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The influence of matrix components on the morphological differentiation of a proliferating hepatocyte line from liver of newborn mice

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Summary. The differentiation behaviour of a liver epithelial cell line of the newborn mouse cultured on various matrix components (Thermanox pure, Thermanox coated with ECM, dried collagen type I and type II, wet collagen type I and type III and on floating collagen) was investigated by electron microscopy. Only during the last few days of pregnancy and up to day 9 p.p. could these cells be isolated using a very delicate method. The cells were smaller than differentiated hepatocytes and proliferated spontaneously. They resembled the so-called oval liver cells. On Thermanox pure or Thermanox coated with ECM, dried collagen type I or type II a confluent monolayer developed after about 6 days that consisted of rather flat extended cells which were characterized by short contacts and the absence of any morphological indications of differentiation. On wet collagen the extension area was smaller and the cells were taller. The length of the contact area and the number and size of gap junctions and cell organelles increased. On floating collagen multi-layered aggregates of polygonal cells developed that were characterized by extended cell contacts, bile capillary-like structures and highly developed cell organelles, especially rough endoplasmic reticulum.

Since differentiation processes can be demonstrated ultrastructurally only on wet collagen, especially on floating collagen, the chemical composition of the substrate and a specific matrix-cell interaction cannot be the only triggering factor. It is assumed that mechanical properties of the substrate, e.g. plasticity, are involved. The change in the shape of the cell, the prolongation or intensification of the cell contact and the adaptation of the cytoskeleton might play a decisive role in this connection.

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Introduction

The increasing use of *in vitro* systems for the testing of possible mutagenic, carcinogenic or teratogenic substances necessitates the development of models that are capable of metabolizing inactive xenobiotics. Hepatocyte cultures and the enzymmes of the P-450 complex are of special interest in this connection (Remmer and Merker, 1963; Neubert et al., 1978; Gelboin, 1980; Lu and West, 1980; Faustman-Watts et al., 1983; Boobis and Davis, 1984; Kremers, 1986). However, all hepatocyte cultures that have been used so far involve one difficulty. The activity of the P-450 enzymes drastically decreases after isolation and during subsequent cultivation, even in non-proliferating adult hepatocytes (Guzelian et al., 1977; Paine and Legg, 1978; Fahl et al., 1979; Sirica and Pitot, 1980; Maslanski and Williams, 1982; Steward et al., 1985; Reid et al., 1986). Therefore, many publications deal with attempts to preserve the activity of these enzymes *in vitro*. For this purpose certain factors (e.g. hemes, selenium, nicotinamide, ascorbic acid, pyridines) were — apart from the common additives (cortisone, insulin etc.) — added to the medium or others were omitted (e.g. serum, certain amino acids) (Guzelian and Bissell, 1976; Laishes and Williams, 1976; Decad et al., 1977; Williams et al., 1978; Paine and Hockin, 1982; Paine et al., 1979, 1980; Lake and Paine, 1982; De Sante et al., 1984; Enat et al., 1984; Forte, 1984; Nakamura et al., 1984; Engelmann et al., 1985; Guillouzo and Gugen-Guillouzo, 1986; Mathis et al., 1986; Reid et al., 1986). Induction *in vitro*, growth on certain substrates (collagen, biomatrix), high cell densities and co-cultures with proliferating epithelial cell strains and other cell types have also been used (Michalopoulos and Pitot, 1975; Michalopoulos et al., 1976, 1979; Rojkind et al., 1980; Elshourbagy et al.,

1981; Freeman et al., 1981; Guggen-Guillouzo et al., 1982, 1983; Newman and Guzelian, 1982; Guguen-Guillouzo et al., 1983; Begue et al., 1984; Clement et al., 1984; Reid et al., 1986; Schuetz et al., 1986).

Despite all these efforts it has so far not been possible to develop a simple and ideal system in which unchanged activity of the P-450 enzymes occurs *in vitro* for a longer period. Therefore, it is desirable to extend our knowledge of triggering and stabilization of the differentiation of hepatocytes *in vitro*. For this purpose we started from the opposite direction. By doing so we investigated the differentiation behaviour of completely dedifferentiated, proliferating liver epithelia of newborn mice on various matrix components. The use of matrix components of the mesenchyme or the connective tissue for such experiments appears to be justified for two reasons:

1) The stimulating influence that the matrix has on differentiation *in vivo* and *in vitro* is well-established (Elsdale and Bard, 1972; Slavkin, 1972; Slavkin and Greulich, 1975; Reddi, 1976, 1984; Emerman and Pitelka, 1977; Hay, 1981; Trelstad, 1984; Wolff et al., 1987).

2) Findings obtained with adult hepatocytes *in vitro* are available that also show at least a stabilizing effect of the matrix on differentiation (Michalopoulos and Pitot, 1975; Rojkind et al., 1980. Reid et al., 1986.)

Materials and methods

Cell Isolation

Liver epithelial cells were prepared from 9-day-old mice (NMRI:Zentralinstitut für Versuchstierkunde, Hannover, W.-Germany). After two washes with Hanks solution (Biochrom KG, Berlin), the livers were cut into large pieces (1 lobe into 3-4 pieces) which were digested for 2 hours with Ca++/Mg++-free Hanks solution (Biochrom KG, Berlin) containing 0.1% trypsin (Boehringer, Mannheim) under gentle shaking at 37° C. The supernatants were collected and centrifuged (82 x g) for 10 min using a Heraeus Christ centrifuge (minifuge 2). Subsequently, the cells were suspended in cold medium (see below) and stored at room temperature for 90 min. Then the cells were washed twice with the selective culture medium and filtered through nylon gauze (mesh width 20 µm) to remove cellular aggregates and tissue debris. The cells were seeded at a density of 1×10^6 per ml as a 1 ml suspension per 35 mm diameter Falcon well. The medium was changed 17 hours after seeding and then every two days.

Culture Conditions

The standard medium for plating and growing was MEM Dulbecco (Zellkultur Boehringer, Mannheim) supplemented with 1% MEM amino acids 50 × (Zellkultur Boehringer, Mannheim), 2 mM L-glutamine (Biochrom KG, Berlin), 0.075% Na-hydrogen carbonate (Zellkultur Boehringer, Mannheim), penicillin at 100 units/ml and streptomycin at 100 µg/ml (Biochrom KG, Berlin) and 10% fetal calf serum (Zellkultur, Boehringer, Mannheim).

The medium was additionally supplemented with 1 mM proline (Sigma Chemical Company), 40 µm/ml fetuin (Sigma) 2 µg/ml glycyl-L-histidyl-L-lysine (Sigma), 5 ng/ml hydrocortisone (Sigma) and 25 ng/ml Epidermal Cell Growth Factor (EGF) (Sigma).

Coating with «Dried» Collagen Type I and III

2.5 mg/ml collagen type I from rat tail (Sigma Chemical Company) and type III from human placenta (kindly provided by Dr. H.-J. Barrach, Berlin) were dissolved in a 0.2% acetic acid solution supplemented with 0.002% phenol red. After stirring for 48 h at 4°C, 0.1 ml of the solution was added to tissue culture dishes (35 mm in diameter) containing Thermanox tissue culture cover slips (Lab-Tek Division). To assure uniform distribution of the solution, the dishes were gently shaken. The opened dishes were then placed in a desiccator that contained a vessel of NH_4OH. The collagen solutions gelated in 5-10 min which was expressed by a colour change. The culture dishes were air-dried for 24 h and washed three times with aqua dest. After the dishes had dried, they could be stored at room temperature for about 2 months. Prior to use, they were exposed for 10 min to ultraviolet light.

Coating with «Wet» Collagen Type I and III

One mg/ml type I collagen (from rat tail) or type III collagen (from human placenta, kindly provided by Dr. Barrach) was dissolved in 0.06% solution of acetic acid in sterile redistilled water supplemented with 100 U/ml/ 100 µl penicillin/streptomycin (Biochrom KG, Berlin). After stirring for 48 h at 4° C 10 mg/ml MEM Dulbecco 1 x dry medium (Zellkultur, Boehringer, Mannheim) and 2 µl/ml Phenol Red Solution 0.5% (Gibco Europe, Glasgow, Scotland) were added and the solution was stirred for 2 more hours at 4° C. Then the acid collagen solution was exposed to raised pH close to the normal physiological level through titration with 1% NH_4OH. The mixture (0.1 ml/dish) was pipetted into tissue culture dishes (35 mm in diameter, Falcon) which were kept at room temperature without moving for 30 min. The dishes contained Thermanox tissue culture cover slips (Lab-Tek). The collagen fibres precipitated fairly fast and formed a hydrated collagen lattice; the final collagen gel. The dishes were then incubated at 37° C in a humidified atmosphere (2.5% CO_2 in air) overnight. The culture medium was added to the gels and the dishes were kept for about 24 hours in the incubator. Before seeding, the medium was changed.

«Floating Collagen» Method

To prepare floating collagen gels, first Falcon dishes

were coated with «wet» collagen type I and type II (see above). Then a sterile syringe was used to cut the gels around the internal periphery of the dishes 4 days after seeding. At this stage the monolayer cultures reach, in most cases, confluency. The collagenous layer with the resting cells contracts and after detachment floats in the medium. Fixation was performed after 2 to 3 days.

Coating with ECM

Bovine cornea endothelial cells were grown on Thermanox tissue culture cover slips (Lab-Tek Division). They produced a matrix (ECM) that resembled a basement membrane (BM) *in vivo*. After removal of the cells, these cover slips which were covered with a BM-like material could be used as a substrate. The ECM-covered slips were kindly provided by Dr. Vlodavsky (Dept. Radiation and Clinical Oncology, Hebrew University, Hadassah Medical School, Jerusalem, Israel).

Electron Microscopy

The cultures were fixed in 0.1 M phosphate buffer (pH 7.2) containing 1% glutaraldehyde and 1% tannic acid, postfixed in a 1% osmium tetroxide solution in buffer, dehydrated and embedded in Epon. Sectioning was carried out on an Ultracut Reichert-Jung microtome and contrasting was performed with uranyl acetate/lead citrate. The sections were examined under a Siemens Elmiskop 101.

Results

The described findings were obtained from 6-day-old cultures. At this developmental stage confluency had largely been reached. Therefore, the age of the cultures will not be mentioned in the following. The sectioning technique was also identical in all cases. The samples were cut perpendicular to the surface. This allows a satisfactory morphological evaluation of all planes of the cell, the contact situations, adhesion at the substrate as well as the situation at the surface. Specific orientation could not be achieved with the floating collagen cultures. Hence, in this case, the cells were sectioned in random planes.

Thermanox

As early as 10 min after addition of the medium a 50 nm electron-dense layer could be demonstrated on the Thermanox cover slips without any additional coating. Since this layer also occurred in cell-free areas, it could be interpreted as a precipitate of serum proteins from the medium. Electron microscopic pictures showed that, using serum-containing media, the cells attached to this layer and not directly to Thermanox. After spreading of the cells, i.e., after approximately 1 h, the basal cell membrane in the contact region had a slightly undulating contour. This promoted the direct contact of some regions with the serum protein layer. Other sites were characterized by an empty gap of varying width (100 nm at the most) between the tannic-acid-positive, that is, the electron-dense surface coat, and the serum protein layer.

The central region of the spread cells, that had a thickness of approximately 3 µm, bulged into the medium. The peripheral portions became flat. Here, the cytoplasm reached a thickness of only 500 to 900 nm. The morphology of the cell contacts varied considerably: 1) Only contacts over short distances occurred between very thin cytoplasmic plates. Despite the limited length of these contacts tight and gap junctions could often be seen. Their demonstration after tannic acid fixation is slightly problematic, but they can be identified according to van Deurs (1975) by a narrowing or the absence of the intercellular space and the occurrence (gap junctions) or absence (tight junctions) of a strongly electron-dense central lamella. 2) In other contact areas the cell periphery was thicker and the developing contacts became longer. Slight indentations and overlappings occurred over short distances. In addition to normal cell contacts with an intercellular space (ICS) of about 20 nm, these areas showed gap and tight junctions. Distinct small dilatations of the ICS were also observed.

The contour of the apical cell membrane, i.e., towards the medium, was smooth over long distances or only slightly undulating. A few short microvilli occurred, especially in the vicinity of the cell contacts and above the nucleus.

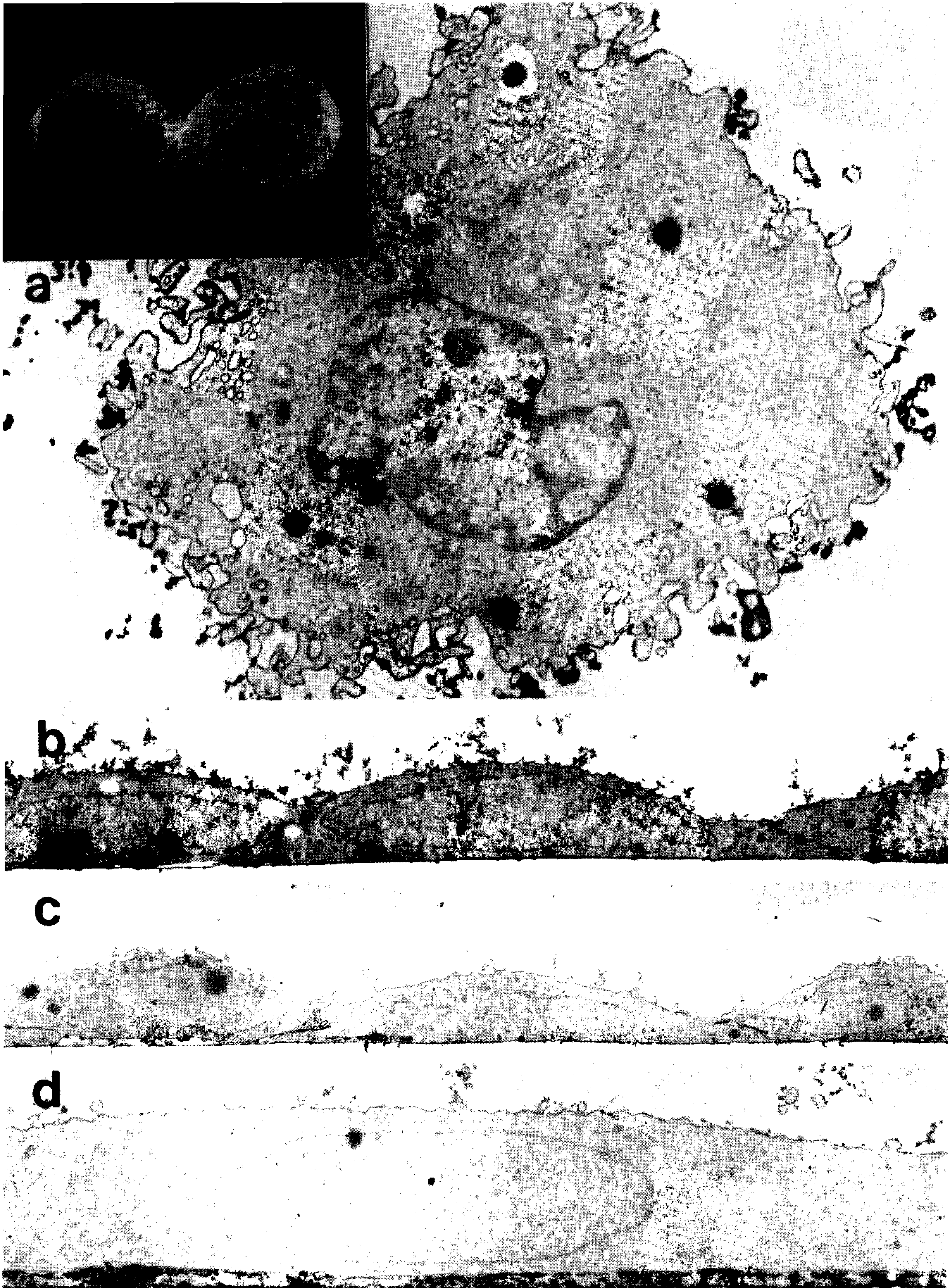
The nucleus was adapted to the flat extended form of the cells, i.e., it was lentiform. Number and size of the other cell organelles was low. Some small mitochondria with a scant matrix and only few cristae were present. Some individual cavities of the rough endoplasmic reticulum (rER) could be demonstrated in addition to a large number of free ribosomes (polysomes). The number of components of the cytoskeleton, such as microtubuli and intermediate filaments, was small. Stress fibres were lacking.

ECM, Dried Collagen Type I and Type III

The morphology of the cells on ECM or dried collagen was not different from controls (growth on pure Thermanox). The described findings also apply to these culture conditions. Naturally, the appearance of the substrate varied.

The thickness of the ECM layer varied between 100 and 300 nm. It consisted of homogeneous material or densely packed granular and filamentous structures. Thicker plaques (up to 1 µm) occurred in some areas that had a diameter of up to 5 µm. The contact situation between cells and ECM corresponded to that which has been described for growth on Thermanox cover slips.

The dried collagen type I and type III was present as a 3 to 5 µm layer in which densely packed and empty regions were irregularly distributed. At high magnification the dense regions revealed a fine-granular and filamentous material into which single and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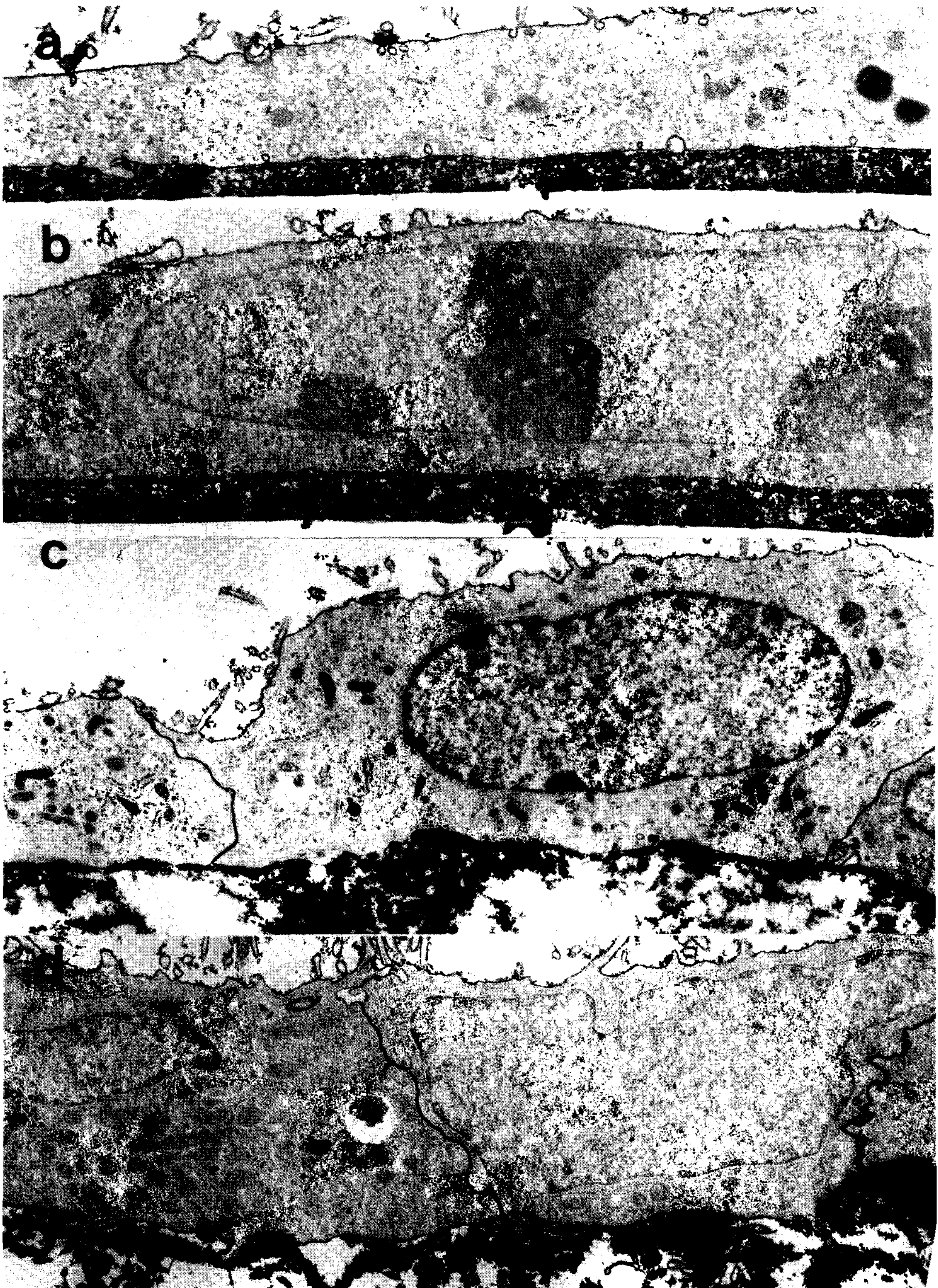
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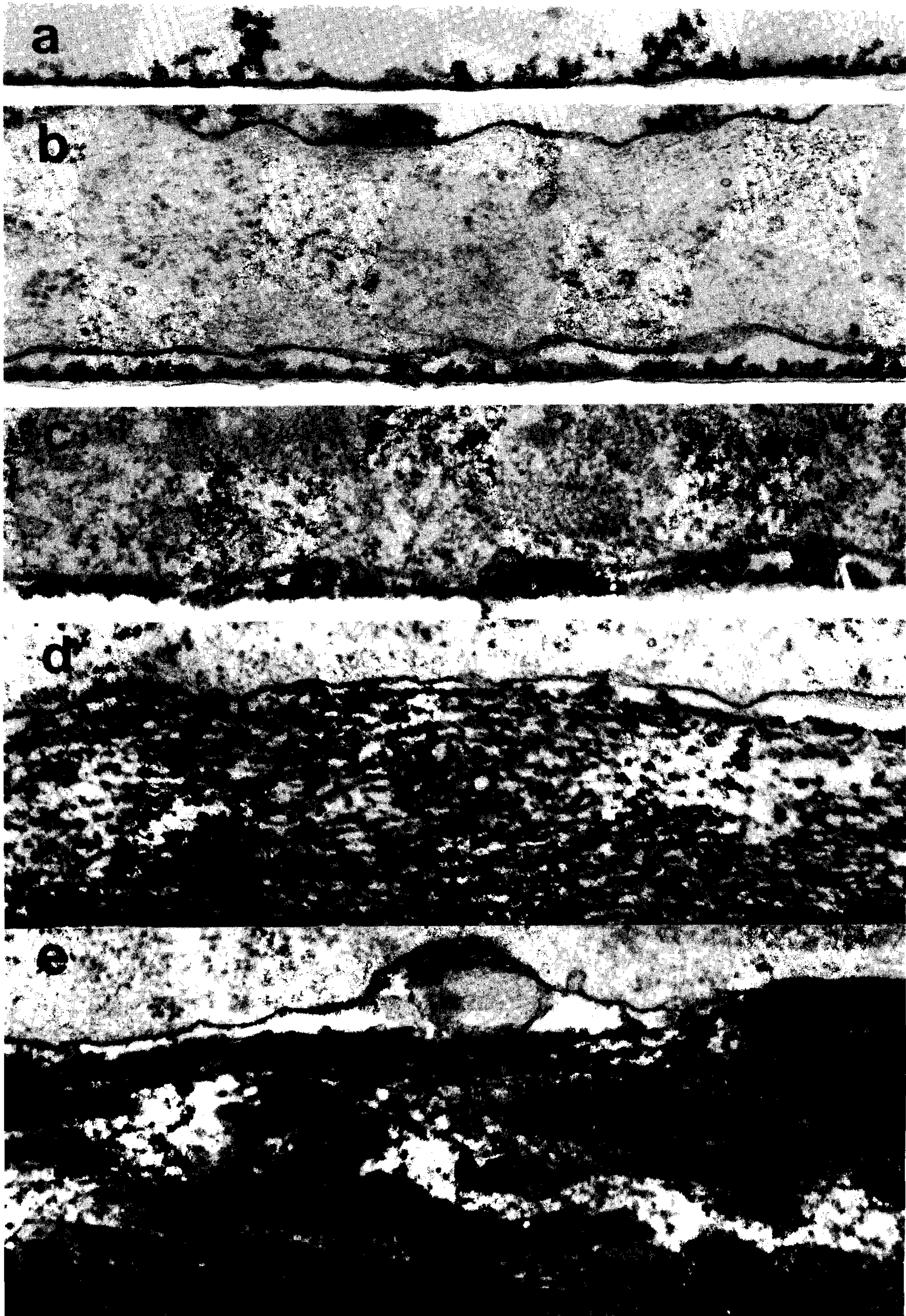
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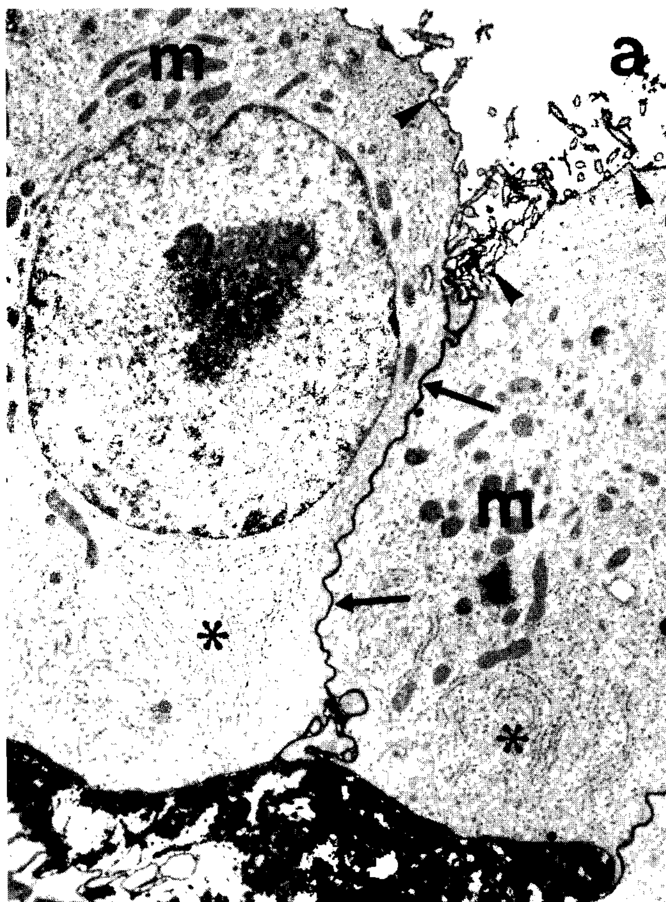
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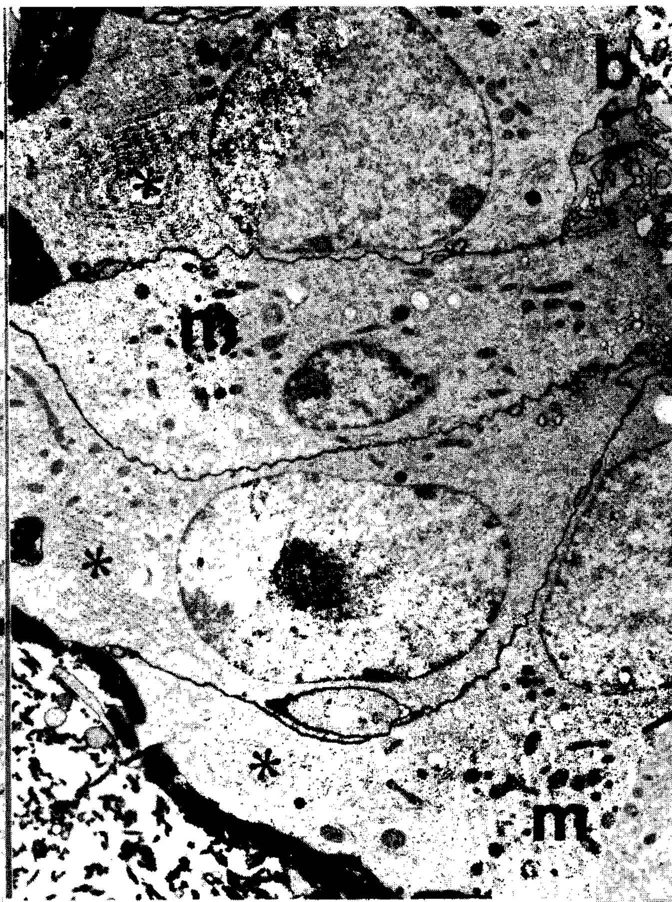
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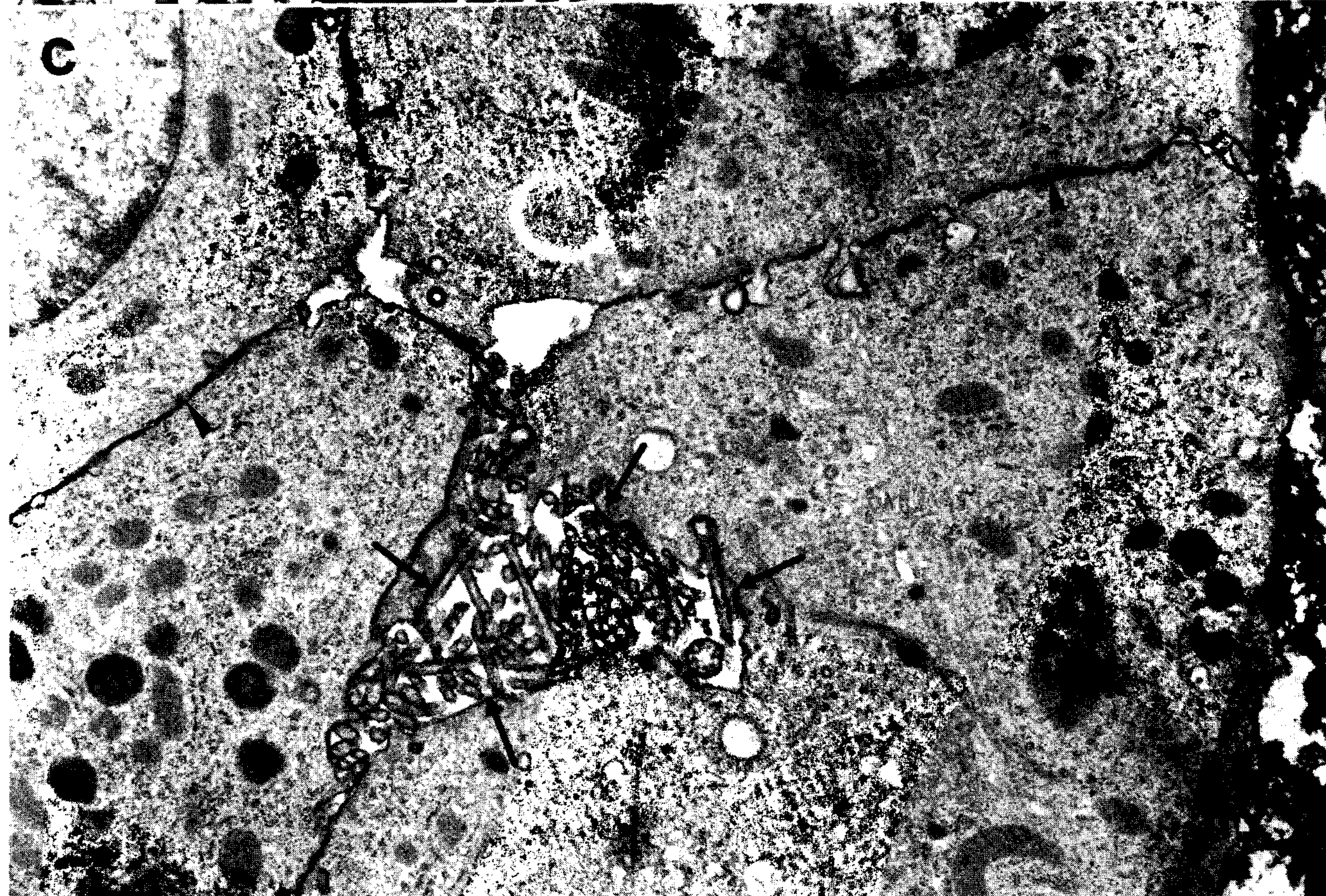
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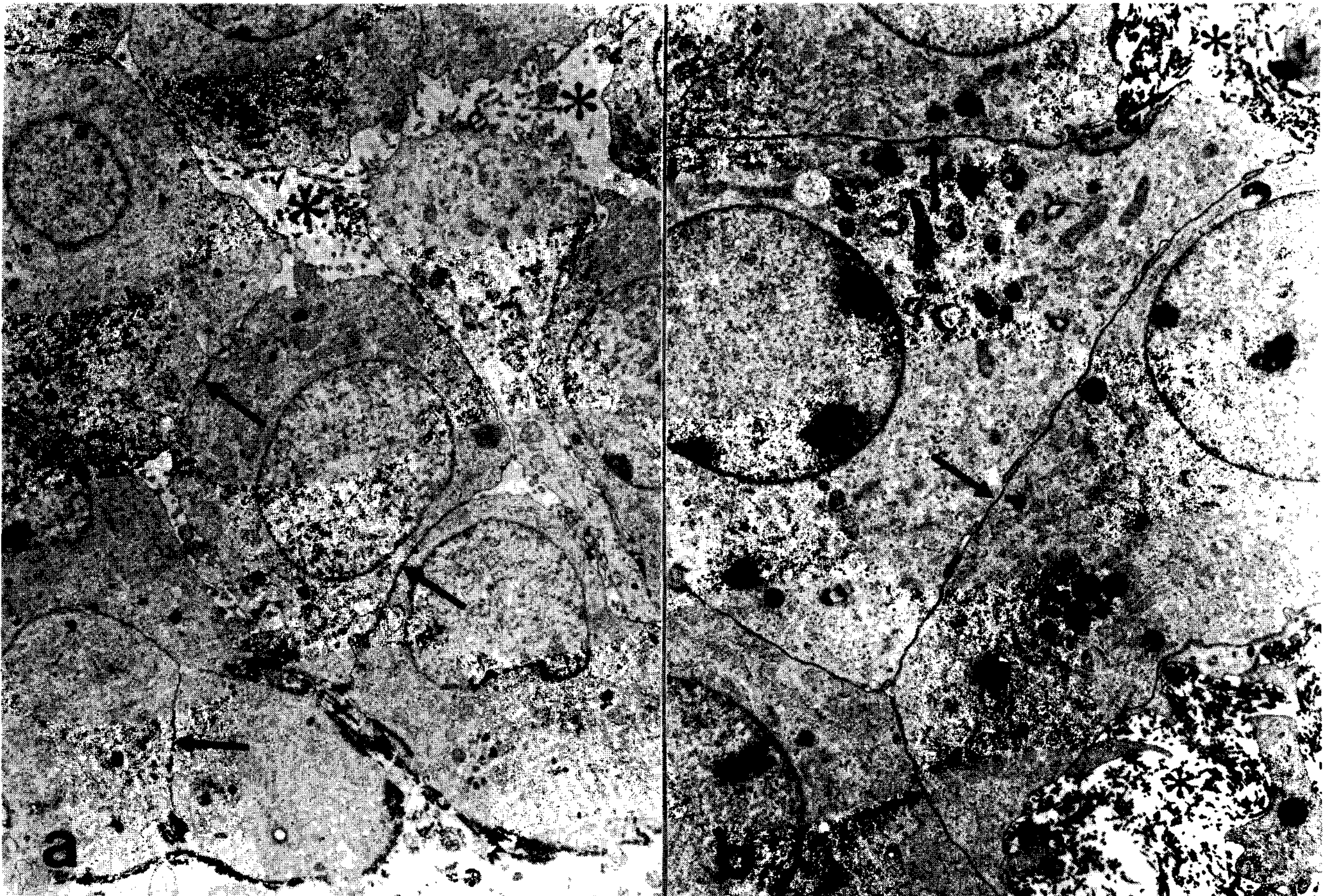
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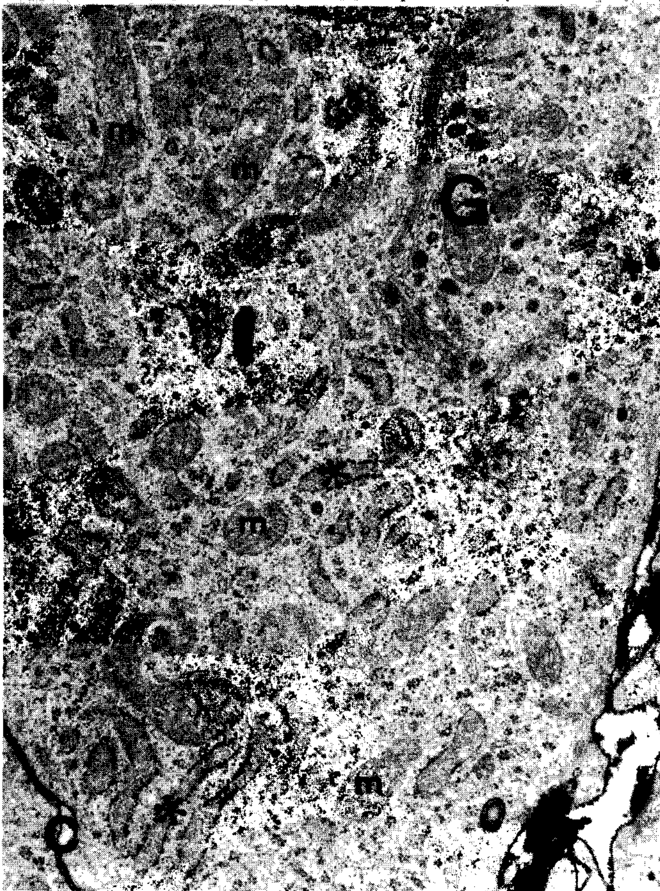
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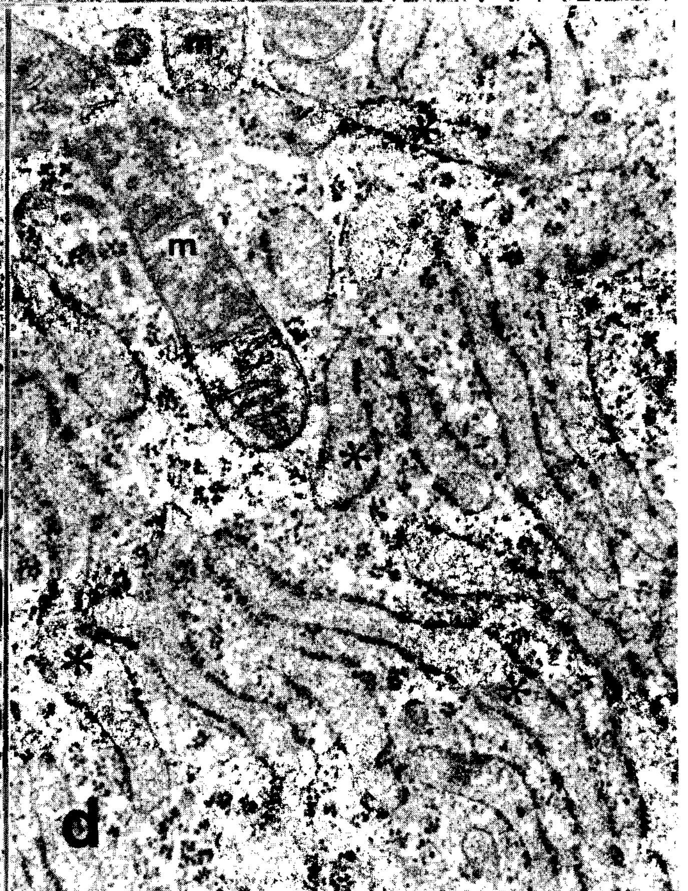
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Fig. 1. a) Liver epithelial cell from 9-day-old mice immediately after isolation. × 15,000 Inset: SEM picture of 2 liver epithelial cells immediately after isolation. **b)** Growth on Thermanox, culture period = 6 days. × 5,000 **c)** Growth on ECM, culture period = 6 days. × 4,000 **d)** Growth on ECM, culture period = 6 days + 14,000

Fig. 2. Liver epithelial cells from 9-day-old mice after a 6-day culture period. **a)** Growth on dried collagen type III. × 21,000 **b)** Growth on dried collagen type I. × 21,000. **c)** Growth on wet collagen type III. × 18,000. **d)** Growth on wet collagen type I. × 18,000 Cell - Substrate connection (arrow).

Fig. 3. Liver epithelial cells from 9-day-old mice after a 6 day-culture period. × 70,000 **a)** Electron-dense layer on Thermanox cover slips in a cell-free region. **b)** Growth on Thermanox. **c)** Growth on ECM **d)** Growth on dried collagen type III. **e)** Growth on dried collagen type I.

Fig. 4. Liver epithelial cells from 9-day-old mice after a 6-day culture period. Cell - substrate connection (arrow). × 60,000 **a)** Growth on wet collagen type III. **b)** Growth on wet collagen type I.

Fig. 5. Liver epithelium from 9-day-old mice after a 6-day culture period on floating collagen. **a)** One-layered epithelial cell with microvilli towards the medium (arrow head), cell contacts over long distances (arrow), numerous mitochondria **(m)** and cavities of the rough endoplasmic reticulum (*). × 8,000. **b)** Incipient multi-layered growth, otherwise see **a)**. **c)** Multi-layered cell aggregate with long cell contacts (arrow head) and a bile capillary-like structure (arrow). × 18,000

Fig. 6. Liver epithelial cells from 9-day-old mice after a 6-day culture period. Growth on floating collagen. **a)** Multi-layered growth with a wide intercellular gap (*) into which microvilli protrude. Narrow cell contacts (arrow). × 4,000 **b)** Multi-layered growth with narrow cell contacts (arrows). Collagen (*). × 6,000. **c)** Detail from a cell of a multi-layered aggregate with Golgi apparatus of the endoplasmic reticulum (*). × 20,000. **d)** Detail from a cell of a multi-layered aggregate with mitochondria **(m)** and numerous cavities of the rough endoplasmic reticulum (*). × 40,000

ducts or intermediate cells that are localized at the junctions between bile duct system and liver lobule. They are obviously able to differentiate into hepatocytes or bile duct epithelia. Due to their capability of proliferation they have also been considered as possible precursors of hepatocellular carcinoma (Farber, 1956; Grisham et al., 1975; Grisham, 1979, 1980; Sells and Leffert, 1982; Tsao et al., 1984).

Three types of behaviour, depending on the substance, can be distinguished on morphological analysis of the differentiation of this cell type *in vitro*. 1) Flat, undifferentiated cells on Thermanox, ECM, dried collagen types I and III; 2) cells with some indications of differentiation (increase in height and contact area, increased number of cell organelles) on wet collagen types I and III, and 3) differentiated cells of polygonal shape, contact over long distances, numerous well-developed cell organelles, especially rough endoplasmic reticulum and mitochondria as well as bile capillary-like tubules on floating collagen. Assuming this interpretation, the question arises whether the described morphological features can be considered as differentiation criteria. In analogy with the morphology of differentiation *in vivo* and on the basis of similarity with the appearance of mature liver cells it appears to be justified to postulate differentiation processes solely on morphological grounds. However, further investigations (e.g. bio- and histochemical) will be necessary for an ultimate validation. Hence, under the used culture conditions the originally small and obviously ambivalent «oval» cells first undergo a dedifferentiation process and, after having reached confluency on wet or floating collagen, they begin to differentiate into hepatocytes.

If this interpretation proves right we must look for the reason or the mechanisms of the differing differentiation behaviour under the culture conditions used. They undoubtedly depend upon the properties of the substrate, because all the other parameters are the same. Since the cell membrane is responsible for the connection to the extracellular space it is reasonable to consider the binding of the matrix to the cell membrane as the triggering factor for differentiation. This mechanism may be due to an unspecific binding of matrix components. This might explain an influence on membrane fluidity and other properties. However, of greater interest is the possibility of a specific binding to receptor-like molecules of the membrane. A sequence of secondary processes might thus occur which finally triggers differentiation processes. Unfortunately, differentiation processes cannot be induced on all collagen layers. Dried layers (collagen and ECM) do not induce any changes in the sense of a morphological differentiation. Such changes do, however, occur on wet collagen and —more pronounced— on floating collagen. Hence, different types of binding of the matrix components to the cell membrane cannot be the only triggering factors for differentiation. We should rather consider the significance of the mechanical properties of the substrate, especially its plasticity (Karst and Merker, 1987).

However, conclusions cannot be drawn from these considerations and findings that would be applicable to the *in vivo* situation. If our cell material consisted of so-called oval cells, *in vivo* the latter would rest on a basement membrane (BM). But the BM does not contain either collagen type I or III, as was used in our experiments. In this case statements cannot be made as to the cell —matrix binding of this cell type *in vivo* that could be related to our *in vitro* system. However, if we are dealing with hepatocyte-like cells, BM-components, as well as collagen types I and III, may be responsible for the contact. All these substances can be demonstrated in the connective tissue of the liver lobule (Hahn et al., 1980; Diegelman, 1986; Reid et al., 1986). Therefore, we cannot draw any definite conclusions from our findings as to the significance of a matrix— cell interaction for the triggering and stabilization of differentiation in the liver *in vivo*. But it is justified to note that properties of the substrate other than its chemical composition influence the differentiation behaviour of the cells. The plasticity of the substrate should be discussed in connection with its involvement in mechanisms triggering differentiation. The plasticity of substrate allows certain changes in cell shape. Changes in cell shape can be the result of cytoskeletal activity or they may be imposed passively on the cell by its mechanical environment. In either case such shape changes may influence cell differentiation by altering intercellular relationships. Each cell type shows a characteristic arrangement of the cytoskeleton and

thus, distribution and localization of the organelles, and the dynamics of the cell membrane are regulated by the cytoskeleton. A pronounced cell contact is obviously important for triggering differentiation processes. These processes could be demonstrated during the differentiation of the blastema *in vitro* and *in vivo* and in so-called high density cultures (Morris and Moscona, 1971; Moscona, 1974; Merker et al., 1980 a,b, 1984; Zimmermann et al., 1982; Guguen-Guillouzo, 1986). It has so far not been possible to demonstrate with certainty whether an unspecific adhesion leading to changes in membrane properties and other secondary events or a linkage via gap junctions with resulting cell - cell communication, is of greater significance. Irrespective of the existing gaps in our knowledge of epithelial cell differentiation *in vitro* it is, from the morphological point of view, certain that distinctly dedifferentiated epithelial cells from the liver of newborn mice can reach a high degree of differentiation when grown on floating collagen.

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