Biochemical and Ultrastructural Characterization of Fluid Transporting LLC-PK1 Microspheres

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Abstract. The established renal epithelial cell line LLC-PK1 (proximal tubule) started to form multicellular spheroids within 24 h when grown in agar overlay culture. The spheroids, average diameter 100 to 350 μm, were free-floating with a butterfly-like structure due to the formation of several hollow microspheres. The microspheres were lined with polarized epithelial cells that had an abundance of microvilli protruding into the external medium and a well developed vacuolar apparatus, including coated pits, endocytotic vacuoles, and lysosomes. The microspheres were sealed between lumen and the surrounding medium by tight junctions and fluctuated in size due to fluid being transported in an apical-to-basal direction. Vasoressin was found to stimulate this transport, whereas the addition of ouabain or HgCl₂ inhibited both spheroid growth and fluctuation in size with time. Biochemical assays of brush-border and lysosomal marker enzymes demonstrated an increase in enzyme activity during spheroid formation and growth. The most dramatic changes were observed for dipeptidyl peptidase IV (two- to threefold after 1 d and 53.5-fold after 15 d), reflecting the cellular polarization and brush-border formation during spheroid formation. When the typical lysosomal enzymes were compared, the activity of peptide bond splitting enzymes increased earlier than others. In conclusion, LLC-PK1 spheroids capable of forming microspheres represent an in vitro manifestation of specialized epithelial properties maintained in cell culture, thus providing a tool for studying renal physiologic mechanisms at a cellular level.

It is well known that functional differentiation is maintained only to a limited degree when cells of normal and malignant epithelial tissues are grown in vitro as monolayer cultures (1). Cultured cells often lose their typical features, including structural and biochemical characteristics, despite many efforts to achieve cell culture conditions which as close as possible resemble the in vivo situation (2-6).

Many cell lines, when grown in suspension culture or in agar overlay culture, will aggregate and grow to form multicellular structures called spheroids (7,8). Multicellular spheroids, grown in vitro as three-dimensional cellular aggregates, represent a biological system of intermediate complexity between the corresponding tissue in vivo and simple monolayer cultures. These structures have also been found to maintain several biochemical and morphologic features similar to the tissue of origin, and represent a model system for studying threedimensional growth and differentiation of both normal and malignant cells in vitro (1,9-11).

Multicellular spheroids are most often obtained from established cell lines. The established renal epithelial cell line LLC-PK1, which has characteristics reminiscent of those of proximal tubule cells (12), has been studied extensively in monolayer cultures. This particular cell line has been reported to possess several unusual morphologic features, including the growth of three-dimensional domelike structures (12), free-floating aggregates, or cysts (13), in which the cells organize to form a polarized epithelium where the apical surface faces the surrounding medium and the basal surface borders a fluid-filled lumen (14). These structures have also been reported to undergo polarity reversal when planted within collagen gels (14), an observation also made in other epithelial cyst culture systems in response to contact with collagen at the apical surface (15).

Three-dimensional growth of proximal tubular cells in vitro would be an excellent model for physiologic studies at the molecular level to get a better understanding of cellular transport processes in epithelial cells in general, and particularly in the renal proximal tubule. In addition, such a model would also be of great importance when studying the morphogenesis of a polarized epithelium.

The structural information available on these structures has been limited mostly to light- and scanning electron microscopic studies. Also, biochemical data are very sparse. The purpose of this study, therefore, was to examine the cell biology of LLC-PK1 cells grown as multicellular spheroids, both with respect to ultrastructure and the presence of enzymes of key importance for reabsorption and transport in the proximal tubule.

The present study shows that LLC-PK1 cells easily form multicellular spheroids in agar overlay culture. The individual growth of different spheroids is studied, and their ultrastructural features are compared with the appearance of the epithelial cells of the proximal tubule in vivo. In addition, several...
physiologic and biochemical features have been determined, including water transport, protein uptake, hormone sensitivity, as well as variations in contents of DNA and cellular protein, and activity of brush-border and lysosomal marker enzymes during spheroid growth.

**Materials and Methods**

**Cell Culture**

LLC-PK1 cells (CRL 1392; American Type Culture Collection, Rockville, MD) were originally obtained from Flow Laboratories (Irvine, United Kingdom) at passage number 202. The cells described in this study were grown from passage numbers 217 to 238 in Eagle’s-Dulbecco’s modified medium (Life Technologies, Grand Island, NY) with 10% newborn calf serum and four times the prescribed concentration of nonessential amino acids: 2% L-glutamine, penicillin (100 IU/ml), and streptomycin (100 mg/ml). Cells were routinely maintained at 37°C at 100% relative humidity in an atmosphere of 5% CO₂/95% air. Cells were grown in 24-well culture flasks (Nunc, Roskilde, Denmark). The dishes and culture of 5% CO₂/95% air. Cells were grown as spheroids in 24-well L-glutamine, penicillin (Irvine, United Kingdom) at passage number 202. The cells described during spheroid growth.

**Spheroid Growth**

The spheroid structure was examined and measured daily over a 2-wk period from day 3 during growth, using a Nikon inverted microscope with a calibrated reticule in the eyepiece. Single multicellular spheroids were also transferred to 15-mm diameter Gel-wells (Costar) to study spheroid growth by time-lapse cinematography. Gel-wells were placed in 83-mm² culture flasks, covered by medium, gassed with air containing 5% CO₂, and kept at 37°C. Micrographs, using Super8 Kodachrome 40 Type A (Kodak-Pathé, France), were then taken at 2- to 3-min intervals by a Leica Special Camera, using a Leitz Aristophot inverted microscope (Wetzlar, Germany).

To study the effect of spheroid age on the activity of typical brush-border and lysosomal marker enzymes, cells were seeded to 10 cells per culture bottle. The number of cells seeded for spheroid growth was 1 to 1.5 (Agar Noble, DIFCO Laboratories, Detroit, MI). The number of cells plated co-cultured with medium, gassed with air containing 5% CO₂, and kept at 37°C. Micrographs, using Super8 Kodachrome 40 Type A (Kodak-Pathé, France), were then taken at 2- to 3-min intervals by a Leica Special Camera, using a Leitz Aristophot inverted microscope (Wetzlar, Germany).

**Treatment with Vasopressin, Ouabain, and HgCl₂**

**Water Transport**

Spheroids (1.1 × 10⁶ cells seeded; 10 d old) were exposed to a final concentration of 10⁻⁸ M vasopressin (deamino-Cys⁵, D-Arg⁸, acetate salt; Sigma Chemical Co., St. Louis, MO), 1 mM HgCl₂, or 1 μM ouabain (G-strophanthin; Sigma).

**Chemical and Enzyme Assays**

Cells and cellular debris in the tissue culture media were sedimented at 2000 rpm for 3 min, resuspended in 8 ml of 0.9% NaCl, and resedimented at the same speed. The pellet thus obtained was resuspended in 7.2 ml of 0.9% NaCl and homogenized in a Turrax homogenizer (Janke & Kunkel, Staufen, Germany) for 20 s at 24,000 rpm. Triton X-100 (Sigma Chemical Co.) was then added to give a final concentration of 1% before homogenization was continued for another 10 s. The homogenate thus obtained was diluted appropriately and used for the assay of intracellular enzyme activity.

Five brush-border enzymes (alkaline phosphatase, γ-glutamyltransferase, leucyl aminopeptidase, neutral α-glucosidase [maltase], dipeptidyl peptidase IV) and eight lysosomal enzymes (dipeptidyl peptidase I [cathepsin C], dipeptidyl peptidase II, tripeptidyl peptidase, pH 4.5, cathepsin B, acid phosphatase, N-acetyl-β-glucosaminidase, acid β-galactosidase, and acid β-d-glucuronidase) were assayed, in addition to DNA and protein. The fluorometric assays for alkaline phosphatase, γ-glutamyltransferase, leucyl aminopeptidase, neutral α-glucosidase, acid phosphatase, N-acetyl-β-glucosaminidase, acid β-galactosidase, acid β-d-glucuronidase, and DNA are described previously in detail (16,17). Dipeptidyl peptidase I was assayed on Gly-Arg-β-naphthylamide at pH 6.0 (10 mM cyclohexamidine) in the presence of 10 mM mercaptoethanol-HCl (18); dipeptidyl peptidase II was assayed on Lys-Ala-7-(4-methyl)coumarylamide at pH 5.5 (0.1 M sodium phosphate) (19); dipeptidyl peptidase IV was assayed on Gly-Pro-β-naphthylamide at pH 7.8 (0.1 M sodium phosphate) (20); tripeptidyl peptidase was assayed on Gly-Pro-Met-β-naphthylamide at pH 4.5 (0.1 M sodium acetate) (21); and cathepsin B was assayed on Z-Ag-Arg-2-(4-methoxy) naphthylamide at pH 6.5 (0.5 M sodium phosphate) (22). Protein was determined by the protein-dye binding method described by Bradford (23). Enzyme activities are expressed as μmol/min per mg protein, where 1 U of enzyme activity is the amount hydrolyzing 1 μmol of substrate per minute under the conditions of assays. DNA is expressed as μg DNA/mg protein. The endo- and exopeptidase substrates were the products of Enzyme System Products (Livermore, CA) or BACHEM Feinchemikalien AG (Bubendorf, Switzerland); substrates for all other marker enzymes were the product of Sigma Chemical Co.

**Electron Microscopy**

Spheroids were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, containing 3.7% sucrose, washed in 0.1 M sodium cacodylate buffer, and embedded in 4% agar. The spheroids were post-fixed in 1% OsO₄ in the same buffer, stained en bloc in 0.5% uranyl acetate in sodium maleate buffer, pH 5.2, dehydrated in graded alcohols, and finally embedded in Epon 812 (TAAB Laboratories, Reading, England). Thin sections, obtained with an LKB Ultratome III (LKB, Bromma, Sweden), were stained with uranyl acetate for 10 min and lead citrate for 2 min, and studied in a Phillips CM 100 electron microscope.

**Immunocytochemistry**

Expression of Na⁺,K⁺-ATPase and aminopeptidase N (CD 13) was studied using immunocytochemical techniques on frozen ultramicrotome sections. The spheroids were fixed with 2 or 4% formaldehyde in 0.1 M sodium cacodylate at 4°C for 30 min, washed and infiltrated in 2.3 M sucrose containing 2% formaldehyde for 30 min at 4°C, and frozen in liquid nitrogen. For electron microscopy, 70- to 90-nm sections were obtained at approximately –100°C from a Reichert Ultratrit S cryoultramicrotome and collected on 300 mesh Ni grids as described before (24). For immunolabeling, the sections were incubated with primary antibodies, either polyclonal rabbit anti-Na⁺,K⁺-ATPase (L21.8, kindly provided by J. Vuust Møller, Department of Biophysics, University of Aarhus, Denmark), mouse monoclonal anti-Na⁺,K⁺-ATPase antibody (H6, kindly provided by M. J. Caplan, Department of Cell Biology, Yale University Medical School, New Haven, CT), or polyclonal rabbit anti-pig aminopeptidase N (kindly provided by O. Norén and H. Sjöström, Department of Medical
Biochemistry and Genetics, The Panum Institute, Copenhagen, Denmark), either at room temperature for 1 h or overnight at 4°C after preincubation in phosphate-buffered saline containing 0.05 M glycine and 0.1% nonfat dry milk. For electron microscopy, the sections were subsequently incubated with protein A-gold (BioCell Research Laboratories, Cardiff, United Kingdom) or with colloidal gold coupled directly to the secondary antibody, goat anti-mouse, or goat anti-rabbit IgG (BioCell). The sections were finally contrasted with methylcellulose containing 0.3% uranyl acetate (25,26).

Ferritin Uptake

Spheroids were exposed to 1 mg/ml cationized ferritin (Sigma) for 30 min and then prepared for conventional electron microscopy as described above.

Results

Light Microscopy

Light microscopy demonstrated that the LLC-PK1 cells formed multiple, free-floating spheroids each consisting of a central dense compact mass of cells from which several microspheres protruded (Figure 1). Although the size of the spheroids varied only slightly with age from day 3 to day 14 with a mean diameter of approximately 0.2 mm, the diameter of the microspheres (cysts) varied extensively in size from approximately 20 to 40 μm to approximately 150 μm (Figure 1). This variation could be due in part to age, but also to the functional state of the individual microspheroid (see below).

Fluid Transport Rates

Vectorial water transport is a significant process of renal proximal tubule cells. Figure 2 demonstrates a variation in volume of microspheres with time. As seen in Figure 3 (top panel), untreated microspheres typically showed a steady increase in diameter with time, from approximately 50 to 90 μm over a period of 2 to 3 h followed by a sudden collapse. After a lag period of 2 to 4 h, they then gradually increased in size.

Figure 1. Light micrograph of LLC-PK1 spheroids. The spheroids are free-floating and have a butterfly-like structure due to the formation of several hollow microspheres in each spheroid. Magnification, ×75.

Figure 2. Time-lapse cinematography of LLC-PK1 spheroid. Note the fluctuation in size of the two microspheres (A and B) observed as a slow increase in sphere diameter, followed by a rapid decrease, which again is followed by a slow increase. The recorded time points, from 0 to 29 h, top left to bottom right, are indicated on each micrograph. Magnification, ×150.
ouabain and HgCl₂ totally stopped the pumping process (Figure 3), and a few hours after addition of ouabain the whole spheroid structure had totally disintegrated.

**Activity of Brush-Border and Lysosomal Enzymes**

To study the effects of cell-cell contacts, spheroid formation, and spheroid growth on cellular enzyme activities, five brush-border and eight lysosomal marker enzymes were assayed, in addition to DNA and protein, during a time course study for 22 d after seeding of cells. The data obtained for DNA and the brush-border enzymes are shown in Figure 4, and the data on the lysosomal enzymes are shown in Figure 5.

Although DNA appeared to remain at the same level (range, 6.9 to 11 μg DNA/mg protein), all enzymes examined showed an increase in activity during spheroid formation and growth. When the activity profiles for the five brush-border enzymes were compared, two major patterns were observed. Alkaline phosphatase and dipeptidyl peptidase IV showed a two- to threefold increase in activity 1 d after seeding. The activity of alkaline phosphatase increased further up to day 7 with an overall maximum at day 18 (13.6-fold increase), whereas dipeptidyl peptidase IV showed an increase up to day 12 with an overall maximum at day 15 (53.5-fold increase). A different

Again reaching a new peak. A representative microspheroid diameter increased from 58 to 83 μm over 150 min (first peak, Figure 3, top panel), corresponding to a volume increase of 1.3 pl · min⁻¹. Assuming an average spheroid diameter of 70.5 μm, the average volume absorption is approximately 0.15 nl · s⁻¹ · cm⁻².

Addition of 10⁻⁸ M of vasopressin significantly increased the rate of fluid accumulation within the microspheres, decreasing the time intervals between peaks to approximately 1 to 2 h and increasing the volume absorption by a factor 3 to 4 (Figure 3). In addition, the variation in diameter between collapsed and extended states was increased. Addition of

**Figure 3.** Variation of microsphere diameter (μm) as a function of time. The first peak in the top panel corresponds to an increase in diameter of the microspheroid from approximately 58 to 83 μm over a period of 150 min. Comparison of the two top panels demonstrates that vasopressin not only stimulated the rate of pumping, but also increased the variation of microsphere diameter between collapsed and fully extended states. Ouabain and HgCl₂ very rapidly stopped the pumping (bottom panels). A few hours after the addition of ouabain, the spheroid structure started to disintegrate.

**Figure 4.** Effect of spheroid age on brush-border enzyme activity and DNA concentration. Aliquots of appropriately diluted spheroid homogenates were incubated for 30 min at 37°C exactly as described in Materials and Methods. Enzyme activities (±SD, n = 10) are expressed as mU/mg protein, where 1 U of enzyme activity is the amount hydrolyzing 1 μmol of substrate per minute under the conditions of assays. DNA is expressed as mg DNA/mg protein.
Renal Epithelial Microspheres

The activity of the eight lysosomal enzymes assayed was also found to increase with increasing time after seeding. However, the shape of the activity profiles for the peptide bond splitting enzymes was different from the others. The activity profiles for dipeptidyl peptidase I and cathepsin B increased more rapidly than the others, showing a two- to threefold increase 1 d after seeding and reaching a plateau after 2 to 4 d, with an overall maximum after 7 to 9 d for dipeptidyl peptidase I (5.0-fold increase) and after 4 d for cathepsin B (10.5-fold increase). The other lysosomal enzymes showed a two- to threefold increase after 2 d, with tripeptidyl peptidase, pH 4.5, and dipeptidyl peptidase II showing maximum values after 7 to 9 d (4.5- to 5.0-fold increase); however, N-acetyl-β-glucosaminidase (11.1-fold increase), acid β-galactosidase (6.0-fold increase), and acid β-D-glucuronidase (9.0-fold increase) did not reach maximum values until 15 to 18 d. Maximum activity for acid phosphatase (8.0-fold increase) was seen after 9 to 12 d.

**Ultrastructure of Microspheroid Cells**

**General Ultrastructure.** The ultrastructural evaluation of the microspheres revealed several striking features. Each microspheroid was formed by a wall consisting of a single layer of epithelial cells that varied considerably in height between microspheres, but only to a smaller extent within each microspheroid. As seen in Figure 6, the cells were highly polarized, but the polarization was reversed compared with renal proximal tubules. Thus, the cells had a well developed brush border consisting of microvilli, with a length of approximately 1 μm protruding into the medium (Figure 7A) and containing longitudinally oriented actin-like filaments with a diameter of approximately 6 to 7 nm (Figure 8A). The basal part of the cells, which faced the lumen of the microspheres, was smooth, although small, finger-like projections occasionally protruded into the central cavity. The population of cells surrounding the microspheres appeared relatively homogeneous, although in some microspheres a single or a few cells appeared flattened, apparently related to the functional state (see above). Occasionally, a cilium was seen protruding into the external medium (Figure 9A), but cilia were also seen, as in Figure 9B, in the intercellular space and oriented toward the lumen of the microspheroid.

**Plasma Membrane.** The plasma membrane in the apical part of the cells formed typical, five-layered tight junctions (Figure 10, A through C) with a length of approximately 50 to 100 nm, which is slightly longer than in normal proximal tubule cells. Below the tight junctions, numerous desmosomes were seen. The lateral intercellular space was quite different from that of genuine mammalian proximal tubules. Thus, the cells had a much simplified form and did not interdigitate like normal mammalian proximal tubule cells. The lateral plasma membranes were only in places closely apposed and, in addition, they formed multiple finger-like or plate-like protrusions into the intercellular space (Figures 7A and 8B). These protrusions contained microfilaments that were not as well organized as in the apical microvilli (Figure 8). At the basolateral aspect, the cells often formed junction-like complexes, and some cells sent long, slender processes under the neighboring cells. The basal plasma membrane was otherwise inconspicuous. The cells were not supported by a thick basement membrane, but occasionally a flocculent and electron-lucent material was seen along the basal plasma membrane (Figure 10D).

**Endocytic Apparatus.** The apical plasma membrane showed numerous coated endocytic invaginations (coated pits)
Figure 6. Electron micrograph of microspheroid protruding from a compact cluster of cells. The cells surrounding the microsphere are cuboidal to columnar with the nucleus placed centrally. The apical pole of the cells is oriented away from the lumen and has a typical brush border (arrows). Inside the lumen, two cells are seen apparently free-floating. Magnification, ×1800.

(Figures 7B and 11). The apical cytoplasm contained both coated and noncoated vesicles, and deeper in the cytoplasm there were numerous small and large lysosomes and multivesicular bodies (Figure 11).

Other Cell Organelles. The cells had a very well developed Golgi apparatus located close to the nucleus (Figure 11). The nuclei generally were rounded, characterized by much euchromatin and relatively little heterochromatin, and exhibited one to two nucleoli in each cross section (Figure 6). Small cisternae of granular endoplasmic reticulum and polyribosomes were scattered throughout the cytoplasm (Figures 7 and 11). The mitochondria were found in all parts of the cells and were usually not elongated as in genuine mammalian proximal tubule cells. Furthermore, they showed no close association to the basolateral plasma membrane, as seen in normal proximal tubule cells. Microfilaments were particularly frequent in the apical and basal part of the cells, but microtubules were abundant throughout the cytoplasm. Occasional lipid-droplets occurred in the basal part of the cells. Sometimes cells showed a less distinct polarization, as illustrated by the cell in Figure 9B, which contains a basal body with its cilium protruding into the intercellular space toward the basal part of the cells and below the tight junction.

Ferritin Uptake

Cells exposed to cationized ferritin demonstrated an extensive uptake of the protein into small and large endosomes and lysosome-like bodies (Figure 12). In addition, these experiments demonstrated that the tight junctions excluded passage of ferritin into the intercellular space and into the lumen of the microspheroid (Figure 12A). Only in one microspheroid was a small cluster of ferritin seen in the lumen, possibly as a result of transcellular transport, but more likely due to diffusion of ferritin through the intercellular space in connection with one of the intermittent disruptions of the tight junctions subsequent to the continuous transepithelial water transport described above.

Immunocytochemistry

Immunocytochemical labeling for Na⁺,K⁺-ATPase was present exclusively on the lateral plasma membrane (Figure 13,
Figure 7. (A) Apical part of LLC-PK1 cells in the wall of a microspheroid. The cells are connected apically with junctional complexes (arrows) and have a well developed brush border (BB). In the intercellular space, small finger-like processes are seen protruding from the lateral aspect of the cells (arrowheads). Several vacuoles (V) are seen in the cytoplasm. RER, granular endoplasmic reticulum; N, nucleus. (B) Apical plasma membrane demonstrating coated pits (arrows). Magnification: ×14,900 in A; ×64,000 in B.

A through C). No labeling was seen on the apical (Figure 13A) or basal plasma membrane or in any intracellular locations. The labeling on the lateral plasma membrane was not confined to the cell body, but was also present on the membrane of the finger-like projections (Figure 13, A through C). No labeling was observed in controls, which included incubations with
Figure 8. (A) Apical microvilli demonstrating longitudinally oriented bundles of actin filaments (arrowheads) with a diameter of 6 to 7 nm. Inset shows two cross-sectioned microvilli with cross-sectioned microfilaments. (B) Lateral intercellular space demonstrating folds and finger-like plasma membrane projections containing microfilaments (arrowheads), which, however, are not oriented strictly parallel as in the apical microvilli. Magnification: \( \times 147,000 \) in A and inset; \( \times 120,000 \) in B.

nonspecific poly- and monoclonal antibodies, as well as incubation without primary antibodies.

Labeling for aminopeptidase N was seen almost exclusively on the apical microvilli and only occasionally in intracellular vacuoles. However, the microvillar expression of this enzyme was intensively increased in 1-wk-old microspheres compared with 2-d-old microspheres (Figure 14).

Discussion
The data presented here demonstrate that LLC-PK1 cells form spheroid structures consisting of numerous microspheres. The microspheres resemble the formation of domes, frequently seen in confluent monolayer cultures of epithelial cells (12,27). It is evident that when the cells come together in the spheroid structure, they organize to form a polarized epithelium where the apical surface faces the surrounding medium. During this process, they regain their physiologic ability to perform vectorial water transport, thereby creating a fluid-filled lumen at the basal side. The intermittent changes in size of the microspheroids are explained by a continuous water transport that increases the size of the lumen to the extent that the luminal hydrostatic pressure exceeds the mechanical strength of the cellular junctions. The sudden leakage of fluid from the lumen back to the culture medium is thus a way to regulate the hydrostatic pressure inside the lumen while maintaining a physiologic transepithelial water transport.

It is well known that LLC-PK1 cells are sensitive to hormones affecting renal tubular functions (14), including receptors for vasopressin (28,29). The three- to fourfold increased volume absorption and the decreased time intervals between peak diameters of microspheres after addition of vasopressin demonstrate that water transport in LLC-PK1 cells is stimulated by vasopressin, and illustrate the suitability of the microsphere model for the study of hormone action on renal epithelial cells. Because vasopressin receptors are usually confined to the basolateral membranes, the hormone probably gets access...
to the receptors during collapse of the microspheres. That an active fluid transport is required for the microsphere formation is also supported by the inhibiting effect of HgCl₂ on water transport and the even stronger effect of ouabain, which creates spheroid collapse.

The fluid transport rate, calculated from Figures 2 and 3, reveals that the microspheroids transport approximately $0.15 \cdot 10^{-9} \, \text{l} \cdot \text{s}^{-1} \cdot \text{cm}^{-2}$ of fluid, and after stimulation with vasopressin $0.45 \cdot 10^{-9} \, \text{l} \cdot \text{s}^{-1} \cdot \text{cm}^{-2}$. These values are approximately one order of magnitude lower than the figures calculated for proximal tubules in *Necturus maculosus* ($1.64 \cdot 10^{-9} \, \text{l} \cdot \text{s}^{-1} \cdot \text{cm}^{-2}$) and *Ambystoma tigrinum* ($1.58 \cdot 10^{-9} \, \text{l} \cdot \text{s}^{-1} \cdot \text{cm}^{-2}$) (30–32), which in turn are much lower than the transport rate determined for mammalian proximal tubules (33). These differences correlate with the different architectures of apical and especially basolateral membranes, as discussed below.

The assays of several typical brush-border and lysosomal marker enzymes show that these enzymes are present in the LLC-PK1 spheroid cells with a specific activity (activity/mg of protein) similar to values found in the rat kidney cortex homogenates (34). Although the DNA (per mg protein) remained almost constant during the period of observation, an increase in activity of all enzymes assayed was observed over time. Why some of the brush-border enzymes increased more (and earlier) than others is not known. The difference in time of expression for some of the enzymes may be caused by sequential events, such as cellular polarization and microvilli formation, which take place early during spheroid and microsphere formation. That the localization of different brush-border enzymes is dependent on the differentiation status of proximal tubule cells in culture (35) is also supported by the present study, demonstrating an increased microvillar expression of aminopeptidase N with time (Figure 14). Microvilli formation itself may be the cause of the fast and most dramatic increase in activity seen for dipeptidyl peptidase IV, because this enzyme is predominantly associated with the membrane of the microvilli and apical invaginations (36), also due to its relatively high concentration per microvillus (37) and the fact that the expression of this enzyme in the kidney is controlled at the transcriptional level only and not regulated through posttranscriptional mechanisms (38). Different routing in the trafficking from the Golgi to the membrane domain known to take place for different enzymes in epithelial cells (11) may also explain some of the differences observed. The drop in activity with time for most enzymes confirms the age-related changes reported previously for brush-border enzymes in the rat kidney cortex (39).

At present there is no explanation why different activity profiles were observed between peptide bond splitting and other lysosomal enzymes. However, it is well known from in
Figure 10. (A through C) Apical regions and D basal regions of microspheroid cells. (A) Two apposed cells have formed a short, five-layered tight junction (arrow). (B) These two cells form a five-layered tight junction that is much longer than the one shown in A (arrows). Bundles of actin filaments are seen in the microvilli core (arrowhead). (C) A five-layered tight junction is seen apically (arrows) and below a desmosome (arrowhead). (D) Basal part of LLC-PK1 cells demonstrating a thin and poorly developed basement membrane (arrows). Magnification: \( \times 92,000 \) in A through C; \( \times 40,000 \) in D.

*vivo* studies that the renal cortex contains very heterogeneous populations of lysosomes (34,40,41), which may also be the case for the microspheres.

At the ultrastructural level, the cells of the microspheroids were in many respects similar to proximal tubule cells despite the fact that the polarization appeared reversed (for a recent
review of proximal tubular ultrastructure, see reference 42) and also despite the functional aspects discussed above, that LLC-PK1 cells express receptors for vasopressin (28,29), which is not a property of the proximal tubule in vivo. However, although most cells had a well developed brush border, the microvilli were generally only about half the length of microvilli in mammalian proximal tubules. The formation of apical tight junctions was obviously a necessary prerequisite for the observed pumping of water. The tightness of these junctions was also demonstrated by the fact that ferritin was very seldom seen inside the microspheroids. The occasional presence of ferritin in the microspheroid lumina may be explained by temporary ruptures during the water pumping processes. The basolateral membrane did not form ridges and grooves as in the mammalian proximal tubule, but instead the basolateral membrane surface was increased by the formation

Figure 11. Electron micrograph illustrating the variety of cell organelles generally found in the microspheroid cells. A very extensive Golgi complex (G) is scattered throughout the cytoplasm. There is an abundance of lysosomes (L), mitochondria (M), polyribosomes (arrowheads), and granular endoplasmic reticulum (RER). In the basal part of the cell, there is an elaborate filamentous network (F). A coated pit is seen at the apical plasma membrane (arrow). Microvilli protrude from the apical plasma membrane (MV), and finger-like projections (P) are present at the basal plasma membrane. N, nucleus. Magnification, ×21,000.
of small lateral folds similar to the folds seen in, e.g., *Ambystoma tigrinum* (32). Also, similar to amphibian proximal tubule cells but in contrast to genuine mammalian proximal tubule cells, the mitochondria are more rounded and not closely associated to the basolateral membranes. The organization of the endocytic apparatus, including coated pits, endocytic vesicles, and lysosomes, was similar to the normal mammalian proximal tubule cell, although less developed. However, these cells still show a high capacity for endocytic uptake of protein as visualized by the extensive vacuolar accumulation of cationized ferritin. The cells exhibited a well developed Golgi apparatus and abundant microtubules and microfilaments.

It is evident from this study that spheroid structures capable of forming cysts, or microspheres, represent an *in vitro* manifestation of specialized epithelial properties maintained in cell culture, in which an active fluid transport is a prerequisite for the formation of numerous microspheres. From the data presented here, it is clear that microspheres may be an excellent candidate for an *in vitro* model to study the regulation of
Figure 13. Immunocytochemical localization of Na\(^+\),K\(^+\)-ATPase, using monoclonal anti-\(\alpha\) subunit antibody and subsequent incubation with 10 nm goat anti-mouse colloidal gold. (A) Apical part showing no labeling of the apical plasma membrane, including the microvilli (MV). Labeling is seen on the lateral plasma membrane (arrows). Virtually no background labeling is present in the cytoplasm. (B and C) Lateral intercellular space limited by labeled lateral plasma membranes, including their finger-like processes (arrows). Magnification: \(\times54,000\) in A; \(\times63,000\) in B; and \(\times64,000\) in C.
Figure 14. Immunocytochemical localization of aminopeptidase N, using rabbit polyclonal anti-pig aminopeptidase N antibody and subsequent incubation with 10 nm goat anti-rabbit colloidal gold. (A) Cell from 2-d-old microspheres expressing the enzyme to a limited extent in the brush border. (B) Cell from 1-wk-old microsphere expressing severalfold higher amounts of the enzyme in the microvilli. Magnification: $\times 63,000$ in A and B.
epithelial cell polarity (43) and transport processes in epithelial cells in which the microspheroid structures may arise in vitro through a process similar to cell differentiation (27). The spheroid formation may also serve as a model to study the morphogenesis of a polarized epithelium (44–46).

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