Human Amniotic Membrane as a Suitable Matrix for Growth of Mouse Urothelial Cells in Comparison With Human Peritoneal and Omentum Membranes

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Introduction: For tissue engineering of the urinary tract system, cell culture requires to be established in vitro and an appropriate matrix acting as cell carrier should be developed. The aim of the present study was to assess the proliferation quality of mouse urothelial cells on 3 natural matrixes of human amniotic membrane (AM), peritoneum, and omentum, and to compare them with collagen matrix.

Materials and Methods: Mouse urothelial cells were isolated by collagenase IV, and the urothelial cells (10^5 cells per milliliter) were cultured on the AM, peritoneum, omentum, and collagen. The pattern of growth and asymmetric unit membrane formation were analyzed by histologic examination and immunocytochemistry on the detached urothelium with pancytokeratin and uroplakin III, respectively. Electron micrographs were taken and cell layers, organelles, desmosomes, and junctions were studied.

Results: Immunocytochemistry of cultivated cells confirmed the urothelial cells phenotype. Up to 4 cell layers were obtained on the AM and 1 to 2 layers on the peritoneum. Distribution of the urothelial cells on the omentum was not favorable, which was due to its large pores. Cell proliferation started later on the AM (7th day) compared to collagen (3rd day). Also, apoptosis started later on the AM (after 14 days) compared to collagen (7 days).

Conclusion: These results showed that the AM can act as a cell carrier for culture of the urothelial cells, and its exceptional properties such as having various growth factors, availability, and cost-effectiveness make it a unique biological matrix for urothelial culture.

Keywords: urothelial cells, amniotic membrane, peritoneum, omentum, mouse, bladder, animal model

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INTRODUCTION

Surgical repair of several congenital and acquired abnormalities of the urinary tract, such as strictures, traumatic defects, and hypospadias, requires additional tissue for reconstruction.[1] Traditionally, the intestinal segment has been used for bladder augmentation or substitution of bladders with abnormalities. However, its benefits are offset by relatively common complications that are potentially serious, including mucus production, calculus formation, metabolic disturbances, and infection.[1,2] Numerous synthetic materials have been investigated for reconstruction of functionally deficient bladder.[1,2] Furthermore, culture of animal urothelial cells provides useful material, especially when human tissue is not available.[1-8]

In the recent years, collagen has been shown to promote in vitro

growth and stratification of the urothelial cell and has been used to repair urinary tract defects.[1-5] Few investigations attempted to use the omentum in urothelial engineering,[6,7] and the peritoneum has also been used for seeding of the urothelial cells by some researchers.[8] Reports on using the amniotic membrane (AM) as a matrix for culturing of the oral, ophthalmic, and skin cells have been encouraging, and the specific characteristic of the AM such as having various growth factors, availability, and cost-effectiveness can make it a valuable biological matrix.[8-12] However, to our knowledge, there is no documented study comparing these biological matrixes, to date. The aim of the present study was to evaluate of the quantity and quality of mouse urothelial cells cultured on the human omentum, peritoneum, and AM matrixes, and to compare them with the previously known material that is collagen.

MATERIALS AND METHODS

Mouse Urothelial Cell Cultures

All animal procedures were carried out in accordance with the Institute of Laboratory Animal Research guide for the care and use of laboratory animals.[13] Congenic NMRI mouse was used and the study was approved by the ethics committees of Royan Institute and the Urology and Nephrology Research Center. Mouse bladder was obtained from the dissected animals. The bladder tissue was frequently rinsed with phosphate-buffered saline (PBS). Then, the apical two-thirds of the mouse bladder was harvested, cut in half, and immersed, and the epithelial layer was peeled. In the first step, the epithelial cells were isolated from fibroblast cells by a collagenase solution (collagenase type IV, 443 U/mL [Gibco-Invitrogen, Grand Island, NY, USA]) in a humidified, 37°C, 95% air/5% carbon dioxide environment within 1 hour. In the next step, the epithelial layer was digested by the use of trypsin/ethylenediamine tetraacetic acid to obtain split epithelial cells in a humidified environment, the same as in the previous process, within 20 minutes.

The obtained cell suspension was plated on a tissue culture plate of bovine dermal collagen (Vitrogen, Cohesion, Palo Alto, CA, USA), serving as control, and on acellularized the AM, peritoneum, and omentum (test groups) at a concentration of approximately 10^5 cells per milliliter with Dulbecco>s Modified Eagle Medium:Nutrient Mix F-12 medium (Gibco-Invitrogen, Grand Island, NY, USA), containing N_2, 15% fetal bovine serum, hydrocortisone, L-glutamin, and β-mercaptoethanol, and incubated at 37°C and 5% CO_2.

Acellular Membranes

The isolated human membranes including amniotic, peritoneum, and omentum membranes were obtained from the patients who underwent operation in Shaheed Labbafinejad Medical Center and Atiyeh Hospital. They were thoroughly washed and placed in phosphate-buffered saline containing penicillin/streptomycin (Gibco-Invitrogen, Grand Island, NY, USA), and ofloxacin (O-8757, Sigma-Aldrich, St Louis, MO, USA). Then, to obtain an acellularized membrane, the epithelial cells were isolated from the membranes by trypsinization

Histology and Immunocytochemistry

For immunostaining, the urothelial cells were washed twice with PBS and fixed with 4% paraformaldehyde for 24 hours at 4ºC. These cells were permeabilized and blocked in PBS containing 0.2% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) and 10% goat serum for 10 minutes and 30 minutes, respectively. Thereafter, the urothelial cells were incubated in primary antibody diluted in 0.5% bovine serum albumin (BSA) at 37ºC for 1 hour. The antibodies used in this study were pancytokeratin (MAB1636, Chemicon, Hofheim, Germany) and uroplakin III (M-17, Santa Cruz Biotechnology, Santa Cruz, CA, USA). At the end of the incubation time, the cells were washed twice with PBS + 0.05% tween 20 and incubated with the fluorescence isothiocyanate-conjugated antimouse IgG (F9006, Sigma-Aldrich, St Louis, MO, USA) diluted in 0.5% BSA for 60 minutes at 37ºC. After washing twice with PBS + 0.05% tween 20, the specimens were examined under fluorescence microscope (BX51, Olympus, Japan) (Figure 1).

Also, for histologic examination, the membranes containing urothelial cells were removed from the medium, washed twice with PBS, and fixed with 4% paraformaldehyde for 24 hours at 4ºC. The samples were then dehydrated in 70% ethanol for 24 hours and embedded in paraffin in a rotary tissue processor. The paraffin-embedded specimens were

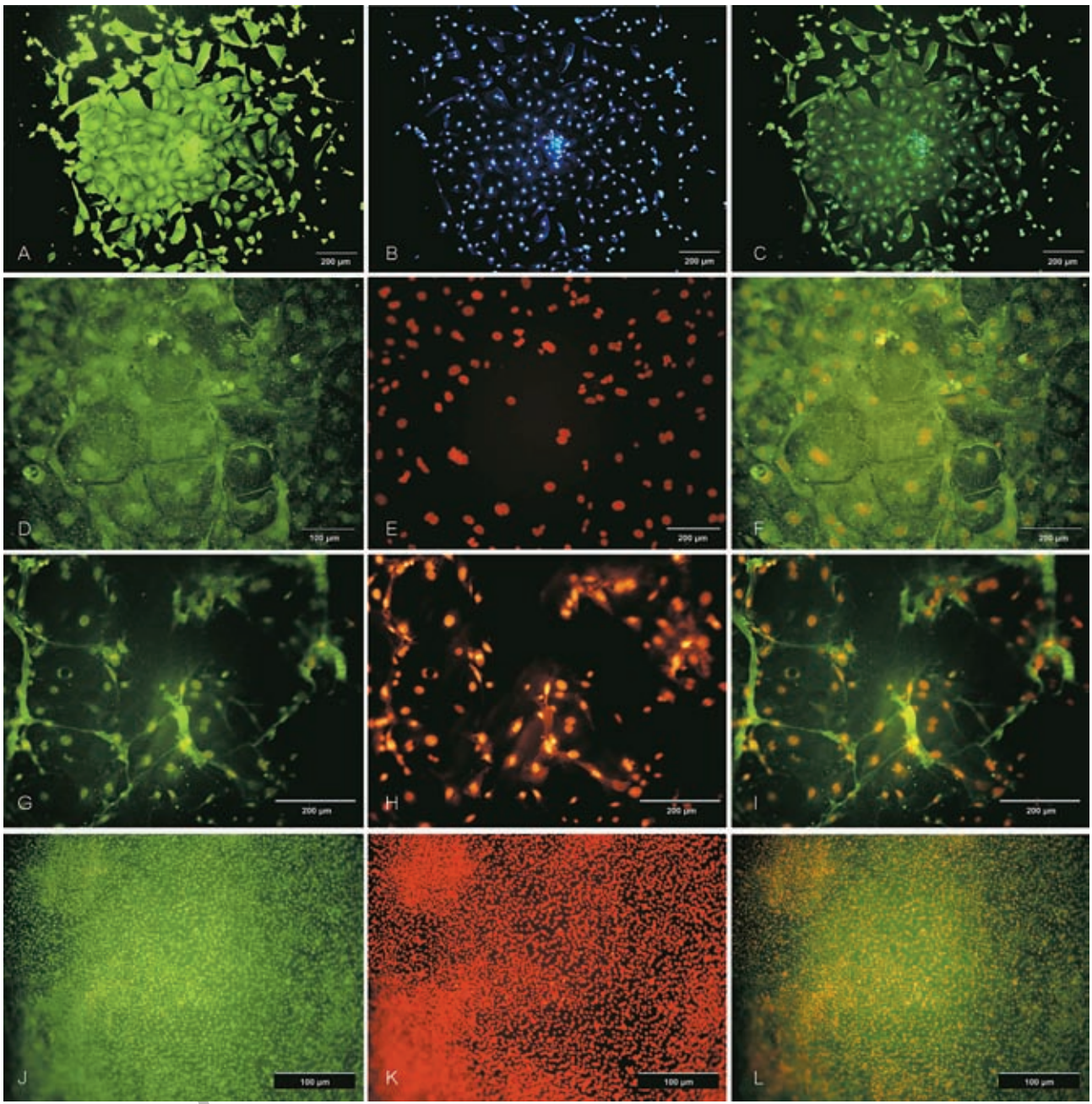

Figure 1. Morphological examination and immunocytological staining with pancytokeratin and uroplakin III of the asymmetric unit membrane antibodies were used to confirm urothelial cells phenotype. **A, B,** and **C.** Pancytokeratin on collagen. **D, E,** and **F.** Uroplakin III on collagen with triton. **G, H,** and **I.** Uroplakin III on collagen without triton. **J, K,** and **L.** Uroplakin III on the amniotic membrane.

sectioned perpendicular to the culture surface at 5-µm thickness. The sections were attached to poly l-lysine-coated glass slides and placed at 60ºC in an oven for 12 hours, dewaxed in xylene, and stained with hematoxylin-eosin.

Transmission Electron Microscopy

For transmission electron microscopy (TEM), the specimens were fixed using 2.5% glutaraldehyde in 0.1 M PBS (pH, 7.4) for 2 hours. After washing with PBS, they were postfixed with 1% osmium tetroxide for 1.5 hour, again washed in PBS, dehydrated in an acetone series, and then embedded in epoxy resin. After resin polymerization, sections of approximately 50 nm were cut and double-stained with uranyl acetate and lead citrate. Electron micrographs were taken using a Zeiss EM 900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany).

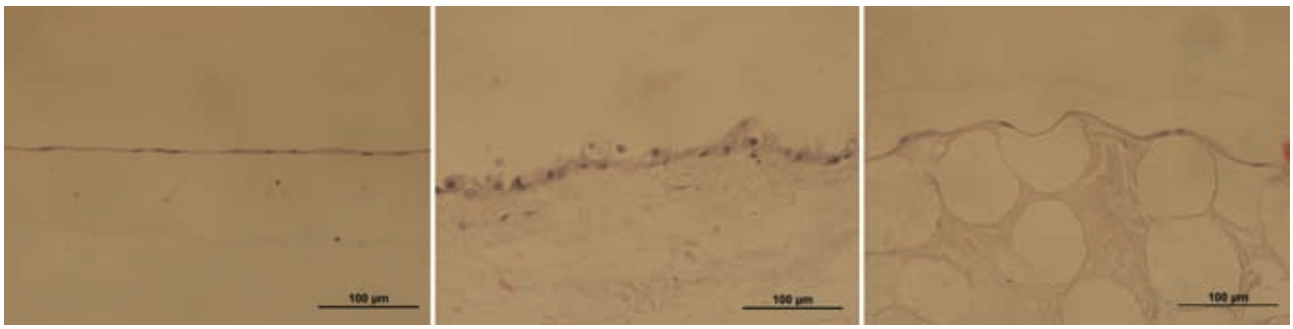

Figure 2. Light microscopy of the urothelial cells on the matrixes (hematoxylin-eosin). **Left.** Urothelial cells constituted a dense layer on the amniotic membrane. **Middle.** A dense layer of cells was not formed on the peritoneum. **Right.** Cells on the omentum could not form a distinct layer.

RESULTS

Primary culture of mouse urothelial cells on human AM, peritoneum, omentum, and collagen were routinely expanded and passaged (Figure 2). There was no incidence of cessation of growth or abnormal changes in morphology. The cells were in culture for about 3 weeks and we followed the growth pattern of cells daily. Morphological examination and immunocytological staining with pancytokeratin (a urothelial cell marker) and uroplakin III of the asymmetric unit membrane (indicating cell differentiation) antibodies were used to confirm urothelial cells phenotype (Figure 1). Histologic examination revealed a multilayered polarized urothelial sheet structure on the AM and 2-layer cells sheet on the peritoneum. But, studies on the omentum confirmed that it has an inappropriate 3-dimentional structure for urothelial cell engineering. Hence, we decided to stop more investigation on the omentum followed by TEM (Figures 2 and 3A).

Light and Immunocytochemistry Staining Analysis

Basically the urothelial cells on the peritoneum had a small round and cuboidal structure. After 3 days, the urothelium appeared well found on the collagen matrix with big flat cells and large nuclei, but on the AM, the cells had a small round shape with unremarkable nucleus, and we found small cuboidal cells with round nucleus on the peritoneum (Figure 1).

Collagen. Growth, colonization, and cell morphology during the first 3 days was superior on the collagen matrix to the AM and the peritoneum matrixes. Cell migration was remarkable on the collagen during the first week and it was more significant than the on the 2 other matrixes (Figure 3B). On the 7th day, the urothelial cells started the process of vacuolization and apoptosis on the collagen matrix, and within the second week, apoptosis was dominant (Figure 4).

Amniotic Membrane. In the first week, cell growth on he AM was not significant, but on the following

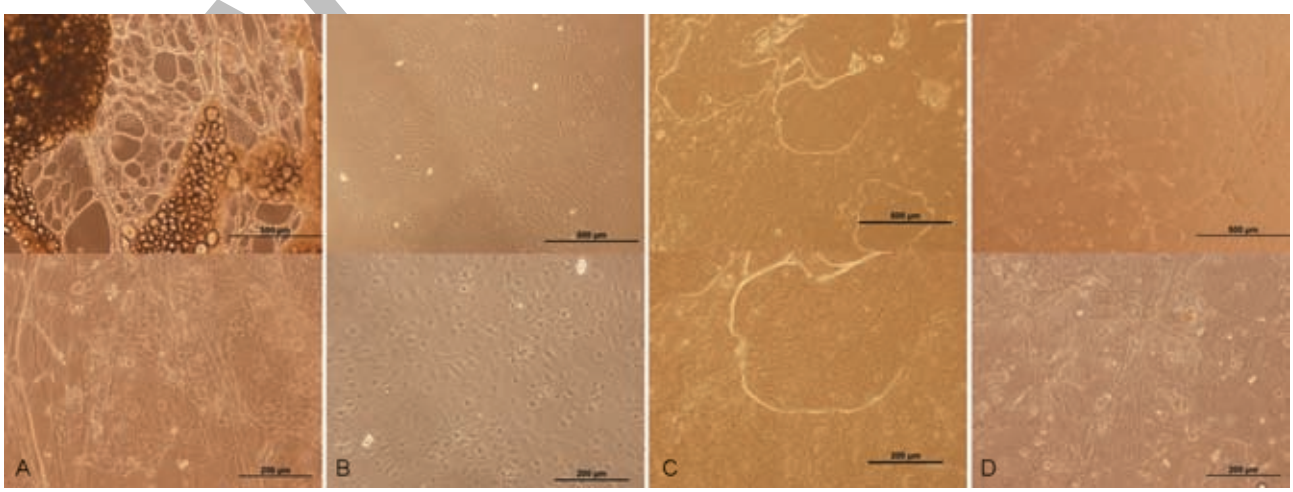

Figure 3. Primary culture of mouse urothelial cells. **A,** omentum. **B,** collagen. **C,** amniotic membrane. **D,** peritoneum.

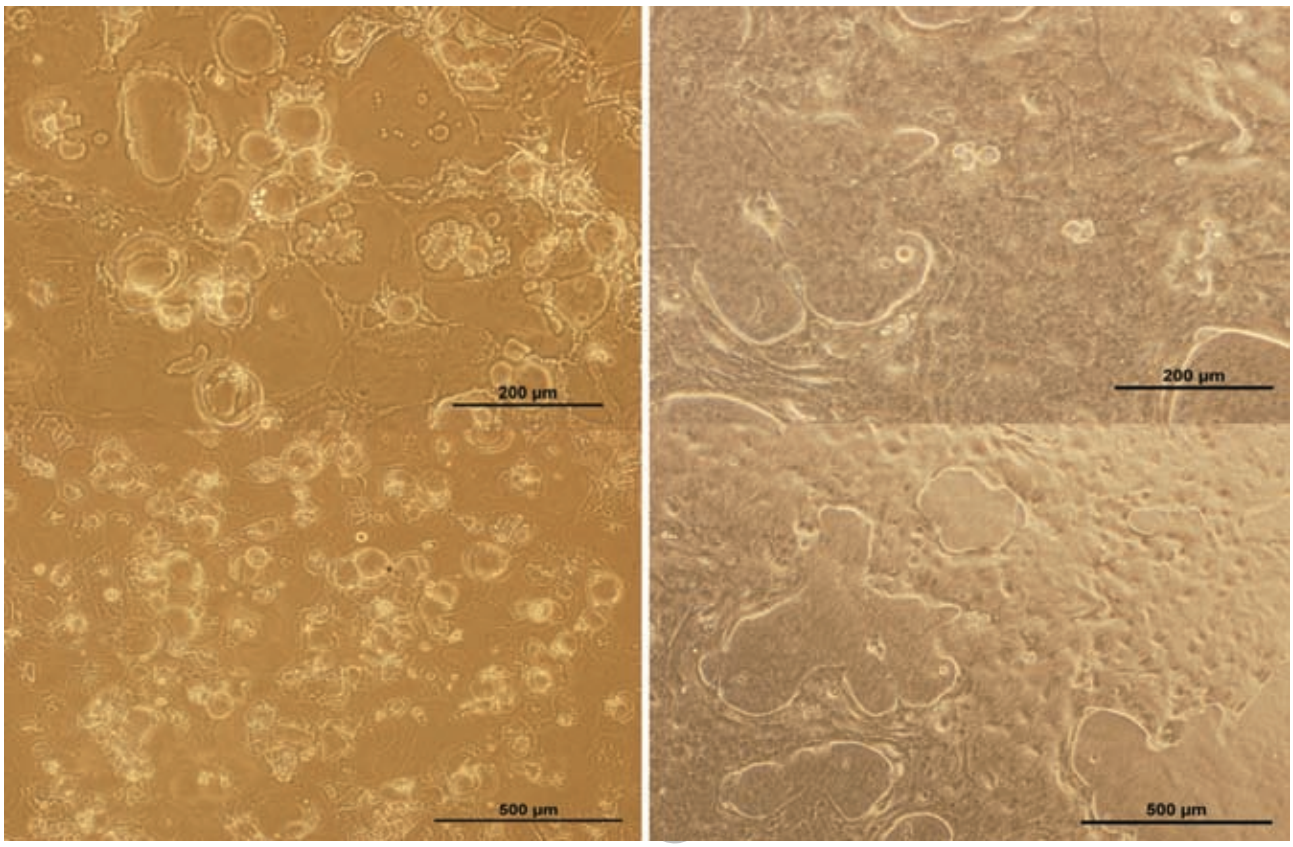

Figure 4. Left, Vacuoles on collagen I on the 7th day. **Right,** Vacuoles on the amniotic membrane on the 14th day.

days (until day 14), cell colonization and morphology on the AM became better than those on the peritoneum and collagen. Furthermore, after 2 weeks, confluent primary urothelial cells were established on the AM (Figure 3C). Fourteen days after culturing, the urothelial cell proliferated actively and reached their peak on the AM. Cell apoptosis occurred on the AM, since day 15, and it gradually improved within the days 16 and 18 (Figure 4).

Peritoneum. Growth and colonization of the cells started on the 7th day. In other words, cell growth pattern, in concert with the AM vacuolization, began on the peritoneum within the second week and was completed by the end of the 14th day. Cell growth pattern on the omentum was the same as it was on the peritoneum matrix, but as we mentioned before, due to its big pores and high density of fat cells, we could not obtain an intact sheet cell layer (on light microscopy), so we excluded it from the rest of the study (Figure 3D).

Transmission Electron Microscopy

Given the poor 3-dimentional condition, we omitted the omentum from the TEM studies. Surprisingly, the urothelial cells on the AM had 3 to 4 layers and the cells were well stratified. The urothelial cells in the basal layer showed various shapes including cuboidal and flat type, but they mostly had remarkable nuclei with all normal cytoplasm organelles. The urothelial cells were tightly attached to each other with desmosomes and microvilli. The cells at the basal layer were tightly adhered to the underlying basement membrane by hemidesmosomes (Figure 5). We found desmosomes and junctions among the cells on the peritoneum. Also, cell organelles were the same as those of the natural urothelial cell. Furthermore, it seemed that the pattern of cell adhesion and attachment to the underlying peritoneum was weak (Figure 6).

DISCUSSION

To the present time, acellular collagen matrix derived from donor bladder submucosa has been successfully used both experimentally and clinically for bladder and urothelial replacement in many centers.[1-4] Collagen has been described as a useful matrix due to

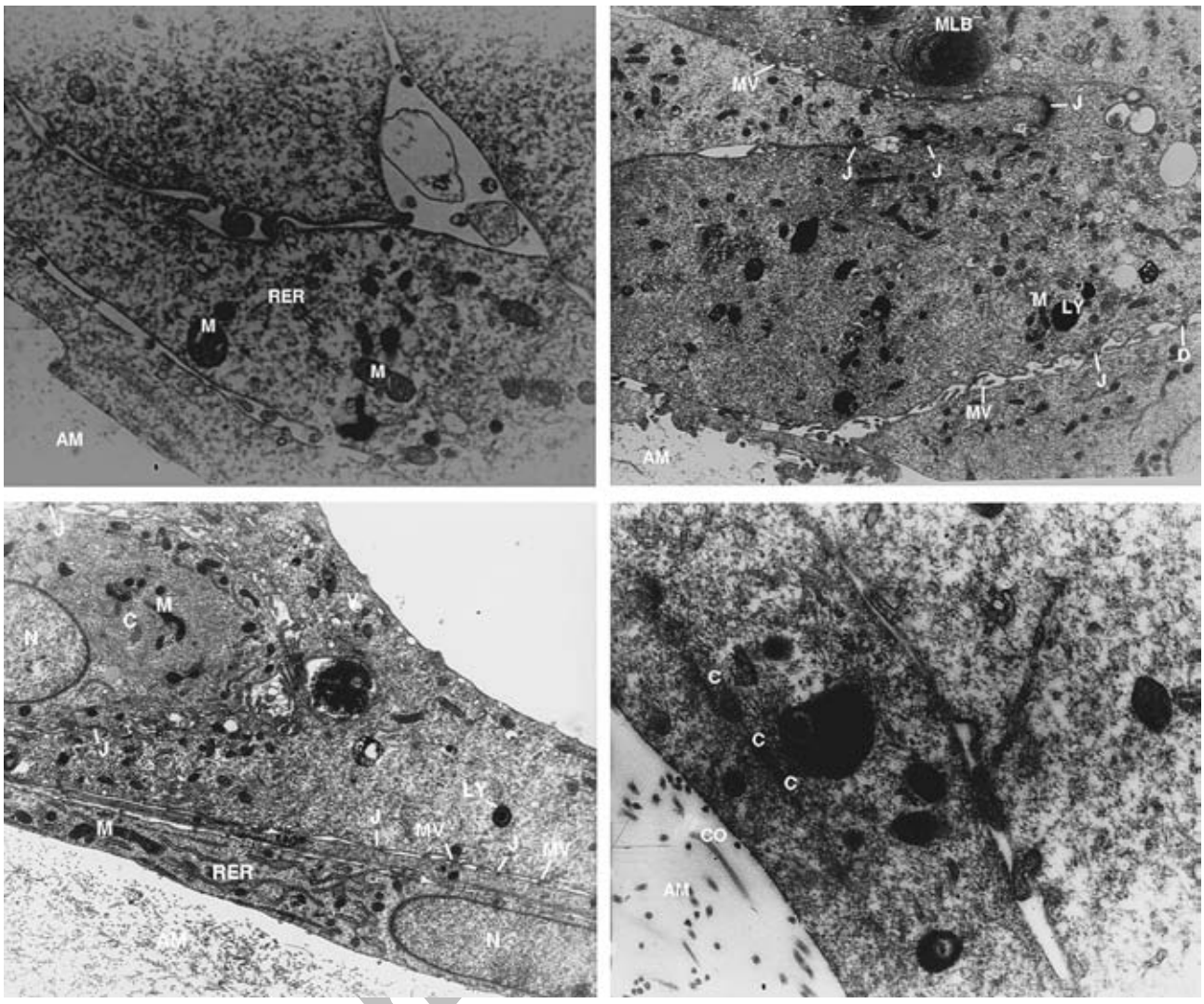

Figure 5. Four urothelial cell layers are seen in the culture on the amniotic membrane by transmission electron microscopy. M, indicates mitochondrion; C, cytokeratin; RER, rough endoplasmic reticulum; J, junction; N, nucleus; AM, amniotic membrane; MV, microvillus; HD, hemidesmosome; V, vacuole; LY, lysosome; CO, collagen; MLB, multilaminar body; and D, desmosome.

its proper characteristics and ease of processing.[1-3] Nevertheless, it is an expensive matrix, encouraging researchers to look for a cost-benefit medium with the same or even better qualities. Collagen sponges support the growth and stratification of the urothelial cells, forming 4 to 8 layers.[5] Sabbagh and colleagues reported that cell proliferation was maximum at days 5 to 10, and collagen sponge remained easy to handle after 3 weeks in culture. On the 15th day, the growth rate reached a plateau.[5] In our study, cell growth was remarkable at the 3rd day. Cells were flat and big, and finally vacuolization was started at the end of the first week which was more significant by the 14th day.

According to our study; the omentum had an inappropriate 3-dimentional structure due to its big pores; thus, it can hardly be used for direct culture of cells. In 2006, Atala and colleagues used the peritoneum for seeding of rat urothelial cell and the cultured cells had the same quality as the natural urothelium did.[7] Comparing with collagen, we had poor cell growth and colonization on the peritoneum within the first 3 days, but on the following days, we obtained better results. Cell apoptosis occurred with delay in the second week; meanwhile, desmosome junctions between the cells did not have features of the normal urothelium, and we just observed up to 2 cell layers in our primary culture which is not desirable.

Cultured cells on the AM enjoyed the features of the normal urothelium. These cells kept growing

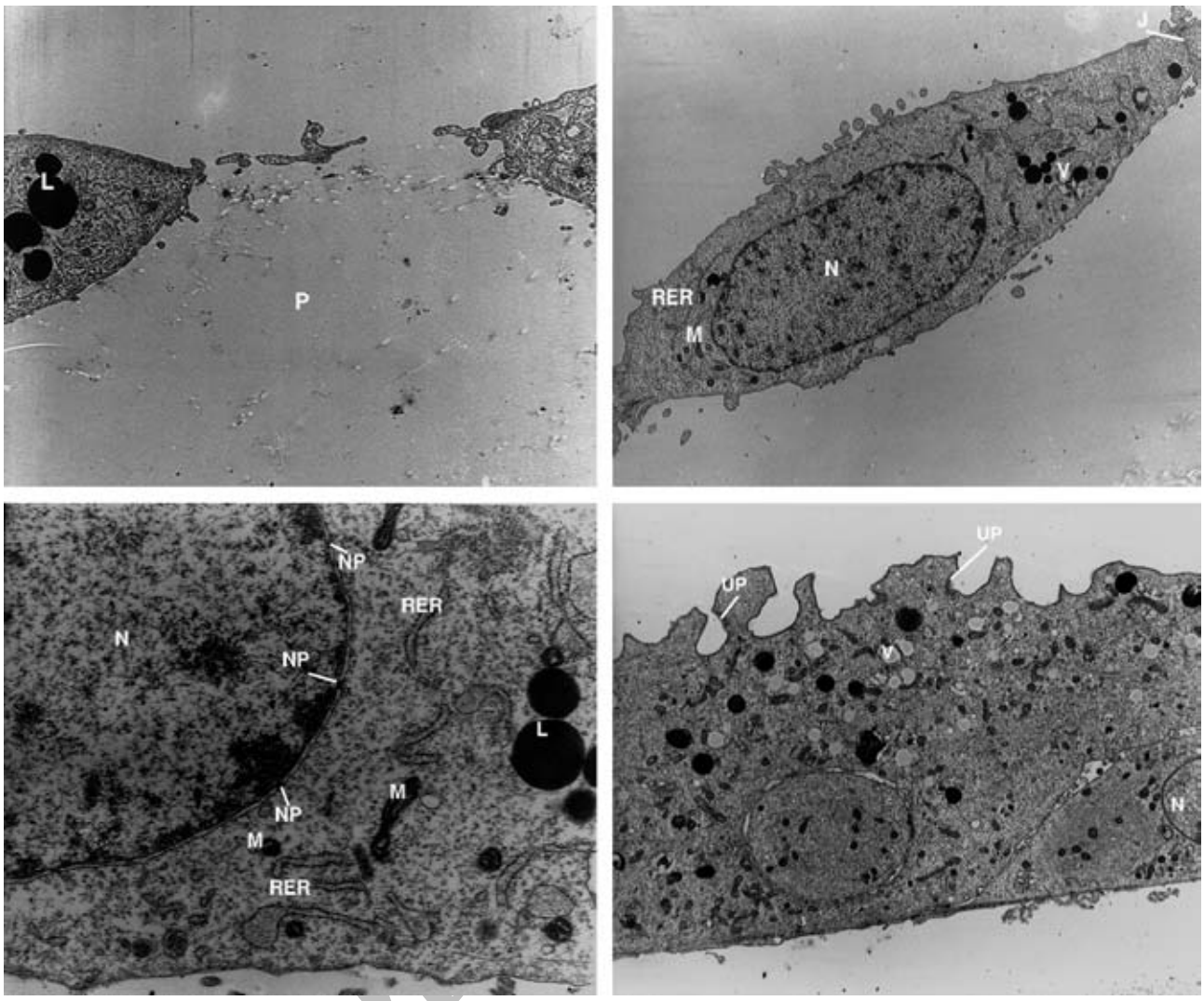

Figure 6. Urothelial cells are seen in the culture on the peritoneum by transmission electron microscopy. L, indicates lipid; P, peritoneum; RER, rough endoplasmic reticulum; M, mitochondrion; N, nucleus; V, vaculole; J, junction; NP, nuclear pore; and UP, uroplakin.

and colonization until the 2nd week which gave us a better chance to preserve it for a longer period of time. Desmosome junction among cells and hemidesmosomes in the basal layer shared features of the normal urothelial. This finding encourages us to use it for urothelial reconstruction. Although there are a few clinical trials on oral, ocular, and skin reconstruction using the AM, this study is pioneering in urology. The AM contains various growth factors and is easy to obtain in large amounts, making it highly accessible and valuable as a biological matrix. In addition, it is a very cost-effective material.[8] In our study, the toughness of the AM was well preserved through out the culture. Therefore, it was easy to transfer and secure the cultured urothelial cells. We used a freeze-dried AM to cover the urothelial layer.

In this study, the cultured urothelial cells survived for 2 weeks, and development of 3 to 4 cell layers gave us a feature like the natural urothelium. We suggest that comparison with the peritoneum and omentum matrixes, the AM has superior results in urothelial engineering. This research can be the first step for future studies concerning urothelial cell culture based on AM matrix.

CONCLUSION

Mouse urothelium cultured on freeze-dried human AM resulted in multilayer urothelial cells with tight intercellular connections by desmosomes. Hemidesmosome junctions between the cells and the AM were observed similar to those of the normal

urothelium. Histological and immunohistochemical examinations also confirmed that the cultured cells had a similar structure to normal urothelial cells, while the peritoneum and omentum did not have the same properties. Overall, the successful culture of the urothelial cells on the AM signifies the possibility of urothelial reconstruction by using this matrix.

CONFLICT OF INTEREST

Dr Hossein Baharvand and Reza Moghadasali are employed at Royan Institute.

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