Hybrid Cell Lines Constitute a Potential Reservoir of Polarized Cells: Isolation and Study of Highly Differentiated Hepatoma-derived Hybrid Cells Able to Form Functional Bile Canaliculi In Vitro

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Abstract. A large number of hepatoma cell lines has been used to study expression and regulation of liver-specific function. However these cells, even the most differentiated, are morphologically far from hepatocytes. In no case is the typical hepatocyte cell polarity well maintained.

Cell hybridization has been used as a potential means for turning on specific genes. From hybrids between well differentiated Fao rat hepatoma cells and WI 38 human fibroblasts, we have attempted to isolate segregated cells that are highly differentiated and polarized. Such cells, detected in aged cultures of only one hybrid (WIF12), were isolated by subcloning. One subclone, WIF12-1 was analyzed.

Expression of liver-specific functions extinguished in the original hybrid is restored in all WIF12-1 cells at a very high level, similar to that of hepatocytes and 5–30 times higher that that of parental cells. Moreover human genes coding for liver-specific proteins (albumin, fibrinogen, and alcohol dehydrogenase) are actively expressed. WIF12-1 cells have acquired a polarized phenotype as attested by the presence of bile canaliculi between adjacent cells and by the asymmetrical localization of apical (Mg$^{2+}$-ATPase, γ-glutamyl transpeptidase) and basolateral membrane markers. The bile canaliculi formed are dynamic and functional structures, characterized by long periods of expansion followed by rapid contractions. The ability to polarize is a general and permanent property of WIF12-1 cells.

These cells appear to constitute a valid model for the in vitro study of hepatocyte cell polarity, membrane domain formation and mechanisms of membrane protein sorting.

PERMANENT cell lines derived from tumors have been widely used to study expression and regulation of cell differentiation. However these cells, even those for which the expression of tissue-specific functions is well maintained, are mostly characterized by morphological traits far from the normal state. Differentiated and polarized epithelial cell lines are in fact not numerous (Simons and Fuller, 1985). The MDCK cell line has been successfully exploited to study polarity (McRoberts et al., 1981). In the case of intestinal cells a few colon carcinoma lines, such as Caco-2 (Pinto et al., 1983) and HT-29 (Zweibaum et al., 1985), have been used as models of polarized cells. Unfortunately, the equivalent for liver cells does not exist. We have previously studied several hepatoma lines, in particular the well-differentiated H4 II EC3 and its derivative Fao, and no real sign of polarity has been observed in these cells (Rogier et al., 1986; Maurice et al., 1988). This could be due to the fact that hepatocytes are complex polarized cells which form a continuous tridimensional lattice; they present thus a unique membrane organization, which could be difficult to maintain or restore in cells growing in monolayers. However this difficulty is not insurmountable, since normal hepatocytes in very short term cultures are able to keep and even to regain a certain degree of polarity (Gautam et al., 1987; Maurice et al., 1988).

Here we report a new approach to obtain stable polarized and differentiated cells of hepatic origin. This approach is based on the fact that in hybrids between cells of different histogenic origin, expression of differentiated functions is subject to different regulations: extinction, reexpression, and activation, according to the parental chromosomes retained (Ringertz and Savage, 1976). Generally, specific functions expressed only by one parent are extinguished in hybrids. However, extinction is reversible since loss of chromosomes of the extinguishing parent leads to reexpression of the extinguished functions and even to activation of previously silent genes (for review see Weiss et al., 1988). Functions that are absent from both parental cells can even be activated (Kao and Puck, 1972; Klee and Nirenberg, 1974), but this phenomenon, known for a long time, has never been systematically exploited. Our strategy was to take advantage of the chromosome segregation that occurs in certain hepatoma-derived hybrids, for obtaining cells even more differentiated and that present the typical hepatocyte ultrastructure.
Materials and Methods

Hepatocyte Isolation and Primary Cultures

Hepatocytes were isolated from Wistar rats after perfusion with collagenase (Worthington Biochemical Co., Freehold, NJ) according to Seglen (1976). Hepatocytes were isolated from Wistar rats after perfusion with collagenase (Worthington Biochemical Co., Freehold, NJ) according to Seglen (1976). Hepatocyte lysate was collected for plasma protein assays and the hepatocytes grown on glass slides or plastic dishes secrete equivalent amount of plasma protein.

Plasma Protein Production

The production of several plasma proteins by different cell lines has been followed using appropriate antisera, by measuring the amounts secreted in spent medium and by immunostaining of cells. These two methods are complementary: the first one gives a direct measurement of the average synthesis rate (Cassio et al., 1981), the second gives information about homogeneity of cell populations.

Indirect immunofluorescent staining of intracellular plasma proteins was performed as detailed previously (Cassio et al., 1986). The amounts of albumin and α1-inhibitor 3 were measured by the electrophoresis method of Laurell (1966). The sensitivity of these methods is such that 0.1 μg/ml of albumin and 0.25 μg/ml of the other proteins is easily detectable. When necessary, spent medium was concentrated up to 150 times by ultrafiltration through CFS40 centriflo membranes (Amicon Corp., Danvers, MA). The amount of protein secreted was expressed in micrograms per 10^6 cells per 24 h taking into account the increase in cell number during the interval studied (Cassio et al., 1980).

Detection of Liver-specific Enzymes

Alcohol dehydrogenase (E.C.1.1.1.1) activity present in cellular crude extracts, was revealed after starch-gel electrophoresis as described by Kiely et al. (1982). This technique permits separation of rat from human isoforms. Tests for the presence of two key enzymes of the gluconeogenic pathway fructose 1-6 bisphosphatase (E.C.3.1.3.1) and phosphoenolpyruvate carboxykinase (E.C.4.1.1.32) were carried out by the use of glucose-free medium supplemented with 2 mM oxaloacetate (Bertolotti, 1977).

Cloning, Karyotyping, and Generation Time

Subcloning of rat hepatoma–human fibroblast hybrids has been performed by inoculation of 20–50 single cells into 10-cm Petri dishes. 4–7 wk later, well isolated colonies (one per dish) were scraped with a micropipette, subcultured, and frozen after about 25 generations (two to five months after subcloning). The cloning efficiency varied from 50–90%, according to the hybrid clone examined. WIF1-2 cells were subcloned by trypsinization exactly as described, and a hundred subclones were examined regularly over a period of several weeks for the formation of bile canalicular-like structures.

Karyotype studies were performed on colcemid-arrested metaphases obtained either by enzymatic detachment and air drying, or by an in situ procedure (Wortman and Duff, 1979). In both cases, cells were swollen in 75 mM KCI at 37°C for 10 min. fixed, and air dried. Analysis of at least 15 metaphases was carried out using various staining techniques. To distinguish human chromosomes from rat chromosomes, the procedure used was staining with Giemsa at pH 11.3 (Buys et al., 1984) preceded or not by G banding (Gallimore and Richardson, 1973).

For generation time determinations, 10^5 cells were plated per 10-cm Petri dish and medium was renewed regularly. The cells in two dishes were enumerated independently, at regular intervals over a period of 7–18 d. Generation times were calculated from the exponential growth phase.
Table I. Reexpression of Rat Albumin and Appearance of Overproducing Cells in Aged WIF12 Cultures*

| Time in culture (d) | Percent of albumin producing cells | Secretion of rat albumin (µg/10⁶ producing cells/24 h) |
|---------------------|-----------------------------------|------------------------------------------------------|
| 8                   | 0                                 | <0.010                                               |
| 17                  | 8                                 | 0.3                                                 |
| 24                  | 13                                | 1.8                                                 |
| 31                  | 21                                | 4.2                                                 |
| 38                  | 26                                | 12.0                                                |
| 46                  | 28                                | 15.3                                                |
| 65                  | 33                                | 22.9                                                |

* WIF12 is a rat hepatoma x human fibroblast hybrid.
† WIF12 cells frozen 25 generations after fusion were re-cultivated for 25 more generations and examined for rat albumin gene expression at regular intervals. The rat parental hepatoma is characterized by a rate of secretion of 2.5 µg albumin/10⁶ cells/24 h, all its cells being engaged in this production.
§ The presence of human albumin in the spent medium of WIF12 cells was first detected after 24 d in culture and the amount secreted after 65 d was 10 µg/10⁶ cells/24 h.

Electron Microscopy Analysis of Bile Canaliculi-like Structures

Cells were fixed directly in plastic dishes with 1.5% glutaraldehyde in 0.13 M sodium phosphate buffer, pH 7.2, for 30 min at room temperature and washed in the same buffer. They were postfixed either in the plastic dishes, or after detachment and centrifugation, for 30 min in 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.2. Cells stained with 0.5% uranyl acetate for 1 h were dehydrated for 10 min using a graded series of ethanol. These preparations were embedded in epoxy resin: epon capsules were inverted and placed on the cells and polymerized at 60°C overnight. After polymerization, cell layers could be easily separated from the bottom of the plastic dishes. For transverse sections, small fragments of the embedded cell layers were reembedded in the correct orientation. Ultrathin sections were contrasted with uranyl acetate and lead citrate, then examined with a Elmiskop 1A (Siemens, Berlin, Germany), at the accelerating voltage of 80 kV.

Localization of Plasma Membrane Markers

Cells were grown on glass cover slides. Mg²⁺-ATPase activity was localized exactly as described by Graf et al. (1984) on cells fixed in cold Karnovsky's fixative (3% paraformaldehyde/0.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4). In most experiments, saponin at a concentration of 0.02% was added from fixation until staining to increase the accessibility to the canalicular membrane domain. The localization of γ-glutamyl transpeptidase activity was performed on cells fixed by cold acetone during 15 min, exactly as described by Rutenburg et al. (1969). Indirect immunofluorescent staining of the basolateral marker CE9 (Hubbard et al., 1985) was performed on permeabilized cells as described for the localization of other membrane antigens (Maurice et al., 1988).

Figure 1. WIF12-1 cells show an hepatocyte-like morphology. Phase contrast images of the rat hepatoma parent Fao (A), the original extinguished rat hepatoma x human fibroblast hybrid WIF12 (B), and the segregated differentiated WIF12-1 cells (C), and rat hepatocytes in primary culture (D). Fao and to a greater extent WIF12 differ strikingly from hepatocytes. Fao cells are very small with little cytoplasm. WIF12 cells are flattened. In contrast WIF12-1 cells have stably acquired typical hepatocyte morphological traits: a fraction of cells is binucleated. Cells have a large and very dense cytoplasm and one observes frequently between adjacent cells clear areas (arrows) of irregular shape that are reminiscent of bile canaliculi. There are at least 14 of these areas in C. Note that WIF12-1 cells (for example the cell surrounded by three arrows) can be implicated in the formation of more than one such area. Similar areas (arrows) are visible between adjacent hepatocytes. Bar, 25 µm.
Appearance of Highly Differentiated Cells in WIF12-1 Progeny

Starting from the WIF hybrid family, we have attempted to isolate segregated hybrids more differentiated than the hepatoma parental cells. A dozen clones, that had been frozen 25 to 30 generations after fusion, were recultivated and reexamined. Most of them were in a differentiated state analogous to that of Fao cells: hepatic functions were stably expressed at the parental level by the majority of cells of each clone and the morphology of the hybrid cells was similar to that of parental hepatoma cells (Fig. 1 A). In contrast, in WIF6 and WIF12 cells, which showed flattened epithelial morphology (Fig. 1 B), hepatic functions, such as albumin, were still extinguished. To favor chromosome segregation, and consequently reexpression and activation of hepatic genes, these two clones were maintained in culture for an extended time. During this period, albumin production was followed at regular intervals, by measuring the amounts of protein secreted, and by staining albumin producing cells in the cellular population.

For both clones, reexpression was observed only in a minority of cells, but, in WIF12 cultures, reexpression was accompanied by overexpression. As shown in Table I, the fraction of WIF12 cells producing albumin increased with time in culture, but more surprisingly, these cells secreted more and more rat albumin, up to 28 times the amounts characteristic of the rat hepatoma parent. In addition, human albumin was also detected in the medium. All these results suggested the presence of highly differentiated cells in WIF12 aged cultures. To isolate such cells, subcloning of WIF12 after 50 d in culture was performed. Among the 13 subclones isolated, four were composed of cells that not only overproduced rat albumin and secreted human albumin, but also presented a morphology reminiscent of normal hepatocytes. One of these subclones, WIF12-1 was analyzed in detail. Subcloning of WIF6 was performed in parallel; WIF6 subclones reexpressing albumin were obtained, but no case of overproduction or acquisition of hepatocyte morphology was observed.

WIF12-1 Cells Are Highly Differentiated

In Fig. 1 are shown representative fields of living cultures of WIF12-1 and its ancestors. Striking differences are observed among the three related cell lines. WIF12-1 cells appear as the most differentiated (Fig. 1 C) and have acquired a morphology very close to that of hepatocytes in primary culture (Fig. 1 D); moreover, in both cases a fraction of cells are binucleated. The most characteristic trait of WIF12-1 cells is the presence of dilated spaces between adjacent cells that are in close contact, spaces that are similar in appearance to bile canaliculi. These spaces are formed in semi-confluent as well as in sparse cultures. In this latter case, they are observed at the junction of cells forming tight couples.

The first question, concerning WIF12-1 cells was whether the acquisition of the typical hepatocyte morphology is accompanied by drastic phenotypic changes. Therefore, the production of hepatic proteins by Fao, WIF12 and WIF12-1 cells, was measured (Table II). For the original hybrid WIF12, the functions examined (plasma proteins and neoglucogenic enzymes) are extinguished with the exception of transferrin. In contrast, WIF12-1 cells reexpress all these proteins: this reexpression occurs not at the parental level, but at a level 6-29 times higher, similar to that of rat hepatocytes in primary cultures (Table II). To evaluate if all cells or only a sub-population are engaged in this overproduction, a cell by cell...
Figure 2. Immunofluorescence analysis of rat \( \alpha_1 \)-antitrypsin synthesis by WIF12-1 cells and its ancestors. The photomicrographs are mounted to show for each clone, the immunofluorescent staining of \( \alpha_1 \)-antitrypsin (right) and the corresponding phase contrast image (left). Fao (A and B), WIF12 (C and D), WIF12-1 (E and F). In positive clones, the staining pattern is similar, the protein being essentially localized in the Golgi apparatus. At least 95% of WIF12-1 cells are positive whereas only 80% of Fao cells are stained as previously reported (Cassio et al., 1986). When adjacent WIF12-1 cells form a canalicular area (400 cases studied), the Golgi apparatus, in 95% of the cases faces this area (arrow). Bar, 25 \( \mu \)m.

analysis was performed. For each of the five plasma proteins, the newly synthesized protein was visualized inside the cells by immunofluorescence. No heterogeneity of the cellular population was observed. As illustrated for \( \alpha_1 \)-antitrypsin in Fig. 2, not only the whole WIF12-1 population synthesizes the protein considered, but it is clear that each cell is equivalent. We can thus conclude that overproduction of hepatic proteins is a general intrinsic property of WIF12-1 cells.

The second question concerned the chromosomal content of these cells. The mean chromosome number of WIF12 cells examined at the 25th generation was estimated at 93 (84-102), that of WIF12-1 was only 76 (70-81). The karyotype of WIF12-1 does not change with time in culture and on average eight (five to nine) human chromosomes are stably retained. The most commonly found were chromosomes 4, 5, 6, 8, 12, 15, 18, and X, the latter being selectively retained owing to the culture conditions (HAT medium); chromosomes 13 and 21 were observed only in a fraction of the metaphases examined (Sue Povey, personal communication).

Among the human chromosomes retained, chromosome 4
carries several genes coding for liver specific functions (Frezal et al., 1989). The expression by WIF12-1 cells of three of them, albumin, fibrinogen, and alcohol dehydrogenase, was investigated. In each instance the presence of the human protein was observed, in addition to the rat form: they were detected either in crude extracts for alcohol dehydrogenase (Sue Povey, personal communication) or in spent medium for the plasma proteins. Thus, activation of genes that are silent in the human fibroblasts occurs in the hybrid cells. The secretion of two human plasma proteins was quantified (Table II) and found to be high. Human albumin was present at 21% of the level of the corresponding rat protein and human fibrinogen at 25%. Since the human genes encoding these proteins are present in only one copy per cell, the human liver-specific genes are very actively expressed in WIF12-1 cells.

**WIF12-1 Cells Are Polarized**

The ultrastructure of WIF12-1 cells was studied with particular attention to the dilated areas existing between adjacent cells. These spaces, highly reminiscent of bile canaliculi, are extremely variable in size. Fig. 3 shows some examples of their electron microscopic appearance. Sections cut in different orientations were examined to provide a three dimensional perspective of these spaces. They present the main characteristics of bile canaliculi: they are closed, lined with more or less abundant microvilli, and delimited by junctional complexes. Experiments are in progress to determine the exact nature of these junctions. Vesicles of different size are frequently present in the vicinity of canaliculi. Microinjections of fluorescein inside these spaces were performed to test their integrity. Generally the fluorescein injected remains stably confined in the canaliculi, as shown in Fig. 4. Occasionally, dye retention is not observed and sometimes cells forming the bile canaliculus become rapidly fluorescent. This reflects either the lack of integrity of such spaces or a defect in the microinjection procedure. Nevertheless, since this situation represents <15% of the cases among the hun-

Figure 3. Electron microscopic appearance of canicular spaces between adjacent WIF12-1 cells. Note the presence of microvilli and junctional complexes in the three examples presented (A–C). Bar, 1 μm.
To further characterize these canicular spaces and to determine the degree to which WIF12-1 cells are polarized, we localized two enzymes that are markers for the canicular domain of the hepatocytes (Simons and Fuller, 1985). First Mg$^{2+}$-ATPase activity was detected, in semi-confluent WIF12-1 cells, as well as in isolated couplets. As shown in Fig. 5, the protein is predominantly located in the canicular membrane domain. This polarized distribution, particularly evident when cells are permeabilized (Fig. 5, B and C) is characteristic of WIF12-1 cells. For Fao and WIF12, Mg$^{2+}$-ATPase activity is detected on the whole membrane which is uniformly and weakly stained. γ-Glutamyl transpeptidase, another hepatocyte apical protein, is also asymmetrically distributed in WIF12-1 cells, since it is present only at the canicular pole (Fig. 5, D and E). In contrast, in Fao and WIF12 cells the enzyme activity is detected in the cytoplasm (Golgi apparatus) and occasionally on the entire plasma membrane. Moreover, a basolateral marker of hepatocyte plasma membrane, CE9 (Hubbard et al., 1985) was also immunolocalized. Whereas, this protein is present on the whole membrane of Fao and WIF12 cells, it is absent from the bile canaliculi formed by WIF12-1 cells (Fig. 5, F and G). Therefore, compared to their parental cells, WIF12-1 cells have acquired a polarized phenotype, as attested not only by their polarized morphology but also by the localization of apical and basolateral membrane markers.

If it is clear that WIF12-1 cells, or at least a fraction of them, are polarized, the question arises whether this property is stably maintained during many generations and whether all cells of WIF12-1 are capable of polarize. Therefore, cloning of WIF12-1 was undertaken, and a hundred colonies were examined over a period of several weeks for the formation of bile canaliculi. The presence of such structures was observed in the great majority of the subclones (95%), four weeks or more after the cloning. At an earlier period, when colonies were formed of about 30 cells, some bile canaliculi were already visible in 75% of the subclones. Compared to Fao cells (generation time: 1 d), WIF12-1 cells grow slowly (generation time: 2 to 3 d). WIF12-1 subclones differing in their growth rates, and their sensitivity to an hepatocyte antiproliferative glycopeptide (Auger et al., 1989) have been isolated (M. Guérin et al., manuscript in preparation). Cells of most of these subclones retain the capacity to form bile canaliculi-like structures, even 30–40 generations after the cloning. This indicates that the ability to be polarized is a general and permanent characteristic of WIF12-1 cells.

### WIF12-1 Bile Canaliculi Are Functional

To assess whether bile canaliculi present between adjacent WIF12-1 cells are functional, several dynamic studies were performed. The ability of cells to transport and concentrate organic anion fluorescent dyes from the medium to the bile canaliculi was tested. As shown in Fig. 6, fluorescein is rapidly accumulated within canicular spaces: this accumulation is visible, as early as 10 min after the addition of fluorescein or fluorescein diacetate. This shows that the canicular excretory system is functional in WIF12-1 cells. However, the number and the intensity of the canaliculi stained were variable and even after several hours in the presence of fluorescent dyes, some canicular spaces remained unstained. Moreover uptake and excretion of these dyes induced the collapse of some bile canaliculi and the rapid disappearance of their luminal fluorescence. This observation has lead us to study in more detail the evolution and maintenance of canicular structures.

The canicular spaces are very sensitive to hypertonic osmotic gradients: addition of sucrose to medium causes a sudden and drastic shrinkage of bile canaliculi, followed by a spontaneous but partial recovery of the canicular volume within 10–20 min. This rapid restoration has been attributed to a process of continuous secretion in the canaliculi, that causes expansion of these spaces. Oshio and Phillips (1981) have isolated normal rat hepatocytes couplets and shown that canicular spaces expand and collapse periodically as a result of continuous secretion. Evolution of bile canaliculi formed between adjacent WIF12-1 cells has thus been examined, either in dense or in sparse cultures, one example being shown in Fig. 7.

WIF12-1 bile canaliculi are in fact dynamic structures, characterized by long periods of expansion followed by rapid collapse. However, compared with isolated rat hepatocytes (Miyiari et al., 1984) the canicular movements are much less frequent in WIF12-1 cells. For the couplet presented in Fig. 7, a succession of five contractions and dilatations occurred during the 17-h period of observation. The length of the dilatation periods is extremely variable, and can attain several hours (Fig. 7). Conversely, canicular contractions are fast and frequently accompanied by fragmentation of the canaliculi, leading to the formation of large vesicles; some of them seem to be resorbed in the cytoplasm of the cells. Generally, there is maintenance of the canaliculi formed, even when one of the cells engaged undergoes mitosis (Fig. 7). Moreover, examination of dense cultures has revealed that the canicular movements are often in phase: in a given field, proximal bile canaliculi contract and dilate simultaneously. For this reason, WIF12-1 cultures examined at different times over a short period can appear either very poor or in contrast enriched in canaliculi. These dynamic pulsations...
support our proposal of the existence of a functional canalicular excretory cycle in WIF12-1 cells.

**Discussion**

Using cell hybridization, we have attempted to isolate cells that fully express liver-specific genes and that are in addition polarized as hepatocytes. The presence of such cells has indeed been detected in the progeny of one rat hepatoma x human fibroblast hybrid, WIF12; from this hybrid, polarized subclones have been isolated and one of them, WIF12-1, was studied in detail.
mRNAs could be more efficient in these hybrid cells than for their Fao hepatoma parent. It is perhaps relevant that, compared with normal liver, the transcription rates of liver-specific genes in hepatoma lines, such as Fao, is very low (1-30%) whereas the concentration of the corresponding mRNAs is not so reduced (Clayton et al., 1985). Finally, restoration of full expression of hepatic functions in WIF12-1 cells could also be linked to the stable acquisition of an hepatocyte-like morphology and ultrastructure.

WIF12-1 cells present many morphological traits reminiscent of hepatocytes that are not observed in Fao parental cells. The most striking is the formation of bile canaliculi but additional features (Fig. 1) are cell size, abundance, and density of the cytoplasm, the presence of two nuclei per cell in a fraction (7-11%) of WIF12-1 cells as well as the position of the Golgi apparatus in cells forming bile canaliculi. All these morphological properties have never been observed together in cells derived, from the H4 II EC3 rat hepatoma cell line, although many clones (differentiated, variants, and revertants) and numerous different hybrids (complete or segregants) have been studied (Deschatrette et al., 1981; Rogier et al., 1986; Weiss et al., 1988). Even in the case of rat hepatoma x mouse hepatoma hybrids that overproduce liver-specific proteins (Cassio et al., 1980) hepatocyte morphological traits have not been acquired. Thus, the highly differentiated morphology of WIF12-1 cells is exceptional.

The acquisition and maintenance of hepatocyte-like ultrastructure and polarity by these cells could be a result of activation of specific genes implicated in the establishment of cellular architecture. This hypothesis is all the more likely, as we have shown that human genes coding for liver-specific functions are activated and permanently expressed in these cells. Experiments are planned to identify the human chromosomes that cells must retain to maintain their highly differentiated phenotype. Moreover a comparative analysis of the chromosome content of WIF12 subclones extinguished for the expression hepatic functions and reexpressing subclones, such as WIF12-1, could lead to the chromosomal localization of regulatory genes implicated in the extinction phenomenon. Until now, only a few extinction loci have been mapped (Killary and Fournier, 1984; Petit et al., 1986; Chin and Fournier, 1989).

The most interesting property of WIF12-1 cells is their ability to polarize, as attested by the formation of functional bile canaliculi and by the polarized localization of the apical and basolateral markers examined. Comprehensive analysis of the degree of differentiation and polarity of the plasma membrane of WIF12-1 and its parental Fao cells is in progress, using antibodies directed against specific markers of each of the three plasma membrane domains of the hepatocyte (Hubbard et al., 1985; Maurice et al., 1985). This analysis is being undertaken first to determine the extent to which WIF12-1 cells constitute a good in vitro model for study of membrane protein sorting and vesicle traffic. Indeed the work of Hubbard’s group (1985) has conclusively established that the sorting pathway for plasma membrane protein in hepatocytes (Bartles et al., 1987) is indirect and differs from that in kidney and even in intestinal epithelial cells. This can be related to the fact that hepatocytes do not have an apically directed secretory pathway (Bartles and Hubbard, 1988). To understand the molecular basis of these differences, a careful comparison of vesicle traffic in the

**Figure 6.** WIF12-1 cells accumulate fluorescein in canalicular spaces; fluorescein diacetate added to the medium of WIF12-1 cultures is transported into cells and hydrolyzed to fluorescein, which is exported and concentrated into the canalicular spaces formed between adjacent cells. Fluorescence of a confluent field of about sixty WIF12-1 cells (A) examined 10-min after an exposure of 1 h in presence of fluorescein diacetate; in this field, there were eight bile canaliculi and seven were fluorescent. Phase (B) and corresponding fluorescent (C) images of two WIF12-1 cells that have formed a tight couplet. Bar, 25 μm.
Figure 7. Evolution of a couplet of WIF12-1 cells forming a bile canaliculus. The evolution was followed during 17 h and photographs taken each hour are presented. Several periods of dilatation followed by rapid contractions of the bile canaliculus are observed. Note that at the 16th hour, one cell of the couplet has entered mitosis. Bar, 25 μm.
three polarized epithelial systems is necessary. WIF12-1 cells could be very useful in such a study.

Moreover, characterization of WIF12-1 cells could give information on bile canaliculi morphogenesis and the primary mechanisms of bile formation. These two processes are indeed still poorly understood because of technical difficulties in vivo studies and from the lack of appropriate in vitro systems. The presence of bile canaliculi-like structures has been observed recently in two human hepatoma lines, HepG2 and HuH7 (Chiu et al., 1990). However, the morphology of these cells is very far from that exhibited by hepatocytes and there is no evidence that the bile canaliculi formed are functional. The only valid in vitro model used to analyze bile secretion and canalicular evolution has been isolated rat hepatocytes couples in short-term monolayer culture (Gautam et al., 1987). There is preservation of the bile canaliculi in this model, but never new formation of them.

In hepatocytes in primary cultures only a progressive polarization of plasma membrane is observed and the process varies in kinetics and extent (Jung et al., 1982; Maurice et al., 1988; McMillan et al., 1988). In contrast WIF12-1 cells are not only able to maintain functional bile canaliculi but they continuously form new ones, even after many generations. Further experiments are necessary to understand why only a fraction of these cells form bile canaliculi. This may be a result of modulations in expression of cytoskeleton elements (Gebhardt, 1983; Kawahara and French, 1990), junctional complex components or of various extracellular matrix proteins, thought to be necessary for the generation of polarity (Rodriguez-Boulan and Nelson, 1989). This could also reflect the existence of different steps in the establishment and maturation of bile canaliculi.

Concerning bile formation, most hepatoma lines, including H4 II EC3 and its derivatives such as Fao, do not synthesize bile acids. However, Polokoff and Everson (1986) have isolated a few hybrids between rat hepatocytes and Fao hepatoma cells that have retained the capacity to synthesize, conjugate, and secrete three major rat bile acid species. Experiments are in progress to establish if WIF12-1 cells have also acquired these properties. Moreover, preliminary studies performed on WIF12-1 cells loaded with the Ca²⁺ indicator fura 2 showed that these cells possess another hepatocyte typical property (Coquil et al., 1991): the ability to release (Ca²⁺) from the ER in response to addition of bile acids (L. Combettes and M. Claret, unpublished results).

The last question that we wish to discuss is the basis of the acquisition of a polarized phenotype by WIF12-1 cells. Are these cells able to polarize because they are hybrids or have we, by chance, isolated a hepatoma clone with more differentiated characteristics? Since any of the numerous differentiated subclones derived from the H4 II EC3 hepatoma line present a polarized phenotype, the first hypothesis appears as the most likely. However, to confirm it, we are searching for the presence of polarized cells in the progeny of various hepatoma-derived hybrids. Three families were briefly examined: rat hepatoma × human fibroblast hybrids (Kiely et al., 1981) similar to WIF hybrids, 2s rat hepatoma × mouse lymphoblastoma hybrids (Sellem et al., 1985), and hybrids between rat hepatoma and microcells of a mouse L-cell (Petit et al., 1986). The presence of a minority of polarized cells was detected in the progeny of one clone of each family. From these clones, polarized cells will be isolated and their degree of differentiation and polarity will be compared. These results show that hepatoma-derived hybrid clones constitute a potential reservoir of polarized cells. Analysis of stable polarized clones of hepatic origin, such as WIF12-1 will certainly contribute to the understanding of the mechanisms implicated in the establishment and maintenance of epithelial cell polarity.

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