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TOPIC PAPER

Tubularized urethral replacement with unseeded matrices: what is the maximum distance for normal tissue regeneration?

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Abstract
Purpose Complete urethral replacement using unseeded matrices has been proposed as a possible therapy in cases of congenital or acquired anomalies producing significant defects. Tissue regeneration involves fibrin deposition, re-epithelialization, and remodeling that are limited by the size of the defect. Scar formation occurs because of an inability of native cells to regenerate over the defect before fibrosis takes place. We investigated the maximum potential distance of normal native tissue regeneration over a range of distances using acellular matrices for tubular grafts as an experimental model.
Materials and methods Tubularized urethroplasties were performed in 12 male rabbits using acellular matrices of bladder submucosa at varying lengths (0.5, 1, 2, and 3 cm). Serial urethrography was performed at 1, 3, and 4 weeks. Animals were sacrificed at 1, 3, and 4 weeks and the grafts harvested. Urothelial and smooth muscle cell regeneration was documented histologically with H&E and Masson's trichrome stains.
Results Urethrograms demonstrated normal urethral calibers in the 0.5 cm group at all time points. The evolution of a stricture was demonstrated in the 1, 2, and 3 cm grafts by 4 weeks. Histologically all grafts demonstrated ingrowth of urothelial cells from the anastomotic sites at 1 week. By 4 weeks, the 0.5 cm grafts had a normal transitional layer of epithelium surrounded by a layer of muscle within the wall of the urethral lumen. The 1, 2, and 3 cm grafts showed ingrowth and normal cellular regeneration only at the anastomotic edges with increased collagen deposition and fibrosis toward the center by 2 weeks, and dense fibrin deposition throughout the grafts by 4 weeks.
Conclusions The maximum defect distance suitable for normal tissue formation using acellular grafts that rely on the native cells for tissue regeneration appears to be 0.5 cm. The indications for the use of acellular matrices in tubularized grafts may therefore be limited by the size of the defect to be repaired.

Keywords Hypospadius · Urethral stricture · Tissue engineering · Urothelium · Urethroplasty · Fibrosis · Collagen · Transitional epithelium

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Introduction

Obtaining adequate biocompatible tissue for extensive surgical reconstruction in patients undergoing hypospadius repair or urethroplasty remains a significant challenge to urologic surgeons today. Past experience has resulted in the successful utilization in selected cases of genital skin, tunica vaginalis, buccal mucosa, and bladder urothelium among other sources. [5–11] However, each of these frequently used donor tissue types has been shown to have a significant rate of complications on long-term follow-up, including stricture, hair growth, bladder prolapse, and donor site morbidity [12–17]. Recently, it has been shown that the use of

acellular tissue matrices composed of processed porcine collagen is a feasible alternative to native tissues in the form of onlay grafts for urethroplasty and hypospadius repairs [3, 19]. This affords the use of a larger quantity of graft tissue without donor site morbidity and with potentially reduced operative times. When applied to the formation of tubularized grafts, however, the use of these acellular matrices has shown significant limitations. In a study published by the Boston Children's group in 2002, all 12 rabbits in whom a 1-cm segment of anterior urethra was replaced by a tubularized graft composed of acellular tissue matrix suffered complete stricture formation of the affected segments at the 1-month mark [7]. Microscopic analysis of these grafts demonstrated complete absence of normal urothelial and submucosal histology as well as extensive scarring. In addition, these grafts did not demonstrate organized smooth muscle or the capacity to contract in response to cholinergic, adrenergic, or electrical stimulation. Conversely, all 12 rabbits in which a 1 cm segment of anterior urethra was replaced by a tubularized graft composed of a similar collagen tissue matrix seeded with bladder urothelial cells demonstrated normal urethral architecture within the graft segment at the 1-month mark. Microscopic analysis of these grafts revealed transitional cell epithelium with underlying organized smooth muscle capable of normal contractility in response to cholinergic, adrenergic, and electrical input. This study was thus very encouraging in that it supported the concept that through seeding collagen scaffolds with living urothelium, physiologically functional tubularized grafts mimicking normal urethra can be reliably created.

One limitation of the aforementioned study is that only grafts of 1 cm length were studied. With the knowledge that acellular collagen scaffolds have been successfully used as onlay type grafts in past studies and in current clinical practice, it seems feasible that the same unseeded scaffolds may be useful in the creation of tubularized grafts of shorter length. Using unseeded grafts, if successful, would potentially be advantageous in terms of cost and labor, providing a simpler, more readily available alternative to the technology of seeded matrices. We thus resolved to study the effects of length on the success rate of tubularized grafts using acellular tissue matrices, and to determine if there is a demonstrable limit to the urethral defect length in which an acellular matrix would suffice for replacement.

Materials and methods

Acellular collagen matrix

The collagen matrix was obtained through previously described methods [1, 2, 4, 7]. 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 h and with 1 l ddH2O and 0.2% Triton X-100 (2 ml) and 0.3 ml ammonium hydroxide for 14 days. The submucosa was then washed for 24 h with 1 l of distilled water and placed in a 10% cefoxitin solution. Random samples were obtained after processing for histological analyses, and 5 cm × 5 cm patches of submucosa cut from these samples. These patches were trimmed to 1, 2, and 3 cm long segments and tubularized around a 16Fr catheter. Several interrupted 6-zero polyglactin sutures were used to fasten the free edges of the graft together. Four marking sutures of nonresorbable 5-zero polypropylene were placed on the external surface of the graft, two at each anastamotic edge on opposite sides of the graft lumen. The grafts were dried and lyophilized for 24 h, and sterilized with ethylene oxide. Before use the grafts were further sterilized with ultraviolet light for 24 h.

Urethral surgery and post-operative evaluation

A total of 12 animals, all New Zealand white rabbits, were used for this study. The animals were anesthetized and maintained with 2–3% isoflurane after pretreatment with 15–20 mg/kg ketamine, 2–3 mg/kg xylazine, and 0.75 mg/kg acepromazine intramuscularly. Penile urethral defects comprising an entire segment of anterior urethra were created of varying lengths (0.5 cm in three animals, 1 cm in three animals, 2 cm in three, and 3 cm in the remaining three). The tubularized acellular matrix graft was then interposed and secured with interrupted 6-zero polyglactin sutures on both ends. The urine was diverted with a 14Fr urethral catheter for 7 days post-operatively. The animals were kept on a fluoroquinolone antibiotic until the catheter was removed. Post-operatively the animals were observed for any voiding difficulties. Retrograde urethrograms and graft harvest were performed at 1, 3 and 4 weeks after implantation. Animals were euthanized at 1, 3, and 4 weeks post-implantation using the anesthetic drug regimen described previously plus a 2 cc intracardiac injection of sodium pentobarbital. 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, native urethral tissue was also recovered in the same fashion from each animal.

Immunocytochemical and histological analyses

Five micrometer sections of formalin-fixed, paraffin-embedded tissues were processed and stained with hematoxylin and eosin, and Masson'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 smooth muscle actin antibodies (Novocastra, Newcastle, United Kingdom). Immunolabeling was performed using the avidin–biotin detection system and sections were counterstained with hematoxylin.

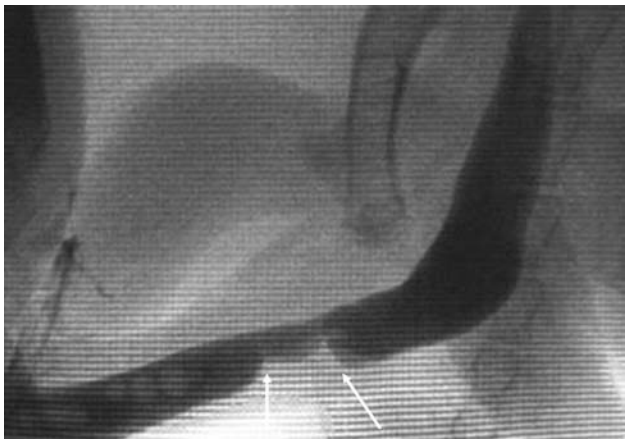

Fig. 1 Representative urethrogram indicating stricture formation in an animal receiving an acellular graft

Results

Retrograde urethrography demonstrated normal urethral calibers in the 0.5 cm group at all time points. The evolution of a stricture was demonstrated in the 1, 2, and 3 cm grafts by 4 weeks post-implantation (see Fig. 1 for a representative urethrogram). Histologically, ingrowth of urothelial cells from the anastomotic sites was evident in all grafts at 1-week post-implantation. By 4 weeks, the 0.5 cm urethral grafts demonstrated a normal transitional layer of epithelium surrounded by a layer of muscle tissue (Fig. 2). The 1, 2, and 3 cm grafts showed cellular ingrowth and regeneration only at the anastomotic edges of the material, with increased collagen deposition and fibrosis toward the center by 2 weeks. Dense fibrotic deposition, visualized using Masson's trichrome staining, was evident throughout each of the long grafts by 4 weeks (Fig. 3). Immunocytochemical and histological analyses indicated that these grafts also did not support the growth of normal smooth muscle tissue (data not shown).

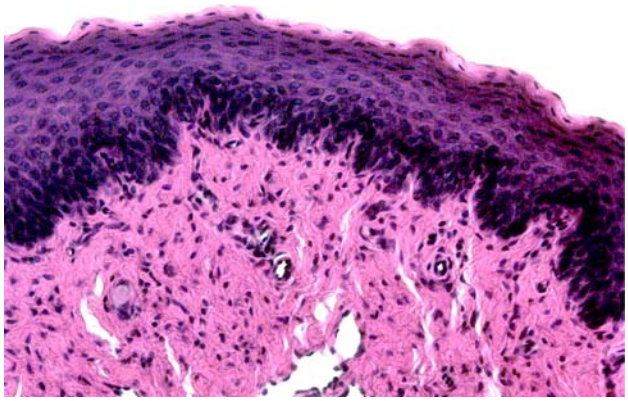

Fig. 2 Hematoxylin and eosin staining of a 0.5 cm acellular urethral graft 4 weeks after implantation. A transitional epithelial layer and a smooth muscle layer can be visualized

Discussion

In recent studies, it has been demonstrated that seeding of collagen matrices with bladder urothelial cells is an effective method of engineering viable tubularized grafts for use in the repair of large urethral abnormalities [7]. Cell seeded matrices seem to allow tissue regeneration across the defect and the maintenance of a nonobstructive outflow from the

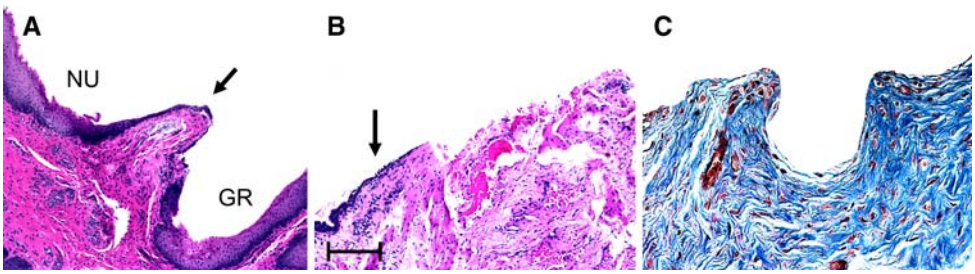

Fig. 3 Histology comparing short and long grafts. **a** Hematoxylin and eosin staining of the anastomosis between normal urethral tissue (NU) and the acellular graft (GR) in the 0.5 cm defect indicates ingrowth of normal cell types. **b** Representative cross section of a longer graft. *Arrow* indicates increased fibrosis and a friable epithelial surface, beginning approximately 0.5 cm from the tissue-to-graft anastomosis. **c** Masson's trichrome staining of a longer graft confirms the increased collagen deposition that is characteristic of fibrosis

bladder. Future research into refining the techniques involved in engineering these seeded grafts appears to be a promising avenue for progress in the field of urethral reconstructive surgery. However, in cases in which a readily available source of autologous bladder tissue for use in seeding the matrix is not available due to disease or injury, a prefabricated, cell-free graft material that could support regeneration would be an ideal solution.

The advantages of using bladder submucosa as a substrate for 1-stage onlay repair for hypospadias and urethral strictures have been previously reported [1, 18]. This type of matrix is easily prepared in large quantities and can be stored until use. The use of prefabricated tissue substitutes eliminates the need to perform additional tissue harvest procedures, reduces operative time and decreases patient morbidity [9]. Our study suggests that the use of unseeded tubularized bladder submucosa collagen scaffolds may be successful when used for repair of relatively small urethral defects. In conjunction with the findings of our prior research [7], this study also suggests that when tubularized grafts over 1 cm are required for repair, use of a cell seeded collagen scaffold may be necessary to allow the growth of healthy urethral tissue and avoid stricture formation.

The well-documented observation that small urethral defects are often successfully repaired with primary reanastamosis would seem to limit the potential value of an acellular tubularized matrix graft that is successful only when applied to similarly small defects, and thus limit the practical value of this study. However, the purpose of this investigation was to determine the largest gap across which regeneration could occur when an acellular tubularized graft was used to repair the urethra. From the results presented above, it may be inferred what the maximum distance for sufficient "auto-regeneration" may be. In spite of the relatively restrictive limits demonstrated in this experiment, unseeded tubularized matrices may still be useful when applied as an adjunct in repair of defects that are slightly above the maximum distance for reliable primary reanastamosis. Additionally, unseeded matrices of other biologic and nonbiologic materials in future investigations may prove more effective than the matrix used in this study. In addition, improvements in the techniques used for the harvest and preparation of tissue-derived matrices may also lead to the development of biological scaffolds that can support tissue regeneration across longer gaps.

Conclusions

The maximum defect distance that can support normal tissue formation using acellular tubularized grafts, which rely on native cells for tissue regeneration, appears to be 0.5 cm. This study may partially explain the discrepancy observed over the last decade regarding the success of acellular matrices for tissue regeneration in small animals and their failure in large animals and patients. The indications for the use of acellular matrices for tubularized grafts produced with present technology may thus be limited by the size of the defect to be repaired. The repair of larger defects would appear to require the use of tubularized grafts manufactured from cell seeded matrices, which have previously been proven to result in higher success rates [7].

Conflict of interest statement None.

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