Tissue Engineering a Complete Vaginal Replacement From a Small Biopsy of Autologous Tissue

Roger E. De Philippo, Colin E. Bishop, Luiz Freitas Filho, James J. Yoo, and Anthony Atala

Background. In women, a healthy, patent vagina is important for the maintenance of a good quality of life. Apart from congenital abnormalities, such as cloacal exstrophy, intersex disorders, and an absence of the posterior two thirds of the organ, individuals may also suffer from cancer, trauma, infection, inflammation, or iatrogenic injuries leading to tissue damage and loss—all of which require vaginal repair or replacement. Of necessity, reconstruction is often performed with nonvaginal tissue substitutes, such as segments of large intestine or skin, which are not anatomically or functionally ideal (Hendren and Atala, *J Urol* 1994; 152: 752; Hendren and Atala, *J Pediatr Surg* 1995; 30: 91). Whenever such tissue is used additional complications often ensue, such as strictures, infection, hair growth, graft shrinkage, diverticuli, and even malignancy (Filipas et al., *BJU Int* 2000; 85: 715; Lai and Chang, *Changgeng Yi Xue Za Zhi* 1999; 22: 253; Parsons et al., *J Pediatr Surg* 2002; 37: 629; Seccia et al., *Ann Plast Surg* 2002; 49: 379; Filipas, *Curr Opin Urol* 2001; 11: 267).

Methods. Using a rabbit model, we report here the construction of a functional vagina using autologous cells expanded from a small vaginal biopsy.

Results. Six months after total vaginal replacement, radiographic analysis of rabbits implanted with the neovagina demonstrated wide, patent vaginal calibers without strictures. Histologic analysis revealed well-organized epithelial and muscle cell layers. Physiologic studies showed normal-range responses to electrical stimulation or to an adrenergic agonist.

Conclusions. These data indicate that a tissue engineering approach to clinical vaginal reconstruction in women is now a realistic possibility.

Keywords: Regenerative medicine, Tissue engineering, Vagina, Replacement organs.

(*Transplantation* 2008;86: 208–214)

Patients with congenital or acquired abnormalities of the vagina and patients with cervical, uterine, ovarian, rectal, and bladder cancer may require partial or total vaginal resection. Because of complications arising from the use of nonvaginal tissue a variety of biodegradable substitutes have been proposed such as decellularized bladder and collagen matrices (*1*). Such attempts at vaginal tissue replacement have usually failed because of mechanical, structural, functional, or biocompatibility problems. It is evident that native vaginal tissue, with its specific functional properties, is preferable for reconstruction. Unfortunately, the limited amount of native vaginal tissue available for reconstruction generally precludes this option. A new approach to the problem has recently been provided by the successful use of bioengineered tissues such as skin, bone, cartilage, and urinary bladder in a number of clinical situations (*2–5*). In an attempt to apply such an approach to vaginal reconstruction, we previously demonstrated the growth and differentiation potential of vaginal cells seeded onto polyglycolic acid (PGA)/poly-(lactide-*co*-glycolide) (PLGA) scaffolds when placed under the skin of immunocompromized nude mice (*6*). Here, we report a total vaginal replacement in the rabbit using a neovagina bioengineered from small fragments of autologous vaginal tissue. Ideally, this approach might allow lost tissue function to be restored or replaced in toto with limited complications such as transplant rejection.

MATERIALS AND METHODS

Cell Source

Vaginal tissue biopsies (~1 cm^2) were harvested from female New Zealand White rabbits using a simple midline transabdominal incision. Smooth muscle cells were extracted by the explant method. Several muscle strips were carefully dissected from the seromuscular layer of the vaginal tissue under loop magnification. These pieces were individually placed in culture dishes and then incubated with Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supple-

Wake Forest Institute for Regenerative Medicine, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina; Currently (R.E.P.) Childrens Hospital Los Angeles, Division of Urology, Keck School of Medicine, University of Southern California, Los Angeles, California; Currently (L.F.F.) Urologia Pediátrica, Hospital Infantil Darcy Vargas, Sao Paulo, Brazil.

Address correspondence to: Anthony Atala, M.D., Wake Forest Institute for Regenerative Medicine, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157.

E-mail: aatala@wfubmc.edu

Received 7 January 2008. Revision requested 29 January 2008.

Accepted 28 March 2008.

ISSN 0041-1337/08/8602-208

DOI: 10.1097/TP.0b013e31817f1686

mented with 10% fetal bovine serum (Life Technologies, Rockville, MD) at 37°C in air and 5% CO_2, and were left undisturbed until a sufficient colony of cells grew from the tissue islets. The explants were removed by gentle suction and the cells were maintained with scheduled replacement of the medium every 48 hr. Epithelial cells were isolated from the vaginal specimens by enzymatic digestion with collagenase type IV (Worthington Biochemical, Lakewood, NJ) and dispase (Boehringer Mannheim, Indianapolis, IN). The vaginal tissue was immersed into the enzymatic solution, and vigorously shaken for 30 min at 37°C. With gentle pipette suction, the cell–fluid suspension was centrifuged at low revolutions for 5 min. The supernatant was resuspended in keratinocyte serum-free medium (Life Technologies), distributed into culture dishes, and maintained in keratinocyte serum-free medium with medium changes for every 24 to 48 hr. Each cell type was expanded to approximately fifty 15-cm polystyrene culture dishes to achieve a desired cell density of 10×10^6 cells/cm^3 for epithelial cells and 20×10^6 cells/cm^3 for smooth muscle cells (*6*). No additives were used to prevent fibroblast growth. Cell doubling times were approximately 36 hr for both cell types; such an expansion could be achieved in less than 4 weeks and cells were used at passage five or less to avoid any cytogenetic abnormalities that could possibly be introduced by extended in vitro culture (*2*, *7*). Epithelial and smooth muscle cell phenotypes were confirmed by morphology and immunofluorescence staining with antibodies to pan cytokeratins (AE1/AE3,) and smooth muscle α-actin, respectively. Immediately before seeding expanded cells were labeled with fluorescent cell linker membrane tracer dyes (*8*) (PKH26, red for muscle; PKH2 green for epithelia), following the manufacturer's protocols (Sigma-Aldrich, MO). Fluorescent microscopy confirmed that green- and red-labeled cells attached to the polymer fibers after dynamic seeding had been completed.

Construction of Neovaginas

Tubular scaffolds approximately 5 cm×2 cm×2 to 3 mm diameter (~6 cm^3 scaffold volume) were constructed using PGA polymers (Albany International, Mansfield, MA) consisting of 15 μm fibers, with an interfiber distance of 100 to 200 μm and a porosity of 95%. Polyglycolic acid was first preconfigured into a three-dimensional vaginal shape with 5-0 polyglycolic sutures. Adjacent PGA fibers in the scaffolds were bonded at cross points by spraying with a solution of PLGA (50:50 copolymer) dissolved in 5% wt/vol chloroform and subsequently dipping into PLGA solution. The scaffolds were kept under vacuum for 2 days to remove any residual solvent, sterilized with ethylene oxide gas, and wetted in Dulbecco's modified Eagle's medium for 24 hr before cell seeding. Labeled vaginal epithelial and smooth muscle cells were seeded sequentially onto the inner and outer surfaces of the coated scaffolds, respectively, by slowly dropping the cell suspension from a pipette. The epithelial cells were seeded at 10^7 cells/cm^3, and the smooth muscle cells at 2×10^7 cells/cm^3. Each type of cell was applied three times over a period of 3 days to ensure appropriate scaffold coverage. The seeded constructs were then cultured in a spinner flask bioreactor for a further 7 days at 37° in a humidified atmosphere with 5% CO_2. This bioreactor contained 500 mL of 1:1 mixture of muscle cell and keratinocyte medium constantly stirred at 100 *g*.

Surgical Implantation

Scaffolds were implanted in place of the native vagina in 15 female New Zealand White rabbits. Unseeded control scaffolds were implanted in six rabbits, whereas cell-seeded scaffolds were implanted into each of nine autologous donor or recipients. The matrices were anastomosed distally to the introitus and proximally to the cervix with interrupted vycril sutures. All of the grafts were covered circumferentially with omentum to increase vascularity. Radiographic vaginograms and tissue analyses were performed preoperatively and at 1, 3, and 6 months after implantation.

Histologic and Immunohistochemical Analysis of Tissue

Normal vaginal and engineered tissues were fixed in 4% paraformaldehyde wax embedded. Immunohistochemical analysis was performed using the Vectastatin Elite ABC peroxidase kit (Vector Laboratories) according to the manufacturer's protocol. Briefly, after blocking endogenous peroxidase activity with DAKO Peroxidase Blocking Reagent (DAKO, CA), sections were incubated overnight at 4°C with primary antibody. Sections were then incubated for 30 min with biotinylated secondary antibody solution diluted in phosphate-buffered saline, followed by Vectastatin Elite ABC Reagent diluted in phosphate-buffered saline for 30 min. Visualization of immunoreactivity was achieved by incubating sections in the DAB peroxidase substrate kit (Vector Laboratories) for approximately 10 min. Identification of PKH fluorescence was performed on frozen sections according to standard procedures. Monoclonal antibodies used were pan-cytokeratin AE1/AE3 (clone 3515, Dakocytomaton, Carpinteria CA), α-actin (clone CGA7, Chemicon, Temecula, CA), desmin (clone DE-R-11 novocastra, Newcastle, UK), neurofilament protein (clone CBL212 Chemicon), and collagen types I, II, and III (clone 1310, 1320, 1330, Southern Biotech, Birmingham, AL). The total collagen content of tissue samples was assessed using the Sircol assay system (Accurate Chemical & Scientific Corporation Westbury, NY) following the manufacturer's protocol.

Biomechanical and Physiological Analysis

Rectangular tissue strips were obtained from normal and engineered vaginal implants. Tensile tests (Instron model 5544, MA) were performed by elongating the tissue strips longitudinally at a speed of 0.05 mm/sec (*9*). Stress or strain curves for each specimen were generated, and the tensile strength and strain forces (MPa) were determined. The maximum tensile strain, which was determined in response to the ultimate strength, was calculated as the elongated displacement ratio to initial length. For organ bath studies longitudinal strips of native vagina and tissue engineered neovaginas were assessed for contractile responses to electrical and pharmacological stimulation as previously described (*6*).

RESULTS AND DISCUSSION

Tubular-shaped biodegradable PGA/PLGA scaffolds, approximately 5 cm in length and 2 cm in diameter were

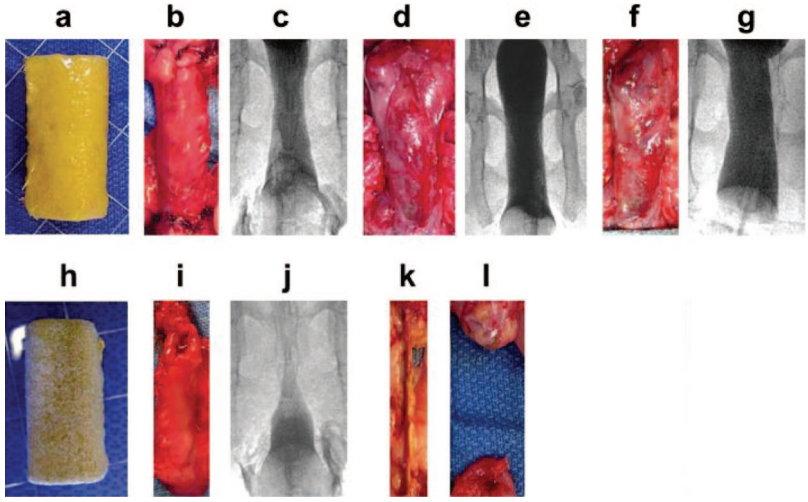

FIGURE 1. Appearance of tissue-engineered, neovaginas. (a) Tubular polymer scaffold after cell-seeding and 1 week in vitro culture, before implantation in vivo. (b, d, f) Gross appearance and (c, e, g) vaginography of cell-seeded constructs 1, 3, and 6 months, postimplantation, respectively. (h) Unseeded control scaffold before implantation. (i, k, l) gross appearance of unseeded construct at 1, 3, and 6 months postimplantation. (j) Vaginography of unseeded graft at 1 month.

seeded with autologous vaginal epithelial and smooth muscle cells that had been expanded separately in culture. After 1 week of growth in bioreactors, the cells grew to cover the polymer scaffolds (Fig. 1a). The seeded scaffolds or control, unseeded scaffolds (Fig. 1h) were then implanted into autologous female rabbits to fully replace the endogenous, normal vagina. Rabbits were killed at 1, 3, and 6 months postoperatively, and the implanted neovaginas were recovered for analysis. Gross examination of the cell-seeded constructs (Fig. 1b, d, f) showed progressive development of healthy,

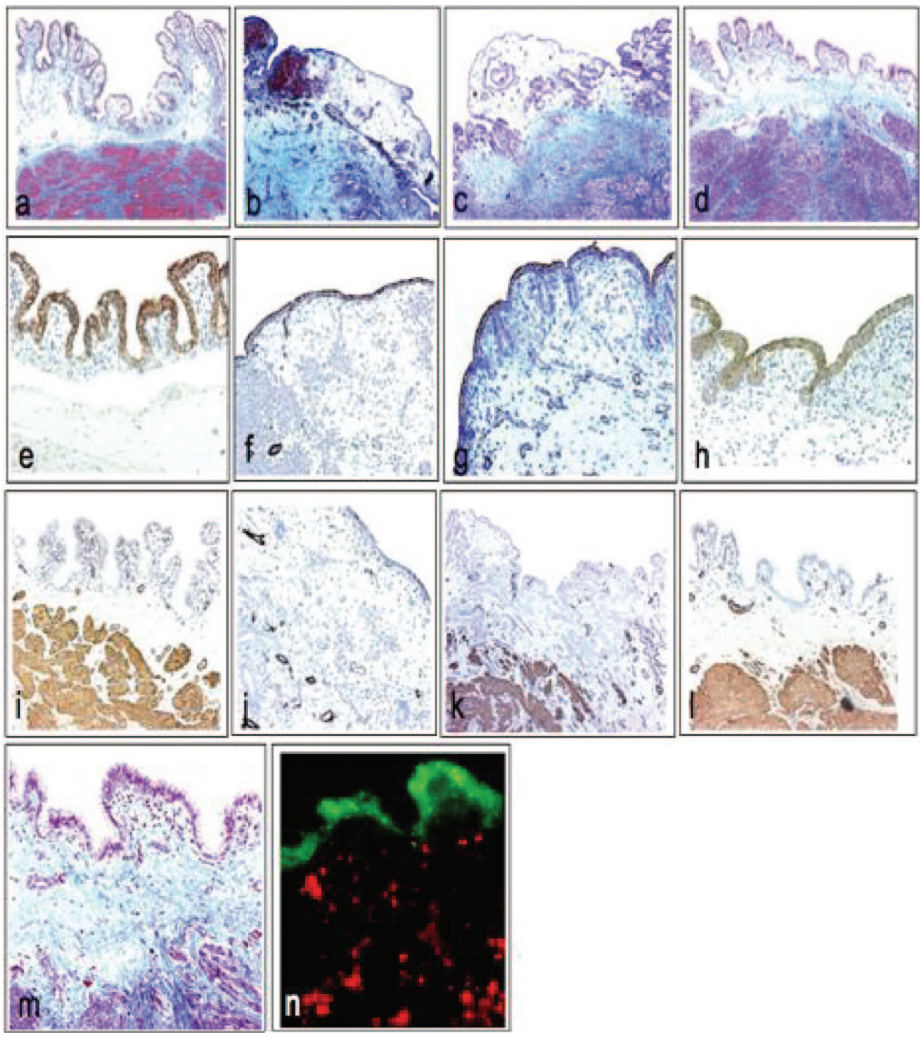

FIGURE 2. Histology and immunohistochemistry (IHC) of neovaginas. (a) Masson's trichrome (MTC) staining of normal vaginal tissue. Red staining indicates keratin and muscle, blue indicates collagen. (b) MTC of unseeded polymer constructs at 1 month postimplantation. (c, d) MTC staining of cell-seeded grafts at 1 and 6 months postimplantation. (e) Expression of cytokeratins AE1/AE3 detected by IHC (brown staining) in normal vagina. (f) Cytokeratin IHC of unseeded polymer constructs at 1 month. (g, h) Cytokeratin IHC of cell-seeded grafts at 1 and 6 months. (i) IHC expression of α-actin in normal vagina. (j) α-actin expression of unseeded graft at 1 month. (k, l) α-actin expression of cell-seeded grafts at 1 and 6 months. (m) MTC staining of cell-seeded graft at 3 months. (n) Prelabeled cells 3 months after engraftment: green—epithelial cells (PKH2); red—smooth muscle cells (PKH26). All cell magnifications are $\times$200.

well-vascularized tissue. As expected, by 3 months the polymer scaffolds had completely degraded. Serial radiographic vaginography of rabbits implanted with the cellularized constructs demonstrated the maintenance of full patency and a wide vaginal caliber at all time points (Fig. 1c, e, g). In fact, all nine seeded constructs maintained wide calibers throughout and good histologies 6 months out.

In contrast, gross morphology and vaginography indicated that all of the unseeded, control grafts had developed strictures or collapsed 1 month after surgery (Fig. 1i, j). At 3 months there was only a remnant thin streak of fibrotic scar tissue (Fig. 1k) and by 6 months all unseeded grafts had been completely resorbed (Fig. 1l).

Histologic analysis of the cell-seeded construct using Masson's trichrome showed that the typical multilayered architecture of a normal vagina (Fig. 2a) was already beginning to form 1 month after implantation (Fig. 2c). Grafts of the unseeded polymer scaffolds alone appeared disorganized and fibrotic (Fig. 2b). Six months after surgery the tissue structure of the seeded constructs closely resembled that of a normal vagina with defined layers of muscle and collagen (red and blue, respectively) and a luminal invaginated epithelium (Fig. 2d). Immunohistochemical staining for cytokeratins A1/A3, a marker of the epithelial cell lineage, showed that a layer of vaginal epithelial cells was apparent by 1 month (Fig. 2g) becoming clearly defined and developed by 6 months (Fig. 2h) and resembling that of a native vagina (Fig. 2e). Only a flattened, atrophied epithelial-like layer could be identified 1 month postimplantation in the unseeded polymer-alone grafts (Fig. 2f). Similarly, staining for smooth muscle specific

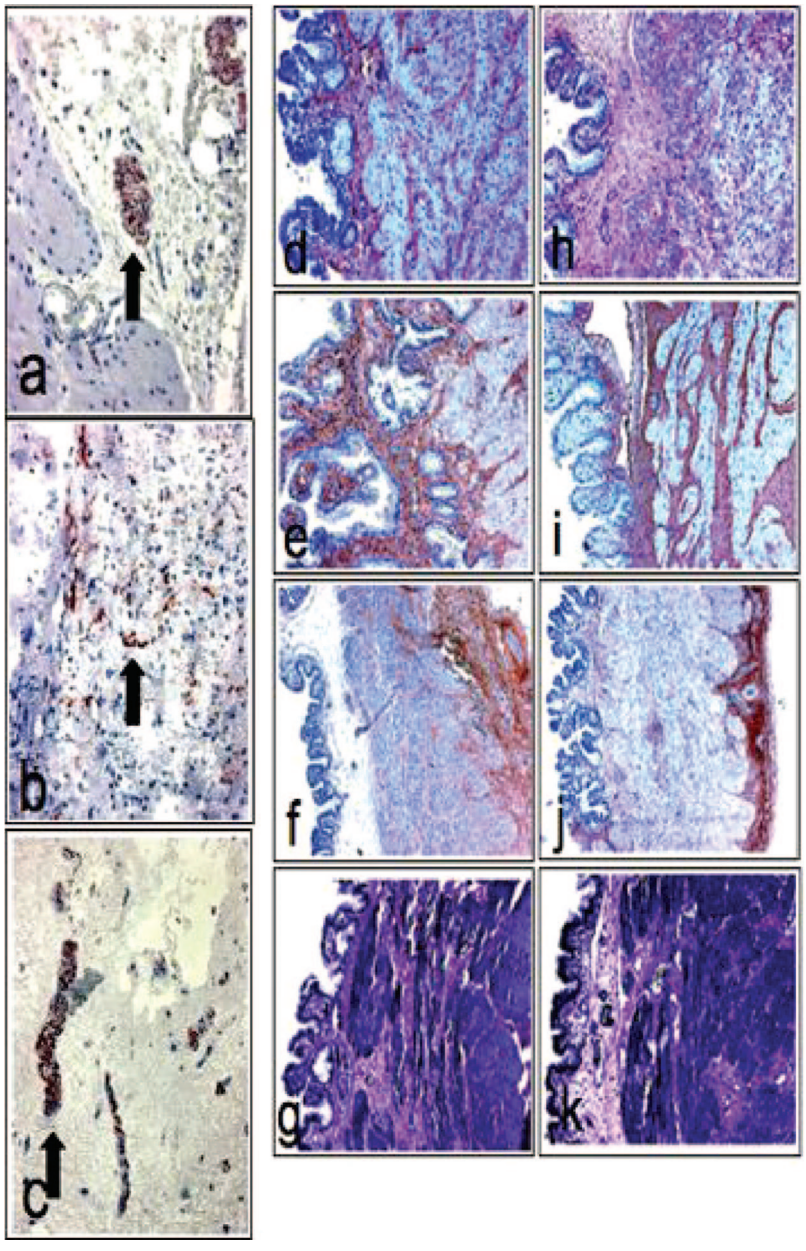

FIGURE 3. Expression of neurofilament protein, collagens, and elastin. (a) Normal vaginal tissue; (b) cell-seeded graft, 1 month postimplantation; (c) cell-seeded graft, 6 months postimplantation (arrows [→] indicate nerve fibers). Immunohistochemistry for collagen types I, II, and III: (d, e, f) cell-seeded graft, 6 months postimplantation, and (h, I, j) normal vaginal tissue, respectively. Red or brown staining indicates positive reaction, (g and h) cell-seeded graft, 6 months postimplantation and normal vaginal tissue stained for elastin by Verhoff-van Gieson staining. Blue or black staining indicates elastin, collagen is red. All cell magnifications are ×200.

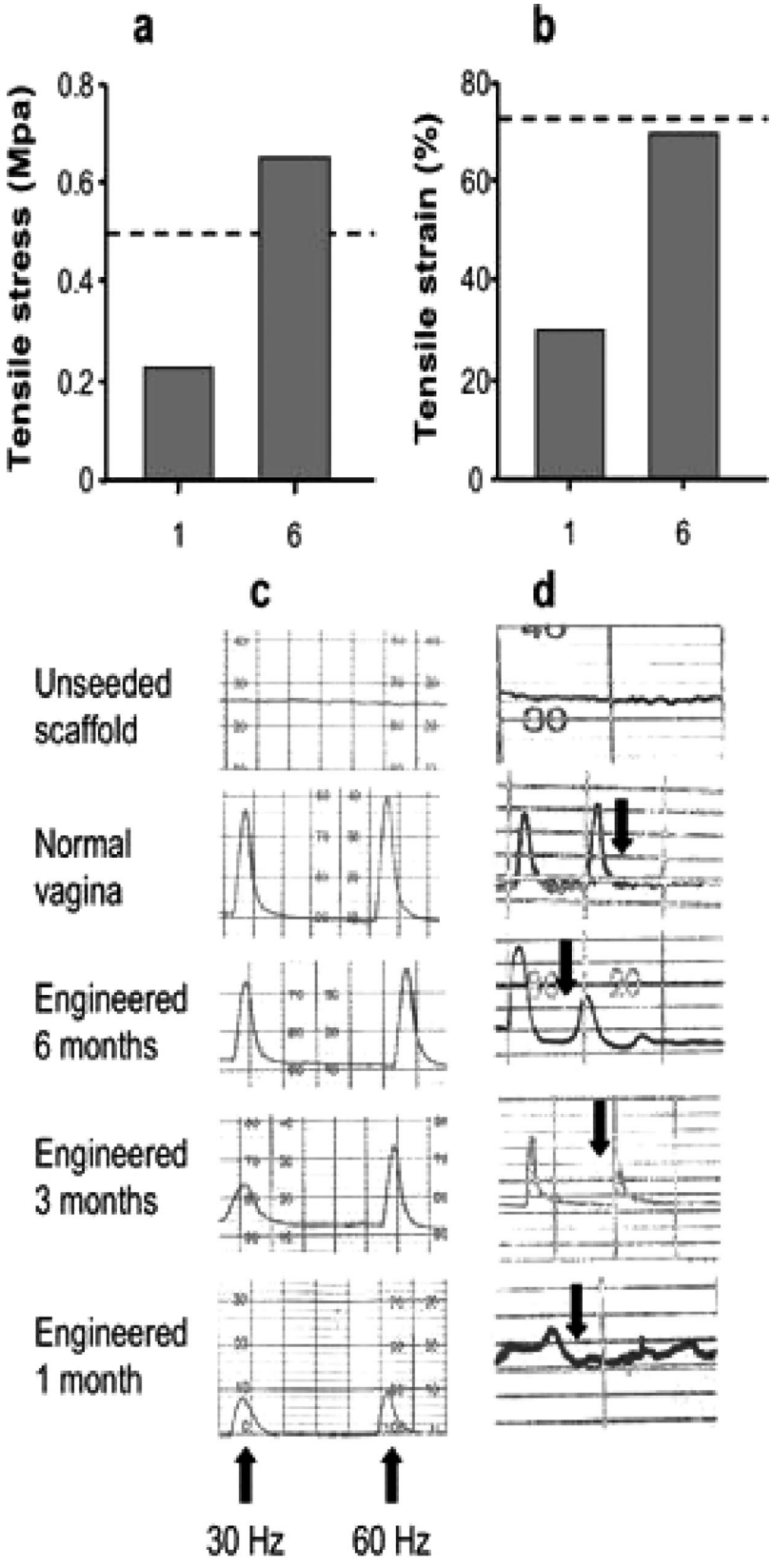

FIGURE 4. Physiology of grafted tissue. (a) Tensile strength and (b) tensile strain of tissue recovered from complete cell-seeded graft at 1 and 6 months after implantation, compared with tissue from native vagina (- - - -). (c) Contractile response of tissue in response to electrical stimulation of 30 or 60 Hz. (d) Contractile response of tissue to adrenergic agonist phenylephrine. Arrows (↓) indicate addition of antagonist phentolamine.

α-actin, revealed that a layer of smooth muscle cells was formed by 1 month posttransplantation (Fig. 2k), with improved development of organized muscle bundles evident by 6 months (Fig. 2l), resembling that of control normal tissue (Fig. 2i). Little or no smooth muscle development was detectable by α-actin staining in tissue recovered from the rabbits that received the unseeded grafts (Fig. 2j).

A key question in tissue engineering is whether the cells that compose the mature neoorgan are derived from the original cell-seeding or whether a substantial number of cells migrate in from the surrounding tissue. To answer this question for the neovaginal constructs, epithelial and smooth muscle cells were separately labeled with different colored membrane-linker fluorochromes. In constructs recovered and analyzed by fluorescence microscopy 3 months postimplantation, labeled cells of both types were easily detected and composed greater than approximately 85% of the organ (Fig. 2n). The labeled vaginal epithelial cells were found in a well-organized layer at the luminal surface (green), coincident with regions that showed expression of cytokeratins. The labeled smooth muscle cells (red) were found within the walls of the tissue-engineered grafts, corresponding to regions that showed expression of smooth muscle α-actin. These data indicate that at 3 months postsurgery most of epithelial and smooth muscle cell populations present in the regenerated vagina derive from the original seeded autologous donor cells. Analysis at later stages with this technique is not possible because of the dilution of the membrane markers. A more accurate, long-term analysis, in an autologous situation, will require permanent cell marking techniques such as lentiviral transduction.

Optimal functionality in an engineered vagina requires a degree of nerve innervation. We therefore examined the presence of nerve fibers in normal and neovaginal tissue by immunohistochemical staining for neurofilament protein. The presence of nerve fibers in normal rabbit tissue is shown in Figure 3 (a, arrowed). Staining for this marker also revealed growth of nerve fibers into the cell-seeded vaginal replacement graft. Some innervation was already apparent at 1 month after implantation (Fig. 3b), and was more extensive at 6 months (Fig. 3c). No innervation could be detected in unseeded constructs (not shown).

The function of vaginal tissue also depends on adequate strength and elasticity. These properties depend on appropriate expression of multiple extracellular matrix proteins, including collagens and elastin (*10*, *11*). After implantation of cell-seeded scaffolds, the total collagen content in the engineered vaginal tissues progressively increased from approximately 1 mg/g tissue at 1 month posttransplantation to 6 mg/g tissue at 6 months. Normal vaginal tissue remained constant at approximately 7 mg/g tissue. Total collagen in unseeded cells remained below 0.25 mg/g tissue at 1 and 6 months. The presence of specific collagen types I, II, and III was assessed by staining with monoclonal antibodies, whereas elastin was identified by Verhoff-van Gieson staining. We observed that by 6 months after implantation the distribution of collagen types I, II, and III (Fig. 3d–f, respectively) and of elastin fibers (Fig. 3g) was similar to that in normal vagina (Fig. 3h–k). In both the normal and the engineered vaginas, the collagen types I and II were uniformly distributed within the connective tissue, whereas collagen type III was localized preferentially in the serosal layer. Finally, the distribution of elastin, visualized by Verhoff-van Gieson staining, was similar in the tissue engineered neovagina at 6 months and normal vaginal tissue.

In addition to the histological studies, we directly assessed the biomechanical properties of strips of the recovered vaginal tissue from engrafted animals. By 6 months after implantation, the tensile stress (Fig. 4a) and tensile strain (Fig. 4b) of the engineered tissue were comparable with that of native vaginal tissue. A critical functional test of the tissue engineered vaginal tissue is the ability to contract in response to electrical or pharmacological signals. Organ bath studies were carried out on strips of muscle tissue from native- and tissue-engineered vagina. In both cases, we observed a graded increase in contractile forces in response to increasing frequency of electrical stimulation (30 vs. 60 Hz, Fig. 4c). The grafted tissue was already responsive at 1 month postsurgery, and by 6 months postimplantation, the amplitude of response was indistinguishable from that of native vagina. Tissue recovered after implantation of a control unseeded scaffold was unresponsive. In response to adrenergic stimulation with the agonist phenylephrine (1 mM), the native vaginal tissue showed a pattern of cyclic contractile response (Fig. 4d), which was interrupted by the administration of the adrenergic antagonist phentolamine (arrows). The results with tissue from the cell-seeded, but not the unseeded, vaginal construct again closely paralleled those with native tissue. The pharmacologic response was already apparent at 1 month postsurgery and by 6 months closely resembled that seen with the native vaginal tissue. This observation implies that a functional neurotransmitter receptor pathway is present in the engineered vaginal tissue.

These data show that 6 months postimplantation, the tissue-engineered replacement vaginal constructs remained patent and the polymer scaffolds on which they were built had degraded as intended. The reconstructed tissue closely resembled native vaginal tissue in its cellular organization, protein expression profiles, and physical properties. The excellent strength and elasticity of the replacement vaginas were consistent with the expression of collagens I, II, and III and elastin. The neovaginal tissue showed appropriate contractility in response to electrical and adrenergic stimulation. In addition, the finding of nerve innervation suggests that the vaginal tissue generated in this way should retain a high degree of functionality. Neovascularization of the grafted structure was facilitated by encasing it with omentum before transplantation. In future, the use of newer "smart scaffolds" incorporating angiogenic factors to stimulate neovascularization and slow oxygen release formulations for increased tissue survival may eliminate this need (*12*, *13*).

Taken together, these results suggest that the use of autologous, engineered vaginal tissue represents an attractive option for the surgical reconstruction of pathological disorders of the vagina in women. Such an approach has several advantages. Only a small biopsy of tissue is needed initially, which is critical in situations where only a limited quantity of native tissue is available. In addition, the fact that the tissue is vaginal in origin should reduce the complications that arise through the use of nonvaginal tissue, and increase functionality dramatically.

ACKNOWLEDGMENTS

The authors thank Dr. Mark Furth for critical reading and editing of this manuscript and all our colleagues at WFIRM for support and advice.

REFERENCES

1. Wefer J, Sekido N, Sievert KD, et al. Homologous acellular matrix graft for vaginal repair in rats: A pilot study for a new reconstructive approach. *World J Urol* 2002; 20: 260.
2. Atala A, Bauer SB, Soker S, et al. Tissue-engineered autologous bladders for patients needing cystoplasty. *Lancet* 2006; 367: 1241.
3. Hohlfeld J, de Buys Roessingh A, Hirt-Burri N, et al. Tissue engineered fetal skin constructs for paediatric burns. *Lancet* 2005; 366: 840.
4. Hollander AP, Dickinson SC, Sims TJ, et al. Maturation of tissue engineered cartilage implanted in injured and osteoarthritic human knees. *Tissue Eng* 2006; 12: 1787.
5. Kershen RT, Fefer SD, Atala A. Tissue-engineered therapies for the treatment of urinary incontinence and vesicoureteral reflux. *World J Urol* 2000; 18: 51.
6. De Filippo RE, Yoo JJ, Atala A. Engineering of vaginal tissue in vivo. *Tissue Eng* 2003; 9: 301.
7. Cilento BG, Freeman MR, Schneck FX, et al. Phenotypic and cytogenetic characterization of human bladder urothelia expanded in vitro. *J Urol* 1994; 152(2 Pt 2): 665.
8. Horan PK, Slezak SE. Stable cell membrane labelling. *Nature* 1989; 340: 167.
9. Dahms SE, Piechota HJ, Dahiya R, et al. Composition and biomechanical properties of the bladder acellular matrix graft: Comparative analysis in rat, pig and human. *Br J Urol* 1998; 82: 411.
10. Goh JT. Biomechanical and biochemical assessments for pelvic organ prolapse. *Curr Opin Obstet Gynecol* 2003; 15: 391.
11. Petros PE, Ulmsten UI. An integral theory of female urinary incontinence. Experimental and clinical considerations. *Acta Obstet Gynecol Scand Suppl* 1990; 153: 7.
12. Rosso F, Marino G, Giordano A, et al. Smart materials as scaffolds for tissue engineering. *J Cell Physiol* 2005; 203: 465.
13. Soker S, Machado M, Atala A. Systems for therapeutic angiogenesis in tissue engineering. *World J Urol* 2000; 18: 10.