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Development and Maintenance of Bile Canaliculi In Vitro and In Vivo

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ABSTRACT The apical surfaces of hepatocytes are specialized to form the boundaries of the bile canaliculi. The canaliculi function to secrete and concentrate components of the bile and to transport the bile out of the interior of the hepatic parenchymal tissue to the epithelium-lined bile ducts. Failure of the canaliculi to form and function properly can lead to biliary stasis or release of bile components into the bloodstream, both potentially life-threatening situations. Experimental analysis of canaliculus development and function has been undertaken in a number of experimental systems, ranging in complexity from intact animals to isolated hepatocyte cell cultures. These approaches each have inherent advantages and disadvantages for studying the various aspects of canaliculus development and function. This article summarizes what is known about how the functional components of the canaliculus develop and the directions that current experimental approaches are leading in analyzing this process. Studies of model epithelial systems have begun to define how interactions between components of the cytoskeleton and plasma membrane regulate the structure of polarized plasma membranes. These results are also discussed in terms of the bile canaliculus. *Microsc. Res. Tech. 39:406–412, 1997.*

INTRODUCTION

The bile canaliculi are a set of narrow intercellular channels between the hepatic parenchymal epithelial cells. Every hepatocyte is polarized and directly participates in the formation and maintenance of the associated canaliculi. This network permeates the liver and connects to epithelium-lined ducts that connect the canalicular network to the gallbladder and hence the lumen of the gut.

The walls of the canaliculus are the apical membranes of two or more adjacent hepatocytes. These membranes form extensive microvilli. The lumen of the canaliculus is separated from the basolateral aspect of the hepatocytes by tight junctions supported by adherens junctions (Farquhar and Palade, 1963). These tight junctions appear to be sufficiently impermeable to ions to support a potential difference between the lumen and the basolateral surface of approximately −10 to −15 mV (Wehner and Guth, 1991). The membrane of the bile canaliculus is associated with a number of tubular membrane structures that appear to represent components of a transcellular membrane transport pathway in the process of fusing with or budding from the plasma membrane (Harada et al., 1992).

The cytoplasmic face of the canalicular membrane is associated with a dense cytoskeletal network consisting of actin microfilaments and cytokeratin intermediate filaments. The actin web consists of two populations of filaments. Directly underlying the apical plasma membrane is a web of microfilaments into which are inserted the microvillar microfilament core. Whereas the microvillar core microfilaments are all oriented in one direction, the microfilaments of the underlying web are oriented in both directions relative to each other (Ishii et al., 1991). More distal is a highly oriented band of microfilaments that insert into the adherens junctions that are just basal to the tight junctions (Ishii et al., 1991). Also associated with this bipartite canalicular sheath of microfilaments is a dense network of keratin intermediate filaments.

The activity of the canalicular membrane can probably be regulated by extracellular signals. The cytoplasmic surface of the canalicular membranes are also enriched in G-proteins relative to the basal and lateral surfaces of the cells (Ali et al., 1989), which suggests that there may be feedback, based on the contents of the lumen of the canaliculus, to the intracellular signaling pathways of the hepatocyte via ligand-receptor mechanisms. The finding that one of the bile salt transport proteins of the canalicular membrane is phosphorylated on tyrosine residues in response to insulin stimulation (Margolis et al., 1988) suggests that the activity of the canalicular membrane can also be regulated by signals acting at the basolateral surface of the hepatocyte.

FUNCTIONAL CHARACTERISTICS OF THE BILE CANALICULI

The bile canaliculi serve one major function in the liver: they sequester materials that are vectorially transported out of the liver to form bile and conduct these materials to epithelium-lined bile ducts, which then conduct the material to the gall bladder. To serve this function, the canaliculi must have a set of polarized transport mechanisms associated with them to remove

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material from the interior of the cell and contractile structures to force the primary bile toward the bile ducts.

The transport function of bile canaliculi has been characterized extensively. There appear to be two main pathways for vectorial secretion via the bile canaliculi: one mediated by vesicular traffic from the sinusoidal surface, and one mediated by transport through the sinusoidal membrane, diffusion or facilitated transport in the cytoplasm, and transport through the canalicular membrane (Wilton et al., 1994). These two general pathways are used to transport a large variety of bile constituents by virtue of an extensive set of transport and processing enzymes that are vectorially inserted in the bile canalicular membrane (reviewed in Arias et al., 1993).

A third secretory pathway into the bile canaliculus is the transcytotic pathway for secretion of IgA. In this case, a specific ligand binds IgA at the sinusoidal surface of the cell, and the complex is then translocated through the cytoplasm in a vesicle to the canalicular surface, where a specific enzyme cleaves the receptor to release the IgA complexed with a receptor fragment called Secretory Component (Musil and Baenziger, 1988).

Once the bile is secreted into the lumen of the canaliculus, it must be moved out of the parenchymal tissue mass and into the bile ducts; failure to do this leads to biliary stasis, usually a prelude to infection. At one time, canaliculi were thought to be essentially passive conduits for bile. There is now extensive evidence that the canaliculi are contractile structures that force their contents to the bile ducts and that the cytoskeletal pericanalicular sheath is essential for forcing the bile out of the parenchyma. First, it is clear that contraction of the canaliculi is a normal phenomenon and that this contraction moves bile through the canalicular network in liver tissue (Watanabe et al., 1991b). The contractility of the bile canaliculi is preserved in hepatocyte doublet preparations (Gautam et al., 1989; Kitamura et al., 1991; Smith et al., 1985; Watanabe et al., 1985) as is the ability to secrete bile vectorially into the canalicular cyst (Gautam et al., 1989; Kitamura et al., 1990). Introduction of Ca^{2+} ions into the cytoplasm of hepatocytes in doublets induces contraction (Kitamura et al., 1991; Watanabe et al., 1985), and introduction of a constitutively activated myosin light chain kinase also induces contraction (Kitamura et al., 1991), indicating that canalicular contractility is based on an actin–myosin ATPase activity. This notion was confirmed by using permeabilized cells (Watanabe et al., 1991a) and cytoskeletal preparations (Tsukada and Phillips, 1993) in which the majority of the plasma membrane and soluble cytoplasmic proteins are eliminated. The role of myosin in calcium-regulated canalicular contraction was also confirmed by inhibition of canalicular contraction with a myosin light chain kinase-specific inhibitor (Watanabe et al., 1991c).

The intermediate filaments and microtubules that form the outer layer of the pericanalicular sheath are not essential for contraction, but disruption with specific drugs causes a virtually complete inhibition of fluorescein diacetate secretion by the bile canaliculi and of secretion of horseradish peroxidase into the canaliculus after uptake from the sinusoidal surface (Kawahara et al., 1989, 1990). Thus, the maintenance of intact polarized transport pathways apparently is dependent on the microtubules and keratin filaments, whereas contraction of the canaliculus is dependent on the actin–myosin cytoskeleton.

Independent evidence for the role of the pericanalicular sheath has been obtained by studying the composition and structure of the sheath in liver tissue in which various pathological alterations are induced. When physical forces are induced that affect canalicular function, by either bile duct ligation or induction of lipid accumulation in alcoholic fatty liver, the microfilament and keratin intermediate filament composition of the sheath are distorted and focally weakened (Ohta et al., 1988). In humans with biliary atresia, improvement in bile flow is associated with formation of continuous, even pericanalicular sheaths (Segawa et al., 1993), indicating that the actin and myosin components of the sheath are essential for normal bile flow in vivo. The triggering event for contraction is not known but may be related to distention of the canaliculus. Colchicine treatment, which disrupts microtubule structure and thus the rate of secretion of bile but not the structure or function of microfilaments, causes a decrease in the rate of canaliculus contraction in doublet preparations, possibly by decreasing the amount of bile secreted into the canalicular cyst and thus the distention of this closed compartment (Oshio et al., 1985). Distention could also explain the sequential triggering of contraction along the length of the canaliculus in vivo (Watanabe et al., 1991b); however, coordinated contraction of canalicular cysts in acute primary preparations has been observed in a situation in which there is no coupled distention of the two canalicular cysts (Smith et al., 1985). This result suggests that a coordination mechanism exists that connects through the lateral membranes, possibly based on gap junctions or mechanical coupling through the cytoskeletal-junction network.

DEVELOPMENT OF THE BILE CANALICULI IN VIVO

Canaliculus development has been studied in a number of organisms, some with radically different temporal patterns of development. However, although the liver may develop at different rates and at different times relative to other organs and relative to time of birth, the order of development and the ultrastructural changes during development are remarkably similar.

During embryonic development, the liver originates as an epithelial bud growing from the newly closed anterior intestine (Kingsbury et al., 1956), and so the connection of the canalicular network to the lumen of the gut might be viewed as a maintained embryonic connection; however, it is not clear that the connection is maintained throughout the early development of the liver, and the connection may well develop de novo at a later stage of development.

The cells of the earliest developing hepatic parenchyma initially appear unpolarized. Although the hepatic diverticulum originates as an inpocketing of the gut epithelium, the bud of epithelial tissue that develops has no visible polarity either at the ultrastructural level or as determined by the presence of antigens that are expressed in a polarized fashion in mature liver tissue (Gallin and Sanders, 1992). As the hepatic

primordium grows, it invades the sinus venosus and is invested by circulation by virtue of the blood flow that it interrupts. At the same time, the first visible signs of polarity arise. In the chicken, this occurs at about day 5 of development, and fully functional and morphologically mature canaliculi are present by day 7 (Gallin and Sanders, 1992). In the mouse, the canaliculi at birth are present but immature and do not attain an adult appearance until 5 days postpartum. In the rat, the earliest canaliculi appear at 12–16 days of gestation but do not attain a mature ultrastructure until 2 days postpartum (Kanamura et al., 1990, and references therein).

In the chicken embryo, antigens that are specific to the canaliculus become visible in the hepatocytes at the core of the primordium at embryonic day 5, and from their first appearance are present in a polarized pattern. Over the next 2 days of development, the network of canaliculi spreads through the rest of the primordium until, by day 7, the canalicular network is present throughout the tissue mass. From this point on, as the liver grows, canaliculi are formed directly by the new cells (Gallin and Sanders, 1992).

Ultrastructurally, this changing antigenic pattern of expression is paralleled by gradual development of the characteristic canalicular structures. The first apparent signs of canaliculi consist of convoluted facing surfaces on adjacent hepatocytes, which appear to be newly forming microvilli (Gallin and Sanders, 1992; Kanamura et al., 1990). These microvilli are immediately followed by development of junction complexes. The lumen of the canaliculus begins to open once the junction complexes have formed, resulting in an open space that is filled by the newly secreted bile components.

The highly polarized structure of the canaliculi appears to arise primarily from directed targeting of a specific subset of proteins to the bile canaliculus immediately after they are synthesized. In the liver, the proteins that are destined for the canalicular membrane are first transported transiently to the basolateral surface and then translocated to the apical surface, probably by a vesicle-mediated transport process (Bartles et al., 1987).

Once the canaliculus-specific proteins reach the apical surface, there appear to be two mechanisms for maintaining their polarized distribution. The presence of tight junctions is paramount in this regard. The tight junctions act not only as gaskets to limit and regulate paracellular transport but also as diffusion barriers in the plane of the membrane that prevent the diffusion of proteins between the apical and lateral surfaces. When hepatocytes are dissociated, some of the canalicular proteins rapidly equilibrate over the plasma membrane, indicating that tight junctions were the major factor maintaining their polarized localization (Mowery and Hixson, 1991; Sawada et al., 1992).

A second mechanism that operates for another subset of the membrane proteins requires the presence of intact cytoskeleton. It appears that some apical proteins are physically linked to an underlying structure and that, even when the tight junctions are dissociated, the proteins will stay associated in a polarized structure (Graf and Boyer, 1990; Terry and Gallin, 1994).

DEVELOPMENT AND MAINTENANCE OF BILE CANALICULI IN VITRO

Analysis of the development and function of bile canaliculi has largely been accomplished in model systems that have a much simpler structure than that of the intact liver. These models include hepatoma cell lines grown in monolayer culture, acutely isolated hepatocyte doublets, primary hepatocyte cultures grown in monolayers, and primary hepatocyte cultures grown in suspension culture. Each of these systems offers experimental advantages, but at the cost of losing important elements of normal liver structure that play a role during development or that are responsible for maintaining a stable phenotype in mature liver tissue.

Primary monolayer cultures of hepatocytes have been used extensively for characterizing bile canaliculus formation and maintenance. Although these cells dedifferentiate in culture on simple glass or plastic substrates, appropriate conditions have been developed for maintaining a polarized phenotype for approximately 1 week. These cultures form extensive connected networks of canaliculi that span tens of cells (Sawada et al., 1992; Terry and Gallin, 1994). The coordinated development of such a connected network is as functionally important as the polarization of individual cells.

Formation and maintenance of the bile canaliculi is critically dependent on the function of the calcium-dependent cell adhesion molecule E-cadherin (also called L-CAM). E-cadherin is the primary cell–cell adhesion molecule in embryonic hepatocytes (Gallin et al., 1983). Although a calcium-independent cell–cell adhesion molecule has also been isolated and characterized (Ocklind and Obrink, 1982), subsequent analysis has shown that this molecule is in fact an apically disposed ATPase (Aurivillius et al., 1990; Ocklind et al., 1983) that appears during development much later than E-cadherin. The activity of this molecule in cell–cell adhesion is probably an artifact of the simple aggregation assays used to characterize it initially, although there is a report that suggests that it may play a role in reinforcing the junction complex at the canaliculus (Mowery and Hixson, 1991).

Disruption of cell–cell contact with antibodies to E-cadherin prevents the formation of intact canaliculi. Ultrastructurally, this treatment prevents the formation of close cell–cell contacts and of junction complexes that are sufficient to maintain the integrity of the canaliculus (Terry and Gallin, 1994). When the treatment is evaluated by determining the location of canaliculus-specific antigens, disruption of cadherin function prevents the formation of intact canaliculi that extend between cells, but many of the antigens are localized to small regions of the cell surface (Terry and Gallin, 1994), indicating that mechanisms other than junctional sequestration are probably involved in the development and maintenance of polarity of membrane proteins.

Primary cultures of hepatocytes suffer from the major disadvantage that they rapidly dedifferentiate. Within minutes of tissue dissociation, transcription of many tissue-specific genes decreases below detectable levels (Clayton and Darnell, 1983). If the cells are grown as simple monolayers, within several days they

stop producing many of the characteristic liver proteins and develop a flattened fibroblastic appearance, although some of the characteristic epithelial proteins are still expressed for much longer.

The effects of tissue dissociation can be partly reversed by culturing the hepatocytes between layers of extracellular matrix, thus mimicking the geometry of cellular organization within the tissue (Ben-Ze'ev, 1991; Ben-Ze'ev et al., 1988; Dunn et al., 1989, 1992; Musat et al., 1993). Collagen, proteoglycan, and complex extracellular matrix (ECM) mixtures have been reported to aid in maintaining hepatocyte differentiation in vitro (Enat et al., 1984; Spray et al., 1987). There also appears to be an interplay between ECM receptor occupancy and cell shape that is essential in maintaining the differentiated polarized hepatocyte phenotype (Singhvi et al., 1994) see Figure 1. Growth of monolayer cultures on collagen-coated permeable membranes (Arterburn et al., 1995; Guery et al., 1995) or in perfused matrix capillary systems (Gerlach et al., 1995) maintain canalicular structures, indicating that nutrient evailability at the basolateral surface also may be a significant factor.

An additional problem for culturing hepatocytes is the fact that serum rapidly promotes dedifferentiation, particularly of the bile canaliculi (Terry and Gallin, 1994). This problem can be partly offset by using defined hormones instead of serum as additions to medium (Terry and Gallin, 1994) or by growing in the presence of STO feeder cell layers (Talbot et al., 1994), but these supplements are probably not providing the complete normal complement of soluble growth modulators. Recently, we have begun characterizing the dedifferentiating activity from serum. There appears to be one factor, a protein with a molecular weight of approximately 60 kD and isoelectric point (pI) of approximately 4.5, that promotes dedifferentiation (W.J. Gallin, unpublished); once this factor is characterized, it will be possible to specifically remove it from serum and then determine whether complete serum can provide better support for primary hepatocyte cultures.

Primary hepatocytes also have been used successfully in acute preparations for studying the function of bile canaliculi, mainly as cell couples that either retain many of the junction contacts present in tissue or rapidly reform them in vitro, within 4–8 hours after tissue dissociation (Gautam et al., 1987). These preparations can be used for characterization of canaliculus physiology, including acute bile production (Gautam et al., 1989), but are not useful for studying development and maintenance of the canaliculi.

Primary hepatocytes also can be grown in suspension and allowed to aggregate, thus forming histotypic aggregates. These aggregates reform and maintain many of the phenotypic properties of liver tissue, and provided they are maintained at a small size and in a fairly rich medium, they will retain those characteristics for days or weeks (Moscona, 1957, 1962). In the case of canaliculi, aggregates appear to be insensitive to the presence of serum and do not dedifferentiate over a period of several weeks, indicating that conditions that maintain cell shape and extensive lateral contacts may be sufficient to support maintenance of a completely differentiated canalicular network (Terry and Gallin, 1994). Thus, the results to date indicate that an intricate interaction between cell shape, cell–cell and cell–substrate interactions, and soluble dedifferentiation and growth factors is responsible for regulating hepatocyte polarity.

A number of permanent cell lines have been derived from hepatomas from a number of species. These cell lines have the advantage that they have developed a stable in vitro phenotype, and many of them maintain at least some phenotypic characteristics of liver. Very few of these permanent lines, however, maintain polarity in a functionally meaningful way. This ability appears to be a function of whether they are derived from differentiated or undifferentiated tumors. HepG2 and HuH-7, both human hepatoma cell lines, form structures that have many of the antigenic and ultrastructural characteristics of bile canaliculi (Chiu et al., 1990), although the means by which the canaliculi form (Chiu et al., 1990; Sormunen et al., 1993) differ from some reports in primary cultures and liver tissue (Gallin and Sanders, 1992), and the number and size of microvilli both appear to be abnormally large.

One approach that has proved successful in generating a cell line that will form and maintain bile canaliculi has been to fuse undifferentiated hepatoma cells with untransformed cells from another species (Cassio et al., 1991; Petzinger et al., 1994). In one case, this has led to the development of a permanent cell line that robustly produces canalicular cysts between cells, with many of the characteristics of the cysts formed in acute primary doublet cultures (Ihrke et al., 1993). However, no lines have been developed that maintain an integrated network of canaliculi that span many cells, as can be obtained in primary culture (Musat et al., 1993; Terry and Gallin, 1994).

EXPERIMENTAL ANALYSIS OF FACTORS REGULATING BILE CANALICULUS DEVELOPMENT

Ultimately, the development of bile canaliculi is a special case of the general problem of development and maintenance of polarity in epithelia. Thus, the insights obtained from the more experimentally manageable epithelial cell lines can be applied to the problem in primary cultures of hepatocytes and can be used as a basis for designing specific experiments.

The development of polarity in epithelia has been best studied in the Madin-Darby canine kidney (MDCK) cell line. This cell line has an extremely robust polarized phenotype that does not rely on the presence of ECM or any soluble factors other than those that are required for cell survival. MDCK cells in confluent monolayer culture form tightly coupled sheets of cells with high transepithelial resistance. Several sublines with different resistance have been isolated, but all form a monolayer with extensive tight junctions and extensive lateral membrane contacts mediated by E-cadherin.

MDCK cells can be grown in medium with low concentration of calcium (5 μM) and maintained as single unpolarized cells. When shifted to a normal calcium concentration (1.8 mM), the cells rapidly form lateral adhesive contacts, form complete junctional complexes, and become polarized, both ultrastructurally and biochemically. One of the earliest events in this polarization is the formation of E-cadherin adhesions

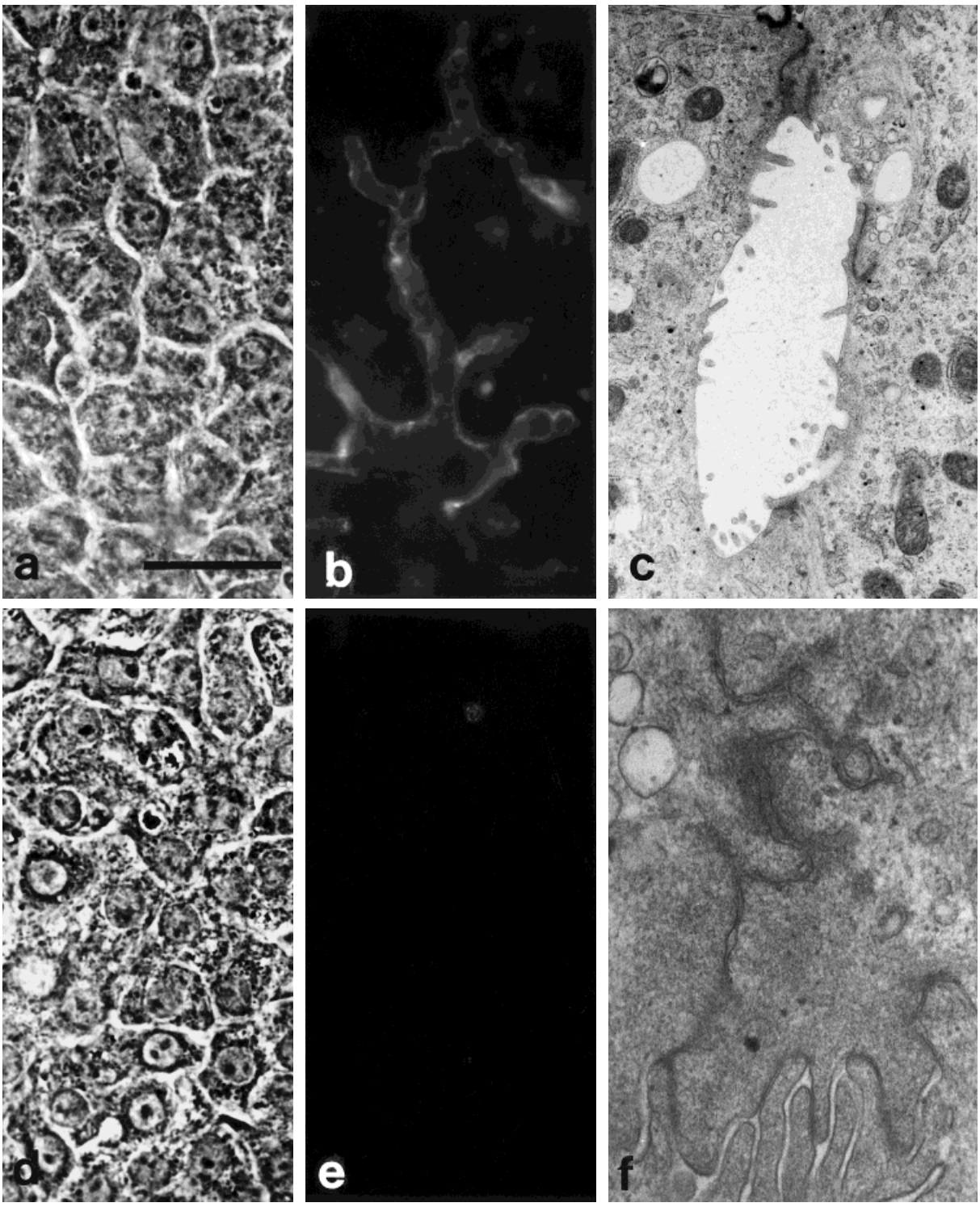

Fig. 1. Hepatocyte monolayer cultures maintained for 5 days in the absence of fetal calf serum in medium suplemented with insulin, dexamethasone, and ornithine (**a–c**) or in the presence of fetal calf serum (**d–f**). Phase-contrast light microscopy (a,d) shows that at 5 days of culture there is no obvious change of cell morphology in the large colonies of cells that form in culture. Immunofluorescent staining for a canaliculus-specific antigen, however, shows that the serum has caused a complete cessation of expression of the canalicular antigen (e), whereas the cells in serum-free medium have formed and have maintained an extensive connected network of canaliculi that extends for at least 10 cell diameters. Transmission electron microscopy of these cultures demonstrates that the cells grown in serum-free medium have maintained canaliculi with open lumens lined with microvilli, with distinctive junctional complexes bounding the canaliculus. The cells grown in serum-containing medium still have remnants of canalicular structures, which are characterized by the interlocked microvilli seen at the bottom of f, but the lumen is almost absent. Close apposition of membranes from adjacent cells is still maintained by E-cadherin in these cells, but the polarized phenotype has almost completely disappeared. Scale bars, 53 μm for a,b,d,e, 1.4 μm for c, 400 nm for f.

and association of ankyrin and fodrin with E-cadherin into a heteromultimeric complex (McNeill et al., 1993).

This association appears to be pivotal for polarization in two ways. First, the formation of stable cadherin-mediated adhesion is essential for formation of functional tight junctions that delineate the apical from the basolateral surfaces (Gumbiner and Simons, 1986). Without the tight junctions, many of the polarized membrane proteins could diffuse in the plane of the membrane into either the apical or basolateral domain. Second, even in the absence of tight junctions, the distribution of some membrane proteins is polarized, and this polarization appears to depend on the presence of cadherins (McNeill et al., 1990). More telling, the identity of the cadherin that is expressed in the cell may determine the pattern of polarization of some membrane proteins (Marrs et al., 1993), suggesting that cadherins may be playing a pivotal role in the development of polarity and thus the formation of bile canaliculi.

Primary cultures of hepatocytes also raise problems that are not addressable in permanent epithelial cell lines, so a reasonable experimental study of the problem of canaliculus development and maintenance must ultimately rest on analysis of the factors that control cell polarity in primary hepatocyte cultures. One major issue that can not be addressed in MDCK cells is how hepatocytes coordinate the location of canaliculus formation on different cells to form a network that extends throughout the tissue or culture. Also, the instability of the polarized phenotype in primary hepatocyte cultures suggests that there are regulatory pathways that are permanently activated or inactivated in MDCK cells but that are subject to modulation in liver tissue and primary culture. Thus, the studies of permanent cell lines can provide insights into the development and maintenance of bile canaliculi but as model systems cannot be expected to tell the whole story.

CONCLUSIONS

The polarization of fully differentiated hepatocytes that results in formation of bile canaliculi is based on cellular mechanisms that are similar to those in other polarized simple epithelia. The junctional complexes, the adhesion molecules, and the intracellular trafficking that vectorially transports the components of the apical and basolateral surfaces to the correct domains are all similar in all polarized simple epithelia studied to date. However, the bile canaliculi possess a number of unique features, in particular the small size of the canalicular lumen relative to the basal and lateral surfaces and the intricate ramifications of the canalicular network. Thus, future analysis of bile canaliculus development and function needs to use the information from studies on polarity in cell lines but will also require analysis of the factors that are unique to real tissue cells.

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