REVIEWS

Morphological Differentiation of Hepatocytes in Different Animal Species During Ontogeny in Relation to the Feeding Patterns

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Morphological differentiation of hepatocytes is determined by the feeding pattern during prenatal life: endogenous (chick embryo utilizing egg yolk and white at the early stages and swallowing amniotic fluid with protein at later stages of embryogenesis) or placental (rat embryo receiving all nutrients via the placenta). In the chick, morphological differentiation of hepatocytes is completed by hatching, while in the rat it is completed by the 30th day of postnatal life. Daily ultrastructural analysis of chick and rat hepatocytes during prenatal development reveals morphologic equivalents for various functional states of the hepatocyte organelles involved in the production and secretion of bile and proteins. Ultrastructural and functional changes in these organelles occur in parallel, and only lipid inclusions are stored in the hepatocyte cytoplasm. Hepatocyte differentiation is stimulated by food digestion (digestive load).

Key Words: *hepatocyte ultrastructure; ontogeny*

The research of the adaptation of organisms to environmental factors is based primarily on the investigation of adaptive reactions providing homeostasis under physiological conditions, since "a common feature of compensatory and adaptive responses is that they represent a particular combination of physiological functions of the organism" [15]. In order to assess the adaptive capabilities of hepatocytes under physiological conditions we studied the ultrastructure of these cells in phylogeny and ontogeny. In vertebrates of different classes and of the same class, hepatic cells have identical sets of organelles and display specific features, which we regard as adaptive because they provide the maximum efficiency of cellular functions [7]. Ultrastructural studies of chick and rat hepatocytes during prenatal and postnatal ontogeny made it possible to observe how hepatocytes adapt to the continuously increasing functional loads during transition from endogenous feeding to mixed feeding during prenatal life and then to exogenous feeding after hatching (the chick) and from endogenous feeding via the placenta to feeding with mother's milk and then to independent feeding after weaning (the rat). In these studies, the ultrastructural equivalents of physiological functions were identified. It was demonstrated that prolonged feeding with mother's milk affects morphological differentiation of hepatocytes. Although there is a large body of evidence on specific changes in the ultrastructure of various organelles and inclusions in chick and rat hepatocytes during embryogeny and postnatal onto-

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geny [13,18-22,25-31], little attention has been given to the Golgi complex, which is involved in accumulation and secretion of bile products, and to analysis of ultrastructural changes occurring in hepatocytes.

The ultrastructural data show that the chick embryo hepatocytes start functioning at the early stages of embryogenesis. The formation of chick liver starts after 3.5 days of incubation [28]. Ultrastructural analysis showed that bile capillaries appear between hepatocytes on day 6 of incubation (endogenous feeding). Small mitochondria with a loose matrix, numerous free ribosomes, occasional short profiles of the rough endoplasmic reticulum (RER), lipid droplets, and glycogen accumulations among the vesicles of the smooth endoplasmic reticulum (SER) were observed at this stage of embryogenesis. Proteins were synthesized by hepatocytes to meet mainly their own needs; glycogen and lipids were also produced. On the 6th day of incubation, the Golgi complex components appear at the outer nuclear membrane [5]. We believe that the origin and location of these components are not casual. It has been generally recognized that the location of cell organelles is determined by their function. Since on day 6 the intensify of protein synthesis in hepatocytes is low, judging from the number of cysternae in the RER, proteins are synthesized primarily in the nucleus and juxtanuclear zone. The proteins are probably transported by the Golgi complex vesicles budding from the outer nuclear membrane. This situation is similar to that observed later, when proteins synthesized and accumulated in the RER cisternae enter the vesicles budding from the smooth areas of the cisternae and then fuse with the Golgi complex cisternae [5]. Throughout the subsequent period of prenatal development (after day 7), great amounts of bubble-like SER profiles with particles resembling very low density lipoproteins (VLDL) were located between the sinusoidal and biliary poles. This ultrastructure reflects the transport of the yolk sac metabolites from the blood to hepatocytes. On day 7 of incubation, blood flowing from the yolk sac and carrying carbohydrates, proteins, amino acids, and minerals passes through the liver [12], where these substances are metabolized. By the 7th day of incubation, when the embryo still receives nutrients from the egg yolk and white, the liver already has a tubular structure, the number of mitochondria and RER cisternae in hepatocytes increase, and two opposite processes occur: clasmatosis of the cytoplasmic fragments with ribosomes, SER vesicles, and lysosome-like granules into sinusoidal capillaries [6] and accumulation and release of bile products into the bile capillary [4]. We have studied the dynamics of both processes.

Clasmatosis occurring from day 7 to day 13 of incubation is an adaptive process ensuring rapid supply of the growing embryo with proteins synthesized by hepatocytes from the amino acids of the yolk globules. The cytoplasm was fragmented along the vesicles extending from one margin of the narrowed portion of a large tongue-like cytoplasmic protrusion to its other margin, or cytoplasmic fragments were lying on a fine stalk along which they were separated from the cell. As a result, wide sinusoids remained filled with cytoplasmic fragments until day 13 of incubation [6], when extraintestinal feeding coincides with intestinal feeding with amniotic fluid containing proteins, while the release of proteins in clasmasomes into the bloodstream gradually decreases.

On day 8 of incubation, a well-developed Golgi complex of hepatocytes was located at the bile capillary. The terminal dilated portions of the Golgi complex cisternae and large vacuoles contained considerable amounts of VLDL particles. Peroxisomes and vacuoles with flaky contents were located in the Golgi complex zone. The mitochondria (0.1-0.2 μ) had a dense matrix. The bile capillary with well-developed microvilli was either closed or had a lumen. The enlargement of the Golgi complex, its approximation to the bile capillary, and saturation with bile products indicated that the hepatocyte was preparing to release bile into the bile capillary, when embryonal feeding was changing from endogenous to mixed (extraintestinal and intestinal). On day 9 of incubation, the embryo starts swallowing the amniotic fluid. On days 9-10, the ultrastructural manifestations of intense release of bile products into the bile capillary and enhanced synthesis of proteins for export were observed. The ultrastructure of the Golgi complex and its position relative to the bile capillary changed. Unlike on day 7, when only occasional hepatocytes released bile products into the bile capillary, virtually all hepatocytes released these products on day 9. The Golgi complex in them was shrunk, being located at some distance from the bile capillary. Its vacuoles formed numerous vesicles arranged in a chain oriented toward the bile capillary; some vesicles fused with the bile capillary membrane and probably discharged their contents into the capillary lumen. Bile capillaries were markedly dilated and contained flocculent material or myelin figures and fragments of microvilli, which were subjected to clasmatosis upon intense secretion of bile products [6]. The mitochondria located near the vesicles and cisternae of the Golgi complex had a clear matrix. In some mitochondria, the outer membranes were disrupted as a result of clasmatosis. We observed these changes in the hepatocyte mitochondria only on day 10 of incubation after a massive release of

bile products, when the energy demand of hepatocytes increases considerably. Presumably, clasmatosis of mitochondrial fragments is associated with intense synthesis of bile acids, since the mitochondria are involved in this process [17,23].

On day 9 of incubation (the beginning of mixed feeding), the energy requirements of growing embryo increase substantially, as evidenced by the appearance of elongated (up to 0.3 μ) and branched mitochondria with dense matrix and numerous cristae. The number of cisternae in the RER near the mitochondria also increased. These changes indicate an enhanced synthesis of proteins for export. Changes in mitochondrial ultrastructure were observed on day 10: all mitochondria had a clear matrix and fewer cristae and were smaller in size (0.1-0.2 μ). Neither elongated nor branched mitochondria were seen. The Golgi complex was still separated from the bile capillary by vesicles, vacuoles with electron dense contents, and mitochondria. On day 11 of incubation, both the size of the Golgi complex and its position in relation to the bile capillary varied from cell to cell, indicating that these cells did not function synchronously. It is interesting to note that glycogen, which present in hepatocytes since the 6th day of incubation, disappeared on day 11 after massive bile release and reappeared on day 13, which is consistent with the findings of others [26]. On day 13, small mitochondria (0.1 μ) predominated; however, larger mitochondria (0.2-0.3 μ) appeared again.

During the fetal period (from day 13 of incubation to hatching), when the chick begins swallowing amniotic fluid containing protein and is still fed endogenously, the ultrastructure of hepatocytes testified to their further adaptation to increased functional load (intensification of protein synthesis). The hepatocyte cytoplasm contained both small (0.1 μ) and elongated (0.6-0.8 μ) mitochondria with dense matrix and numerous cristae. The mitochondria were surrounded by the RER. Glycogen reappeared in the cytoplasm. We observed no clasmatosis of cytoplasmic fragments with ribosomes into the sinusoidal lumen. On days 14-15 of incubation, mitochondrial and RER ultrastructure varied from cell to cell: in some hepatocytes the mitochondria had a loose matrix, and the RER cisternae were dilated, while in others the mitochondria had a dense matrix, and the RER cisternae were closed. Small mitochondria (0.2 μ) predominated, although larger mitochondria (0.3 μ) were also seen. The ultrastructure and location of the Golgi complex were also changed. These findings suggest that the hepatocytes function asynchronously. On day 15, the number of peroxisomes increased, probably due to intense bile production and enhanced protein and lipid metabolism, the processes involving peroxisomes [17,23]. During the fetal period, hepatocytes contained considerable amounts of glycogen and lipids. Lipids were present in the nucleus until day 15 of incubation. On days 20-21, the wide lumens of bile capillaries contained a flaky fine-grained material and structures resembling myelin figures, which was indicative of intense release of bile products into bile capillaries to prepare the chick for active exogenous feeding soon after hatching. On days 20-21, the mitochondria were 0.1-0.2 μ in size, no larger mitochondria were observed.

Detailed ultrastructural analysis of prenatal chick hepatocytes enabled us to describe for the first time the behavior of organelles in these cells during accumulation and release of bile products. We have followed quantitative and qualitative modifications occurring in the ultrastructure of hepatocyte organelles and reflecting adaptation of the hepatocyte to the particular stage of functioning. The modifications manifest themselves as hypertrophy and hyperplasia of the organelles, i.e., are of the same type as those observed in the process of physiological regeneration.

The ultrastructural alterations observed in the hepatocytes attest to active functioning of these cells at the early stages of embryogeny. These alterations proved to be similar to those occurring in the hepatocytes of other animals utilizing egg yolk and white during embryogeny. For example, the hepatocytes of the Sevan trout (*Salmo ischchan*) [8] and of the chaser (*Elaphe schrencki*) synthesize bile products and proteins, deposit glycogen and fat in the cytoplasm, and discharge synthesized products into the sinusoid. Based on ultrastructural studies, we have concluded that in fishes, reptilians, and birds the liver differentiates at the early stages of embryogeny and metabolizes yolk and white, thus performing the function that the maternal organism plays in mammals.

In mammals, the embryo is fed via the placenta, receiving from the mother all the metabolites it requires for growth and development. In the rat embryo, the liver is laid down later than in the chick (9.5 days after fertilization) [28], although the duration of prenatal development in the same. On day 13 of prenatal life, rat hepatocytes contain numerous free ribosomes, small mitochondria with dense matrix, and occasional RER cisternae and lipid droplets. Like in a 6-day-old chick embryo, in a 13-day-old rat embryo the Golgi complex develops from the outer nuclear membrane [5], which may be associated with protein synthesis occurring on the outer nuclear membrane. In the rat liver, bile capillaries form on day 14, but glycogen did not appear until day 18, which agrees with the published data [13,25]. Our electron microscopic findings indicate that adapta-

tion of rat hepatocytes to protein synthesis starts on day 14 of embryogenesis, when RER and mitochondria are well developed. Rhythmic ultrastructural modifications in the RER cisternae, mitochondria, and Golgi complex were similar in the vast majority of hepatocytes. The presence of mitochondria with swollen matrix, dilated RER cisternae, and Golgi complex with widened cisternae and vacuoles pointed to a high functional activity of hepatocytes. The phase of physiological recovery (day 15) was characterized by the presence of mitochondria with dense matrix and flattened cisternae of RER and Golgi complex [3]. The Golgi complex remained rather small throughout prenatal life, being represented by 1-2 short cisternae and several vesicles. The release of bile products was observed only before birth, which was supported by biochemical evidence [18, 29]. Bile capillaries were dilated and had few microvilli. Numerous small vesicles were seen between the Golgi complex and bile capillary. Mitochondria with the signs of clasmatosis were located near the capillary, i.e., the picture was similar to that observed in the chick embryo hepatocytes, when large amounts of bile products were first released into the bile capillaries. By the time of birth, rat hepatocytes contained all organelles necessary for normal functioning, although their morphological differentiation was not complete.

Between days 1 and 30 of postnatal life, ultrastructural changes in rat hepatocytes were determined by the diet (feeding with mother's milk substituted by active independent feeding). Hepatocytes of newborn pups were much larger than embryonal or fetal hepatocytes and contained numerous free ribosomes and a small number of RER cisternae. On the first day of postnatal life, hepatocytes contained no glycogen, which is consistent with the findings of other researchers [27,30]. This probably reflects adaptation to new conditions of thermoregulation. The hepatocytes of newborn rats contained a small number of mitochondria varying in size and shape (large, oval, round, and open ring with few cristae). This polymorphism points to considerable changes underwent by the mitochondria in order to increase the area of the contact with the RER cisternae (which are not numerous at this time) and to provide effective synthesis of proteins discharged from the cell. In addition to unchanged mitochondria, hepatocytes contained swollen mitochondria with a clear matrix and few cristae, which may be associated with the maximum cell activity leading to dystrophy [2]. On day 1 of postnatal development, peroxisomes and cytosegresomes appeared in the cytoplasm, and a small Golgi complex was located near the bile capillary. Presumably, numerous cytosegresomes with electron-dense contents resembling ferritin utilized hemosiderin and ferritin released from dead embryonal erythrocytes, which were replaced by erythrocytes typical of the adult organism [24].

On postnatal days 4 and 14, when the pup receives mother's milk, the functional load on its liver increases, and the ultrastructure of hepatocytes changes. The number of the RER cisternae and mitochondria as well as the size of the Golgi complex increased, and glycogen and lipid droplets were accumulated in the cytoplasm. It should be noted that lipid droplets were also identified in the nucleus, which agrees with the findings of others [21]. The close topographic contact between mitochondria and lipid droplets implies that the mitochondria are involved in lipid metabolism. During suckling, the enlarged Golgi complex located near the bile capillaries had widened cisternae and vacuoles with particles resembling VLDL. Mitochondrial polymorphism (the presence of large, small, and swollen organelles with dense or loose matrix) occurring in different cells and within the same cell indicated that not only cells but also the mitochondria are in different functional states. Moreover, "dark" and "light" cells were seen. Although the functional state of most hepatocytes is the same in the embryonal liver, after birth not only hepatocytes but even organelles in the same hepatocyte occur in different functional states, which is a striking demonstration of the "law of intermittent activity of functional structures" [10,14]. On day 30, when the young rat actively feeds itself, morphological differentiation of hepatocytes is complete. The hepatocyte cytoplasm contained numerous RER cisternae arranged in parallel rows, forming so-called "working units" with mitochondria. The Golgi complex consisted of flat cisternae, large vacuoles, and small vesicles, increased in size, and was located in the bile capillary area; lysosomes and peroxisomes had the typical structure. The cytoplasm contained great amounts of glycogen, while the number of lipid droplets decreased considerably compared with that during the suckling period.

The development and differentiation of hepatocytes are stimulated by food digestion (digestive load). This was confirmed by experiment in which the suckling period was prolonged: the mother of weaned rat pups was replaced by a lactating rat. Although the pups received milk *ad libitum*, their hepatocytes failed to differentiate; their ultrastructure remained similar to that of cells with a low level of protein synthesis. Ribosomes disappeared from the RER cisternae, and the cytoplasm contained no glycogen. Accumulations of cytosegresomes with fragments of cell organelles testified to the death and utilization of these organelles. It is interesting to note that the

Golgi complex was located near the outer nuclear membrane, as in the early embryogeny, when little protein was synthesized in the cytoplasm. After the pups had been weaned again, the typical ultrastructure of hepatocytes was restored and their morphological differentiation completed [9].

From the ultrastructural changes observed in hepatocytes during ontogeny we have concluded that:

1. The liver of the animals developing by utilizing the nutrients stored in the egg plays the key role in the metabolism of the egg yolk and white, which accounts for the facts that the liver is laid down at an early stage of embryogenesis and hepatocytes undergo rapid morphological differentiation.

2. Our findings support the concept formulated by A. N. Severtsov [16] and P. K. Anokhin [1] that "the systems providing the existence of animal species in their ecological niches develop by the time of birth". Morphological differentiation of hepatocytes in chick embryo is completed by the time of hatching, since the chick feeds itself soon after birth, while in the rat morphological differentiation of hepatocytes continues within a 30-day period of postnatal life.

3. Our studies confirm the close relationship between morphogenesis and environmental factors and show that morphological differentiation of hepatocytes is stimulated by increased food digestion (digestive load) and inhibited at a lower digestive load.

4. Daily quantitative or quantitative (or both) ultrastructural modifications of differentiating hepatocytes reflect the principle of structural and functional unity, i.e., each functional modification is accompanied by morphological changes.

5. At the ultrastructural level, the functional activity of rat embryo hepatocytes is characterized by a pronounced cyclicity. After the first simultaneous release of bile products into bile capillaries by most chick embryo hepatocytes, their functioning is asynchronous, and individual ultrastructural changes occur not only in cells but also in organelles, which confirms the "law of intermittent activity of functional structures" [10,14]. In the rat liver this law does not operate until birth.

6. Daily ultrastructural analysis of developing hepatocytes provided more insight into the following issues:

- generation of the Golgi complex from the outer nuclear membrane;
- development of the Golgi complex and other organelles during the accumulation and release of bile products;
- ultrastructural changes in the hepatocyte organelles in relation to functional activity of hepatocytes (polymorphism of the mitochondria and the RER cisternae);
- the existence of morphological equivalents of particular functional states: for example, the presence of long mitochondria with numerous cristae surrounded by long cisternae of the RER reflects the intense protein synthesis, while large Golgi complex cisternae and vacuoles with VLDL-like particles reflect the accumulation of bile product.

In the chick, we observed ultrastructural manifestations of both bile-forming and bile-excreting functions at the early stages of ontogeny, while in the rat the manifestations of only protein-synthesizing function. The intensity of bile production in the rat embryo increased before birth; such differences exemplify the heterochrony resulting from different needs of the developing organism.

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