Special Characteristics of Culturing Mature Human Bladder Smooth Muscle Cells on Human Amniotic Membrane as a Suitable Matrix

Farzaneh Sharifiaghdas,[1,2] Reza Moghadasali,[2,3] Hossein Baharvand,[3]
Seyed Mohammadmehdi Hosseini-Moghaddam,[2,4] Nastaran Mahmoudnejad[1]

Introduction: Our aim was to evaluate the natural behavior, growth pattern, morphology, and specific features of human bladder smooth muscle cells (HBSMCs) on two different matrixes, including human amniotic membrane (HAM) and collagen.

Materials and Methods: The HBSMCs were obtained from 6 children with primary vesicoureteral reflux undergoing open antireflux surgery, and they were isolated from the anterior wall of the bladder. The specimens were cultured on a tissue culture plate of bovine dermal collagen serving as control and on decellularized HAM. Histological, transmission electron microscopy, and immunocytochemical examinations were done, thereafter.

Results: On HAM, very few HBSMCs slowly migrated from explant tissue on the 7th day of culture. All the cells were placed at the same direction, and in some parts, formed multilayer. After 35 to 40 days, the confluency rate was 75% and the cells were orderly arranged. On collagen, cell migration from explant culture took place as rapidly as the 3rd to 4th day of culturing. On days 30 to 40, the confluency rate was 100%. Immunocytochemical staining was positive for anti-actin and antidesmin antibodies. On transmission electron microscopy, cell organelles of HBSMCs exhibited the same features of the natural smooth muscle cells. They were tightly attached to each other and the underlying layer basement membrane.

Conclusion: A well-designed growth pattern of HBSMCs on HAM with abundant cell-to-cell adhesions encourages us to use it as a competent tissue for reconstruction of relatively damaged or diseased bladders. Undoubtedly, further clinical studies should be performed to replicate our results.

Urol J. 2009;6:283-8.
www.uj.unrc.ir

Keywords: cell culture techniques, bladder, smooth muscles, amnion

[1]Department of Urology, Shahid Labbafinejad Medical Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Urology and Nephrology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]Department of Stem Cells, Royan Institute, Tehran, Iran
[4]Department of Infectious Diseases and Tropical Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Corresponding Author:
Farzaneh Sharifiaghdas, MD
Urology and Nephrology Research Center, No 103, 9th Boustan St, Pasdaran Ave, Tehran, Iran
Tel: +98 21 2256 7222
Fax: +98 21 2277 0954
E-mail: fsharifiaghdas@yahoo.com

Received June 2009
Accepted August 2009

INTRODUCTION

Congenital abnormalities, cancer, trauma, infection, inflammation, iatrogenic injuries, and several other conditions may lead to genitourinary organ damage or loss, requiring eventual reconstruction.[1] Finding a suitable material to reconstruct the genitourinary tract has been a challenging task. Over the past few decades, the use of several bladder-wall substitutes has been attempted with both synthetic and organic materials.[2] The first application of a free tissue graft for bladder replacement was reported by Neuhof in 1917, when fascia was used to augment bladders in dogs.[3] Since that first report, numerous other free graft materials have been

used experimentally and clinically including the bladder allograft, pericardium, dura, placenta, peritoneum, and omentum.[4-9]

Generally, 3 classes of biomaterials have been utilized for engineering genitourinary tissues: naturally derived materials, acellular tissue matrices, and synthetic polymers.[10] Most of the attempts have usually failed due to either mechanical, structural, functional, or biocompatibility problems.[2] Our goal in present study is to evaluate and compare the natural behaviors, growth pattern, morphology, and specific features of human bladder smooth muscle cells (HBSMCs) on 2 different matrixes including human amniotic membrane (HAM) and collagen, in order to achieve a suitable, cost-effective, abundant matrix for further studies and reconstruction of the bladder.

MATERIALS AND METHODS

Harvesting and Preparation of Bladder Cells

Bladder smooth muscle cells were obtained from 6 children with a mean age of 6 years old (range, 2 to 10 years) with primary vesicoureteral reflux undergoing open antireflux surgery. They had no evidence of bladder dysfunction on history, physical examination, imaging studies, and urodynamic studies. Informed consent was obtained from the parents before the operation.

At the time of operation, a 0.5 × 0.5-cm full-thickness biopsy of the anterior wall of the bladder was obtained. The adherent perivesical fat and urothelium were removed with fine scissors. The sample was immediately put in the cold phosphate-buffered saline (PBS; Gibco, Cat No 21600-051) containing penicillin, 100 U/mL; streptomycin, 100 μg/mL (Gibco, Cat No 15070); and ofloxacin, 0.3% (0-8757, Sigma-Aldrich, St Louis, MO, USA), and transported to the laboratory. After 2 times washing of the specimen with PBS, the muscle cubes were divided into 1 × 1-mm pieces, washed again with PBS, and put in a humidified incubator at 37°C with 5% CO_2.

The cells were reefed with fresh medium M199 (Gibco, Cat No 31150), 10% fetal bovine serum (Gibco, Cat No 10270-106), and L-glutamine, 2 mM (Gibco, Cat No 25030), 2 days thereafter, and subsequently, on alternate days. Passaging and subculturing of the cells was performed as per routine. The HBSMCs were cultured on a tissue culture plate of bovine dermal collagen (Nalgene, Cat No 5409), serving as control and on decellularized HAM as test.

Preparation of Decellularized Human Amniotic Membrane

The sample of HAM was harvested from seronegative maternal donors. Under sterile conditions, the placental membrane was thoroughly washed and placed in PBS containing penicillin/streptomycin (Gibco-Invitrogen, Grand Island, NY, USA) and ofloxacin (o-8757, Sigma-Aldrich, St Louis, MO, USA). Then, to obtain a decellularized membrane, the epithelial cells were isolated by trypsinization (trypsin-ethylenediamine tetraacetic acid, Gibco, Cat No 25300).

Immunocytochemistry

For immunostaining, the HBSMCs were washed twice with PBS and fixed with 4% paraformaldehyde for 24 hours at 4°C. These cells were permeabilized and blocked in PBS containing 0.2% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA; Cat No T8787) and 10% goat serum for 10 minutes and 30 minutes, respectively. Thereafter, the HBSMCs were incubated in primary antibody diluted in 0.5% bovine serum albumin at 37°C for 1 hour. The antibodies used in this study were desmin (Sigma, Cat No D1033) and α-smooth muscle actin (Sigma, Cat No A5228). At the end of the incubation time, the cells were washed twice with PBS plus 0.05% tween 20 and incubated with the fluorescence isothiocyanate-conjugated antimouse immunoglobulin (FITC, Chemicon, AP308F and abcam, ab6785-1), respectively diluted in 0.5% bovine serum albumin for 60 minutes at 37°C. After washing twice with PBS plus 0.05% tween 20, the specimens were examined under fluorescence microscope (BX51, Olympus, Japan).

Transmission Electron Microscopy

For transmission electron microscopy, 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). [11]

RESULTS

Culture

Primary cultures of HBSMCs were successfully established and passaged from biopsies obtained before. We did not inspect any abnormal morphologic features or cessation of growth in both matrixes. Culturing was continued for 40 days. During this time, we observed growth pattern, confluency rate, and cell morphology, on a daily basis. Immunocytochemical staining was performed on each cell strain periodically for the duration of these experiments and in all instances, staining was positive for all strains utilized. Morphological examination and immunocytological staining with desmin and α-smooth muscles actin antibodies were used to confirm smooth muscle cells phenotype (Figure 1).

Collagen Matrix

Cell migration from explant culture took place as rapidly as the third to fourth day. All the cells had the typical spindle-shaped morphology with centrally located round-to-oval nuclei. They continued to migrate from the explant tissue till the second week and had a confluency rate of approximately 25% at this time. Multiple pseudopodia emanated from the cells until they made contact with the adjacent cells, forming a "hill and valley" appearance. Multilayer formation was clearly seen in different parts (Figure 2). On the 30th to 40th day, the confluency rate was

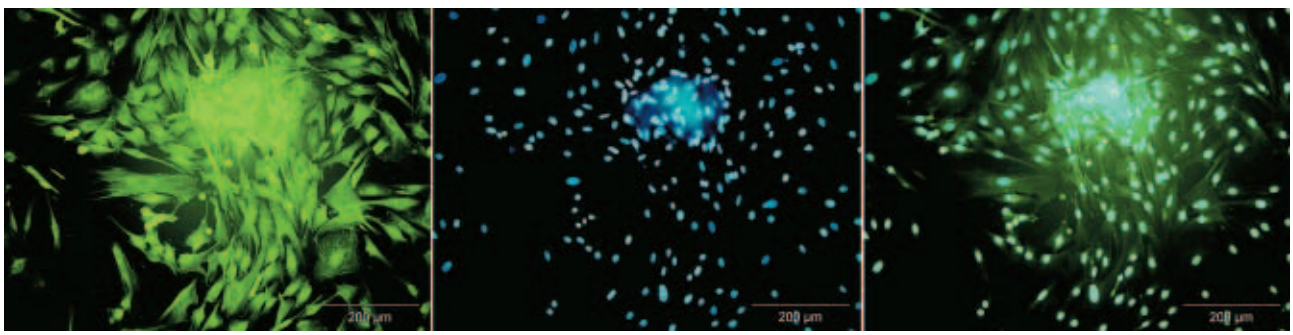

Figure 1. Morphological examination and immunocytological staining with desmin antibody was used to confirm smooth muscle cells phenotype (15th day). **Left,** Desmine. **Middle,** Hoechst. **Right,** Merge.

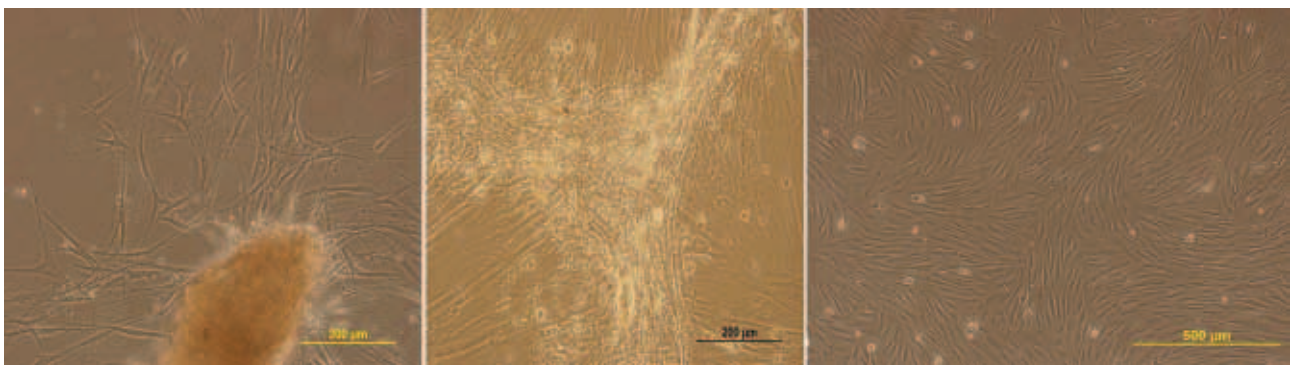

Figure 2. Human bladder smooth muscle cells on collagen matrix. **Left,** Migration of smooth muscle cells from explant tissue on the 7th day. **Middle,** Multilayer formation of cells on the 14th day. **Right,** Final appearance of cells on collagen matrix on the 35th day.

100%. The cells did not exhibit contact inhibition.

Human Amniotic Membrane

Very few HBSMCs slowly migrated from the explant tissue on the 7th day. The confluency rate after 3 weeks reached to 25% and the cells displayed characteristic spindle-shaped morphology as discussed earlier. Surprisingly, all of the cells were placed at the same direction, and in some parts, formed multilayer. After about 35 to 40 days, the confluency rate was 75%, and we had a well-designed sheet of cells (Figure 3).

Transmission Electron Microscopy

Cell organelles in the HBSMCs, both on HAM and the collagen matrix, exhibited the same features of the natural smooth muscle cells. The HBSMCs on HAM had formed 2 to 3 layers in some parts. They were tightly attached to each other and also to the underlying basement membrane layer by cellular junctions. Interdigit formation of cells on this matrix was seen as well (Figure 4).

DISCUSSION

Many injuries may lead to damage or loss of the bladder, necessitating eventual replacement or repair of the organ.[12] These injuries include congenital abnormalities, iatrogenic injuries (eg, vesicovaginal fistula due to a pelvic surgery), cancer, trauma, infection, and inflammations.[1] Gastrointestinal segments are frequently used as donor tissue for augmentation cystoplasty or for making urinary reservoirs (pouches) after radical cystectomy for bladder cancer. However, several complications can ensue, such as metabolic disturbances, urolithiasis, increased mucous production, and malignant transformation.[13, 14] These complications made many investigators attempt to use alternative methods, materials, and tissues for replacement or repair of the bladder.[12] Many materials have been used for free grafts including the skin, bladder, submucosa, omentum, dura, peritoneum, placenta, seromuscular graft, and small intestinal submucosa. Synthetic materials like polyvinyl sponge matrixes, vicryl matrixes, resin-sprayed paper, and silicon were used, as well.[12] However, as mentioned before, these biomaterials have

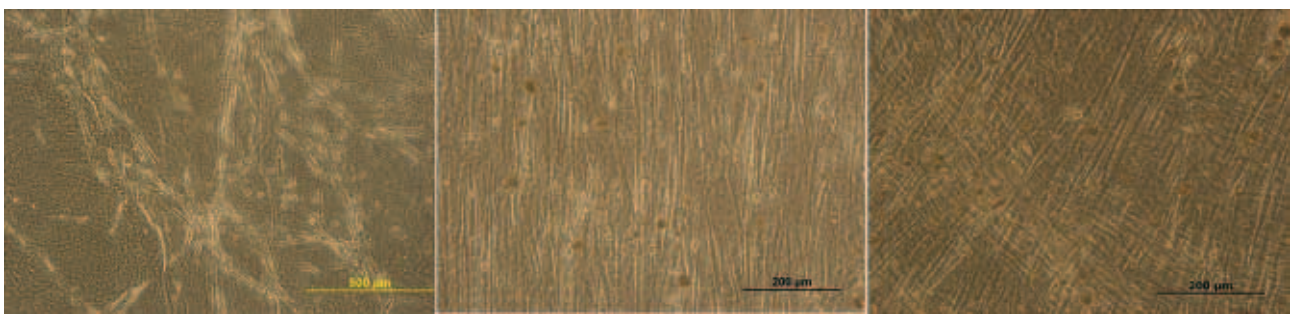

Figure 3. Human bladder smooth muscle cells on human amniotic membrane matrix. **Left,** Primary culture of smooth muscle cells on the 14th day. **Middle** and **Right,** Final appearance and orderly arrangement of the cells on the 40th day.

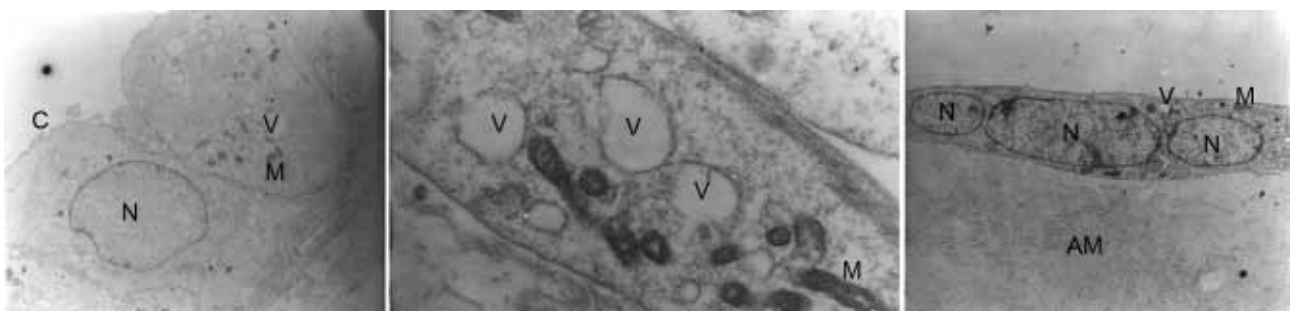

Figure 4. Transmission electron microscopy (35th day). **Left,** The HBSMCs on collagen matrix. **Middle,** The HBSMCs on human amniotic membrane matrix matrix. **Right,** Interdigit formation of cells on human amniotic membrane matrix matrix. C, indicates caveola; M, mitochondrion; N, nucleus; V, vacuole; and AM, amniotic membrane.

many mechanical, structural, functional, or biocompatibility problems. Permanent synthetic materials will have mechanical failure; urinary calculus formation and using degradable materials lead to fibroblast, scaring, graft contracture and reduced reservoir volume.[12]

The ideal biomaterial used to engineer a tissue should be biocompatible, since the formation of new tissue would strongly depend on the interaction of the biomaterial with the transplanted or ingrowing cells. The biomaterial should not only be nontoxic to the cells, but also elicit bioactive cellular responses.[15] It must also be capable of controlling the structure and function of the engineered tissue in a predesigned manner, by interacting with transplanted cells and/or the host cells. This ideal biomaterial should be biocompatible, promote cellular interaction and tissue development, and possess mechanical and physical properties.[10] To the present time, a cellular collagen matrix derived from donor bladder submucosa has been successfully used both experimentally and clinically for bladder and urothelial replacement in many centers. It has been described as a useful matrix due to its proper characteristics and ease of processing. Nevertheless, it is an expensive matrix, encouraging researchers to look for a cost-benefit medium with the same or even better qualities.[11]

In 1993, Baskin and colleagues[16] separated and cultured HBSMCs for the first time. Since then, several studies have evaluated the morphology and pattern of growth of smooth muscle cells on collagen matrix.[15, 17] According to the good results of these studies in utilizing collagen, we decided to choose it as a control matrix in our study. Our HBSMC culture on collagen had promising results. The cell migration from explant tissue, morphology, and growth pattern were all acceptable. We may simply use this semiliquid material (collagen plus HBSMC) for treatment of vesicoureteral reflux or incontinence as a "viable bulking agent"; however, the high cost of collagen serves as a limiting factor.

The amniotic membrane comprises the innermost layer of the placenta. Amniotic membrane transplantation has been used in many different types of reconstructive surgeries.[18] Amniotic membrane transplantation became important because of its ability to diminish the occurrence of adhesions and scarring, its ability to enhance wound healing and epithelialization, and its antimicrobial potential. The amniotic membrane expresses incomplete human leukocyte antigen classes A, B, C, and DR,[19] which may account for the fact that immunological rejection after transplantation has not been observed. In 1940, De Rotth used a fresh fetal membrane as a graft for conjunctival surface reconstruction with limited success.[20] Sorsby and colleagues reported in 1946 and 1947 the successful use of amniotic membrane as a patch graft in the treatment of acute ocular burns.[21,22]

Using the HAM for urological purposes is a young technique. The HAM was successfully used for culturing mouse urothelial cells.[11] The present study, to our knowledge, is the first report of successful HBSMC culturing on HAM. The classic spindle-shaped morphology, abundant cell-to-cell and cell-o-basement membrane junctions exactly resembled a normal bladder smooth muscle layer. We could see multilayer and interdigit formation of cells in some parts. Surprisingly, HBSMCs cultured on HAM were all located at the same direction in good order, but the same cells were randomly arranged on collagen matrix. This may encourage us to use the HAM as a competent tissue for reconstruction of relatively damaged or diseased bladders. Whether this special arrangement or configuration helps us in achieving better results in vivo remains unclear and needs to be examined in future studies.

CONCLUSION

A well-designed growth pattern of HBSMCs cultured on HAM with abundant cell-to-cell adhesions as well as a tight adherence of cells to the underlying basement membrane were detected.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Atala A. Tissue engineering in urology. Curr Urol Rep. 2001;2:83-92.

2. Falke G, Caffaratti J, Atala A. Tissue engineering of the bladder. World J Urol. 2000;18:36-43.
3. Neuhof H. Fascial transplantation into visceral defects an experimental and clinical study. Surg Gynecol Obstet. 1917;25:383.
4. Fishman IJ, Flores FN, Scott FB, Spjut HJ, Morrow B. Use of fresh placental membranes for bladder reconstruction. J Urol. 1987;138:1291-4.
5. Kambic H, Kay R, Chen JF, Matsushita M, Harasaki H, Zilber S. Biodegradable pericardial implants for bladder augmentation: a 2.5-year study in dogs. J Urol. 1992;148:539-43.
6. Kelami A, Ludtke-Handjery A, Korb G, Rolle J, Schnell J, Danigel KH. Alloplastic replacement of the urinary bladder wall with lyophilized human dura. Eur Surg Res. 1970;2:195-202.
7. Tsuji I, Ishida H, Fujieda J. Experimental cystoplasty using preserved bladder graft. J Urol. 1961;85:42-4.
8. Moriya K, Kakizaki H, Murakumo M, et al. Creation of luminal tissue covered with urothelium by implantation of cultured urothelial cells into the peritoneal cavity. J Urol. 2003;170:2480-5.
9. Baumert H, Simon P, Hekmati M, et al. Development of a seeded scaffold in the great omentum: feasibility of an in vivo bioreactor for bladder tissue engineering. Eur Urol. 2007;52:884-90.
10. Atala A. Tissue engineering for the replacement of organ function in the genitourinary system. Am J Transplant. 2004;4 Suppl 6:58-73.
11. Sharifiaghdas F, Hamzehiesfahani N, Moghadasali R, Ghaemimanesh F, Baharvand H. Human amniotic membrane as a suitable matrix for growth of mouse urothelial cells in comparison with human peritoneal and omentum membranes. Urol J. 2007;4:71-8.
12. Atala A, Bauer SB, Soker S, Yoo JJ, Retik AB. Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet. 2006;367:1241-6.
13. McDougal WS. Metabolic complications of urinary intestinal diversion. J Urol. 1992;147:1199-208.
14. Soergel TM, Cain MP, Misseri R, Gardner TA, Koch MO, Rink RC. Transitional cell carcinoma of the bladder following augmentation cystoplasty for the neuropathic bladder. J Urol. 2004;172:1649-51.
15. Pariente JL, Kim BS, Atala A. In vitro biocompatibility evaluation of naturally derived and synthetic biomaterials using normal human bladder smooth muscle cells. J Urol. 2002;167:1867-71.
16. Baskin LS, Howard PS, Duckett JW, Snyder HM, Macarak EJ. Bladder smooth muscle cells in culture: I. Identification and characterization. J Urol. 1993;149:190-7.
17. Kropp BP, Zhang Y, Tomasek JJ, et al. Characterization of cultured bladder smooth muscle cells: assessment of in vitro contractility. J Urol. 1999;162:1779-84.
18. Trelford JD, Trelford-Sauder M. The amnion in surgery, past and present. Am J Obstet Gynecol. 1979;134:833-45.
19. Akle CA, Adinolfi M, Welsh KI, Leibowitz S, McColl I. Immunogenicity of human amniotic epithelial cells after transplantation into volunteers. Lancet. 1981;2:1003-5.
20. De Rotth A. Plastic repair of conjunctival defects with fetal membranes. Arch Ophthalmol. 1940;23:522.
21. Sorsby A, Symons HM. Amniotic membrane grafts in caustic burns of the eye: (Burns of the second degree). Br J Ophthalmol. 1946;30:337-45.
22. Sorsby A, Haythorne J, Reed H. Further Experience with Amniotic Membrane Grafts in Caustic Burns of the Eye. Br J Ophthalmol. 1947;31:409-18.