IN VIVO ASSEMBLY OF TIGHT JUNCTIONS IN FETAL RAT LIVER

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ABSTRACT

Examination of glutaraldehyde-fixed, freeze-fractured livers from 14-15-day rat fetuses provided the basis for the following observations. Membrane particles align in otherwise poorly particulated areas of the presumptive pericanalicular plasma membrane (A face), frequently forming a discontinuous "honey-comb" network joining small particle islands. Even at this early stage, contiguous B-fracture faces contain furrows, rather than rows of pits, distinguishing the linear particle aggregates on the A face as developing tight junctions rather than gap junctions. Short segments of these linear arrays merge with smooth ridges clearly identifiable as segments of discontinuous tight junctions. With the continuing confluence of particulate and smooth ridge segments, mature tight junctions become fully appreciable. We conclude that tight junctions form de novo by the alignment and fusion of separate particles into beaded ridges which, in turn, become confluent and are transformed into continuous smooth ones. At 21 days of fetal life, most of the images of assembly have disappeared, and the liver reveals well-formed bile canaliculi sealed by mature tight junctions.

The tight junction (zonula occludens) is the outermost element of the junctional complex in virtually all mammalian epithelia lining the cavity tissues and organs (3, 5, 6, 14, 18, 19). Architecturally, it is now considered to be composed of a series of single fibrils conjoining the hydrophobic interiors of the plasma membranes of adjacent cells (20). The shared fibril consequently contributes to the functional properties of the junction; cell attachment and selective sealing of the intercellular space (3, 6, 7, 14). In thin-section, the tight junction is usually recognized between neighboring cells as a sequence of focal pentalaminar fusions, and in freeze-fracture, as fibrils or ridges on the A face, with complementary furrows on the B face (2, 6, 9, 10, 12, 17-19).

The mode of formation of the basic detectable unit of the tight junction, the smooth-contoured fibril or ridge, is unknown. Some investigators have discussed the possibility of its growth from the discontinuous fragments of former tight junctions undergoing turnover in rapidly evolving tissues (16). Others have fostered the interpretation that it is the product of membrane particle fusion (6, 8, 13). But convincing documentation of the latter viewpoint has awaited observations on a suitable model of de novo tight junction assembly, where the discrimination between linear particle aggregates of both presumptive gap and tight junctions could be realized. The 14-15-gestational-day fetal rat hepatocyte is a favorable model for such scrutiny, and examination of its junctional assembly constitutes the basis of this report.

MATERIALS AND METHODS

Pregnant rats with known mating dates were subjected to laparotomy under light ether anesthesia at daily intervals
between days 14 and 21 of gestation. The fetuses were delivered by cesarean section and their livers were quickly removed and minced in 4% glutaraldehyde in phosphate buffer, pH 7.4, at room temperature. Other fetuses were briefly perfusion-fixed by injection of the same fixative through the heart. After 2 h of fixation, small pieces of tissue (fixed by either method) were immersed in 30% glycerol in phosphate buffer for at least 30 min, immediately frozen in Freon 22, cooled in liquid nitrogen, fractured, and shadowed in Balzers BAF 301 apparatus (Balzers High Vacuum Corp., Balzers, Liechtenstein) according to the technique of Moor and Mühlethaler (11). After thawing of the tissues, the replicas were cleansed in a sodium hypochlorite solution for 2 h, then soaked overnight in dimethyl formamide, rinsed in distilled water, mounted on copper grids, and examined in a Philips EM 300 electron microscope.

For thin-section electron microscopy, the glutaraldehyde-fixed fragments were briefly washed in phosphate buffer, post-fixed for 2 h in 2% phosphate-buffered OsO4, then dehydrated in graded ethanols and embedded in Epon. Part of the pieces were stained en bloc with maleate-buffered uranyl acetate. Thin sections were stained with aqueous uranyl acetate and lead citrate.

**OBSERVATIONS**

**Thin-section**

In the fetal liver, the developing parenchymal cells form an irregular network, the interstices of which are occupied by hematopoietic cells. On gestational day 14, intercellular contacts between adjacent hepatocyte plasma membranes can be detected (Figs. 1 and 2), although well-delineated bile canaliculi are infrequent and usually lack typical junctional complexes (5) as observed in adult liver. Intercellular contacts visible in thin-section consist of both pentalaminar fusions (tight junctions) (Fig. 1) and gap junctions (Fig. 2). In the last gestational days, bile canaliculi, now clearly identifiable, are sealed by mature junctional complexes.

**Freeze-fracture**

In replicas of freeze-etched fetal rat liver, hepatocytes are recognizable by the fact that fracture of the cytoplasm reveals abundant rough endoplasmic...
mic reticulum and other organelles. Hematopoietic cells, in contrast, possess relatively few organelles. The cytoplasmic face (A face) of the fetal hepatocyte plasma membrane contains randomly distributed particles averaging 11 nm in diameter. Membrane specializations, such as tight and gap junctions, are only occasionally observed in the 14-day fetal hepatocyte, but become increasingly apparent during successive days of liver maturation. Tight junctions, as previously described in adult liver (2, 6, 9, 10), comprise a network of anastomosing ridges or fibrils on the A face, with complementary furrows on the B face. The tight junction separates the lumen of the bile canaliculus from the intercellular space, which is, in turn, confluent with the space of Disse. More often than in adult liver, gap junctions reside within the tight junction domain, but they are also present as isolated plaques at considerable distances from tight junctions. These gap junctions progressively enlarge during the course of development, until in the last days of gestation they occupy sizable areas of the plasma membrane.

Besides the membrane specializations which can be readily distinguished as tight or gap junctions, other intramembranous particulate distributions which we consider to reflect stages in tight-junctional assembly are encountered. These are commonly observed in the membranes of the 14–15-day old fetal hepatocytes and diminish in number during successive days of liver development, coincident with the increasing emergence of mature tight junctions. Thus, the following description is mainly concerned with the 14–15-day fetal hepatocyte plasma membrane. We should emphasize that this particular period encompasses all stages to be defined and characterized, including “mature” junctions.

**Junction Formation**

In addition to plasma membranes exhibiting only random particles and those with distinct cell junctions (A face), the alternate patterns of intramembranous particles are as follows. The simplest occurs in pericanalicular regions nearly devoid of scattered particles; it is represented by linear aggregates or chains of closely-packed particles (Fig. 3). The chains generally tend to be disposed at the edges of polygonal depressions in the membrane face and are almost always associated with small islands of densely-packed particles (Figs. 4 and 5). Besides short, single chains, the membranes may contain longer, anastomosing linear arrays which may interconnect with particle aggregates (Figs. 6–8). In the latter instances, the characteristic pattern of the tight junction, namely, that of a network, is evident, although the structural element of the tight junction, the smooth ridge or fibril, is absent. Still other regions of the plasma membrane manifest linear arrays which can be seen merging with short segments of smooth ridges (Fig. 11). Conversely, long, slick ridges may be interrupted at irregular intervals by abbreviated chains of particles (Fig. 12). When discontinuities in the anastomosing ridges are lacking (Fig. 13), the typical architecture of the tight junction becomes unmistakable. B faces of the plasma membranes disclose various-sized furrows corresponding to the lines of particles and/or to the continuous ridges (Figs. 7, 9, 11); transitional zones from A faces containing linear arrays to B faces with furrows “in register” are easily demonstrable (Fig. 9), whereas B faces corresponding to particulate islands are more difficult to find. Only rarely is a pitted surface detectable, especially in the case of the larger aggregates (Fig. 10).

**FIGURES 3–13** are freeze-fracture preparations of 14–15-day old fetal hepatocytes in the region of forming bile canaliculi. The sequence of micrographs reflects our interpretation of steps in tight junction formation.

**FIGURE 3** Short chains of particles (arrowheads) appear in a poorly-particulated region of the plasma membrane. The membrane face shows circular or polygonal depressions. × 82,500.

**FIGURES 4 and 5** Multiple chains of particles and diminutive particle islands tend to be disposed around the hollows in the membrane face, forming a discontinuous honeycomb network. Fig. 4, × 82,000. Fig. 5, × 82,500.

**FIGURE 6** Longer particle chains become continuous with one another, as well as with the particle islands, forming interconnecting arrays. × 88,000.
FIGURES 7 and 8  Anastomosing chains join irregular particle islands in a honeycomb pattern around a bile canaliculus (BC). The chains of particles on the A face clearly correspond to a network of shallow but easily identifiable furrows on the contiguous B face. Fig. 7, × 36,500. Fig. 8, × 82,500.
FIGURE 9 A- to B-fracture-face transition showing a chain of particles on the A-face in register with a furrow on the B-face (arrow). × 105,000.

FIGURE 10 A- to B-fracture-face transition including two particle islands. The B-face of the island to the left displays a barely visible finely pitted surface. The pitted surface, suggestive of a gap junction, stands out better on the B-face of the island shown in the inset. × 91,000. Inset, × 105,000.

FIGURE 11 Images suggestive of particle fusion or coating are indicated by arrowheads. × 69,000.

In most other instances, the B face over particle islands, even in A-to-B transitional zones, demonstrates merely slight depressions and appears for the most part to be evenly smooth.

On day 21 of the gestational period, plasma membranes (A faces) seem to lack the pattern of short, disconnected chains of particles (see Fig. 5), and the bile canaliculi are usually sealed by tight junctions with mature configuration (Fig. 14).

DISCUSSION

At particular stages in development, fetal cells afford useful models for studying de novo generation of specialized structures. The hepatocyte of the 14–15-day old rat fetus, in the present instance, is well-suited for our purposes, witnessing the sequential construction of the tight junction, for this is the stage of bile canaliculus development.
when emergence of such junctions is most exuberant.\(^1\) At no later time (even after partial hepatectomy of the adult liver) does the character of the event appear the same.\(^2\) Therefore our interpretation of the sequence of intramembranous activities which culminate in formation of tight junctions in the liver applies primarily to the fetus.

\(^1\) Before 14 days of gestation, the liver is not reliably recognized visually, since it is extremely small. We did not consider it essential to study earlier stages, for at 14 days the fetal hepatocyte presents a complete spectrum of differentiation of the membrane face.

\(^2\) R. Montesano, Unpublished observation.

In our view, the whole process begins with multiple short alignments of particles, all rather homogeneous in size and appearance, within the presumptive pericanalicular regions of the plasma membrane, otherwise poor in particles. Subsequently, simultaneous outgrowth and branching of single chains results in the construction of a network of anastomosing particulate strands which include or interconnect with small particle islands. The next step appears to involve the fusion or partial coating of adjacent particles to form short segments of smooth ridges, many of which later become confluent. The finale is a discontinu-
ous yet clearly delineated meshwork of anastomosing strands in which short segments of smooth-surfaced ridges alternate with beaded rows with the overall configuration of the tight junction as it is seen in adult liver. With progressive emergence of the smooth, continuous ridges, the newly-formed tight junction becomes complete. In our model, tight junctions appeared to arise from myriad discrete centers of growth which, at the beginning, represented nodal points in a prospective network, rather than originating from the peripheral elongation and branching of a newly-formed plexus of particulate strands or uninterrupted ridges. The interpretation we have given here, i.e., that certain specific intramembranous events reflect the ultimate formation of the complete tight junction, is based upon three direct observations: (a) the presence of pentalaminar fusions in thin sections, (b) the characteristic arrangement of particulate chains in a tight-junction-like network, and (c) the occurrence of furrows in membrane B-fracture faces. Precisely because the B faces are furrowed instead of pocked by aligned pits, we can assume that the particulate chains, rather than linear gap junctions (1, 4, 6, 15), are the skeletal structures from which the smooth ridges of the tight junction eventually emerge. And if we now propose (in spite of lacking double-replica images) that these furrows on the B face correspond to lines of particles on the A face, we may consider the membrane asymmetrical and the "material" which later coats the A-face particles more adherent to the B face in this early stage of development. Subsequently, this coating substance would cover the A-face particles and impart the images of "mature" smooth ridges. Examination of thin sections, however, does not serve to resolve this issue, since they reveal only a single type of pentalaminar fusion, not the coexisting particulate lines and smooth ridges apparent in freeze-fracture.

An intriguing feature of the forming junctions we observed was their association with particle islands. Again, examination of the B faces proved valuable, disclosing finely pitted surfaces over the larger aggregates. This landscape, together with the finding of typical 20–30-A gaps between hepatocyte plasma membranes in thin-section, would seem to support the concept that 14–15-day embryonic liver does contain true gap junctions. What the smaller arrays with no demonstrable pits on B faces represent is doubtful at present. But we hesitate to dismiss entirely the possibility that at

FIGURE 14 Bile canaliculus (BC) from a 21-day rat fetus. Image of a "mature" tight junction composed of the characteristic branching and anastomosing fibrils. × 44,000.

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least some of them are also gap junctions which differentiate simultaneously with tight junctions.

The sequence interpreted as tight-junctional assembly may perhaps be elucidated with greater clarity in fetal liver than in regenerating adult liver because of several factors. Foremost, the events may simply be more synchronous and hence more easily discerned. Second, images of tight junction assembly during cell-to-cell tactility (which is probably the case with the 14-day fetal model) may not be quite the same as those observed in tight junction formation after cell division as it occurs late in gestation and in regeneration. Thus, there must be an element of variance between the two processes. Third, tight junction formation may require that the cell membrane perform two separate functions: alignment of particles and their fusion or coating. Possibly, liver development, at this early stage, includes a time-lag between the stage of particle alignment and the attainment of cellular ability to transform this pattern to that of smooth-contoured fibrils.

Tight junctions could disintegrate as well in rapidly growing fetal liver as in other proliferating tissues (16), so that we must take into account the potentiality that we may be witnessing junctional degradation and dispersion. Another configuration of junctional units, however, appears to reflect such a process here, as in other tissues (16, 18). We assume that the remnants of former tight junctions consist of discontinuous strands, often with peculiar contour (16), in regions of the plasma membrane far from the bile canaliculus. In order to accommodate both points of view as reflecting junction "disintegration", we must introduce the theory that two different coexistent lytic mechanisms are operative: (a) fragmentation into isolated strands, subsequently to be "diluted" (16) and/or removed from the plasma membrane via endocytosis and lysosomal digestion; and (b) breakdown of the junctional strands into their own constituent subunits. On the basis of observations in other systems and organs, the second phenomenon would seem unlikely (16, 17).

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