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Technical report

A compatible support system for cell culture in biomedical research

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Abstract

The lack of a suitable system to culture epithelial cells for a long period under a luminal-antiluminal medium gradient, was the reason to develop a new system. It consists of an interchangeable sheet of permeable support material, which is set in place by two tight fitting holding rings. For special demands the supports can be coated with extracellular matrix proteins improving cellular attachment and terminal differentiation. The handling of the sheets by forceps proceeds easily and quickly, thus fastening the transfer of cultured cells without additional manipulations. The sheets can be transferred to a newly developed microperfusion chamber on which an apical and a basal perfusion over a long culture period parallel to a transepithelial electrophysiological registration becomes possible. The chamber has an extremely low amount of fluid dead space. The separate perfusion of cultured cells under isotonic, hypotonic or hypertonic conditions opens new possibilities. Thus, culture can be performed under most natural conditions e.g., that found within the kidney.

Introduction

In biomedical and biotechnical experiments, the cell culture technique has two defined aims: First, the culture protocol used should possess excellent proliferation characteristics, providing for a satisfactory harvest and reproducibility of cells. Second, if in addition, organospecific questions are to be investigated, the cultured cells should exhibit typical features of that organ from which they derived, e.g., they should reveal a high degree of terminal differentiation characteristics.

The state of differentiation in cultured cells is regulated by both gene expression and environmental factors such as extracellular matrix proteins (ECM) (Opas, 1989; Karst and Merker, 1988; Yang *et al.*, 1986; Wissmann and Jacobson, 1985; Reid and Jefferson, 1984; Bernanke and Markwald, 1982). While continuous cell lines are – more or less – independent of their proliferation characteristics from these substrate proteins, defined primary cell cultures are extremely sensitive to those environmental factors (Minuth, 1987; Strom and Michalopoulos, 1982). Because ECM-coated cell culture tools are offered to a very limited degree, we developed a multipurpose system for the culture of principally all kinds of adherent cells. It comprises that:

1. Cultured cells are maintained in a highly differentiated state on specific supports.
2. Cells can be exposed for a long period to a luminal-antiluminal medium gradient.
3. Experimental procedures are not to be hind-

ered by a high lateral wall of the culture dish or a membrane inlet.

4. The transfer of the cultured cells under sterile conditions from a culture dish to the experimental set up and vice versa is possible without trypsination or other chemical treatment.
5. It is possible to culture cells on several identical support sheets within one culture dish.
6. The technique is compatible to a wide range of specific demands such as physiological demands.
7. The new system may be a suitable tool for the scaling up procedure in biotechnological experiments.

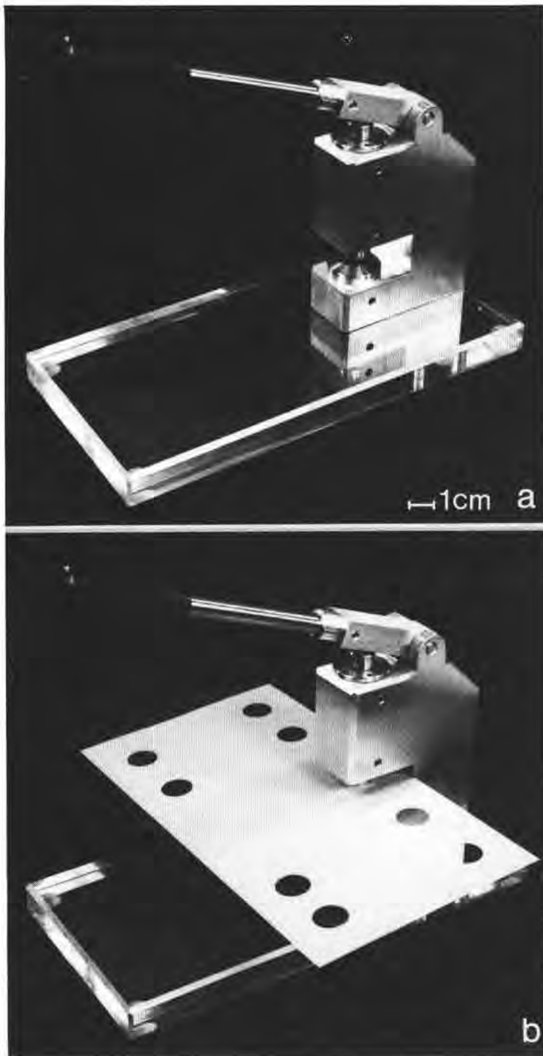


Fig. 1. a) Photograph of a punching tool with excised support material. b) The diameter of punched sheets in this specific case is 13 mm, but it may vary in size.

Construction of Minusheets (patent pending and registered trade mark)

Aided by a punching tool (Fig. 1), the suitable support material for cell culture is cut to the desired diameter. The punched pieces may have a diameter of few millimeters, although a larger scale may be used. The small sizes of sheets renders them suitable for all commercially available culture dishes with an inner diameter of 9, 15, 34, 52, or 85 mm or even larger (Fig. 4). The punched out sheets are then framed by suitable,

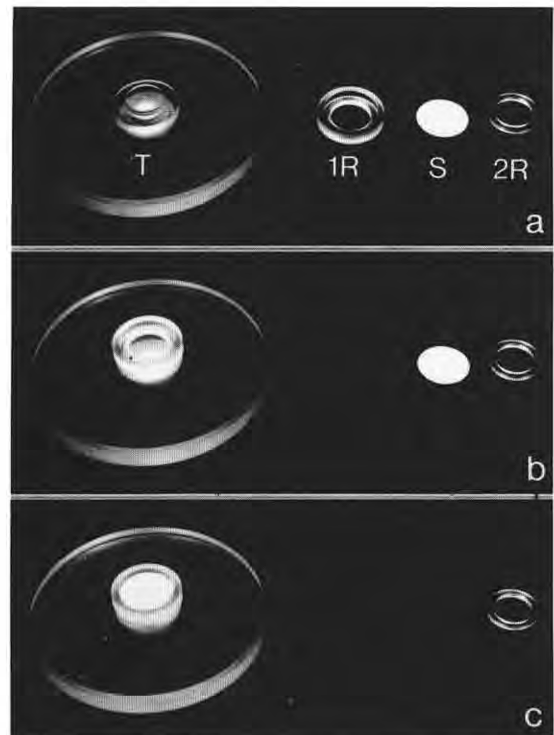


Fig. 2. Mounting of Minusheets in photographic illustration: a) for mounting of sheets, a cylindrical mounting tool (T), a first ring (1 R), a punched out support (S) of 13 mm diameter and a second ring (2 R) are needed. b) The first ring (1 R) is set on the mounting tool (T). c) The support (S) is put into the first ring (1 R). Finally, the second ring (2 R) is inserted into the first ring (1 R).

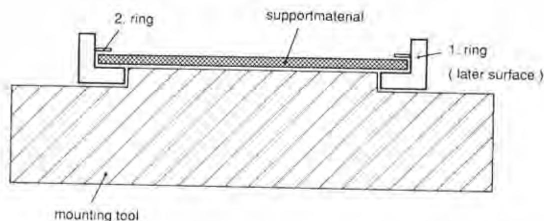
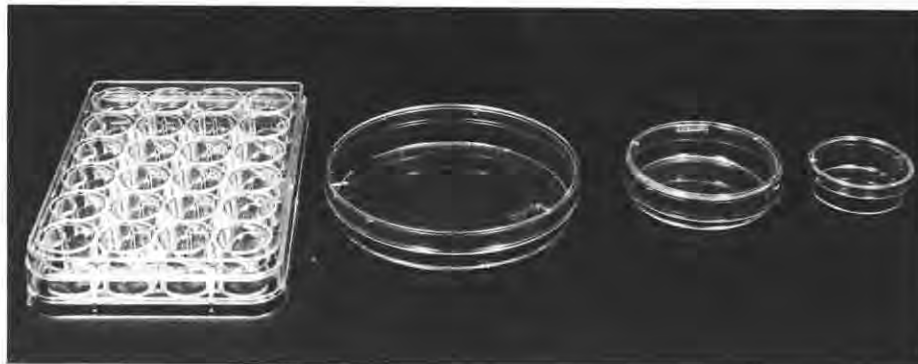
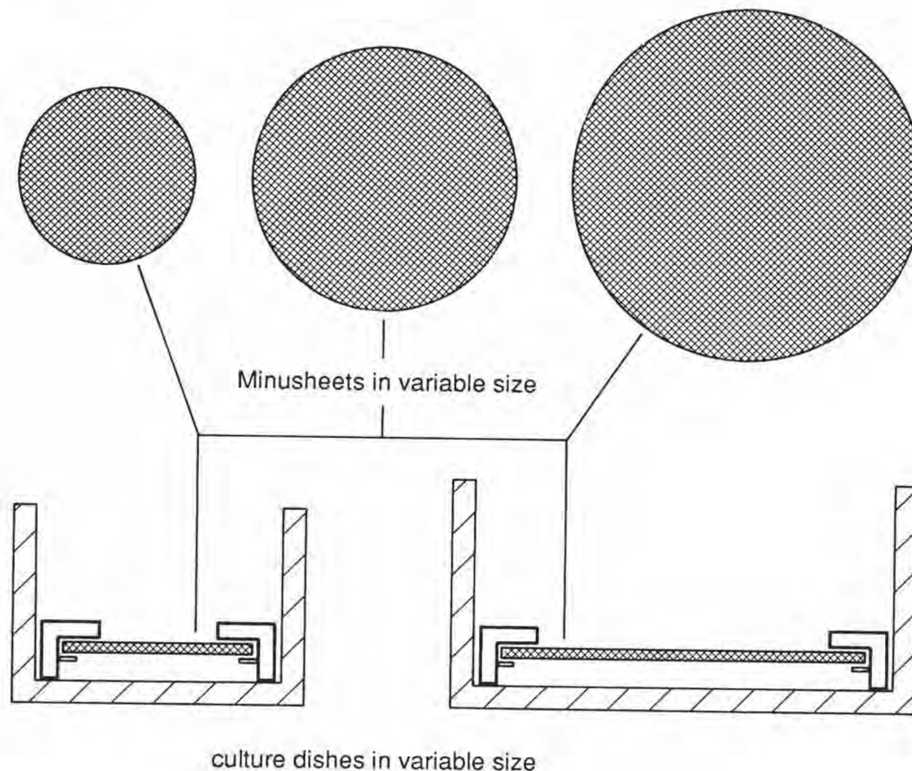


Fig. 3. Schematic cross section of Minusheet mounting: On the mounting tool (M), the first ring (1 R) serves as a holder; the support material and the second ring (2 R) are inserted into the first ring. Finally, the sheet is turned over.

tight-fitting holding rings in the following manner (Fig. 2): First, a set-up ring is installed (Fig. 2b) with a cylindrical mounting tool (Fig. 2a). The punched support is set into this ring (Fig. 2c) and, with a smaller tight-fitting ring, the support material is held and stretched like a drumhead

Fig. 4. Flexibility of Minusheets: The constructed sheets are able to improve the base of commercially available culture dishes by being flexible in diameter and allowing variation in the quality of individual support material.





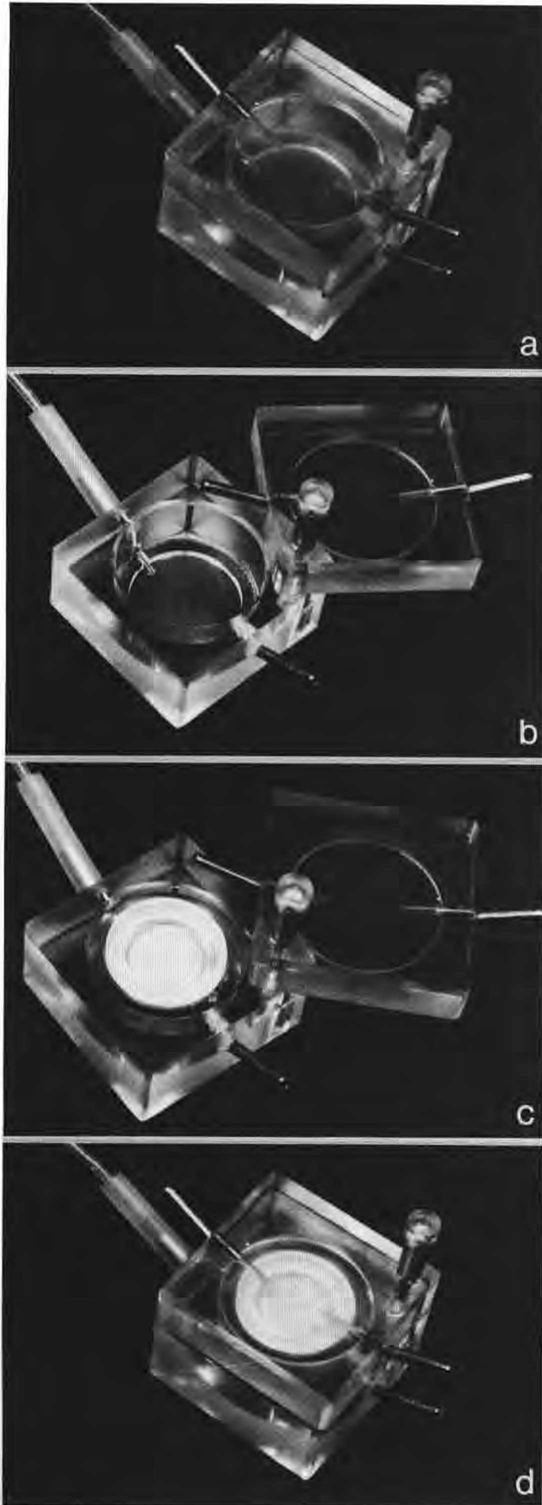


Fig. 5. Microperfusion system for Minusheets: a) The perfusion chamber can be perfused from the apical and basal side with individual media. b) By turning the lid, the chamber is opened. c) A Minusheet (13 mm in diameter) can be inserted via forceps into the chamber. d) After inserting the sheet, the chamber is closed by turning the lid in a backward direction.

(Fig. 3). After pressing the small ring into the set-up ring, the mounting procedure is finished. The support sheet is turned and the so called 'Minusheet' is ready to be used for further experiments.

Support material of Minusheets

Virtually all kinds of permeable of impermeable materials such as filters, nets, biological membranes, or glassware may serve as a support material for minusheet mounting. In addition, depending on individual demands, all of the supports can be coated with a variety of ECM-biomolecules. This possibility of combining various membranes with varying coats opens a wide range of experimental opportunities.

Sterilizing of Minusheets

Depending on the quality of the support material used, the sheets may be sterilized by heat, radiation or chemical treatment.

Applications of Minusheets

Microperfusion

The lack of a microperfusion system suitable for all kinds of adherent cells or tissues grown in culture was one of the reasons that minusheets were developed. The sheets can be easily removed from a culture dish and transferred to a newly developed microperfusion chamber (Fig. 5) in which both apical and basal perfusion of cell sheet becomes possible (Fig. 6). An O-ring gasket on the top and base of the sheets seals the

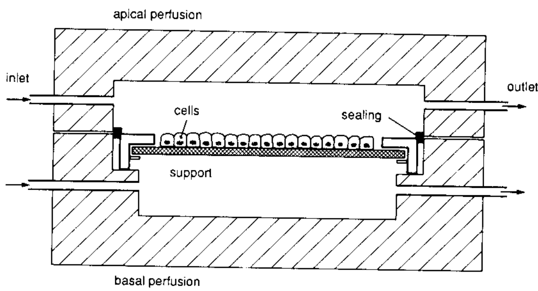


Fig. 6. Schematic cross-section through a microperfusion chamber. The chamber consists of an apical and a basal part. After Minusheet insertion, the chamber can be perfused from the apical and basal side by individual media.

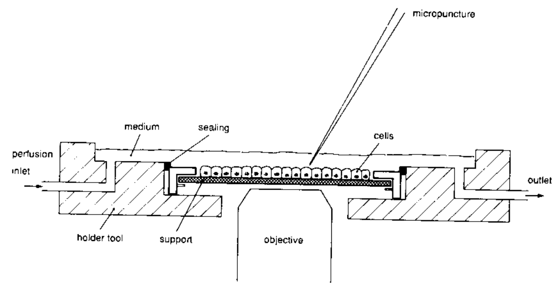


Fig. 8. Schematic illustration of a micropuncture experiment with living cells grown on a Minusheet. The cells can be perfused from the apical side. With a microscopic objective, the living cells can be observed from the basal side.

system from the apical to the basal side, preventing leakage. In addition, the system works with an extremely low amount of dead space.It is possible to simulate gradients *in vitro* over a long period of time (Fig. 7). Thus, culture can be performed under most natural conditions found, for example, in the kidney. This technique of culturing cells under permanent fluid gradient conditions provides new possibilities in the field of cell biological research.

Micropuncture

Inserting Minusheets with living cells to a newly constructed microscopic holder (Fig. 8), it is

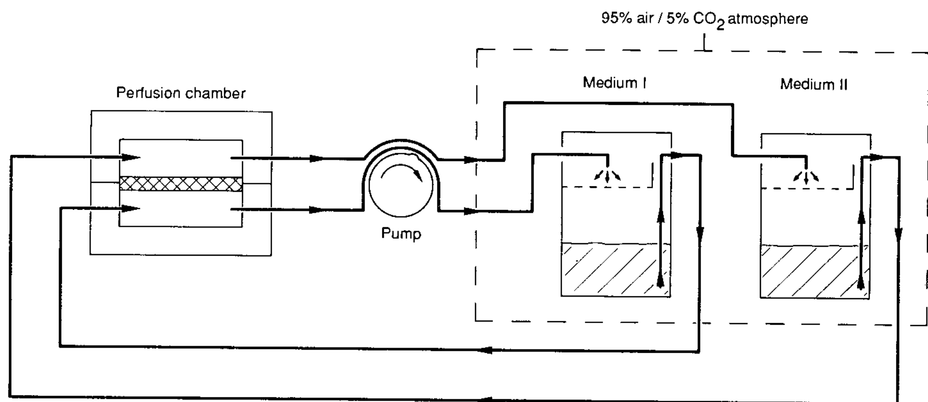


Fig. 7. Schematic illustration of a perfusion cell culture system. Living cells are held in the perfusion chamber on a Minusheet. The chamber is permanently perfused by a luminal-antiluminal culture medium gradient. The media are pumped into filtration units. Within the described system, only the filtration units are in a 95% air/5% CO_2 atmosphere: the rest of the system is free for experimental manipulations.

possible to penetrate the cells from the apical side easily with micropipettes without any hindrance by the lateral dish wall. In addition, an apical perfusion of cells becomes possible. The temperature of the assembly may be raised simply by prewarming the perfusion fluid. In a case where it is necessary to change the cells, the support sheet can be lifted easily by a forceps and replaced by a new one.

Possible biotechnological approach

Support sheets may be used in bioreactor technology to improve the scaling up procedure in a module-like manner: The sheets can be successively stacked (Fig. 9). They may vary in diameter and the quantity of sheets may be adjusted according to need. The sheets can be perfused,

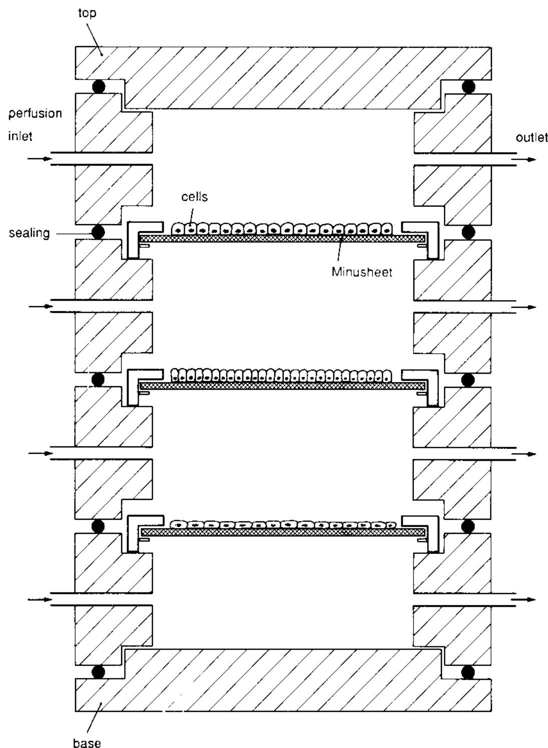


Fig. 9. Schematic illustration of a bioreactor cartridge with inserted Minusheets. Each inserted sheet can be perfused from the apical and basal side separately.

namely, each from apical and basal side and with an extremely low fluid dead space. It will be possible to juxtaposition one cell type against another, or, alternatively, one sheet may serve as a cell feeder layer; the other may contain a different cell type.

A further improvement is that each sheet can be individually controlled for infection or biomaterial secretion rate, Since each sheet accepts a fluid inlet and outlet from the luminal or basal side. The reactor tube can be opened easily at a particular sheet site, facilitating the substitution. An advantage of the sheet technique is the possible 'scaling ups' procedure of cells in the bioreactor under almost identical conditions: It is possible to culture cells on a small size sheet on the laboratory scale. If the experiments are successful on a small scale level, the sheet size can be successfully increased in diameter. Consequently, the same quality of support sheet can always be used, independent of the size. Thereby, it is possible to control the growth of cells on either a small or large-scale basis.

Representative cell culture experiment

Origin of cells, support and culture conditions

In the present experiments, we used small renal cortex explants from newborn New Zealand rabbits, isolated as described previously (Minuth, 1987). A thin cortical tissue layer, consisting of nephrogenic blastema containing collecting duct anlagen and S-shaped bodies, was placed onto a Minusheet (inner diameter of 9 mm). The support was capsula fibrosa from pig fixed in ethanol (70%) and affixed on cellophane. Subsequent cultures were prepared with Dulbecco's modified Eagle medium (DMEM/HEPES), supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic solution (Gibco-BRL Life Technology, Eggenstein, FRG).

Aldosterone (1×10^{-6} M; Aldocorten, Ciba Geigy AG, Wehr, FRG) was added to all culture dishes, starting on the third day of cultivation. The culture was grown for 20 days at 37°C with

5% CO_2/85% air in a humid atmosphere as was described previously (Minuth, 1987).

Histology

For histological examination, the Minusheets with living collecting duct cells on it were re-

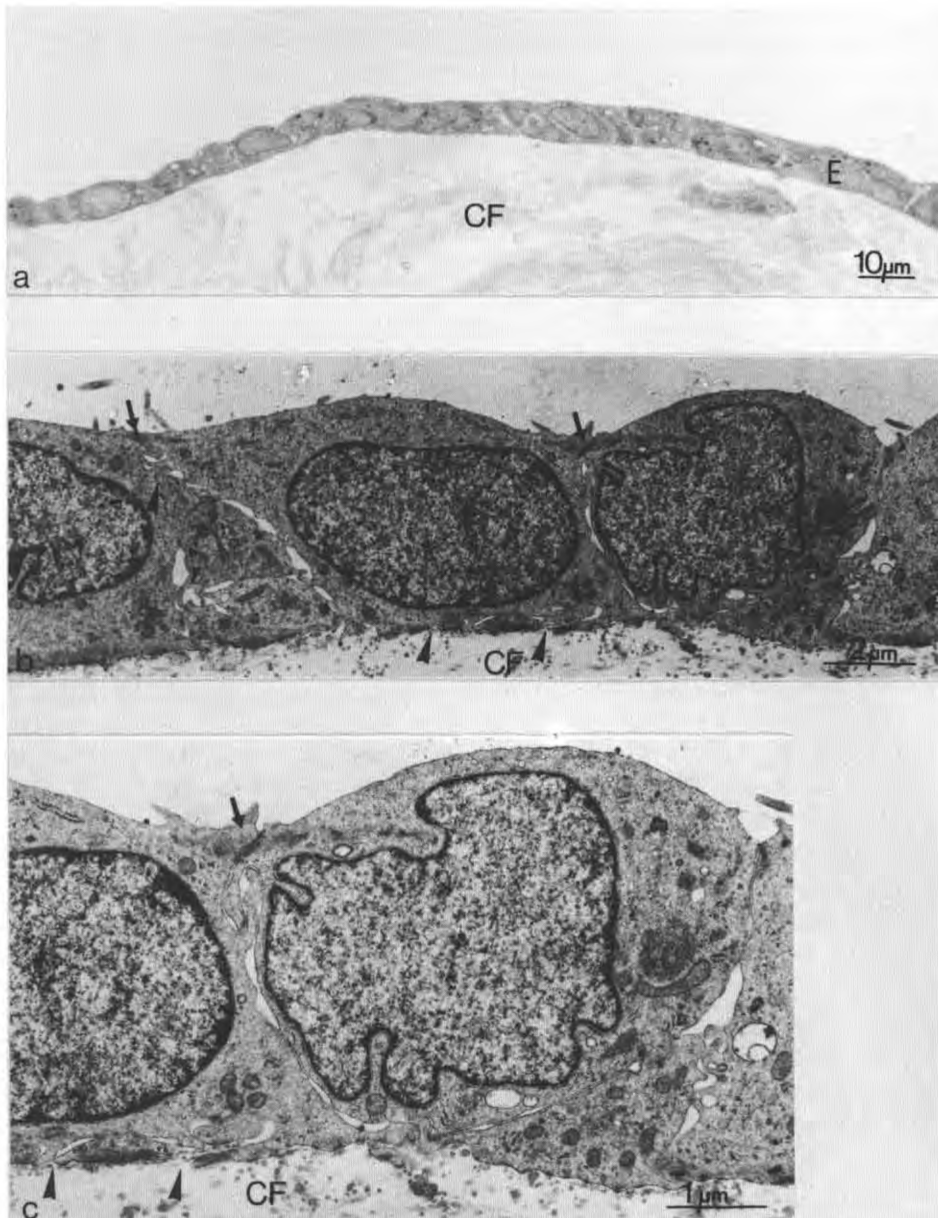


Fig. 10. Photomicrograph of individual cultured renal collecting duct cells grown on a Minusheet. The support consisted of an alcohol fixed capsula fibrosa from pig kidney (CF). The cells are maintained for 20 days on a 13 mm support sheet. a) Semi-thin section: Monolayer of polar differentiated renal collecting duct principal cells E = epithelium (rabbit); CF = capsula fibrosa (pig) × 1.000; b) Ultrathin section: The cells show basal and lateral infolding (arrowhead) and apical tight junctions (arrow) × 8.100; c) Detail of Fig. 10b at higher magnification × 17,000.

moved from the culture dishes with forceps and fixed for 20 min. in glutaraldehyde solution (3% glutaraldehyde in DMEM, pH 7.4). Following the initial fixation, the support sheets were removed from the holder rings and postfixed with 1% osmium tetroxide in PBS (pH 7.4) for 15 minutes. The tissue was washed in PBS and dehydrated in a graded alcohol series, passed through propylene oxide, and embedded in Epon. Semi-thin sections were stained with Richardson solution; ultrathin sections were contrasted by uranyl acetate and lead citrate, and then examined with a Phillips 301 electron microscope.

Results

Within 24 hours after isolation, the surface of the renal explants began to be covered with proliferating collecting duct principal cells. The cells spread then from the explant toward the periphery of the support. Until the 20th day the emigrating cells covered an area continuously increasing in diameter. Light microscopic observations of semi-thin sections revealed a monolayer of polar-differentiated epithelium cells (Fig. 10a). When observed with transmission electron microscopy, the cells exhibited typical features of collecting duct principal cells such as basal and lateral membrane infolding and apical tight junctions (Fig. 10b, c). Beneath the monolayer, we found only collagen structures and no fibroblasts. The epithelial monolayer strongly resembled the collecting duct epithelium as described previously by Minuth (1987). However, with our new and improved culture technique, we were able to prevent the growth of fibroblasts underneath the epithelium and we greatly increased the amount of cells as compared to earlier experiments.

Conclusions

Organo-typic cell cultures following experimental applications are often hindered by dedifferentiation. The isolated cells loose morphological characteristics, physiological properties and biochemical traits within a short time after isolation (Minuth and Gilbert, 1988; Minuth *et al.*, 1988). This process can be improved by offering the cells interchangeable supports with ECM-coating. For this demand we constructed specific sheets to improve the unspecific bottom of culture dishes. The sheets are easy to transfer in microperfusion chambers, in which a further improvement of terminal differentiation may be obtained by isotonic, hypotonic or hypertonic media perfusion.

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References

1. Bernanke DH and Markwald RR (1982) Migratory behaviour of cardiac cushion tissue cells in a collagen-lattice culture system. Dev. Biol. 91: 235–245.
2. Karst W and Merker HJ (1988) The differentiation behaviour of MDCK cells grown on matrix components and in collagen gels. Cell Differentiation 22: 211–223.
3. Minuth WW and Gilbert P (1988) The expression of specific proteins in cultured renal collecting duct cells. Histochem. 88: 435–441.
4. Minuth WW, Gilbert P and Gross P (1988) Appearance of specific proteins in the apical plasma membrane of cultured renal collecting duct principal cell epithelium after chronic administration and arginine vasopressin. Differentiation 38: 194–202.
5. Minuth WW (1987) Neonatal rabbit kidney cortex in culture as tool for the study of collecting duct formation and nephron differentiation. Differentiation 36: 12–22.
6. Opas M (1989) Expression of the differentiated phenotype by epithelial cells in vitro is regulated by both biochemistry and mechanisms of the substratum. Dev. Biol. 131: 281–293.
7. Reid LM and Jefferson DM (1984) Cell culture studies using extracts of extracellular matrix to study growth and differentiation in mammalian cells, pp. 239–272. In: Mammalian cell culture – the use of serum free hormone-supplemented media by JP Mather Plenum Press, New York and London.
8. Strom SL, Michalopoulos G (1982) Collagen as a substrate for cell growth and differentiation. Methods Enzymol. 82: 544–555.

9. Wissemann KW and Jacobson BS (1985) Pure gelatin microcarriers: Synthesis and use in cell attachment and growth of fibroblast and endothelial cells. In Vitro Cell Dev. Biol. 21: 391–401.
10. Yang AH, Gould-Kostka J and Oberley TD (1987) In vitro growth and differentiation of human kidney tubular cells on a basement membrane substrate. In Vitro Cell Dev. Biol. 23: 34–46.

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