I LC-3-positive cells is small as compared with the number of apoptotic cells. It is possible that autophagy is switched on in cells with impaired apoptotic machinery, but this theory needs further evaluation (70). Several attempts were undertaken to enhance the mitosis-to-apoptosis ratio in the developing gut. Blum & Baumrucker (56) proved that supplementation of single growth factors and tissue hormones alone to milk formula was not enough to increase the mitotic ratio in calves. Positive effects were observed only when growth factors were supplemented to colostrum or milk. Mimicking the effect of feeding milk and colostrum, in particular, to neonates is very difficult, as they contain a species-specific composition of regulatory peptides, hormones, cytokines and growth factors as well as albumins capable of protecting these bioactive substances from enzymatic digestion (76). So far only leptin (Table 2) and IGF (77) were proved to be potent in increasing the mitotic ratio to some extent and in reducing the apoptosis of enterocytes in neonates when
supplemented to milk formula (35, 77). Furthermore, preventing enterocyte apoptosis does not mean enhancement of adaptation and development of the intestinal mucosa, as shown by Juno et al. (78). Investigation of gut epithelium apoptosis may be of help in constructing new artificial milk formulas (79).

In conclusion, there are two major stages of gut development during postnatal life, easily distinguishable by the mitosis-to-apoptosis ratio. The first stage of intensive development is characterized by a two-fold increase in this ratio. This stage occurs immediately after the first meal and is associated with a significant reduction in apoptosis with a parallel increase in the mitotic index. The second stage, remodeling, is associated with a corresponding increase in the mitosis and apoptosis indexes with no change in the mitosis-to-apoptosis ratio. The packet pattern of cell death via apoptosis pathways suggests involvement of some auto- or paracrine factor(s) controlling the process. The ubiquitous expression of TGF-β1 in the gut of neonatal piglets suggests its role as one of the apoptosis-inducing auto/paracrine factors in the intestinal mucosa. The intensity of apoptosis in gut mucosa of piglets fed milk formula suggests a powerful influence of bioactive factors from colostrums and milk on gut development, especially during the first few days of neonatal life. Finally, although the presence of autophagy was confirmed during intestinal mucosa development, it seems to play only a complementary role to apoptosis.

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REFERENCES


