

URETHRAL REPLACEMENT USING CELL SEEDED TUBULARIZED COLLAGEN MATRICES

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ABSTRACT

Purpose: Acellular collagen matrices derived from bladder submucosa have been used successfully as an off-the-shelf biomaterial for urethral replacement, experimentally and clinically in an onlay fashion. We investigated whether collagen matrices, either alone or with autologous cells, could be used for tubularized urethral replacement.

Materials and Methods: Acellular collagen matrices were processed and tubularized. Ten rabbits underwent an open bladder biopsy with subsequent cell expansion. Autologous bladder cells were grown and seeded onto the pre-configured tubular matrices. A 1 cm. long urethral segment was excised in 24 male rabbits. Urethroplasty was performed with the tubularized collagen matrices seeded with cells in 12 animals and without cells in 12. Serial urethrography was performed preoperatively and at 1, 2, 3 and 6 months postoperatively. Retrieved urethras were analyzed grossly, histologically, immunocytochemically and with Western blots. Contractility and the presence of neurotransmitter receptors were confirmed with organ bath studies.

Results: Serial urethrography confirmed the maintenance of a wide urethral caliber without any signs of strictures in animals implanted with the cell seeded matrices. The urethral segments replaced with the collagen scaffolds without cells demonstrated strictures and graft collapse at all time points. The implanted cell seeded matrices had a normal urethral architecture by 1 month, consisting of a transitional cell layer surrounded by muscle cell fiber bundles with increasing cellular organization with time. Epithelial and smooth muscle phenotypes were confirmed immunocytochemically and with Western blot analyses using pancytokeratins AE1/AE3 and smooth muscle specific α -actin antibodies. Formation of a transitional cell layer was confirmed in the matrices implanted without cells but only scant unorganized muscle fiber bundles were present, mostly at the anastomotic sites. Organ bath studies demonstrated the capacity for contractility along with cholinergic and adrenergic specific receptors in the tissue engineered scaffolds compared to controls.

Conclusions: These results show that collagen matrices seeded with cells form normal urethral tissue can be used for tubularized replacement, whereas tubularized collagen matrices alone without cells lead to poor tissue formation and strictures. The collagen matrices seeded with cells may offer a useful alternative in the future for patients requiring a tubularized urethral segment replacement.

KEY WORDS: hypospadias, urethral stricture; tissue engineering; muscles, urothelium

Collagen constructs have already been successfully applied experimentally and clinically for urethral repair but only as an onlay.^{1,2} Several investigations involving replacement of ureters and urethras have demonstrated the nature of cylindrical biodegradable structures functioning as graft substitutes in cell seeded and unseeded conditions.^{1,3} The data suggest that in cases of entire segmental replacement cells were necessary to prevent strictures from occurring, whereas if the area replaced was small in at least 1 dimension, as in the case of an onlay graft, cells were not essential for adequate healing. Many pathological conditions may necessitate replacement of a significant urethral segment for which onlay grafts would not be suitable. The next challenge in the area of tissue engineering is to perform tubularized urethral replacement with a scaffold that demonstrates functional compatibility to the native urethra. We investigated the feasibility of using a cell seeded or nonseeded scaffold for tubularized urethral replacement in an animal model.

MATERIALS AND METHODS

Acellular collagen matrix. The collagen matrix was obtained through previously described methods.^{1,2,4} Porcine bladder tissue was harvested from sacrificed animals and rinsed with phosphate buffered saline. The submucosa was microdissected from the muscular and serosal layers of the bladder. Under continuous agitation the submucosa was washed with 1 l. ddH₂O for 24 hours and with 1 l. ddH₂O and 0.2% Triton X-100 (2 ml.) + 0.3 ml. ammonium hydroxide for 14 days. The submucosa was washed for 24 hours with 1 l. distilled water and placed in a 10% cefoxitin solution. Random samples were obtained after processing for histological analyses. The submucosa was trimmed to 1 cm. and tubularized around a 16Fr catheter. Several interrupted 6-zero polyglactin sutures were used to fasten the free edges of the graft together. Marking sutures were placed in the external surface of the graft at each of the 4 corners with nonresorbable 5-zero polypropylene. The grafts were dried and lyophilized for 24 hours, and sterilized with ethylene oxide. Before use the grafts were further sterilized with ultraviolet light for 24 hours.

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Harvesting and cell culture. Bladder biopsies were procured from 12 male New Zealand white rabbits. The animals were anesthetized and maintained with 2% to 3% isoflurane after pretreatment with 15 to 20 mg/kg. ketamine, 2 to 3 mg/kg. xylazine and 0.75 mg/kg. acepromazine intramuscularly. A small laparotomy incision was made above the pubic symphysis exposing the bladder. A 2 × 2 cm. biopsy specimen was excised from the bladder wall, and the defect was closed in 2 layers with 2-zero and 3-zero polyglactin sutures. The animals were kept on 5 mg/kg. enrofloxacin intramuscularly for 3 days postoperatively for prophylaxis against infection.

Microdissection under loop magnification was used to remove the seromuscular layer of the bladder tissue carefully. Primary cultures of smooth muscle cells were obtained by explanting several pieces of seromuscular tissue on 10 cc culture plates in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Enzymatic dissection with collagenase and dispase was used to isolate epithelial cells in solution which were plated onto 25 cc culture plates in keratinocyte serum free medium. The cells were expanded to a density of 1.0 to 3.0×10^7 cells per cm^2 for seeding onto the urethral grafts.

Seeding the collagen scaffold. The cells were incubated with trypsin ethylenediaminetetraacetic acid solution until cell separation and inactivated with serum. The cells were centrifuged and the cell pellet was resuspended to a concentration of 1×10^7 cells per cc of fresh medium. The scaffolds were sequentially seeded with epithelial cells within the lumen and smooth muscle cells on the outer surface with the resuspended cell medium solution. The seeded grafts were incubated for 7 days. The unseeded grafts, as controls, were incubated for 24 hours in Dulbecco's modified Eagle's medium/fetal bovine serum medium before implantation (fig. 1).

Urethral surgery and postoperative evaluation. A total of 24 animals were used for this study, 12 including that received unseeded and 12 that received cell seeded tubularized grafts. The animals were anesthetized according to the same drug regimen described previously. A 1 cm. penile urethral defect which comprised the entire anterior urethra was created, and the graft was interposed and secured with interrupted 6-zero polyglactin sutures on both ends. The urine was diverted with a 14Fr urethral catheter for 7 days postoperatively. The animals were kept on a fluoroquinolone antibiotic until the catheter was removed. Postoperatively the animals were observed for any voiding difficulties. Retrograde ure-

thograms and graft harvest were performed at 1, 2, 3 and 6 months after implantation.

Animals were euthanized using the anesthetic drug regimen described previously plus a 2 cc intracardiac injection of sodium pentobarbital solution at 1 (6), 2 (6), 3 (6) and 6 (6) months after implantation. The entire urethra and corporal bodies were circumscribed with sharp dissection and removed en bloc for sectioning. The nonabsorbable sutures were identified to demarcate the graft margins. Several sections of the graft were obtained for histological and immunohistochemical analyses as well as molecular and organ bath studies. Normal urethral tissue was also recovered in the same fashion from each animal.

Immunocytochemical and histological analyses. Five μm . sections of formalin fixed, paraffin embedded tissues were processed and stained with hematoxylin and eosin, and Mason's trichrome. Epithelial cell layers were identified using broadly reacting monoclonal anti-pancytokeratins AE1/AE3 (Boehringer Mannheim, Indianapolis, Indiana). Smooth muscle fibers were labeled with monoclonal α smooth muscle actin antibodies (Novocastra, Newcastle, United Kingdom). Immunolabeling was performed using the avidin-biotin detection system and sections were counterstained with hematoxylin.

Organ bath studies. A longitudinal strip of the urethral graft and normal urethra were retrieved for organ bath analysis. The strips were attached by 4-zero silk sutures to a tissue support hook at 1 end and an isometric force transducer (Radnoti Glass Technology, Monrovia, California) at the other end. The specimens were mounted in isolated baths containing 50 ml. Tyrode's solution equilibrated with 95% O_2 , 5% CO_2 supplied by a bubbling chamber and maintained at 37°C. Peak contractions were recorded for each individual strip of tissue exposed to a variety of electrical and chemical stimuli. Transducer signals were fed into a recorder. For electrical field stimulation 2, 10 mm. diameter ring platinum iridium electrodes were used with a Grass S48 stimulator (AstroMed, Inc., Warick, Rhode Island). The tissues were mounted at a preload tension of 20 mN. (2.0 gm.), resulting in a resting tension of about 8 mN. (0.8 gm.) at the end of a 60-minute equilibration period. Serial field stimulation was applied (2, 4, 8, 16 and 32 Hz., 80 volts, 1 millisecond duration square pulses) with 3 minutes of resting intervals between stimulations, and active tension was measured. To confirm the expression and function of neurotransmitter receptors, specific autonomic agonists, carbachol (10^{-6} M.) and phenylephrine (10^{-3} M.), were used.

RESULTS

Histological analyses of the in vitro processed collagen matrices confirmed their acellularity. After implantation all of the unseeded grafts developed fistulas or collapsed by 1 month. Serial urethrography of the unseeded collagen matrices demonstrated the presence of strictures. In contrast, serial urethrography confirmed maintenance of a wide urethral caliber without any signs of strictures in animals implanted with cell seeded tubularized matrices (fig. 2). Gross examination of the cell seeded grafts at the time of retrieval showed normally appearing tissue without any evidence of fibrosis and equidistant marking sutures indicating preservation of graft diameter.

Hematoxylin and eosin, and Mason's trichrome analysis of the specimens indicated that the cell seeded matrices had a normal urethral architecture tissue by 1 month, consisting of a transitional cell layer surrounded by increasingly organized muscle fiber bundles that resembled normal urethra by 6 months. The unseeded grafts demonstrated an organized surface epithelial layer with a paucity of disorganized muscle fiber bundles with large amounts of fibrosis surrounding the urethral lumen. Using anti-CD-31 antibodies, angiogenesis

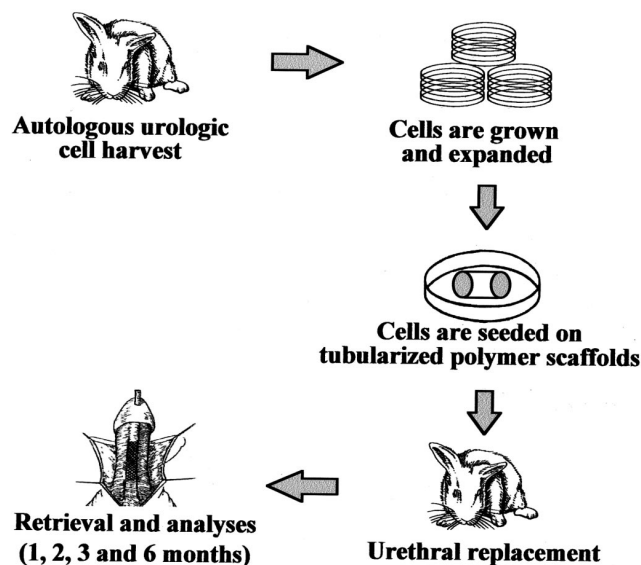


FIG. 1. Study overview

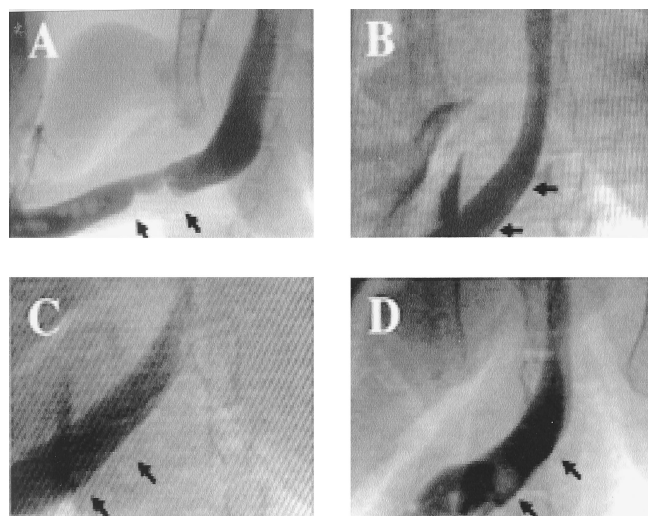


FIG. 2. Retrograde urethrograms. A, stricture with unseeded graft. All seeded constructs remained open at all time points. B, 1 month. C, 3 months. D, 6 months.

was noted on the cell seeded scaffolds within the organizing seromuscular layers. However, the unseeded grafts demonstrated a scarcity of vascular organization in comparison to their seeded counterparts.

The presence of a complete transitional cell layer of urothelial cells was confirmed with the broadly reacting anti-pancytokeratins AE1/AE3 in all implants. Immunohistochemical analyses with smooth muscle specific α -actin antibodies demonstrated an increasing number of organized muscle bundles with time in the cell seeded matrices. Evidence of only scant unorganized muscle fiber bundles was present with α -actin stains, mostly at the anastomotic sites (fig. 3). The same primary antibodies were used for Western blots which confirmed the presence of normally differentiated epithelial and smooth muscle cells in the tissue engineered scaffolds seeded with cells (fig. 4).

Specific agonists (carbachol and phenylephrine) were used to confirm functionally the presence of muscarinic and adrenergic receptors in the engineered and normal urethral walls. Addition of carbachol (10^{-6} M.) and phenylephrine (10^{-3} M.) elicited qualitatively identical contractions in the engineered and normal bladder strips (fig. 5). The tissue engineered urethral tissue proved capable of generating contractile forces through neurotransmitter based mechanisms.

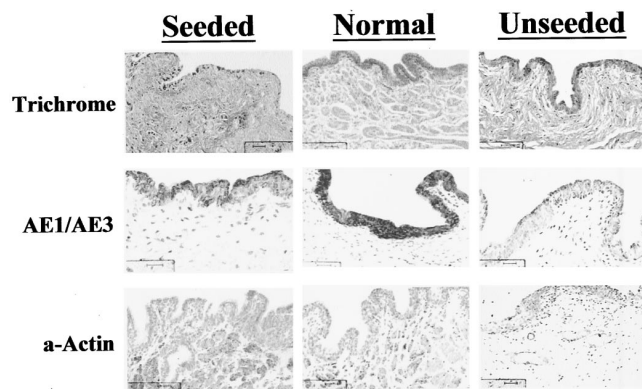


FIG. 3. Histological and immunocytochemical analyses of normal urethra compared to seeded and unseeded urethral scaffolds. Seeded scaffolds 6 months after surgery illustrate well differentiated epithelial and smooth muscle cells and increasing architectural organization approaching normal urethral histology.

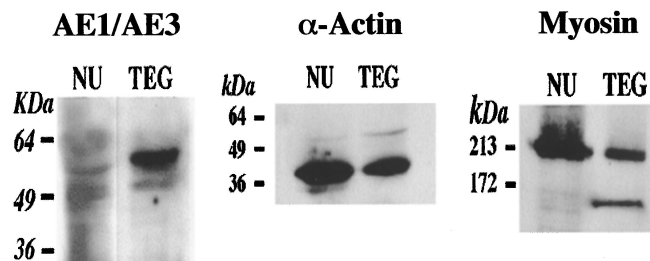


FIG. 4. Western blot analyses of tissue engineered grafts (TEG) compared to normal urethra illustrate similar concentrations of cytokeratin (AE1/AE3) and smooth muscle (α -actin, myosin) proteins.

Electrical field stimulation also demonstrated similar responses in contraction in the tissue engineered constructs compared to normal urethra (fig. 6).

DISCUSSION

Various urethral conditions, such as inflammatory and posttraumatic strictures, congenital defects and malignancy, often require extensive urethral reconstruction. When local tissue is limited other sources have been used, such as genital and extra genital skin flaps, bladder and buccal mucosa, tunica vaginalis, bowel and temporary or biodegradable stents as alternative conduits for urethral substitution.⁵⁻¹⁸

Initial results with genital and extragenital skin grafts had been encouraging but significant complications with fistulas, hair growth, stone formation and graft contracture were reported with long-term followup.^{19,20} Significant donor site morbidity has also been reported after harvesting these grafts.^{20,21} Buccal mucosa remains a widely used source of tissue replacement at many centers especially in cases of complex urethral reconstruction but anastomotic strictures and fistulas are overwhelmingly the most common complications reported.²²⁻²⁴ Oral paraesthesias and pain have also been associated with the use of buccal mucosa grafts.²⁵ Bladder mucosa grafts have been associated with glandular protrusion of bladder epithelium resulting in secondary meatal stenosis and urinary obstruction.²⁶ The clinical experience with intestinal segments for urethral substitution has been limited and largely confined to laboratory investigations.²⁷ Free grafts or flaps of tunica vaginalis for hypospadias repair have resulted in meatal stenosis.

The techniques involved with tissue engineering may allow for creation of a reservoir of biocompatible tissue that can be used when pathological conditions mandate extensive urethral reconstruction, especially when there is a paucity of local epithelial tissue available. This study shows that tissue engineered tubularized grafts seeded with autologous cells can be used successfully for urethral replacement in an ani-

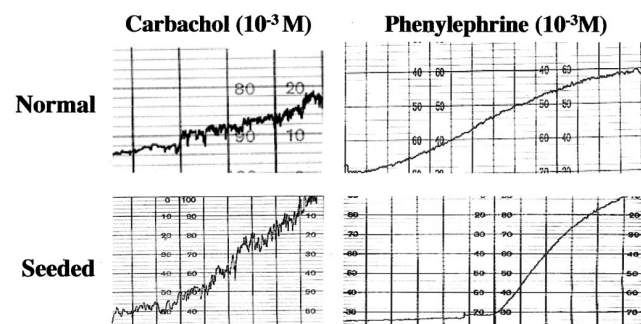


FIG. 5. Organ bath study. Contractile response to muscarinic (carbachol) and adrenergic (phenylephrine) stimuli are seen on normal urethra and tissue engineered (seeded) grafts retrieved 6 months after surgery.

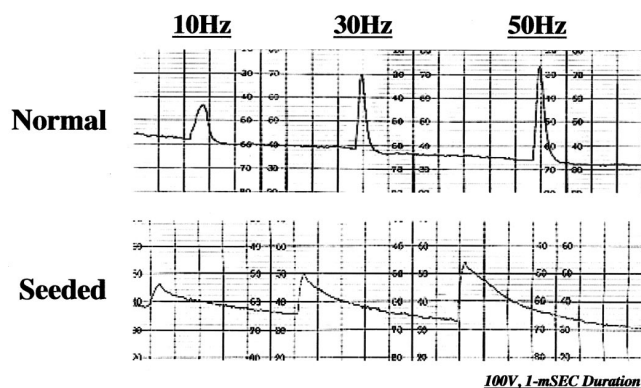


FIG. 6. Electrical field stimulation (100 v, 1 millisecond in duration) demonstrates similar contractile potentials with seeded grafts compared to normal urethra at various electrical frequencies.

mal model. Our results support previously documented findings that unseeded tubularized bladder submucosa scaffolds have a propensity for stricture formation.^{1,3} The seeded implants demonstrated similar phenotypic and histological makeup to native urethra with a normal uroepithelium surrounded by increasingly well developed muscle bundle fibers and evolving neovascularity. Results from serial urethrography demonstrated preservation of functional integrity while organ bath studies provided evidence of an intact neuromotor pathway.

The advantages of using bladder submucosa as a substrate for 1-stage onlay repair for hypospadias have been previously reported.^{1,28} These matrices are easily prepared in large quantities and stored until use. They eliminate the need to perform additional tissue harvest procedures because the tissue engineered grafts offer the advantage of already being prefabricated in the laboratory, thus reducing operative time and decreasing morbidity.

Acellular collagen matrices have been used in an onlay fashion successfully in patients with hypospadias and urethral strictures.^{1,29} Our study suggests that use of unseeded tubularized bladder submucosa collagen scaffolds would lead to increased fibrosis and urethral strictures. When tubularized urethral replacement is required, use of a cell seeded collagen scaffold may be necessary. Tubularized cell seeded constructs can be tailored to various dimensions and could be applied to a variety of conditions afflicting the urethra when an onlay repair may not be optimal.

CONCLUSIONS

The results of this study show that collagen matrices seeded with cells can be used for tubularized urethral replacement, whereas tubularized bladder submucosa collagen matrices alone without cells lead to poor tissue formation and strictures. The advantages afforded by these cell seeded matrices are maintenance of a nonobstructive outflow from the bladder and a readily available source of autologous tissue already prefabricated in the laboratory for direct implementation. The cell seeded matrices form new tissue that is histologically and functionally similar to native urethra. This technology may be useful clinically in the future.

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EDITORIAL COMMENT

The authors present a potentially clinically useful technology for urethral replacement using seeded acellular bladder submucosa matrices. They also demonstrate that this type of collagen matrix in an unseeded form does not promote urethral regeneration. From this

article one could speculate that all forms of unseeded acellular collagen matrices do not promote urethral regeneration but this may not necessarily be true. It is important to remember that all biomaterials and collagen matrices are not the same, and that the results presented in this article cannot be applied to other similar materials. It is well known that the method of decellularization and sterilization can significantly change the intrinsic properties of any biomaterial. A harsh decellularization process, as that described in this article, clearly renders a collagen matrix somewhat inert, thereby making regeneration without cells unsuccessful because the decellularization process inactivates the intrinsic growth factors or regulatory proteins that may play a significant role in the successful regeneration. In our laboratory we have worked with several different collagen matrices. We have found that the exact same collagen matrix can be altered with regard to its regenerative properties by processing it in different ways. Therefore, even if 1 investigator is using the same collagen matrix, the method of decellularization and sterilization of that collagen matrix must also be evaluated as a potential variable that can affect the regenerative results. Thus, until we understand the basic mechanism of action for the regenerative process, tissue engineering research will continue to be inhibited by varying and discrepant results from different materials and different methods of processing.

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REPLY BY AUTHORS

We agree that the porcine derived collagen matrix used in our study is somewhat inert. We have tried several noninert collagen matrices in the past, including bladder submucosa and small intes-

tinal submucosa, and were disappointed in the regenerative variability observed, even when the same decellularization and sterilization protocols were used.^{1,2} The variability observed with the collagen matrix regenerative process is also largely due to the different growth factor milieu present in each animal. Unfortunately, the individual growth factor milieu varies widely from one animal to the next, even within the same herd of animals.

The only way to avoid the animal-to-animal variability of the collagen matrices, regardless of their source, would be to genetically engineer animals with a specified growth factor milieu and through nuclear transfer clone a genetically identical herd of animals, clearly not an economically viable option today.³ Therefore, we believe that with the matrices currently available for tissue engineering, the best option involves the use of relatively inert matrices. Accurate concentrations of growth factors can be added to the defined inert matrices for the induction of controlled cell regeneration.

Regardless of the matrix used, we have also found that the maximum distance for normal regeneration in vivo is approximately 1 cm. from the edge of normal native tissue. Therefore, for large tissue defects (1 cm. or longer), growth factor are helpful for regeneration but the presence of transplanted cells is essential for the engineering of normal tissues.

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