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Morphology, differentiation and matrix production of liver cells in organoid cultures (high density cultures) of fetal rat livers

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Summary. The aim of this study was to demonstrate the morphology and matrix synthesis of embryonic rat liver cells (day 18 of gestation) in organoid cultures (high density cultures) with electron microscopic and immunomorphological techniques. For this purpose the cells of embryonic rat livers were isolated enzymatically and grown in an organoid culture (high density culture) for 3 weeks in a Trowell system. During the first 48 h a sorting-out process took place, i.e. liver and blood-forming cells met to form aggregates. In between mesenchymal cells were seen. Vessel-like cavities developed. Electron microscopic inspection of the hepatocytes did not reveal any lesions of the cell organelles after 14 days in culture. As late as after a 3-week culture period mitochondrial swellings and an increased number of autophagic vacuoles were observed. A rim of collagenous fibrils or fibrillar bundles and granular matrix structures was perceptible as early as after 7 days in culture. Immunofluorescence microscopic techniques revealed collagen types III, IV and VI as well as laminin, nidogen, heparansulfate-proteoglycan and fibronectin in these areas. Thus, the composition of the matrix in this culture system corresponds (apart from the absence of collagen type I) to the embryonic situation. Therefore, the organoid culture appears to be an appropriate technique to study the behaviour of hepatocytes in vitro. It is especially suited to demonstrate the formation of matrix components in liver cells and their extracellular occurrence.

Key words: Fetal rat, Liver, Organoid culture

Introduction

The cultivation of hepatocytes has involved a number of difficulties, such as the viability of these cells during isolation and their rapid dedifferentiation. The first problem could largely be solved by improving the technique, e.g. optimization of the perfusion technique and the perfusion solution as well as by application of the gradient centrifugation method (Berry and Friend, 1969; Segeln, 1973; Williams et al., 1977; Schaeffer and Kessler, 1980; Sirica and Pitot, 1980; Carlsen et al., 1981; Kreamer et al., 1986). For a better understanding of the second point the term «dedifferentiation» should be defined more closely. Proliferating and spreading cells quickly lose most of their typical hepatocyte features and functions (Leffert and Paul, 1972; Michalopoulos and Pitot, 1975; Guzelian et al., 1977; Schwarz et al., 1979; Bissell and Guzelian, 1980; Paine et al., 1980; Maslansky and Williams, 1982; Paine and Hockin, 1982; Lake and Paine, 1983; Nakamura et al., 1983; Begue et al., 1984; Steward et al., 1985; Ratanasavanh et al., 1986). But also non-proliferating hepatocytes show the phenomenon of a dedifferentiation after isolation. A certain classification of their functions in vitro becomes obvious. First the P-450 system (mixed function oxidases) disappears within hours or days, i.e., the capability to metabolize endogenous or exogenous substances is lost. A prolongation of the capability of metabolization is achieved by the use of substrates from matrix components of the connective tissue and so-called co-cultures (Michalopoulos and Pitot, 1975; Michalopoulos et al., 1976, 1979; Sattler et al., 1978; Reid and Rojkind, 1979; Rojkind et al., 1980; Guguen-Guillouzo et al., 1982, 1983a,b; Marceau et al., 1982; Lescoat et al., 1985). Nevertheless, it has so far not been possible to maintain the P-450 system for several days in routine experiments. But it is above all because of this capability that the cultivation of hepatocytes is of special importance. The availability of a metabolization system is a prerequisite for testing xenobiotics in vitro.

One of the reasons for an insufficient differentiation of hepatocytes in culture over an extended period of time may be the missing interrelationship between epithelium and connective tissue. The connective tissue is able to

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influence or even to stimulate the differentiation behaviour of epithelial cells via a direct contact, via the secretion of diffusible substances or via matrix components (Fleischmajer and Billingham, 1968; Le Dourin, 1975; Rojkind et al., 1980; Kleinman et al., 1981; Riso, 1983; Sawada et al., 1987; Bissell and Choun, 1988). This interaction is missing in monolayer cultures. To a certain degree it may be present in co-cultures. In organ cultures as in vivo-like situation is observed, but this culture technique involves other difficulties, e.g. lesions during cutting and problems in the supply of central areas (Sipes et al., 1987).

The search for hepatocyte cultures that have the capability of metabolization over a long enough period has therefore still a great topicality. For this purpose mainly monolayer cultures have so far been used. Organ or slice cultures have been applied to a negligible extent only (Sipes et al., 1987). The organoid or high density culture has so far not been used in this connection. However, in the case of other tissues the high density technique has proved to be very suitable for the study of differentiation processes, for example with blastemal cells (chondrogenesis), with calvarial osteoblasts (osteogenesis) and fetal lung cells (differentiation into pneumocytes type II) (Merker et al., 1981; Kistler et al., 1985; Flint, 1986; Zimmermann, 1987; Merker, 1991). We therefore studied the behaviour of hepatocytes in this culture system using morphological means.

Several possibilities exist to study the functions of hepatocytes in culture. Firstly, we have to consider the demonstration of the metabolization capability (Lake and Paine, 1983; Begue et al., 1984; Mennes et al., 1988). Secondly, the production of plasma proteins can be measured (Hannah et al., 1980; Guguen-Guillouzo et al., 1983a; Guillouzo, 1986). And finally, hepatocytes are involved in the formation of the connective tissue matrix (Hata and Ninomiya, 1984; Diegelmann, 1986; Xu et al., 1989, 1991). The aim of this study is to demonstrate the morphology of fetal hepatocytes in high density cultures depending on time and to investigate the production of connective tissue matrix components.

Materials and methods

Livers from 18-day-old rat fetuses (Wistar strain) were used for the experiments. The animals were kept at a reversed day/night cycle. Two female animals each, at estrus, were mated with one male between 7 a.m. and 9 a.m. Only animals showing copulatory plugs served for our investigations. The first 24 h after mating was designated as day 0 of gestation. Immediately after removal the fetal livers were mechanically minced and the pieces rinsed 3 times with Hanks solution. Subsequently, the pieces were placed for 15 min at 4° C into a buffer solution consisting of 1 mM EDTA, 10 mM $KHCO_3$ and 155 mM NH_4Cl_2 in distilled water, pH 7.4 to remove the blood cells. This was followed by enzymatic isolation of the cells with 0.1% dispase (grade II) in buffer solution (see above) for 30 min in a water-bath (37° C) using a magnetic stirrer, and filtration through a nylon network (mesh width 20 µm). The isolated cells were centrifuged for 10 min at 700 rpm. One drop each of the sediment was dropped onto the filter paper resting on a KV4A steel bridge. The medium reached the filter from beneath (Trowell technique). The ISCOVE-Modified-Dulbecco solution with the addition of 10% fetal calf serum, 1% penicillin/streptomycin and 0.1% ampholin B, 1% vitamin C, glutamine and NEA served as medium.

The culture period was 7, 14 or 21 days. The cultures were fixed in a modified Karnovsky solution (3% glutaraldehyde, 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2) or in a 2% glutaraldehyde solution with the addition of 0.5% tannic acid. Postfixation in buffered 1% OsO_4 and dehydration in alcohol series were followed by embedding in Epon. The sections were cut on Reichert Ultracut microtomes and contrasted with uranyl acetate and lead citrate. Pictures were taken and evaluated on a Siemens Elmiskop 101 or a Zeiss EM10.

For the immunofluorescence microscopic investigation the cultures on the filters were coated with Histosol, frozen in liquid nitrogen and cut in an almost transverse direction on a cryotome. Antibodies against the following antigens were used: collagen type I, III, IV, VI, laminin, nidogen, heparan sulfate proteoglycan and fibronectin. Collagen type I and type III were obtained from fetal mouse skin (Trelstad et al., 1976), collagen type IV from human placenta according to Sage et al. (1979), collagen type VI was a gift from Fa. Heyl (Berlin, FRG), laminin, nidogen and heparan sulfate proteoglycan were obtained from ESH tumour according to Hassell et al. (1980) and Timpl et al. (1979), and fibronectin from rat serum (Engvall and Ruoslahti, 1977).

Antisera were prepared in rabbits following standard protocols (Kittelberger-Ewert et al., 1988) using 0.5 mg antigen per injection. The sera were purified by cross-absorption on affinity columns consisting of each of the other antigens and additionally collagen type I and type II and fibronectin coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) and processed according to March et al. (1974).

The monospecificity of the antibodies was established by ELISA (Engvall and Perlman, 1971) on microtiter plates, coated with laminin, nidogen, fibronectin and collagen types I, II, III, IV, V and VI. The antibodies against laminin, collagen type IV and type VI reacted only with their corresponding antigens. A cross-reaction of the anti-nidogen antibody with laminin in ELISA could be attributed to contaminations of the laminin preparation with nidogen by immunoblotting (Towbin et al., 1979).

The unfixed cryosections (frozen in liquid nitrogen) were covered with the antibodies and their binding was demonstrated with FITC-labelled IgG-antibodies (Merker et al., 1987).

Results

Electron microscopy

The mixture of isolated cells obtained after enzyme

treatment and filtration, which is the basic material used for the organoid cultures, shows all the cell types that can also be demonstrated in vivo in the fetal liver: hepatocytes, blood cells and their precursors, endothelial cells, fibrocytes, Kupffer's cells, ITO-cells, epithelial cells of bile ducts and smooth muscle cells. Many of these cell types obviously die or are not able to adhere. Other cell types, especially hepatocytes, are characterized by the phenomenon of a «sorting-out» process, i.e., the cells approach one another and adhere to form aggregates. Endothelial and fibroblast-like cells are localized between and around these aggregates.

This situation was observed as early as 48 h after the beginning of the culture period and remained in this form for principally 3 weeks (period of the study). However, some morphologically demonstrable alterations occurred depending on the time.

After 6 days (Figs. 2, 3) in vitro the hepatocytes did not show any lesions. The nucleus was round and the karyoplasm had a loose appearance except for some small plaques of marginal chromatin and 1-2 large nucleoli. The cytoplasm exhibited numerous profiles of the rough endoplasmic reticulum. Structures of smooth endoplasmic reticulum could only rarely be identified with certainty. The mitochondria contained an electron-dense matrix; swellings were not observed. The other cell organelles did not show any changes either. Numerous polysomes and some glycogen granules were located between the organelles. According to this morphological structure the described cells can easily be identified as hepatocytes. Only some membrane-bordered inclusions of 300-600 nm in diameter that contained cytoplasmic components or other poorly identifiable material did not show the morphology of a normal liver cell. In analogy to similar structures in other cultured cells these inclusions may be considered as autophagic vacuoles (cytolysosomes) that develop due to an adaptation to the new in vitro conditions.

Polarization of the hepatocytes was pronounced. In the periphery of the epithelial cell aggregates the hepatocytes with their smooth cell membranes were resting on an almost continuous matrix rim of varying thickness (50-300 nm). After tannic acid fixation this rim showed a high electron density. Higher magnification revealed densely packed fine-filamentous and fine-granular material in which densely packed collagenous fibrils were embedded. Cavities were perceptible in the central areas of the aggregates. These cavities resembled bile capillaries. Stump-like microvilli reached into them. The adjacent intercellular gap was sealed by tight junctions. The diameter of these cavities varied considerably, which was due to the number of hepatocytes that were involved in their demarcation. Smaller cavities contained 3 to 10 hepatocytes, the apical cell membrane of which reached as far as the lumen. In these cases the hepatocyte aggregates showed a one-layered structure. The cells stretched from the matrix rim in the periphery up to the bile capillaries in the centre. In other areas this one-layered structure could not be demonstrated with certainty and the structure of such aggregates was not clear.

Around and often rather close to the aggregates wide, capillary-like cavities occurred. The endothelium had a thickness of 200-500 nm and did not show any pores or gaps. A basal lamina (BL) could not be demonstrated. In the vicinity of the hepatocyte aggregates the endothelium often attached to the described electron-dense matrix rim. Outside these regions a comparable structure was missing or collagenous fibrils were in direct contact with the endothelium. Outside and inside the vessel-like cavities colonies of blood cells of various maturation stages of the red and the white series (neutrophil, basophil and eosinophil and megakaryocytes precursors included) occurred. A clear-cut morphological demarcation of these colonies was rare. They were occasionally surrounded by endothelium-like cells.

Two other cell types were recognizable in the organoid cultures of fetal rat livers in addition to these hepatocyte aggregates, endothelial cells and blood cells: mesenchymal cells and histiocytes. The mesenchymal cells were characterized by a greatly varying shape with plump processes and a well-pronounced rough endoplasmic reticulum, thus resembling fibroblast-like cells. These cells occurred singly or in a group filling areas of varying size between the hepatocyte aggregates. The histiocytes also occurred singly or in groups. They were characterized by an irregular surface with numerous processes and ridges of varying size and by numerous inclusions. The morphology of the 500 to 3000 nm-big membrane-bordered inclusions varied from cell to cell. Their content may be of average electron density and mainly homogeneous. In other cells granular or membrane-like structures predominated. Inclusions with a distinct polymorph content also occurred.

Electron microscopic inspection revealed matrix structures between the different cell types. Using the Karnovsky or tannic acid fixation technique mainly collagenous fibrils were observed in addition to the rim around the hepatocyte aggregates (Figs. 5c, 6b). They were 20-30 nm thick, had a cross-striation and appeared singly and irregularly or occurred in bundles, i.e., parallel to one another. Moreover, filaments of 12-18 nm in diameter could be demonstrated. These filaments occurred also singly or in bundles and often showed a clear-cut spatial relationship to the collagenous fibrillar bundles. Finally, irregularly bordered, electron-dense plaques of 100 nm to 1 µm were seen. They consisted of a network of fine filaments (less than 12 nm) and fine granular material.

The described picture of the organoid cultures was also valid for 2-week-old cultures (Figs. 4,6). Only minor changes were seen. The number of inclusions in the hepatocytes, described as cytolysosomes, had slightly risen. The amount of tannic acid-positive matrix with its various structures had also somewhat increased. The size of the blood cell colonies and of the number of histiocytes had increased. Single cell necroses could be demonstrated in the different cell types.

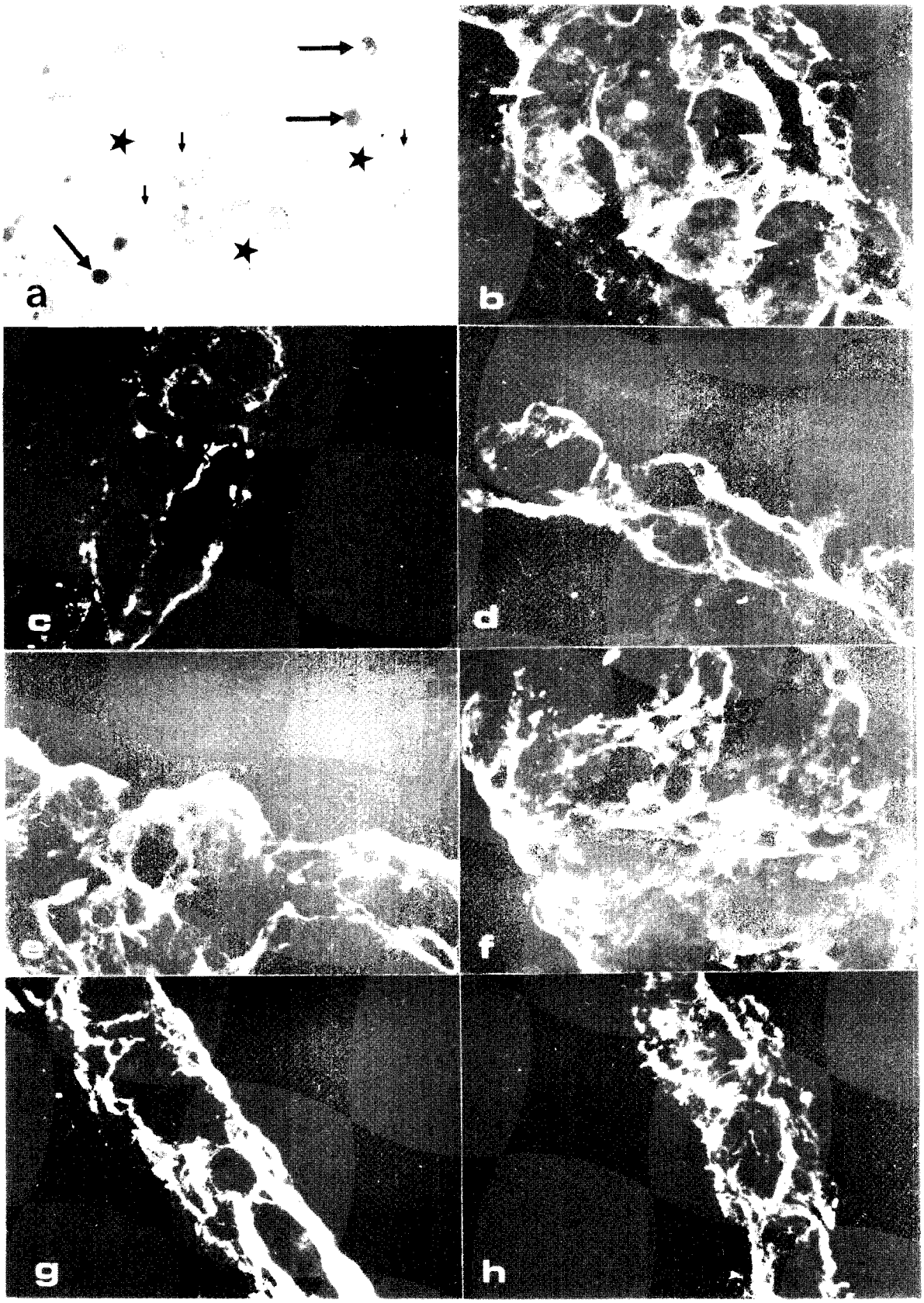

Fig. 1. Organoid cultures of fetal rat livers, oblique sections (a, b, e, f) or cross-sections (c, d, g, h). **a.** Culture period = 3 weeks; plastic embedding, 1 μm section, Giemsa staining. Liver cell aggregates (*) with numerous vacuoles (small arrow = swollen mitochondria and autophagic vacuoles), single necroses (arrow) × 250. **(b-h).** Frozen sections of 2-week-old cultures. Immunofluorescence microscopic demonstration of connective tissue matrix components **b.** Collagen type III. **c.** Collagen type VI. **d.** Collagen type IV. **e.** Nidogen. **f.** Laminin. **g.** Heparan sulfate proteoglycan. **h.** Fibronectin. × 150

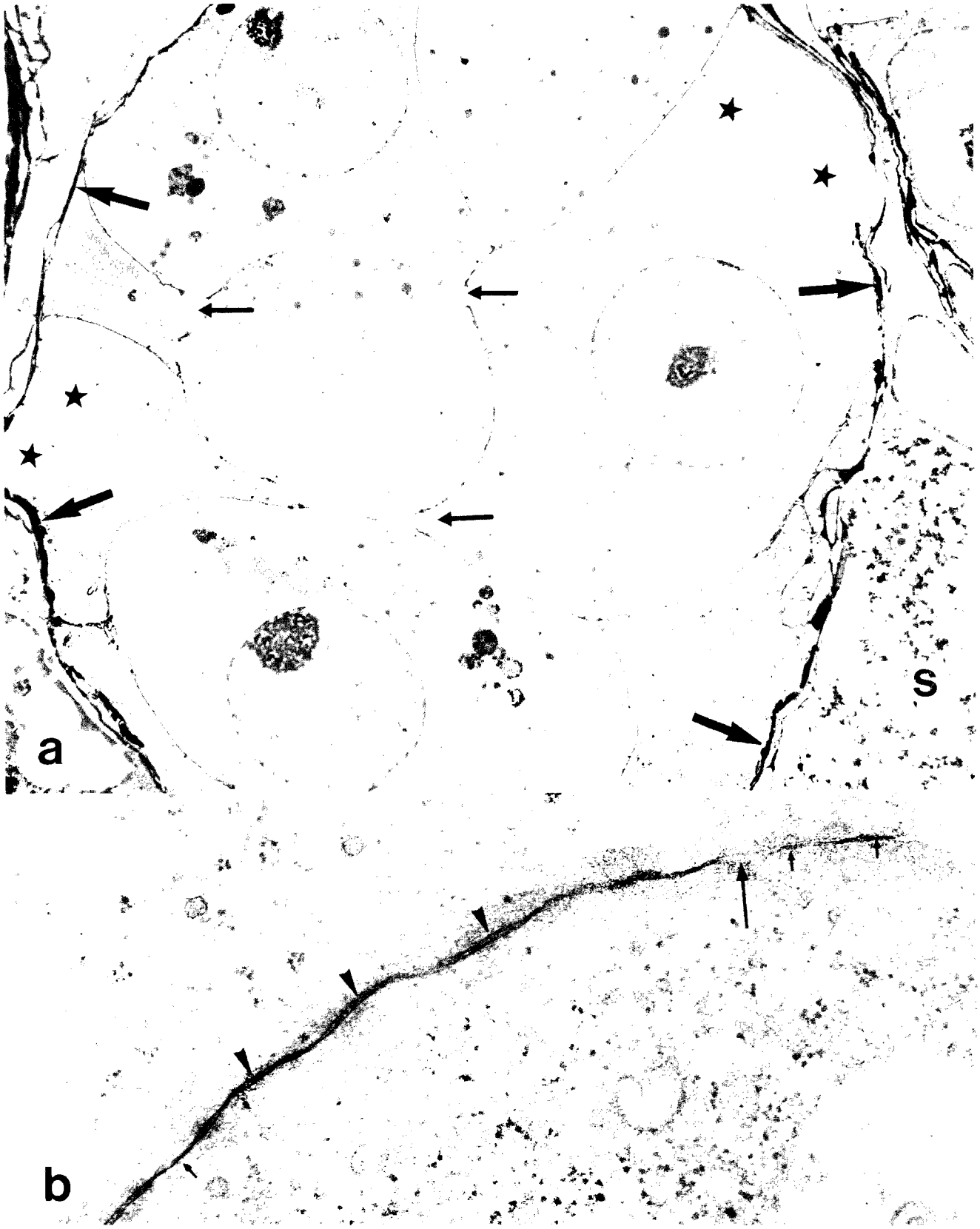

Fig. 2. Culture period = 7 days. **a.** Hepatocyte aggregate with tannic acid-positive matrix coat (arrow). Bile capillaries in the centre (small arrow), glycogen areas (*) with indications of loosening. S = sinus-like cavity. × 6,000. **b.** Cell contact between two hepatocytes in an aggregate. Next to contact areas with an intercellular space of 20 nm (arrow head), gap junction (small arrow) and tannic acid-negative tight junction (arrow) are seen. × 60,000

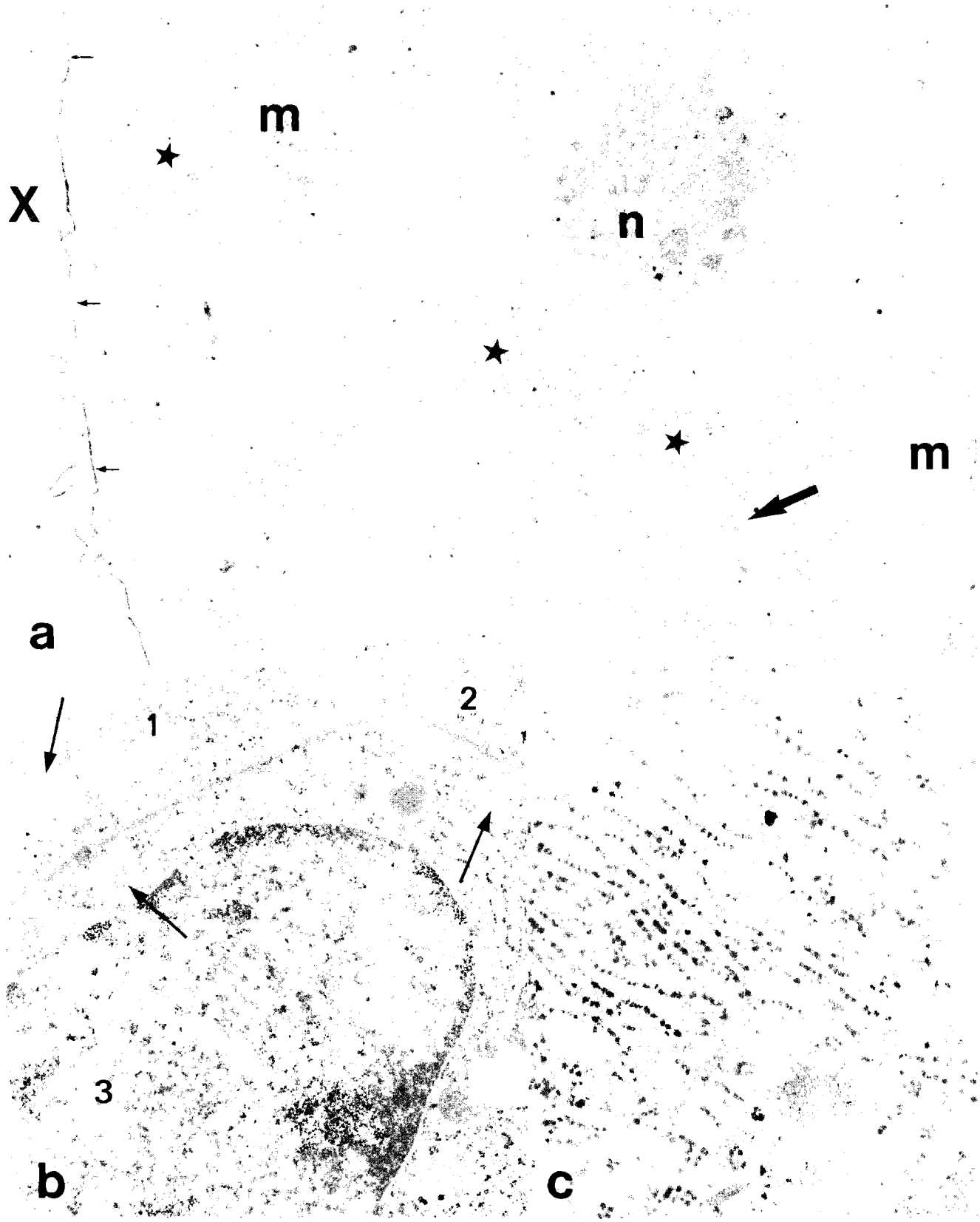

Fig. 3. Culture period = 7 days. **a.** Hepatocyte with two nuclei in an aggregate showing mitochondria (m), rough ER (') and an autophagic vacuole (arrow). One nucleus with a large nucleolus (n) in a loosened karyoplasm, cell contacts (small arrow) and a bile capillary (X) in a neighbouring cell. × 12,000. **b.** Secretion granules (arrow) in the apical region of 3 contacting hepatocytes (1, 2, 3). × 30,000. **c.** Rough ER of a hepatocyte from an aggregate. × 60,000

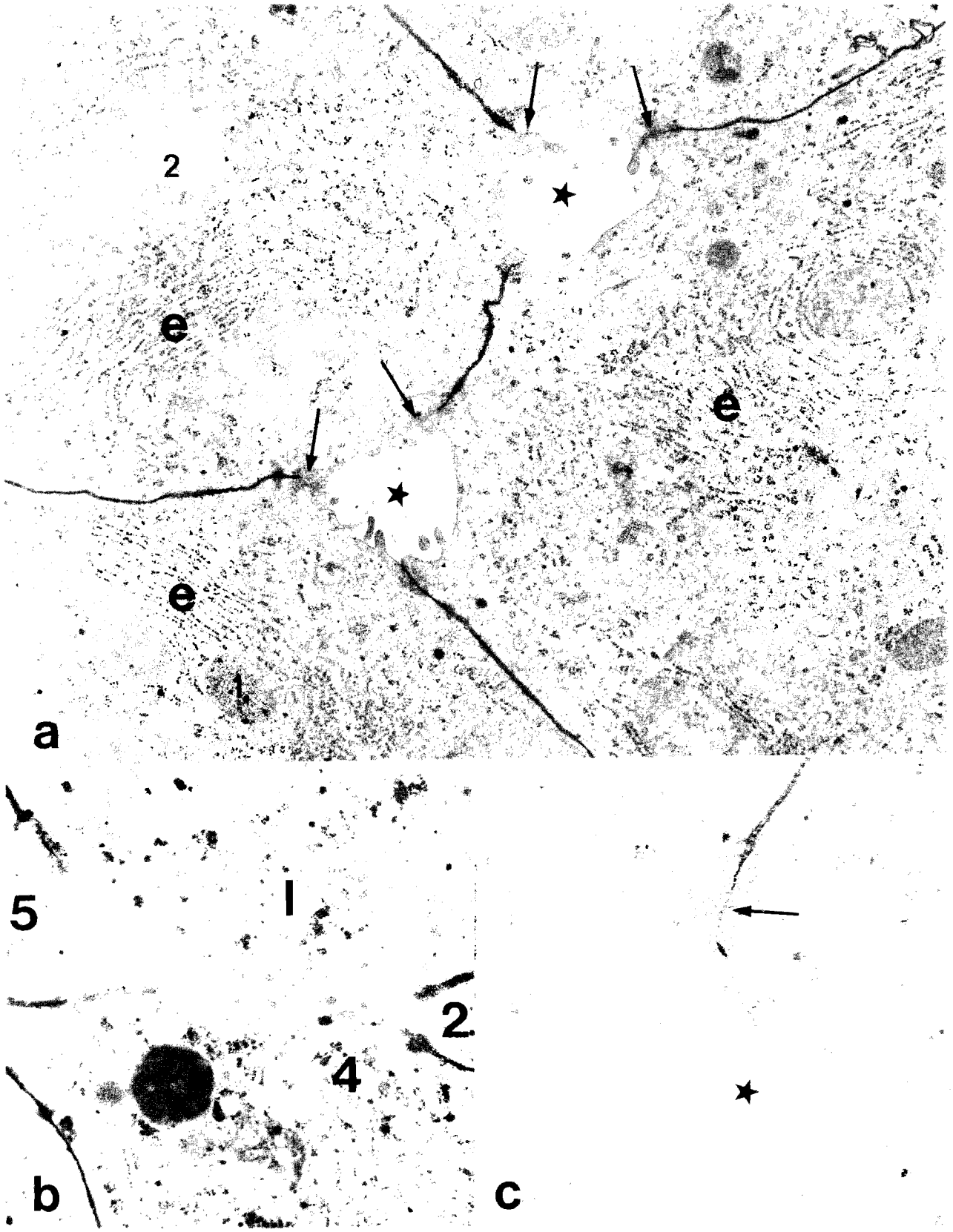

Fig. 4. Culture period = 2 weeks. **a.** Centre of a hepatocyte aggregate with two bile capillaries (*). Narrowing of the intercellular space and reduction in the tannic acid staining (arrow) in the vicinity of the bile capillaries. A well-developed rough ER (e) and normal (1) and swollen (2) mitochondria. × 24,000. **b.** Bile capillaries in the contact areas of four hepatocytes (1-4) in an aggregate. × 35.000. **c.** Contact area between two hepatocytes in the vicinity of a bile capillary (*). No tannic acid-positive staining (arrow = tight junction). × 60.000

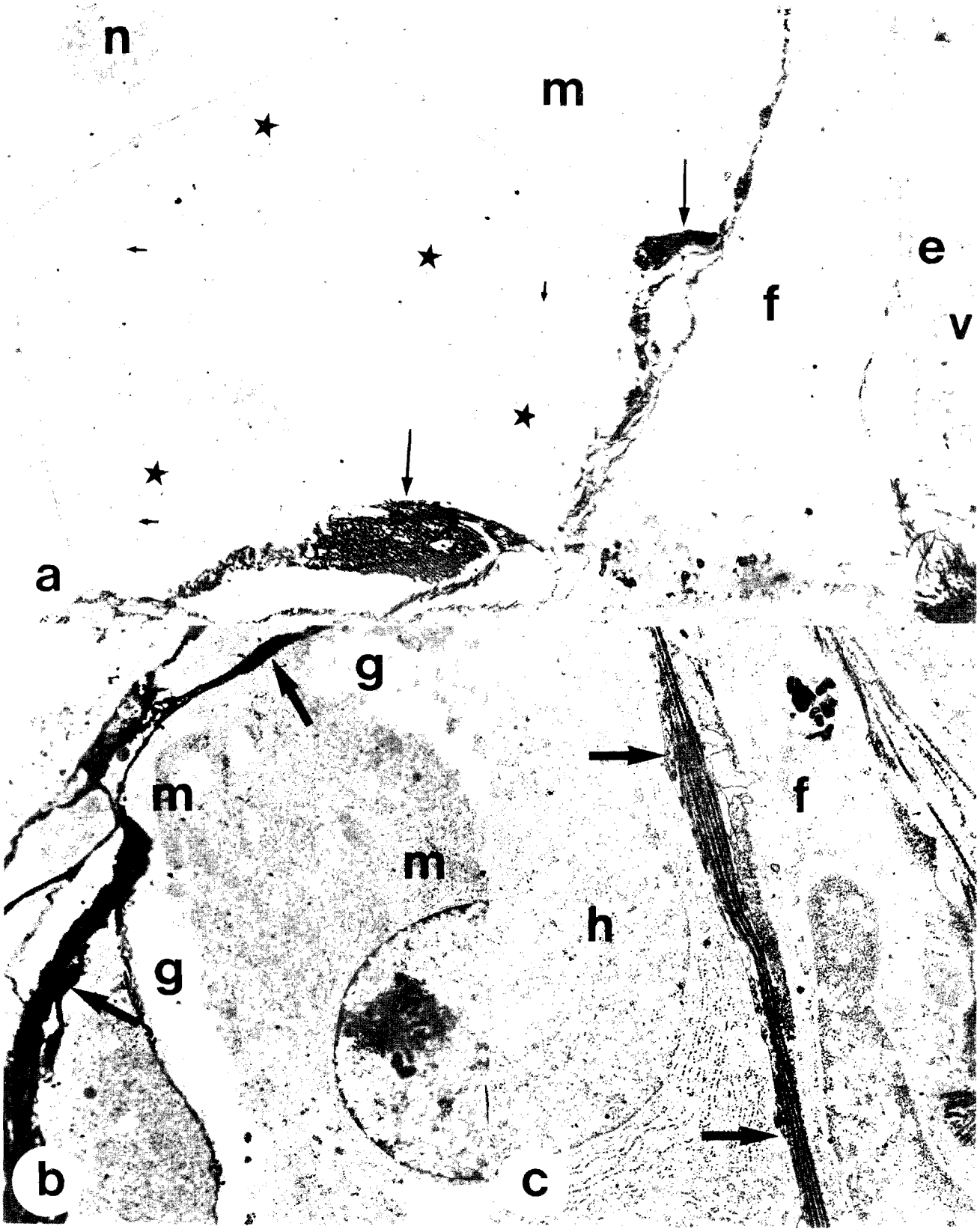

Fig. 5a. Culture period = 3 weeks. Typical polygonal liver cell with well-developed rough ER (*) and mitochondria (small arrow). In neighbouring cells nucleus (n) with large nucleolus and numerous swollen mitochondria (m). In the periphery closely attached bundles of collagenous fibrils (arrow). At the edge a vessel-like cavity (v) with endothelial lining (e). Between hepatocyte aggregates and endothelium a fibroblast-like cell (f). × 9,000. **b.** Culture period = 7 days. Hepatocytes in an aggregate with mitochondria (m) and rough ER. At the periphery of the cell glycogen areas (g). The aggregate surrounded by a highly tannic acid-positive matrix rim (arrow). l = lumen of a vessel-like cavity lined by endothelium (e). × 9,000. **c.** Culture period = 2 weeks. Periphery of a hepatocyte aggregate (h) with closely attached collagenous fibrils (arrow). Fibroblast-like cell (f) bordering on this aggregate from the outside. × 25,000

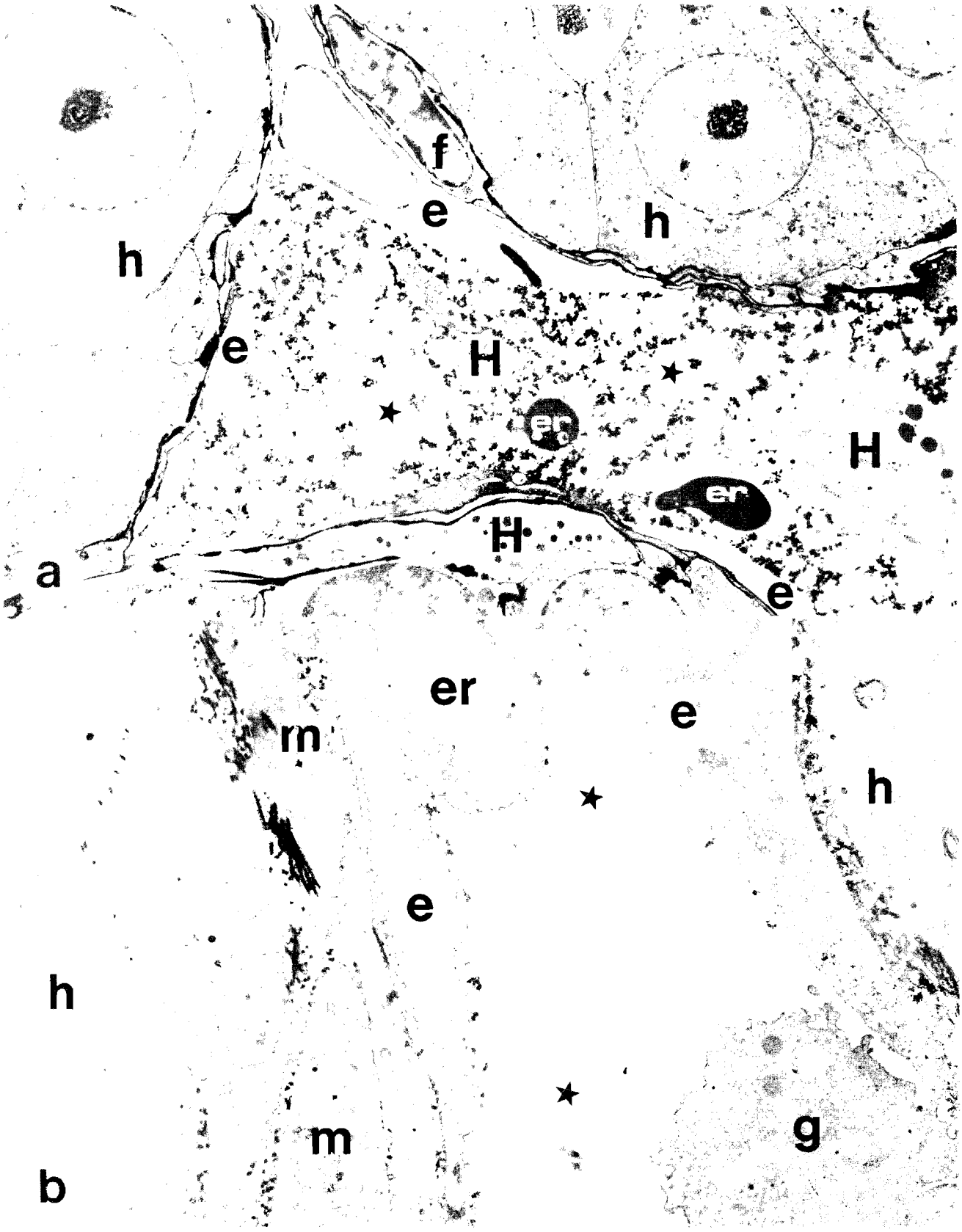

Fig. 6. a. Culture period = 2 weeks. Vessel-like cavity (*) between hepatocyte aggregates (h) with granular tannic acid-positive structures (serum proteins?), erythrocytes (arrow) and sections of histiocytes (H). Continuous lining by endothelium-like cells (e). Between hepatocytes and endothelium fibroblast-like cells (f) or histiocytes (H). × 6,000. **b.** Vessel-like cavity (*) showing a neutrophil granulocyte (G) and an immature erythrocyte (er). Lining by endothelial cells (e). At the outside mesenchymal cells (m) with collagenous fibrils in the intercellular space. h = hepatocytes. × 12,000

After a 3-week culture period (Fig. 5) the picture of these cultures had principally not changed. But swollen mitochondria occurred to an increasing extent in the hepatocytes. The number of cell necroses, especially in the hematopoietic foci, had risen and the amount of connective tissue matrix had increased further as had the number of cytolysosomes in the hepatocytes.

Immunofluorescence microscopy

As early as after 6 days in culture different matrix components could be demonstrated immunomorphologically. Their quality and distribution did not change during the 3-week investigated period (Fig. 1). It was only the amount that increased during the culture period. The distribution was similar for all antigens. They surrounded the hepatocyte aggregates as a clear-cut rim, occasionally and less clearly defining the haematopoietic foci. Smaller amounts could also be demonstrated within the foci. Between the rims around the aggregates antibody binding was seen in the form of loosely distributed, network-like fibres or more homogeneous areas. The following matrix antigens could be demonstrated: collagens type III, IV, VI, laminin, nidogen, heparan sulfate proteoglycan, fibronectin.

Discussion

The organoid culture (high density culture) of the various enzymatically isolated cell types of fetal rat liver yielded in 100% of the cases a liver-like tissue as early as after a 2-day culture period given a sufficient or optimal cell density. An important mechanism for this histiotypic picture is a sorting-out process where the hepatocytes are attached to one another to form aggregates (Zimmerman, 1987). Although the composition of these aggregates is often difficult to follow in the thin slices, we are in most cases obviously dealing with a one-layered unit of polarized cells. In the periphery they usually border on the connective tissue and apically they are involved in the composition of the wall of the central cavities. These central cavities do not basically differ from the bile capillaries *in vivo*.

The morphology of the border between epithelium and connective tissue *in vivo* and *in vitro* shows clear-cut differences. In vivo a rim-like densification of the matrix at the sinus pole of hepatocytes is missing. But this rim does not represent a typical basal lamina. A clearly defined Lamina rara and Lamina densa do not develop. A striking observation is a varying thickness. Collagenous fibrils radiate into this rim. Higher magnification reveals collagen fibrils and a basal lamina-like material that consists of fine filaments and granular material. We are, therefore, obviously dealing with a mixture of BL-material and the other components of the connective tissue matrix. This hypothesis is supported by the occurrence of the different matrix and BL-antigens, as revealed by immunomorphological means. Immunofluorescence microscopic inspection has shown that they are mainly located at the border of the aggregates. BL-material can undoubtedly be demonstrated immunomorphologically in other areas also. In these we obviously see the electron-dense plaques of filamentous and granular material. The matrix components demonstrated with the immunomorphological technique correspond in many aspects to the findings obtained for intact rat livers. Collagen type I is missing, types III, V, VI and fibronectin are present. The BL components are also demonstrable in sufficient amounts: collagen type IV, laminin, nidogen and heparan sulfate proteoglycan. Some differences do, however, exist. Adult liver contains small amounts of collagen type I; laminin is missing. These differences may be due to the fetal origin of the hepatocytes. Our previous results (Xu et al., 1989, 1991) have shown that in the new-world primate *Callithrix jacchus* the qualitative spectrum of the matrix components of the liver changes time-dependently: before birth collagen type I is missing, but laminin is expressed. In the organoid culture the hepatocytes maintain their fetal character as far as this aspect is concerned. Another problem is the allocation of the matrix production to certain cell types. Studies performed during the last few years have shown that most cell types are able to produce matrix (Xu et al., 1989, 1991). The hepatocyte is especially capable of this function, but fibroblasts, ITO-cells and endothelial cells must also be mentioned. The number of fibroblast-like cells is very low; ITO-cells could not be demonstrated. Since neither the number nor the morphology of the endothelial cells suggest a pronounced matrix production, the epithelial cells in the aggregates must be responsible for the formation of the major part of the epithelial cells in the aggregates. This concept is also supported by the accumulation of matrix material at the periphery of the aggregates.

The electron microscopic picture of the epithelial cells in the aggregates largely corresponds to that of normal hepatocytes. After 6 days or 2 weeks lesions are not yet observed in the cell organelles. Inclusions (cytolysosomes, autophagic vacuoles) however, are not detectable *in vivo*. It can be assumed that we are dealing with processes of adaptation to the culture conditions and to the non-optimal conditions *in vitro*. The increase of inclusions after 3 weeks and the start of the formation of necroses suggest that the temporal limit of this *in vitro* model is reached after 3 weeks. Until this stage the typical morphology of a hepatocyte is largely maintained; dedifferentiation processes are not perceptible. From this point of view the organoid culture has an advantage over the other culture techniques of hepatocytes. The main aim of these cultivation experiments is undoubtedly to achieve and maintain a metabolization *in vitro* for an extended period of time. Based on the electron microscopic picture this aim could be achieved with the described organoid culture technique. But one problem must first be solved. It is not possible to establish an organoid culture system using hepatocytes from adult animals. This is only possible with liver cells from fetal livers. This raises the question

whether these hepatocytes are able to metabolize, i.e., whether they already have the P-450 system. According to the hitherto obtained findings this system develops in rats shortly before or after birth. However, more recent findings indicate that the capability to metabolize can already be induced before birth (Cresteil et al., 1982; Schulz and Neubert, 1988). First results indicate the possibility of an induction with barbiturates. If these findings can be confirmed, a stable *in vitro* metabolization system would be available. Since the hepatocytes produce a large amount of matrix material, it can be assumed that at least this step of differentiation can be achieved with this organoid culture technique.

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References

Begue J.M., Guguen-Guillouzcco C., Pasdeloup N. and Guillouzco A. (1984). Prolonged maintenance of active cytochrome P-450 in adult rat hepatocytes cocultered with another liver cell type. Hepatology 4, 839-842.

Berry M.N. and Friend D.S. (1969). High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. J. Cell Biol. 43, 506-520.

Bissell D.M. and Guzelian P.S. (1980). Phenotypic stability of adult rat hepatocytes in primary monolayer culture. Ann. N.Y. Acad. Sci. 349, 85-98.

Bissell D.M. and Choun M.O. (1988). The role of extracellular matrix in normal liver. Scand. J. Gastroenterol. 23 (Suppl. 151), 1-7.

Carlsen S.A., Schmell E., Weigel P.H. and Roseman S. (1981). The effect of the method of isolation on the surface properties of isolated rat hepatocytes. J. Biol. Chem. 256, 8058-8062.

Cresteil T., Beaune P., Kremers P., Flinois J.P. and Leroux J.P. (1982). Drug metabolizing enzymes in human foetal liver: partial resolution of multiple cytochrome P. 450. Pediatr. Pharmacol. 2, 199-207.

Diegelmann R.F. (1986). Synthesis of extracellular matrix components by cultured hepatocytes. In: Research in Isolated and Cultured Hepatoyctes. Gillouzo A. and Guguen-Guillouzo C. (eds). John Libbey Eurotext Ltd./INSERM. pp 209-224.

Engvall E. and Perlmann P. (1971). Enzyme-linked immunosorbent assay. Quantitative assay of immunoglobulin G. Immunochemistry 8, 871-874.

Engvall E. and Ruoslahti E. (1977). Binding of a soluble form of fibroblast surface protein fibronectin to collagen. Int. J. Cancer 20, 1-5.

Fleischmajer R. and Billingham R. (1968). Epithelial-Mesenchymal Interactions. Williams and Williams. Baltimore.

Flint O.P. (1986). An in vitro test for teratogens: Its practical application. Food. Chem. Toxicol. 24, 627-631.

Guguen-Guillouzo C., Seignoux D., Courtois Y., Brissot P., Marceau N., Glaise D. and Guillouzo A. (1982). Amplified phenotypic changes in adult rat and baboon hepatocytes cultured on a complex biomatrix. Biol. Cell 46, 11-20.

Guguen-Guillouzo C., Clement B., Baffet C., Beaummont C., Morel-Chany E., Glaise D. and Guillouzo A. (1983a). Maintenance and reversibility of active albumin secretion by adult rat hepatocytes co-cultured with another liver epithelial cell type. Exp. Cell Res. 143, 47-54.

Guguen-Guillouzo C., Baffet G., Clement B., Begue J.M., Glaise D. and Guillouzo A. (1983b). Human adult hepatocytes: isolation and maintenance at high levels of specific functions in a co-cultured system. In: Isolation, characterization and use of hepatocytes. Harris R.A. and Cornell N.W. (eds). Elsevier. New York. pp 105-110.

Guillouzo A. (1986). Plasma protein production by cultured adult hepatocytes. In: Research in isolated and cultured hepatocytes. Guillouzo A. and Guguen-Guillouzo C. (eds). John Libbey Eurotext LTD./INSERM. pp 155-170.

Guzelian P.S., Bissell D.M. and Meyer U.A. (1977). Drug metabolism in adult rat hepatocytes in primary monolayer culture. Gastroenterology 72, 1232-1239.

Hannah R., Simkins R. and Eisen H.J. (1980). Synthesis of α-fetoprotein and albumin by fetal mouse liver cultured in chemically defined medium. Dev. Biol. 77, 244-252.

Hassell J.R., Gehron R.P., Barrach H.J., Wilszek J., Rennard S.J. and Martin G.R. (1980). Isolation of heparan sulfate-containing proteoglycan from basement membrane. Proc. Natl. Acad. Sci. USA 77, 4494-4498.

Hata R. and Ninomiya Y. (1984). Hepatocytes (hepatic parenchymal cells) produce a major part of liver collagen in vivo. Biochem. Int. 8, 181-186.

Kistler A., Mislin M. and Gehring A. (1985). Chondrogenesis of limb bud cells: improved culture method and the effect of the potent teratogen retinoic acid. Xenobiotika 15, 673-679.

Kittelberger-Ewert R., Hinz N., Oeschner I., Schröter-Kermani C. and Barrach H.J. (1988). Production and specificity of antibodies against the central region of type II collagen. Immunol. Invest. 17, 49-61.

Kleinman H.K., Klebe R.J. and Martin G.R. (1981). Role of collagenous matrices in the adhesion and growth of the cells. J. Cell Biol. 88, 473-485.

Kreamer B.L., Staecker J.L., Sawada N., Sattler G.L., Stephen Hsia M.T. and Pitot H.C. (1986). Use of a low-speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. In vitro 22, 201-211.

Lake B.G. and Paine A.J. (1983). Induction of hepatic cytochrome P-450 and drug metabolism by metyrapone in the rat: relevance to its effects in rat liver cell culture. Xenobiotika 13. 725-730.

Le Dourin N.M. (1975). An experimental analysis of liver development. Med. Biol. 53, 427-455.

Leffert H.L. and Paul D. (1972). Studies on primary cultures of differentiated fetal liver cells. J. Cell Biol. 52, 559-568.

Lescoat G., Theze N., Clement B., Guillouzo A. and Guguen-Guillouzo C. (1985). Control of albumin and α-fetoprotein secretion by fetal and neonatal rat hepatocytes maintained in co-culture. Cell Differ. 16, 259-269.

Marceau N., Noel M. and Deschenes J. (1982). Growth and functional activities of neonatal and adult rat hepatocytes cultured on fibronectin-coated substratum in serum-free medium. In Vitro 18, 1-11.

March S.C., Parikh J. and Cuatrecasas P. (1974). A simplified

method for cyanogen bromide activation of agarose for affinity chromatography. Anal. Biochem. 60, 149-152.

Maslansky C.J. and Williams G.M. (1982). Primary cultures and the levels of cytochrome P-450 in hepatocytes from mouse, rat, hamster and rabbit liver. In vitro 18, 683-693.

Mennes W.C., Blaanboer B.J. and Van Holsteijn C.W.M. (1988). Biotransformation and cytotoxicity studies in primary hepatocyte cultures derived from different mammalian species. In: Liver Cells and Drugs. Vol. 164. Guillouzo A. and Guguen-Guillouzo. C. (eds). John Libbey Eurotext. Ltd./ INSERM. pp. 351-355.

Merker H.-J. (1986). Die Bedeutung der sog. «limb bud»-Kultur für die experimentelle Embryologie. Verh. Anat. Ges. 80, 137-149.

Merker H.-J. (1991). Testung von Teratogenen in vitro. In: Teratologie, die Lehre von den Fehlbildungen. VEB Verlag für Medizin. Berlin. (in press).

Merker H.-J., Krüger U. and Zimmermann B. (1981). Simulation of limb bud skeletogenesis in vitro. In: Culture Techniques. Neubert D. and Merker H.-J. (eds). Walter de Gryuter Verlag. Berlin. pp 119-133.

Merker H.-J., Bremer D., Barrach H.-J. and Gossrau R. (1987). The basement membrane of the persisting maternal blood vessels in the placenta of *Callithrix jacchus*. Anat. Embryol. 176, 87-97.

Michalopoulos G. and Pitot H.C. (1975). Primary culture of parenchymal cells on collagen membranes: morphological and biochemical observations. Exp. Cell Res. 94, 70-78.

Michalopoulos G., Sattler C.L. and Pitot H.C. (1976). Maintenance of microsomal cytochromes b5 and P-450 in primary cultures of parenchymal liver cells on collagen membranes. Life Sci. 18, 1139-1144.

Michalopoulos G., Russel F. and Bile C. (1979). Primary cultures of hepatocytes on human fibroblasts. In Vitro 15, 796-806.

Nakamura T., Yoshimoto K., Nakayama Y., Tomita Y. and Ichihara A. (1983). Reciprocal modulation of growth and differentiated functions of mature rat hepatocytes in primary culture by cell-cell contact and cell membranes. Proc. Natl. Acad. Sci. USA 80, 7229-7233.

Paine A.J. and Hockin L.J. (1982). The maintenance of cytochrome P-450 in liver cell culture: recent studies of P-450 mediated mechanisms of toxicity. Toxicology. 25, 41-45.

Paine A.J., Villa P. and Hockin L.J. (1980). Evidence that ligand formation is a mechanism underlying the maintenance of cytochrome P-450 in rat liver cell culture. Biochem. J. 188, 937-939.

Ratanasavanh D., Beaune P., Baffet G., Rissel M., Kremers P., Guengerich F.P. and Guillouzo A. (1986). Immunocytochemical evidence for the maintenance of cytochrome P-450 isozymes, NADPH cytochrome C reductase and epoxide hydratase in pure and mixed primary cultures of adult human hepatocytes. Food Chem. Toxicol. 24, 577-578.

Reid L.M. and Rojkind M. (1979). New techniques for culturing differentiated cells: reconstituted basement membrane rafts. Methods Enzymol. 58, 263-303.

Riso B. (1983). Morphology of the epitelio-mesenchymal interaction during lung development of the mouse. Cell Differ 13, 309-318.

Rojkind M., Gatmaitan Z., Mackensen S., Giambrone M.N., Ponce P. and Reid L.M. (1980). Connective tissue biomatrix. Its isolation and utilization for long-term cultures of normal rat hepatocytes. J. Cell Biol. 87, 255-263.

Sage H., Woodbury R.G. and Bornstein P. (1979). Structural studies on human type IV collagen. J. Biol. Chem. 254, 9893-9900.

Sattler C.A., Michalopoulos G., Sattler C.L., and Pitot H.C. (1978). Ultrastructure of adult rat hepatocytes cultured on floating collagen membranes. Cancer Res. 38, 1539-1549.

Sawada N., Tomomura A., Sattler C.A., Sattler C.L., Kleinman H.K. and Pitot H.C. (1987). Effects of extracellular matrix components on the growth and differentiation of cultured rat hepatocytes. In Vitro 23, 267-273.

Schaeffer W.I. and Kessler D.J. (1980). Human liver cells in culture. Methods Cell Biol. 21B, 457-473.

Schulz T. and Neubert D. (1988). Comparative studies on the activity of monooxygenases during the perinatal period in the marmoset and rat. in: Non-Human Primates-Developmental Bioology and Toxicology. Neubert D., Merker H.-J. Hendrickx A.G. (eds). Ueberreuter Wissenschaftsverlag. Wien - Berlin. pp 353.

Schwarz L.R., Götz R., Wolff Th. and Wiebel F.J. (1979). Monooxygenase and glucuronyl transferase activities in short-term cultures of isolated rat hepatocytes. FEBS Lett. 98, 203-206.

Segeln P.O. (1973). Preparation of rat liver cells. III. Enzymatic requirements for tissue dispersion. Exp. Cell Res. 82, 391-398.

Sipes I.G., Fisher R.L., Smith P.F., Stine E.R., Gandolfi A.J. and Brendel K. (1987). A dynamic liver culture system: a tool for studying chemical biotransformation and toxicity. Arch. Toxicol. Suppl. 11, 20-33.

Sirica E.A. and Pitot H.C. (1980). Drug metabolism and effects of carcinogens in cultured hepatic cells. Pharmacol. Rev. 31. 205-228.

Steward A.R., Dannan G.A., Guzelian P. and Guengerich F.P. (1985). Changes in the concentration of seven forms of cytochrome P-450 in primary cultures of adult rat hepatocytes. Mol. Pharmacol. 27, 125-132.

Timpl R., Rohde H., Geron R.P., Rennard S.J., Foidart J.-M. and Martin G.R. (1979). Laminin- a glycoprotein from basement membranes. J. Biol. Chem. 254, 9933-9937.

Towbin H., Staehelin T. and Gordon T. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350-4354.

Trelstad R.L., Catanase V.M. and Rubin D.F. (1976). Collagen fractionation: separation of native types I, II and III by differential precipitation. Anat. Biochem. 71, 114-118.

Williams G.M., Bermúdez E. and Scarmuzzino D. (1977). Rat hepatocyte primary cell cultures. III. Improved dissociation and attachment techniques and the enhancement of survival by culture medium. In Vitro 13, 809-817.

Xu Daqun, Schröter-Kermani C., Hinz N. and Merker H.-J. (1989). Connective tissue of the livers of newborn and adult marmosets (*jacchus*). Histol. Histopath. 4, 479-492.

Xu Daqun, Schröeter-Kermani C., Hinz N. and Merker H.-J. (1991). Immunofluorescence microscopic localization of collagen type IV and VI, laminin and nidogen in the livers of *Callithrix jacchus* during pre- and postnatal development. Acta Anat. (in press).

Zimmermann B. (1987). Lung organoid culture. Differentiation 36. 86-109.

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