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# The morphology of mesangial cells cultured at high density and in collagen gels

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Summary. Primary mesangial cells (rat) from monolayer cultures of the 6th to 12th passage and permanent SV40 Mes13 cells were grown at high density in organoid culture at the medium/air interphase. After adaptation to the in vitro conditions, both mesangial cell types developed after 7 days a synthesis apparatus (endoplasmic reticulum, Golgi apparatus) and produced matrix which consisted of Lamina densa-like material, collagenous fibrils and filaments. Unspecific contacts, gap junctions and adhesion belts could be demonstrated in the contact areas. Additionally, some cells exhibited thick bundles of actin filaments. A close resemblance of the mesangial cells in high density culture to those in vivo can, therefore, be stated. Hence, they differentiated with regard to their matrix formation, contraction and contact behaviour and can therefore be used for experimental studies within a short culture period of 7 days. Cell aggregates in monolayer culture and in cultures in collagen gels had not differentiated at this stage.

Key words: Morphology, High density cells cultures, Collagen gels

## Introduction

The low density of mesangial cells and their diffuse distribution in the glomeruli of the kidney complicate the demonstration and evaluation of their function and behaviour. The in vitro technique and its possibility to grow pure cell types is especially suited to overcome these problems. Therefore, in vitro techniques were also developed for the investigation of mesangial cells. These included primary cultures of different species as well as permanent, i.e. transformed, cell types (Foidart et al., 1979, 1980; Striker et al., 1980; Kreisburg and Karnovsky, 1983; Striker and Striker, 1985; Lovett and Sterzel, 1986; Davies, 1994). Analysing the literature it can even be claimed that a great part of our knowledge about the function of the mesangial cells under normal and pathological conditions is based on investigations using mesangial cell cultures.

Mesangial cells have so far been grown predominantly in monolayer cultures at low density. After a certain period, however, aggregates are formed in which multilayered cellular units develop in some areas (Sterzel et al., 1986; Abrass et al., 1995; Glass et al., 1996; Kitamura et al., 1996). All cell types are at risk of being dedifferentiated during cultivation. Variations of the medium, addition of factors and hormones have been used to guarantee a sufficient degree of differentiation of mesangial cells. Two techniques that are able to stimulate differentiation in other cell types have obviously not yet been discussed: (1) cultivation at high cell density (high density, micromass or organoid cultures); and (2) cultivation in gels of matrix components (Zimmermann, 1987; Karst and Merker, 1988; Lilja et al., 1988; Zimmermann et al., 1992; Shakibaei, 1998). The functions of mesangial cells that are necessary for a high degree of differentiation are based on the capability to contract, to form matrix, to synthesize numerous mediators and factors, to respond to these (autocrine) or other substances (para- and endocrine), to phagocytose blood components and to establish a mechanical and electrical connection between the juxtaglomerular apparatus and the glomerular capillaries via specific cell contacts (Michael et al., 1980; Kreisberg et al., 1985; Schlondorff, 1987, 1996; Hawkins et al., 1989; Mené et al., 1989; Kriz et al., 1990; Kashgarian and Sterzel, 1992; Veis, 1993). Electron microscopic investigations are suitable to demonstrate and evaluate contraction by the demonstration of actin filaments, the synthesis of matrix and other compounds by the occurrence of the appropriate cell organelles (smooth and rough endoplasmic recticulum, Golgi apparatus, specific secretion granules) and the occurrence of matrix structures between the cells, phagocytosis by the demonstration of endocytotic processes and phagolysosomes, and electrical and mechanical communications by the occurrence of gap junctions and adhesion

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contacts.

The aim of this study was to investigate the behaviour of mesangial cells in primary cultures (rat) and permanent cultures (SV40 Mes 13) at high density in organoid culture and in collagen gels in order to characterize their degree of differentiation and thus their suitability for in vitro experiments.

## Materials and methods

We used primary cultures of mesangial cells from the rat after the 6th to 12th passage as well as SV40 Mes13-cells (glomerular mesangial cells from SV40 transgenic mice, ATCC CRL-1927).

The cells were grown, as usual, at low density ($10^4$/ml medium) in monolayer culture in MEM/F12 (1/1) medium until confluency had been reached. Organoid cultures were prepared as follows: the cells were detached with trypsin, rinsed with medium and sedimented by centrifugation. Eight $\mu$l of the sediment was placed onto a piece of membrane filter (cellulose nitrate, pore size 0.2 $\mu$m, type SM 13307, Sartorius, Göttingen, Germany) resting on a stainless grid and grown at the medium/air interphase. Medium consisted of DMEM/F12 (1:1) supplemented with 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 $\mu$g/ml) and ascorbic acid (50 $\mu$g/ml). It was changed every second day (Zimmermann et al., 1992). Another part of the sediment was suspended in a collagen solution (collagen type I, 2.4 mg cells/ml medium) at a density of $10^6$/ml medium and placed onto multiwell plates (0.2 ml per well) (Medium and all medium components were purchased from Biochrom, Berlin). After polymerisation of collagen gel at 37 °C for 12 h an additional 0.2 ml of medium was given on top of the gel. Medium changes were done every 2nd day.

After 1, 3 or 7 days, fixation was performed either in Karnovsky's solution (3% paraformaldehyde plus 3% glutaraldehyde in 0.1M phosphate buffer, pH 7.2) or in 2% glutaraldehyde in phosphate buffer with the addition of 0.5% tannic acid. Rinsing with buffer solution was followed by post-fixation in 1% OsO4 (in phosphate buffer) and dehydration in the ascending alcohol series, embedding in Epon, sectioning with a Leitz Ultracut E, contrasting in uranyl acetate/lead citrate, and examination with an electron microscope (Zeiss EM 10).

## Results

The primary mesangial cells as well as the permanent SV40 Mes13 cells formed multilayered cellular units immediately after seeding at high density at the medium/air interphase. The development of both cell types over the next few days was identical. Separate description and discussion are therefore not necessary.

After a 1-day culture period the cellular aggregates consisted of densely-packed cells without large intercellular spaces (not shown). Some cells showed indications of cell lesions with swelling of cell organelles, occasional fragmentation of the cell membrane and loosening of the karyoplasm (necroses). We additionally observed apoptoses with densification of the karyo- and cytoplasm, blebbing at the surface and incipient constriction, i.e. fragmentation, of these cells. Necroses and apoptotic cells occurred singly and were diffusely distributed or in groups. Debris was found between the cells.

After a 3-day culture period the necrotic cells and cell debris had largely disappeared. Only apoptotic cells and their fragments inside and outside vital cells of the vicinity were still present. The cells that were still densely packed, i.e. without intercellular spaces and matrix, began to form cavities of the rough endoplasmic reticulum and larger Golgi apparatuses. Many cells exhibited dense inclusions (0.3 to 0.5 $\mu$m) that often contained membranous structures. These might be lysosomal residual bodies, the orgin of which (autophagic vacuoles or phagolysosomes) is not quite clear.

After a 7-day culture period, the cellular aggregates exhibited distinct intercellular spaces (ICS) that were filled with matrix structures (Fig. 1). The shape of the cells was oval to elongated. Long, in most cases terminal processes of varying width and length occurred. Apart from these situations the cell membrane proceeded in a regular manner over long distances. The dilated, matrix-filled ICS of up to 3 $\mu$m in width did not surround the cells continuously, but were interrupted by long direct cell-cell contacts. Four types of matrix structures could be distinguished: (1) A mixture of granular structures (15 to 25 nm) and very irregularly running filaments (5 to 10 nm); the individual filaments could be recognized only with difficulty because of their dense packing, irregular course and electron density after tannic acid fixation. This material closely resembled the Lamina densa of the basal lamina (Figs. 1a, 2a). (2) Fibrils showing cross-striations and a thickness of 30 to 40 nm. They were arranged singly or in bundles (up to 10 fibrils per bundle) without any preference in direction (Fig. 2b). They obviously represented collagen fibrils of types I or III. (3) Filaments (10 to 15 nm), arranged predominantly in bundles and coated with fine-granular material after tannic acid fixation (Fig. 2c). (4) A mixture of these three matrix types (Fig. 2d).

Regions with direct contact of the cell membranes exhibited three types of contact: (1) the so-called unspecific contact with an ICS of 17 to 20 nm width (Fig. 3c, f); (2) other areas showed gap junctions with a typical 5-layered structure, i.e. with a direct contact of the outer lamella of the cell membrane (Fig. 3a,e); and (3) another type of contact occurred rather frequently where an ICS remained. It was clearly narrower (10 nm) (Fig. 3b-e). In the vicinity of these contacts micro-filaments (actin) appeared intracellularly that radiated into the membrane. They might represent adhesion belts.

The appearance of the cytoplasm varied. Cells were found that showed few cell organelles. Often they had numerous morphologically very heterogeneous inclusions (0.3 to 0.7 $\mu$m) that resembled lysosomes or

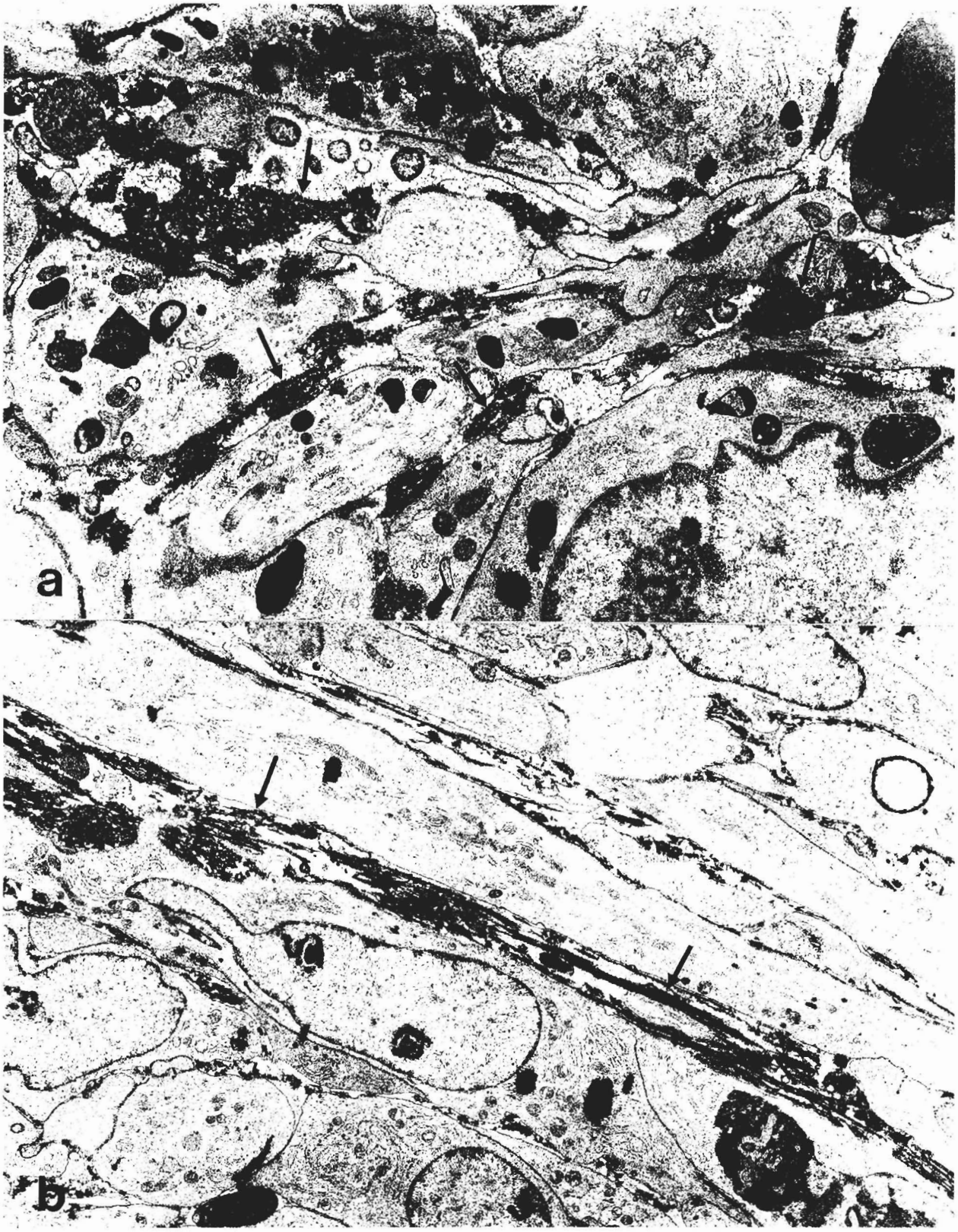


**Fig. 1. a.** Mesangial cells of a normal rat kidney (primary culture) grown at high density (high density or organoid culture), 8th passage. Culture period: 7 days. Cells with numerous electron-dense inclusions. Tannic acid-positive, i.e. electron-dense matrix (arrow) between the cells. *: apoptotic body. x 10,000. **b.** SV40 Mes 13-cells grown in high density (organoid) culture after 7 days. Mainly elongated cells with matrix in the intercellular space (arrow). x 6,500


**Fig. 2.** Sections from intercellular spaces with matrix components after 7 days in vitro. **a.** Lamina densa-like material. Primary culture, 8th passage. x 61,000 **b.** Collagenous fibrils in longitudinal, oblique and cross sections. SV40 Mes13-cells. x 61,000. **c.** Filament bundle in the vicinity of a cell process. SV40 Mes13-cells. x 45,000. **d.** Mixed, i.e. granular-filamentous material. Primary culture, 8th passage. x 52,000

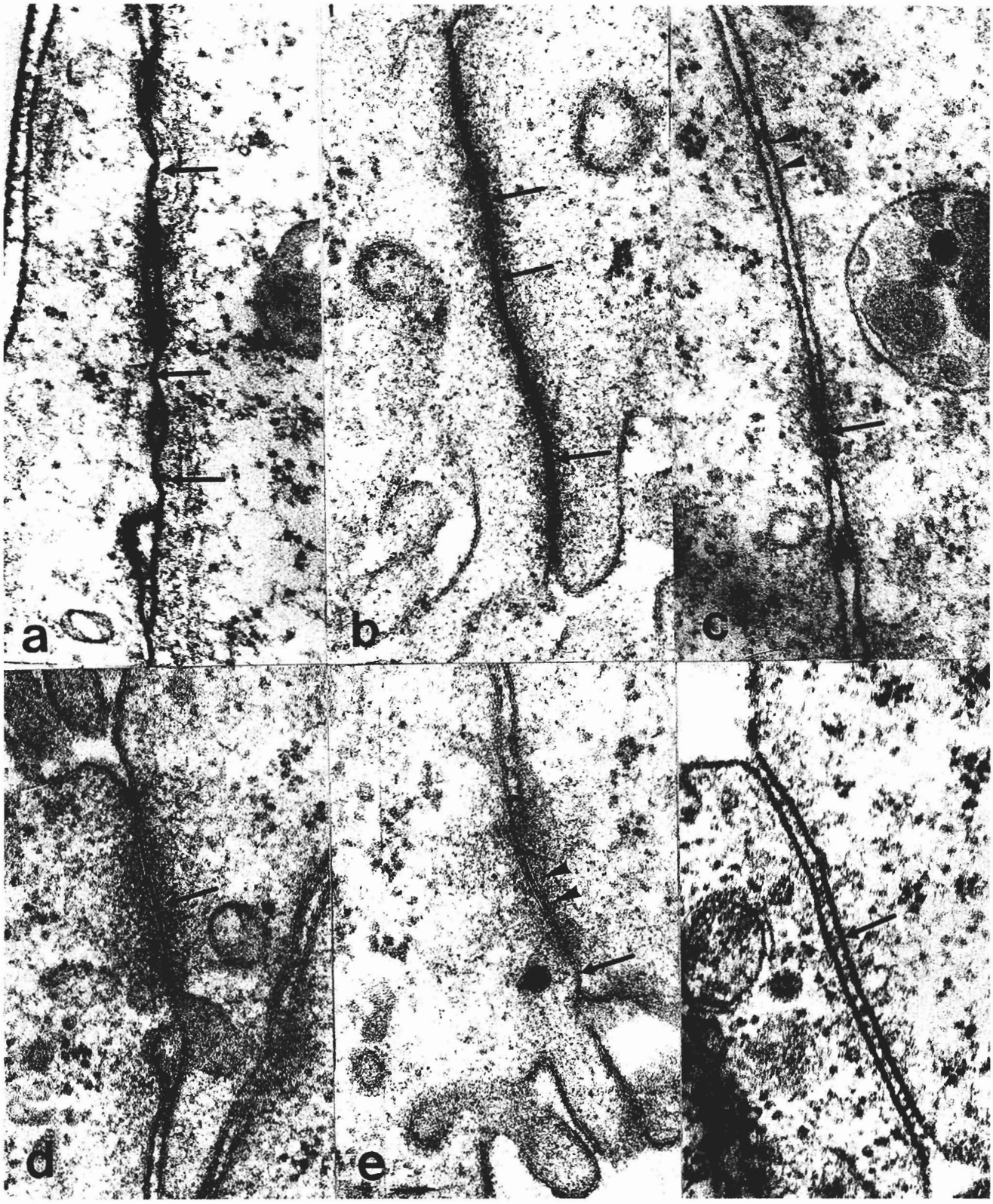


**Fig. 3.** Contacts between mesangial cells grown at high density (high density or organoid culture) for 7 days. x 70,000. **a.** Primary culture, 8th passage. Segments with gap junctions (arrow) alternating with segments with unspecific contacts. **b.** SV40 Mes13-cells. Narrower contacts (arrow), probably representing adhesion belts. **c.** SV40 Mes13-cells. Normal, i.e. unspecific contacts (arrowhead) and a segment with narrower intercellular space (arrow), probably adhesion belt. **d.** Primary culture, 10th passage. Strongly narrowed cell contacts (arrow), probably representing adhesion belts. **e.** SV40 Mes13-cells with gap junction (arrow) and adhesion belt (arrowhead). **f.** Primary culture, 8th passage. Normal, i.e. unspecific cell contact (arrow).

lysosomal residual bodies. They contained packed membrane structures, granular material or a mixture (Fig. 4c) of both structures. Other organelle-poor cells or processes contained bundles of parallel running microfilaments (actin) (Fig. 4b,c). The cells showing organelles contained oval to stretched mitochondria of the crista type (Fig. 6b). Long, parallel running or shorter cavities of the rough endoplasmic reticulum could often be demonstrated (Figs. 4a, 6b); next to these cavities varying amounts of free ribosomes were always present. A striking finding was the large Golgi apparatuses (Fig. 5a,b) that consisted of 2 to 4 membrane staples of varying length of 3 to 6 flat, membrane-bordered cavities surrounded by a halo of many vesicles. In most cases they were empty and only a few contained fine-granular material of medium electron density. The empty vesicles were either smooth or coated. Despite this abundance of vesicles in the vicinity of a Golgi apparatus the remaining cytoplasm was rather poor in vesicles, even underneath the cell membrane. Endocytotic processes were rarely visible. The shape of the nucleus adapted itself to the shape of the cell, i.e. it was round to elongated. The karyoplasm was less densely packed showing a narrow rim of chromatin. One to two nucleoli of medium size were clearly discernible (Figs. 1a,b, 2a). Mitoses could occasionally be demonstrated even after a 7-day culture period (Fig. 6a).

The cultures enclosed in collagen gel showed a different, less favourable picture. Using this technique, difficult adaptation processes could be observed. As late as after 7 days in vitro numerous cell necroses and occasionally also apoptoses occurred. The few surviving cells remained single or formed aggregates without showing any indication of differentiation, such as matrix formation, contact specialisation and occurrence of contractile structures (Fig. 6c).

Cell aggregates developed in monolayer cultures. Differentiation on day 7 could so far not be shown.

## Discussion

The aim of this study was to investigate the morphological behaviour of mesangial cells in high-density organoid cultures or embedded in collagen gels in order to ascertain the degree of their differentiation. Electron microscopic parameters for the evaluation of the degree of differentiation were the presence of the structures necessary for the synthesis of the mesangial matrix (endoplasmic reticulum, Golgi apparatus) and the occurrence of matrix in the extracellular space, the demonstration of contractile filaments (actin) and gap junctions as well as other types of contact required for the attachment and communication of the cells among one another. Indications of a normal mesangial function and a certain degree of differentiation could undoubtedly be demonstrated in numerous cells. However, the heterogeneity in this respect is considerable. Cells exist that have developed one of these indications well, others show different structures or lack them completely, and finally there are several features of differentiation that occur jointly in one cell. The reasons for the differences in cell differentiation are not known. They might be due to a heterogenicity of the cells with regard to the differentiation capacity, to local influences, such as the supply with nutrients, distance to the surface or properties determined in vivo and persisting in vitro.

The occurrence of a well-developed rough endoplasmic reticulum and large Golgi apparatuses corresponds to synthesis and secretion of proteins. As matrix structures also occur in the extracellular space, some of these proteins certainly are matrix components (for Lit. see Alberts et al., 1990). The appearance of the matrix varies considerably. There are structures that resemble the Lamina densa of the basal lamina (Merker, 1994). Other areas exhibit typical collagen fibrils with cross-striation, probably collagen types I and III. In addition, there are filaments (12 nm) without cross-striation. Their classification is slightly more difficult. Some of these filaments could be pre-stages of the formation of elastic structures. The absence of elastic fibres in the mesangial matrix speaks against this notion. Recently, a separate filament type has been demonstrated and characterized: fibrillin. Also fibronectin can aggregate and occur in the form of filaments. Furthermore, each collagen fibril has to pass a filamentous stage during its formation. Collagen types V and VI occur predominantly in the form of filaments (Schwarz, 1957; Carmichael and Fullmer, 1966; Fullmer et al., 1974; Goldfischer et al., 1983; Böck and Stockinger, 1984; Broek et al., 1985; Trueb et al., 1987; Gibson et al., 1989; Hagiwara et al., 1993; Hewett et al., 1993; Spiro and Crowley, 1993; Saulnier et al., 1996). The chemical composition of filaments cannot, therefore, be defined by descriptive morphological means alone. It must, however, be pointed out that mesangial cells at high density are able to form larger amounts of matrix that resemble the matrix in vivo and in vitro (Courtoy et al., 1980; Linder et al., 1980; Bruneval et al., 1985; Haralson et al., 1987; Abrass et al., 1988, 1995; Mohan et al., 1990; Saito et al., 1993; Truong et al., 1994; Groggel and Hughes, 1995; Takeuchi et al., 1995; Trachtman et al., 1995; Gomez-Garre et al., 1996; Hadad et al., 1996; Wahab et al., 1996).

The behaviour of mesangial cells embedded in collagen type I is different. The developing cell aggregates do not yet exhibit any intercellular spaces with matrix structures in the same culture period of 7 days. It must, however, be kept in mind that the fate of these cultures, i.e. after a culture period of 1 week and more, is not known. In collagen gel many cells obviously perish so that the surviving cells require more time for proliferation and differentiation. Hence, a time factor might play a role in this respect. Whether this behaviour that differs from that of cells grown at high density is due to the gel technique or to the employed unphysiological matrix components (collagen type I) cannot be decided by our experimental set-up (Saito et

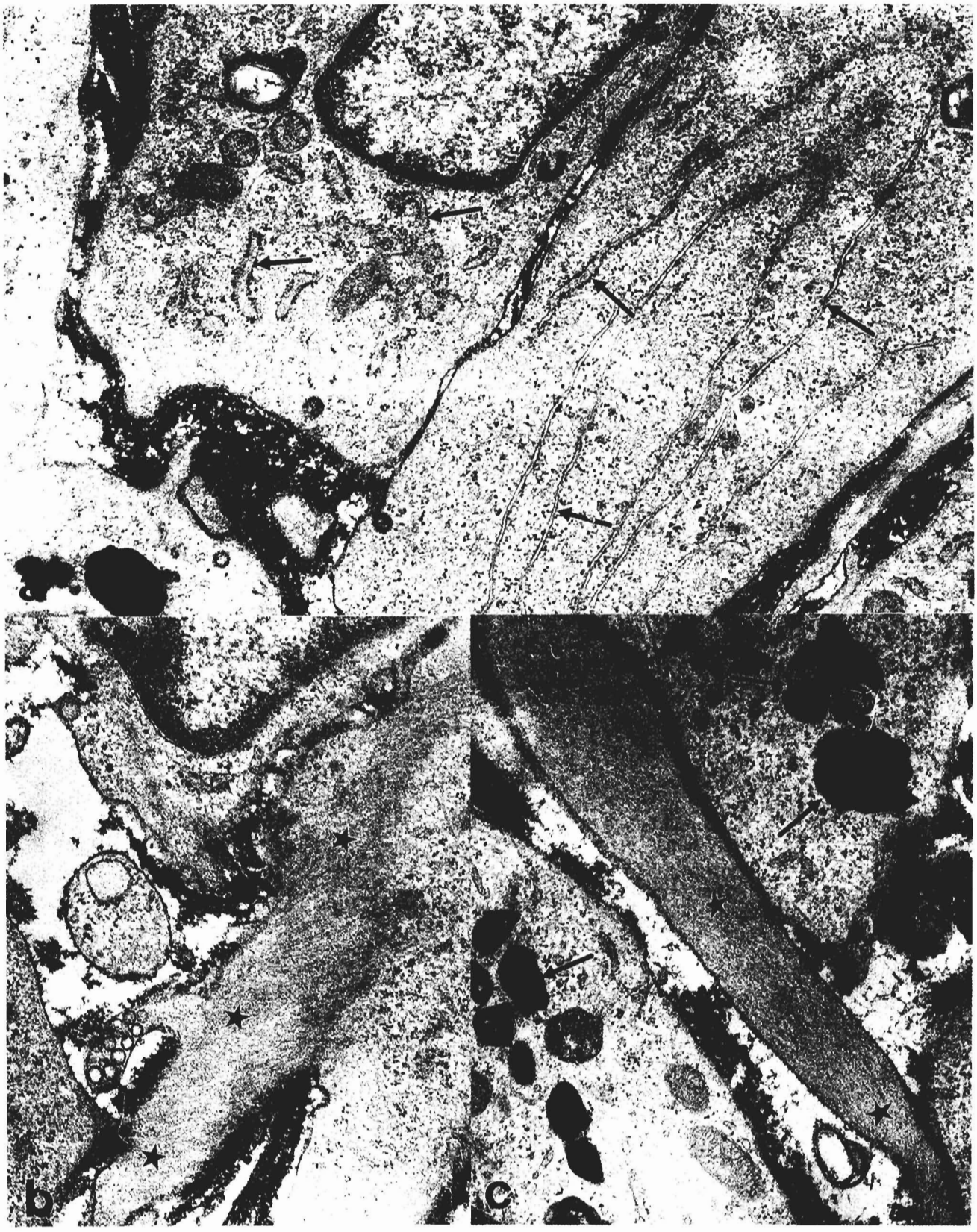


**Fig. 4.** Mesangial cells grown for 7 days at high density. **a.** SV40 Mes13-cells with numerous, partly elongated cavities of the rough endoplasmic reticulum (arrow), intercellular space with matrix (*). x 30,000. **b.** Primary culture, 8th passage. Cell with actin bundle in oblique section (*). x 27,000. **c.** SV40 Mes13-cell. Process (*) filled with actin filaments. Neighbouring cells with lysosome-like inclusions (arrow). x 27,000

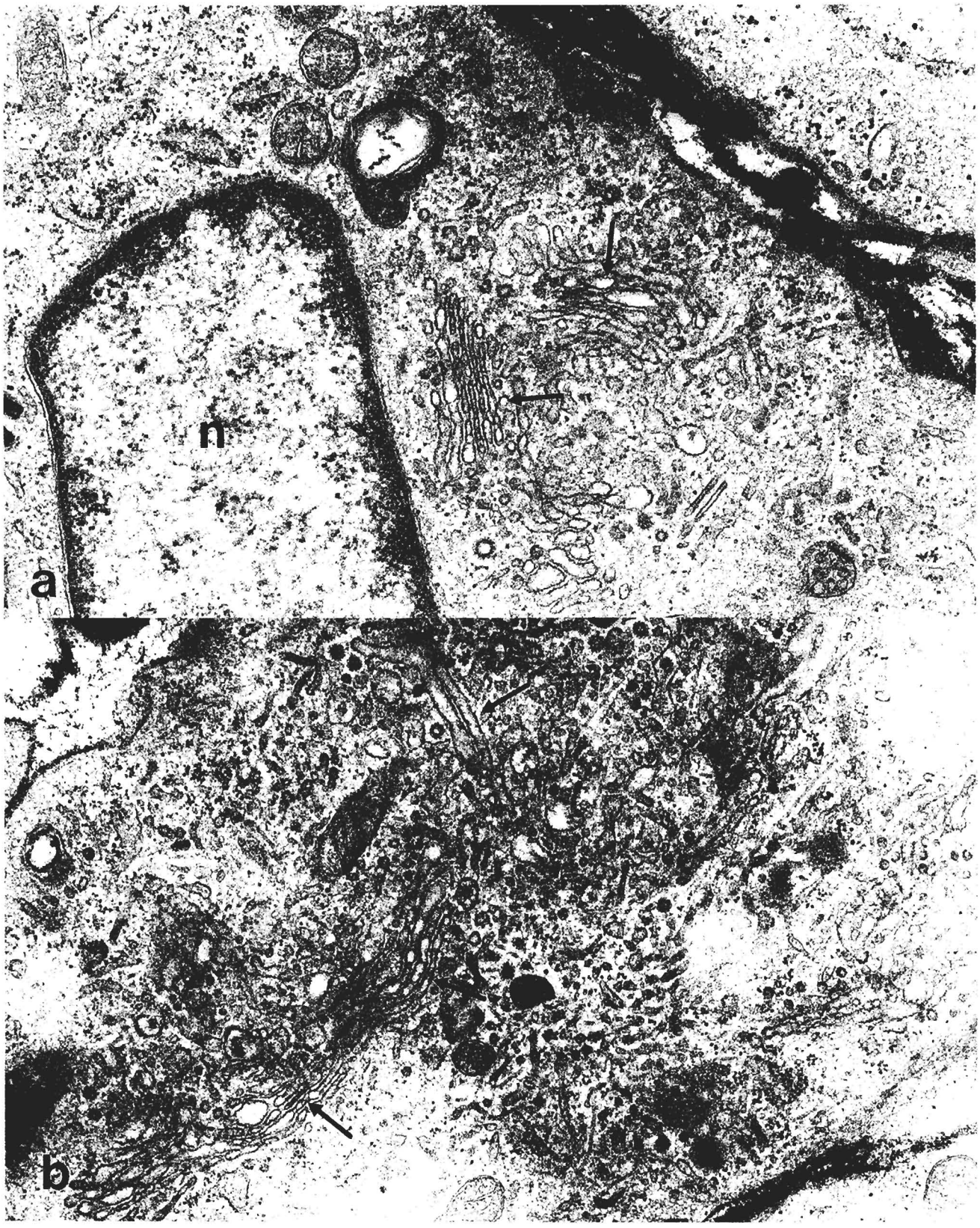


**Fig. 5.** Culture period 7 days. Golgi apparatus of mesangial cells from primary culture, 8th passage **(a)** and from cultures of SV40 Mes13-cells **(b)**. Staples of flat cavities (arrow) and large amounts of vesicles (also coated vesicles) and a few granules in the vicinity. x 32,000

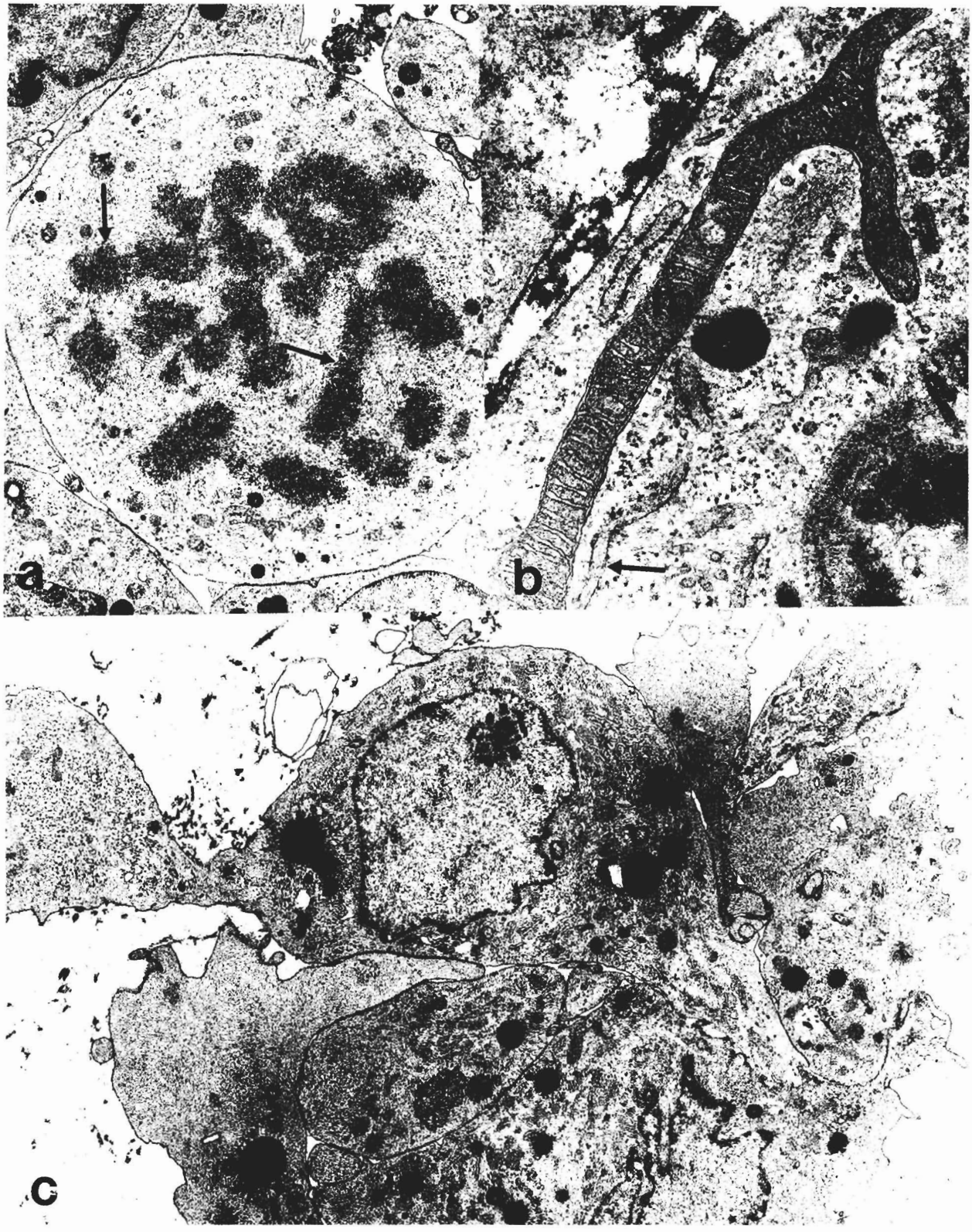


**Fig. 6. a.** Mitotic figure with chromosomal sections (arrow) in a high density culture, 8th passage after 7 days. x 10,000. **b.** Elongated mitochondrium (m) from a high density (organoid) culture of SV40 Mes13-cells after 7 days, in the vicinity cavities of the rough endoplasmic reticulum (arrow). x 25,000. **c.** Cell aggregate of SV40 Mes13-cells grown for 7 days in collagen gel. Dense packing of cells, but without matrix formation. x 6,000

al., 1993; Sakatsume et al., 1995; Miralem et al., 1996). The other features of differentiation (specific contacts) are not very pronounced either in the cells grown in collagen gels.

Another function of mesangial cells is based on their capability for contraction (Kreisberg et al., 1985; Iehara et al., 1996; Schlondorff, 1996; Stockand and Sansom, 1996). Indeed, some cells occur in high density cultures that contain densely-packed actin filaments as a morphological indication of this function. A rough calculation shows that the ratio of the number of cells with actin bundles to the number of cells without actin bundles is approximately 1:10.

Finally, we must mention the specific cell contacts between mesangial cells in high density culture. In addition to unspecific contacts, gap junctions and connexin 43 as well as adhesion contacts (adhesion belts) have been described to occur in vivo and in vitro (Pricam et al., 1974; Iijima et al., 1991; Barajas et al., 1994; Goligorsky et al., 1997; Hillis et al., 1997). Adhesion belts are necessary for the mechanical connection of cells and are of importance for joint contractions of the mesangial cells in one glomerulus and for the acceptance of traction and tension forces during changes in blood pressure and the blood supply of the glomerulus. The communication among mesangial cells via gap junctions has an important function in the transmission of information from the juxtaglomerular artery to the glomerular capillaries and vice versa. Only if such possibilities of communication exist can mesangial cells respond jointly in a glomerus and interlink the juxtaglomerular artery with the periphery of the glomerulus in a functionally sensible manner (Taugner et al., 1978).

In summary, it can be stated that mesangial cells in high density cultures (primary as well as permanent cultures) reach a degree of differentiation which can be compared with that reached in vivo. Using this in vitro technique differentiation of mesangial cells is advanced compared to cells in gel or monolayer culture. In the latter culture type they form multilayered cell aggregates only days later. An interesting finding is that in organoid culture this differentiation also occurs when the cells originate from primary cultures after several passages, i.e. the mesangium-specific reactions and differentiations gradually decrease (Ouardani et al., 1996). Hence, this in vitro technique is suitable for "revitalisation" of older primary cultures. Finally, we want to mention the disadvantages of high density cultures. Apart from the greater technical expenditure (use of steel bridges and filters, additional centrifugation step) it is not possible to establish the stage of communication by the use of the stain-injection technique. Finally, it must be pointed out that it is not possible to demonstrate the synthesis of factors, prostaglandins, etc., by electron microscopy alone although it would represent another valuable indication of the behaviour and differentiation of cells in vitro.

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