Tissue Engineering of a Genitourinary Tubular Tissue Graft Resistant to Suturing and High Internal Pressures


The aim of this study was to evaluate the possibility of constructing a fully autologous tissue-engineered tubular genitourinary graft (TTGG) and to determine its mechanical and physiological properties. Dermal fibroblasts (DFs) were expanded and cultured in vitro with sodium ascorbate to form fibroblast sheets. The sheets were then wrapped around a tubular support to form a cylinder. After maturation, urothelial cells (UCs) were seeded inside the DF tubes, and the constructs were placed in a bioreactor. The TTGGs were then characterized according to histology, immuno-histochemistry, Western blot, cell viability, resistance to suture, and burst pressure. Results obtained were encouraging on all levels. All layers of the TTGGs had merged, and a pluristratified urothelium coated the luminal surface of the tubes. The burst pressure of non-sutured TTGGs was measured and found to be, on average, three times as resistant as that of porcine urethras. Suturing was accomplished without difficulty. Results have shown that our construct can sustain an entire week of pulsatile stimulation without loss of mechanical or histological integrity. The tissue-engineering technique used to produce this model seems promising for bioengineering a urethra or ureter graft and could open a doorway to new possibilities for their reconstruction.

Introduction

Various conditions such as congenital defects and acquired disorders of the urethra or ureter may lead to damage or loss, often requiring tissue reconstruction. Only a small quantity of genitourinary tissue or mucosa is available for grafting, and other sources of tissue are frequently used (e.g., skin flaps, buccal mucosa, bowel). Initial results are encouraging, but long-term follow-up shows significant complications, such as fistulas, hair growth, and graft contracture. To overcome these problems, several investigators have used cylindrical biodegradable biomaterials such as acellular scaffolds for the replacement or reconstruction of urethras. The studies using unseeded acellular matrices were experimentally and clinically successful for onlay urethral repairs (half-pipe). When a tubulized repair was attempted, tissue regeneration was not achieved. The results obtained established a minor functional compatibility with the native urethra, and it was demonstrated that the presence of cells was necessary to prevent strictures from occurring.

The need to develop other treatment options and a more thorough understanding of cell biology has led us toward tissue engineering for therapeutic use. The clinical need to find a suitable and reliable method to replace original urogenital tissue is essential. Therefore, we investigated the feasibility of constructing a tissue-engineered tubular genitourinary graft (TTGG) made entirely with autologous cells and their own cell-produced extracellular matrix, using the self-assembly technique developed at our laboratory. Our goal is to create functional tissues such as corneal, blood vessel, skin, and now urogenital constructs without the use of exogenous matrices. A histological analysis and evaluation of the constructs’ mechanical properties assessed the method’s capability of producing structurally adequate grafts with acceptable mechanical properties. To our knowledge, this technique is a novel approach that could eventually bring important changes to urethral and ureteral reconstructions.

Materials and Methods

Cell isolation

Dermal fibroblasts (DFs) were isolated from human skin biopsies as previously described using 0.2 U/mL collagenase H (Roche Diagnostics Canada, Laval, Canada) and cultured in Dulbecco-Vogt modified Eagle medium (DMEM; Invitrogen, Burlington, Canada) supplemented with 10% fetal bovine serum (FBS; HyClone, Fisher Scientific, Ottawa, Canada) and antibiotics, 100 U/mL penicillin (Sigma,
Oakville, Canada) and 25 µg/mL gentamicin (Schering, Pointe-Claire, Canada), hereinafter referred to as DF medium. The cells were placed in a humidified incubator at 37°C with 8% carbon dioxide (CO₂), and the medium was changed three times a week.

The urothelial cells (UCs) were obtained as previously described from a small bladder biopsy and cultured with 1.5 × 10⁸ irradiated fibroblasts (as a feeding layer for the UCs) in DMEM-Ham containing 10% FBS, 5 µg/mL insulin (Sigma), 0.4 µg/mL hydrocortisone (Calbiochem, San Diego, CA), 10⁻¹⁰ M cholera toxin (ICN, Saint-Laurent, Canada), 10 µg/mL epidermal growth factor (Austral Biologicals, San Ramon, CA), 100 U/mL penicillin, and 25 µg/mL gentamicin, hereinafter referred to as UC medium. The cells were cultured in a humidified incubator with 8% CO₂ at 37°C, and the medium was changed three times a week.

Genitourinary tubular tissue construct

DFs were seeded on gelatin-coated 500-cm² cell culture dishes (Corning Inc., Corning, NY) at a concentration of 1 × 10⁴ cells/cm². They were cultured in DF medium supplemented with 50 µg/mL ascorbic acid (Sigma) for 27 days, and the medium was changed three times a week. On day 27, the DF sheets (comprising cells and extracellular matrix) were separated into two sections, and each segment was tightly rolled around a sterile plastic tubular support (6.5 mm in diameter) to produce a cylinder of approximately 10 concentric sheet layers. The DF cylinder was cultured in DMEM-Ham containing 10% FetalClone II serum (Hyclone), 100 U/mL penicillin, 25 µg/mL gentamicin, and 50 µg/mL ascorbic acid for 21 more days to allow the layers to merge. Burst resistance and histology were assessed on some of the constructs at maturation days 14 and 21.

At day 21, the plastic tubular supports were slipped out of the DF tubes to allow them to be cannulated at both ends (Fig. 1A) and placed in a sterile chamber, allowing for separation of the luminal and external culture media. UCs were removed from their culture flasks using trypsin/ethylenediaminetetraacetic acid (Valent Canada Limited, Montreal, Canada), centrifuged for 10 min at 1180 rpm, and suspended at a concentration of 1 million/suspension volume in UC medium. Intraluminal cell seeding was performed similarly to a technique previously described. Briefly, 3 mL of the UC suspension was inserted into the luminal region of the DF tube. Some DF tubes remained without seeding to allow for comparison of the mechanical properties and histology with the seeded tubes. The chambers were placed onto a bottle-roller (Wheaton Instruments, Millville, NJ) at low speed (~1 rpm) for 2 h to allow uniform seeding of the UCs. The DF tubes, with or without UC seeding, were then placed in a custom-made bioreactor (Fig. 1D) to allow intraluminal flow (Ismatek pump, Cole-Parmer Canada Inc., Montreal, Canada) of UC medium and positioned in a humidified incubator with 8% CO₂ at 37°C. Other DF tubes, with or without UC seeding, were kept under the same conditions, but without intraluminal perfusion, to evaluate the effects of the bioreactor on the constructs. The extraluminal medium was the same as that used for the maturation of the DF tubes, whereas the internal medium was the one for the UCs. The constructs were kept in these conditions for a week, with a renewal of the internal and external media after 4 days.

Mechanical strength

DF tubes or TTGGs at maturation periods of 14, 21, and 28 days (28 being 21 days of maturation plus 1 week with or without perfusion in bioreactor) were mounted in an apparatus that exposed the tissue-engineered urethra or ureter to hydrostatic loading using phosphate buffered saline (PBS) while monitoring its internal pressure (Fig. 2). Through a computerized automation program designed using LabView (National Instruments, Austin, TX), a stepper motor (Excitron Corp, Boulder, CO) was used to compress a syringe inserted between the two plates of a vise. Flow rate was kept constant.
at 8 mL/min until the construct burst. To eliminate system compliance, rigid tubing (Cole-Parmer Canada Inc.) was used to connect the set-up to a pressure transducer (Cole-Parmer Canada Inc.). The pressure transducer was used to monitor the pressure variations inside the graft via a data acquisition card (National Instruments, TX). Burst pressure was considered to be the maximum recorded pressure before failure of the graft. Visual inspection of the plotted pressure curves allowed for easy reading of burst pressure, analysis of construct behavior, and the assessment of proper test conditions. Freshly obtained porcine urethras were used to determine the gold standard for the constructs.

Resistance to suture

Sections of the TTGGs, after 28 days of maturation (including 1 week in the bioreactor) were sutured together using a 6-0 synthetic absorbable suture (Tyco, Pointe-Claire, Canada) (Fig. 1B). They were then mounted on the same apparatus as described previously to determine the maximum pressure that the suture could be exposed to before leaking.

Histological characterization

Sections of the DF tubes and of the TTGGs were fixed in 1x Histochoice tissue fixative (Amresco, Solon, OH) and embedded in paraffin. Histology sections (4 μm) were stained with Masson’s Trichrome. Internal diameters of the constructs were measured at the same time points as the mechanical resistance testing. For indirect immunofluorescence staining, frozen sections of the reconstructed tubular genitourinary tissue (5 μm) were fixed in 100% methanol, blocked in phosphate buffered saline containing 1% (w/v) bovine serum albumin, and then incubated with monoclonal antibodies. The primary antibodies used were guinea pig immunoglobulin (IgG) anti-keratin 8/18 (dilution 1:1000) (ARP, ME) to visualize the urothelium and rabbit IgG anti-human collagen I (dilution 1:100) (Cedarlane Laboratories Ltd., ON, Canada) to stain the collagen produced by the DFs. The secondary antibodies used were coupled to rhodamine fluorochrome. For controls, the primary antibody was omitted. Nuclei were stained with Hoechst nuclear dye. The results were viewed using a Nikon eclipse E600 epifluorescence microscope (Nikon, Tokyo, Japan), and images were processed with Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA).

Cell viability

Two different methods were performed to evaluate the viability of the cells after the complete culture time. First, we extracted the cells from the reconstructed tissue, using the same protocols as for the extraction of the DFs or the UCs from the biopsies. The second method used for the DF was the explant method. Small pieces of the DF tube were placed in a cell culture dish with DF medium. Observation of the migration and of the multiplication of the cells was done at different time points. For both methods, the cells were placed in a 37°C humidified incubator with 8% CO₂, and the medium was changed three times a week until confluence was obtained.

Immunoblotting

For Western blotting, samples were ground manually in liquid nitrogen using a mortar and pestle; cell extracts were collected and analyzed using spectrophotometry. The samples were then boiled for 5 min to denature them. Electrophoresis of the samples (20 μg/lane) was done on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, Tokyo, Japan). Membranes were blocked with tris buffered saline-Tween 0.05% containing 5% skimmed milk and incubated with a 1:3000 dilution of the same IgG anti-keratin 8/18 (ARP) antibody used for the immunohistochemistry. The membranes were then treated with peroxidase-conjugated anti-mouse IgG-IgM, and signals were detected using electrogenerated chemiluminescent solution and exposing the blot to an X-ray film in a darkroom. As loading control, membranes were blotted with an anti-alpha-tubulin antibody.

Results

Morphology and histology

From a macroscopic point of view, the engineered tubular genitourinary tissue grafts were uniform and presented enough resistance to retain the medium pressure while in the bioreactor. The internal diameters of the cylindrical constructs were 6 mm (18Fr) before UC seeding and approximately 8 mm (24Fr) after 1 week in the bioreactor. The histology observed after Masson’s Trichrome staining of the tissue-engineered urethra or ureter graft showed that the DFs had formed a cohesive tubular tissue (Fig. 3A, B). Furthermore, a pluristratified urothelium, which contained four to six layers of cells, could be identified in the luminal area of the constructs (Fig. 3A, B). Some of the apical cells showed a differentiated morphology resembling umbrella cells.

The urothelium of the reconstructed genitourinary tube, stained with anti-keratin 8/18 antibodies (Fig. 3D), had the appearance of a normal porcine urethra epithelium. The keratin 8/18 is a specific marker of transitional epithelium which is found throughout the full urothelial thickness. The extracellular matrix, produced entirely by the fibroblasts, presented a uniform staining of collagen I (Fig. 3F), which is, along with collagen III, the most common type of collagen in the extracellular matrix.

The results obtained using Western blot confirmed the presence of urothelial cells inside the constructs and therefore supports the specificity of the marker used for immunohistochemistry (Fig. 4). Furthermore, the semi-quantitative results suggest that perfusion of the constructs might help urothelial growth or differentiation. The fact that there is a more important amount of keratin in the lanes where perfusion of the constructs occurred (lanes 3, 4, 5) supports this hypothesis. These results will be confirmed and studied more thoroughly in further experiments.

Mechanical testing

The apparatus developed in our laboratory allowed us to evaluate with accuracy the burst strength of the reconstructed genitourinary tissue grafts at rupture point (Fig. 5). Porcine urethras were tested as well to allow a comparison. After a maturation period of 2 weeks, the DF tubes were capable of resisting until a mean burst pressure of 803 ± 22 mmHg (n = 6). After 3 weeks of maturation, the DF tubes gained in resistance and displayed a mean burst pressure of 1133 ± 76 mmHg (n = 5), which represents an average augmentation.
of 330 mmHg. After adding a 1-week period in a bioreactor, the DF tubes with UC seeding obtained a mean rupture pressure of $1703 \pm 103$ mmHg ($n = 5$), and those without UC seeding obtained a rupture point of $1761 \pm 248$ mmHg ($n = 3$). The DF tubes that had a 4-week maturation period, without the bioreactor, obtained a rupture pressure of $1801 \pm 158$ mmHg ($n = 6$). On the other hand, the porcine urethras had a maximum-recorded burst pressure of 583 and a mean burst pressure of $418 \pm 66$ mmHg ($n = 4$), which is inferior to all maturation periods of the tissue-engineered grafts. For all of the maturation periods, the layers of the TTGGs remained firmly fixed to one another, forming a continuous tubular tissue. Furthermore, after 1 week in the bioreactor, the mechanical strength of the TTGGs and their histological integrity was maintained. Knowing that the constructs can retain their properties for at least 1 week in a pulsatile mode is appealing. These results show that UC seeding does not significantly improve or reduce the overall resistance of the constructs.

**Suturing**

Suturing of the TTGGs was easily performed, and the tissue did not tear where the stitches were made. After joining together two full sections of TTGGs, we obtained a long tubular construct approximately 14 cm long (Fig. 1C). When submitted to pressure, the freshly sutured constructs leaked at an internal pressure of approximately 40 mmHg (54 cm H2O).

**Viability**

Having to sustain a long period in culture, cell viability had to be confirmed to evaluate the potential of the TTGGs. Using the extraction method, both cellular types, UC and DF, were isolated and plated with a mortality rate of less than 2%, which is a percentage regularly obtained for any cell extraction. With the explant method, migration of the DFs from the reconstructed tissue was visible after a few hours, and cells started to colonize the dish after 24 h (Fig. 4B).
Discussion

The limited amount of autologous genitourinary tissue available for reconstructive surgery and the multiple complications associated with the use of existing materials have brought an increasing interest in developing new genitourinary tissue substitutes. In this article, we have demonstrated that it is possible to recreate a genitourinary tubular tissue graft using the self-assembly technique, without the use of exogenous material.

Furthermore, every tissue-engineered construct included in this study displayed better mechanical strength than our gold standard (porcine urethras), which shows our capacity to produce in vitro tissues suitable for grafting. Adding urothelial cells to our DF tubes did not decrease their mechanical resistance. Moreover, results have shown that our constructs can sustain an entire week of pulsatile stimulation without loss of mechanical or histological integrity. The development of this technique brings an alternative method for the reconstruction of the ureters and urethras that require reconstruction for a wide variety of congenital and acquired clinical conditions. Our tissue-engineered grafts contain no other material than the cells and their extracellular matrix. The cellular elements used to form these grafts are obtained from small biopsies acquired from the individual who needs to be grafted, which allows the production of a fully autologous substitute. An additional advantage that this type of graft offers is that it avoids the need for cellular ingrowth after implantation.

In the animal model, we are planning to replace portions of urethras or ureters. We will apply basic surgical principals for reconstruction in urology: end-to-end spatulated watertight anastomosis, tension-free stenting, and secondary coverage with vascularized tissue (retroperitoneal fat for ureters and dartos fascia for urethras). Because we have documented that our constructs are made of living cells and have the characteristics of a functional urologic tube, we foresee that the graft will take rapidly and act not only as a biological dressing. Fibrosis could still occur if vascularization of the graft is inadequate. Therefore, we want to optimize the model by adding endothelial cells within the fibroblasts layers, which would accelerate the inosculation of the model by secondary coverage. Our group has already documented this technique in the skin model, in which inosculation took place in 4 days instead of 14.18

In our upcoming studies, permeability and more histological characteristics of the urothelium will be evaluated to determine the functionality of the urothelial cells. These evaluations will reveal whether the tissue-engineered grafts have the ability to form an effective barrier that can retain urine. Ultimate tensile strength measurements will also be assessed to corroborate the burst pressure results, because we are limited to one burst measurement per construct. This technique will allow us to perform stretching until failure to multiple cylindrical sections for the same construct in its circumferential direction. On a more clinical level, grafting our model in vivo will also be a next step in our studies. We are presently evaluating the possibility of implanting completely autologous grafts to replace ureters in a porcine model and urethras in a rabbit model.

Conclusion

The development of this new reconstructive technique for the replacement of ureters or urethras represents a major
breakthrough and could have considerable clinical effect in urology. This innovative method could open a doorway to new possibilities of reconstruction and solve part of the problem related to insufficient quantities of urinary tissue. Moreover, tissue engineering of the ureter or urethra without the use of scaffolds offers an ideal replacement for these essential structures.

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