Impact of fibrin glue and urinary bladder cell spraying on the in-vivo acellular matrix cellularization: a porcine pilot study

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Purpose: *Urinary bladder tissue engineering utilizing autologous cell-seeded scaffolds requires enough bladder cells to populate a large surface area which may be difficult to obtain from abnormal bladders. We evaluated whether a fibrin glue spray technique enhances cell seeded acellular matrix (ACM) repopulation in a porcine bladder model.*
Materials and methods: *Porcine urothelial and smooth muscle cells cultured from open bladder biopsy were sprayed with or without fibrin glue onto porcine bladder ACM. After 10 days in vitro, constructs were implanted onto porcine bladders (4/group) and harvested after 1 or 6 weeks for H&E and immunohistochemical staining.*
Results: *In vitro, fibrin glue was associated with more continuous cell growth and enhanced cellular organization, maintained particularly in the periphery in vivo, where both groups demonstrated central fibrosis.*
Conclusions: *While fibrin glue enhanced cellular organization on ACM in vitro, central fibrosis in vivo suggests that factors supporting seeded cell survival are lacking.*

Key Words: fibrin tissue adhesion, extracellular matrix, bladder, tissue engineering, cell culture

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Introduction

Currently, tissue-engineered constructs consisting of autologous cells seeded on a supporting scaffold represent an area of expanding interest. However, this approach strongly depends on the scaffold biocompatibility, the propensity for autologous cells to expand, and the interactions between the scaffold

and the seeded cells. In addition, there is a challenge in generating sufficient expansion of urinary bladder cells from biopsies taken from bladder with supposedly limited regenerative capacity,[1] hence we have been investigating practical methods to induce homogeneous cellular coverage of urinary bladder acellular matrix prior to implantation. This bladder acellular matrix has been developed in our laboratory using a patented detergent-based acellularization technique[2,3] for use as urinary bladder substitute.

Fibrin glue possesses several important features as an ideal cell delivery vehicle given that it is biocompatible, biodegradable, and helps cells adhere to a variety of biomaterials.[4,5] Fibrin glue had been shown to be a favorable cell delivery vehicle for urological applications.[5,6] Additionally, an aerosol technique, which provides homogeneous cellular distribution over the biomaterial surface, had been previously developed.[7] The proof of concept for the fibrin glue/spraying technology had been confirmed whereby Hafez et al[8] covered demucosalized colonic segments using mixed urothelial and smooth muscle cells. Herein we assess whether fibrin glue, as a transport vehicle utilizing an aerosol technique to seed ACM with bladder cells harvested from a 2 cm^2 x 2 cm^2 small biopsy, facilitates the ultimate goal of producing a larger *in vitro* urinary bladder construct amenable to bladder replacement.

Materials and methods

Protocol for porcine urinary bladder acellularization

Pigs weighed between 20 kg and 28 kg. Whole porcine urinary bladders were harvested and cut in half. Fresh bladders were washed in sterile phosphate buffer saline (PBS) and then stirred in a hypotonic solution of 10 mM Tris HCl pH 8.0, 5 mM EDTA, 1% Triton X-100, Petabloc Plus^{tm} (protease inhibitor) 0.1 mg/ml and antibiotics/antimycotic at 4°C for 24 hours to lyse all cellular components. On the second day, the tissue was placed in a hypertonic solution containing 10 mM Tris HCl pH 8.0, 5 mM EDTA, 1% Triton X-100 and 1.5 M KCl and stirred for 24 hours at 4°C to denature residual proteins. Tissue was then washed in Hanks' Balanced Salt solution for 1 hour at room temperature twice prior to a 6 hour enzymatic digestion with DNAse/RNAse solution at 37°C with shaking. A final 24 hour extraction was performed at 4°C in 50 mM Tris HCl pH 8.0, 0.25% CHAPS, 1% Triton X-100, and antibiotics/antimycotic with shaking. The resulting ACM was finally washed four times in sterile dH_2O at 4°C and then stored in physiological saline.

Routine H&E sections of detergent extracted ACM showed no detectable urothelial, bladder smooth muscle, vascular smooth muscle or endothelial cells. However, residual cytoskeletal profiles of cells, in particular smooth muscle cells, provided anatomical reference points for the acellular bladder wall and the basis for further immunohistochemical and western blot analysis. In order to determine whether all DNA had been extracted during processing we used a sensitive fluorescence based quantification method. The mean DNA contents of fresh and processed tissues were 0.217 μg/mg and 0.0075 μg/mg tissue wet weight respectively; t-test analysis showed a

TABLE 1. **The experimental groups at a glance**

Pig bladders → Decellularization → Bladder ACM

Porcine bladder and serum collection → Bladder cells isolation and characterization → SMC and urothelial cells in vitro culture

IN VITRO $5x10^4$ cells per cm^2 Left for 10 days

1. ACM & PUC & fibrin glue
2. ACM & SMC & fibrin glue
3. ACM & PUC/SMC & fibrin glue

Assessment of cell proliferation

IN VIVO $5x10^4$ cells per cm^2 **4X4 cm**^{2} Left for 10 days

A.ACM & PUC/SMC (4 pigs)

B.ACM & PUC/SMC & fibrin glue (4 pigs)

1 week, bladders harvested

6 week, bladders harvested

Cell specific histology and immunohistochemistry

significant difference in the DNA content between the two samples ($p<0.001$). Thus for all intents and purposes the ACM was devoid of residual DNA. The summary of the experimental protocol can be found in Table 1.

Establishment of primary cell cultures

Ten days prior to implanting grafts, 2 cm^2 x 2 cm^2 urinary bladder segments were excised from the bladder of each study pig for cell harvest. Using microscissors, the mucosal layer was dissected off the underlining muscle layer, and tissues were cut into 2 mm-3 mm fragments.

Primary urothelial cell culture[9,10]

Mucosal fragments were washed in Minimum Essential Medium (MEM) and centrifuged. After removing the supernatant, the remaining tissue was immersed in Type IV collagenase in MEM (1 mg/ml) and incubated at 37°C for 60 minutes. Cells were resuspended in keratinocyte serum-free medium (KSFM) supplemented with recombinant epidermal growth factor, bovine pituitary and 0.5% penicillin/streptomycin (P/S). Primary urothelial cell (UC) cultures were established in T75 culture flasks and incubated in a humidified 5% CO_2 air atmosphere at 37°C. Medium was changed every 2 days.

Primary smooth muscle cell culture[11]

Muscle fragments were washed in MEM and centrifuged. After removing the supernatant, the remaining tissue was immersed in Type I collagenase in MEM (1 mg/ml) and digested at 37°C with shaking for 30 minutes. The cell digest was vortexed and then settled for 30 seconds. Supernatant containing liberated cells was collected and centrifuged. Cell pellets were washed twice with MEM and resuspended in MEM with 10% Fetal Bovine Serum and 1% P/S. Primary smooth muscle cell (SMC) cultures were established in T75 culture flasks and incubated in a humidified 5% CO_2 air atmosphere at 37°C. The medium was changed every 2 days.

Once the UC and SMC cultures achieved 90%-100% confluence, they were harvested with trypsin/EDTA and routinely passaged. Cell culture phenotypes were confirmed using immunohistochemical methods, and only cells between passages 2 and 5 were used for *in vitro* assessment.

Fibrin glue was prepared using autologous serum from pigs, and for aerosolization an air compressor apparatus was used as described by Fraulin et al.[12]

In vitro protocol

Direct seeding method

In the first two groups, single UC or SMC suspensions were seeded with a spray technique separately on the ACM luminal or abluminal surfaces respectively, at a density of 5 x 10^4 cells per cm^2. In the third group (co-culture seeding), a cell suspension of mixed UC and SMC was prepared in 5 ml of a 50/50 media of KSFM and MEM and 5 x 10^4 cells/cm^2 were sprayed over the ACM luminal side. Cells were left to adhere for 15 minutes prior to addition of culture medium.

Fibrin glue method

Cell combinations as in experiment A were mixed with fibrin glue and sprayed on the ACM. Suspensions of UC and/or SMC were prepared in 5 ml of 50/50 media of KSFM and/or MEM, mixed with 5 ml fibrin glue, and sprayed over the luminal or abluminal side of the ACM pieces. Cells were left to adhere for 15 minutes prior to addition of culture medium.

In both arms, cell-ACM constructs were maintained for 10 days at 37°C and 5% CO_2 and medium (KSFM for UC [Ca^{++} 0.9mM], MEM for SMC, 50/50 KSFM/MEM for mixed cell cultures) was changed every 2 days.

In vivo protocol

The experimental protocol was reviewed and approved by the Animal Research Committee and in accordance with the public health service policy on humane care and use of laboratory animals (CCAC guidelines). Animals were fasted overnight. Anesthesia was induced with Akmezine followed by endotracheal intubation, automatic ventilatory support, and maintenance anesthesia with a combination of 1.5% Halothane and oxygen. I.V. penicillin was administered at induction.

Grafts were implanted 10 days after ACM was seeded with bladder cells. The mixed co-culture technique, which demonstrated the best growth pattern *in vitro*, was used. A 4 cm^2 x 4 cm^2 segment incorporating the site of the initial harvest was removed from the anterior bladder wall, and the resultant defect was replaced with a 4 cm^2 x 4 cm^2 graft. ACM grafts seeded with fibrin glue were implanted in group A (four pigs) and without fibrin glue were implanted in group B (four pigs). Orientation of the ACM luminal and abluminal surfaces was maintained. Bladders were closed in two layers, with prolene for the second layer to allow graft identification. The repair was tested for water tightness, and the abdominal wall was closed. Animals were recovered

in the animal facility recovery room and returned to the regular animal pens. They received a veterinary antibiotic preparation of (P/S) and analgesia in the form of Tamgesic I.M. for 1 week postoperatively.

Recovery of grafts and evaluation

At 1 and 6 weeks post-augmentation, bladders were harvested from two pigs per group using a similar anesthetic and surgical technique as described above.

Histology and immunohistochemistry

After harvest ACM pieces were formalin fixed for 24 hours prior to processing them for routine histology (H&E) and immunohistochemical staining for smooth muscle (ALPHA actin) and urothelial cells (cytokeratin 7). Cellular growth characteristics were studied with attention to cell morphology and proliferation, and ACM penetration.

Immunoperoxidase staining for smooth muscle actin and cytokeratin 7 was completed using the vectastain ABC kit (Vector Laboratories, Burlinton, CA) according to manufacturer's instructions. Tissue sections were deparaffinized, rehydrated through xylene and graded alcohol to distilled water, and incubated for 30 minutes in 0.3% H_2O_2 in water to block endogenous peroxidase. After washing for 5 minutes in PBS (pH 7.4), sections were incubated for 1 hour in 1.5% normal goat serum and

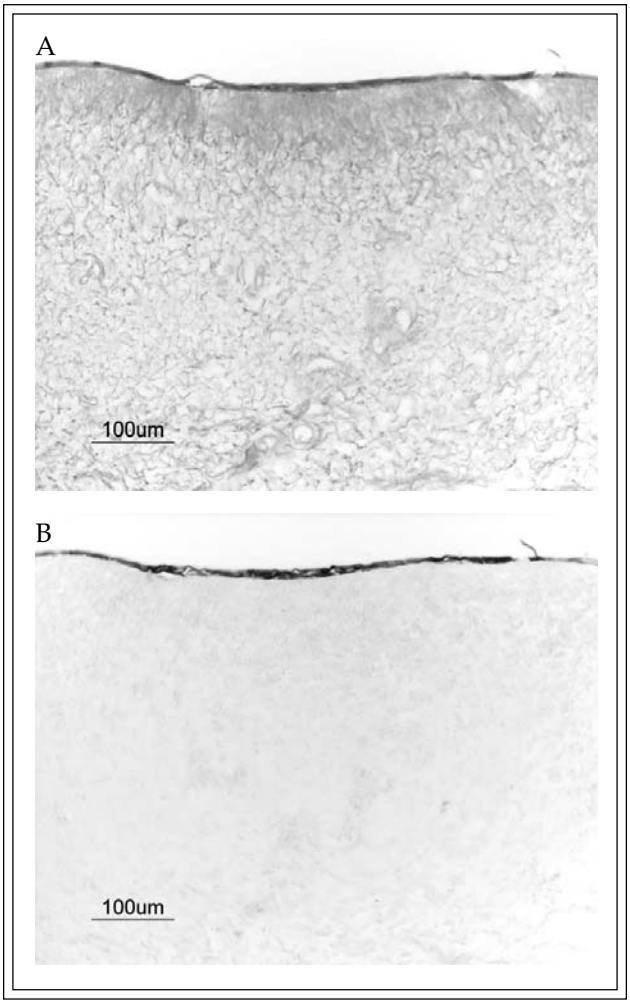

Figure 1. When seeded alone on ACM, few urothelial cells have attached and only an occasional continuous single cell layer was observed after 10 days in culture.
A. H& E staining
B. CK 7 immunostaining

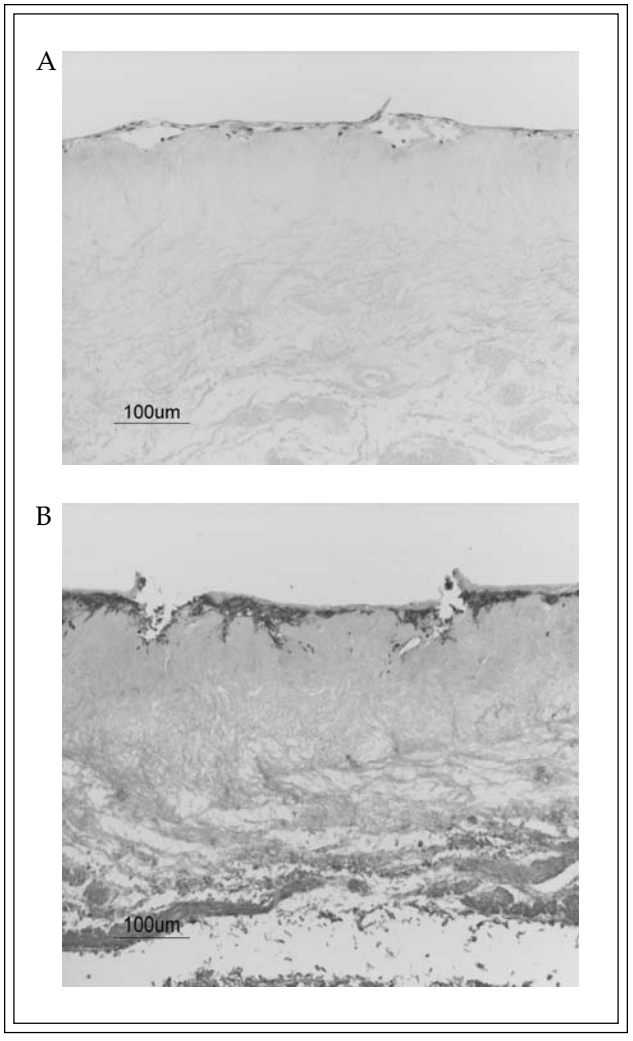

Figure 2. Seeded smooth muscle cells (SMC) readily attached to ACM and grew as one to two layers by day 10.
A. H& E staining
B. alpha-actin immunostaining

then overnight at 4ºC with primary antibodies. Subsequently, sections were washed three times for 5 minutes in PBS and incubated for 30 minutes with diluted (1:100) biotinylated secondary antibodies (horse anti-mouse secondary antibody for monoclonal primary antibodies, goat anti-rabbit secondary antibody for polyclonal primary antibody). Afterwards, they were washed for 5 minutes in PBS and then incubated for 30 minutes with vectastain ABC Reagent.

Finally, sections were incubated in DAB substrate solution until the desired stain intensity developed. Slides were counterstained with Hematoxylin and then mounted with Pristine Mount (Research Genetics). Images were recorded using a digital camera (Nikon E 400) and a Nikon DXM1200 light microscope.

Results

In vitro

With single cell seeding after 10 days, UC scarcely attached with minimal evidence of a continuous single layer Figure 1 while SMC readily attached and grew into one to two cell layers Figure 2. With the co-culture seeding method, at 10 days and without fibrin glue there was distinct cell sorting, whereby SMC preferentially adhered to and formed multiple layers on the luminal surface below stratified UC Figure 3.

Addition of fibrin glue to cells resulted in a more uniform cell distribution for all three methods of cell seeding. The most pronounced proliferation was in the cell co-culture group which also demonstrated distinct cell sorting confirmed by immunohistochemical analysis Figure 4. SMC attached to the luminal surface with minimal matrix penetration while UC grew on top of the SMC. In summary, addition of fibrin glue facilitated better and more even dispersion and retention of UC and SMC.

In vivo

Demonstrating better cellular dispersion and proliferation, the spray technique with cell co-culture provided a good three-dimensional construct for the *in vivo* model. At 1 week after implantation, both groups (with and without fibrin glue) demonstrated acute inflammation with minimal cellular infiltration of both UC and SMC Figure 5. However, at the 6-week interval the acute inflammation subsided completely with clear evidence that the fibrin glue group showed enhanced cellular repopulation, manifested by well-defined multilayered UC and SMC compared to the group without fibrin glue Figure 6. Cellular repopulation was more accentuated in the ACM

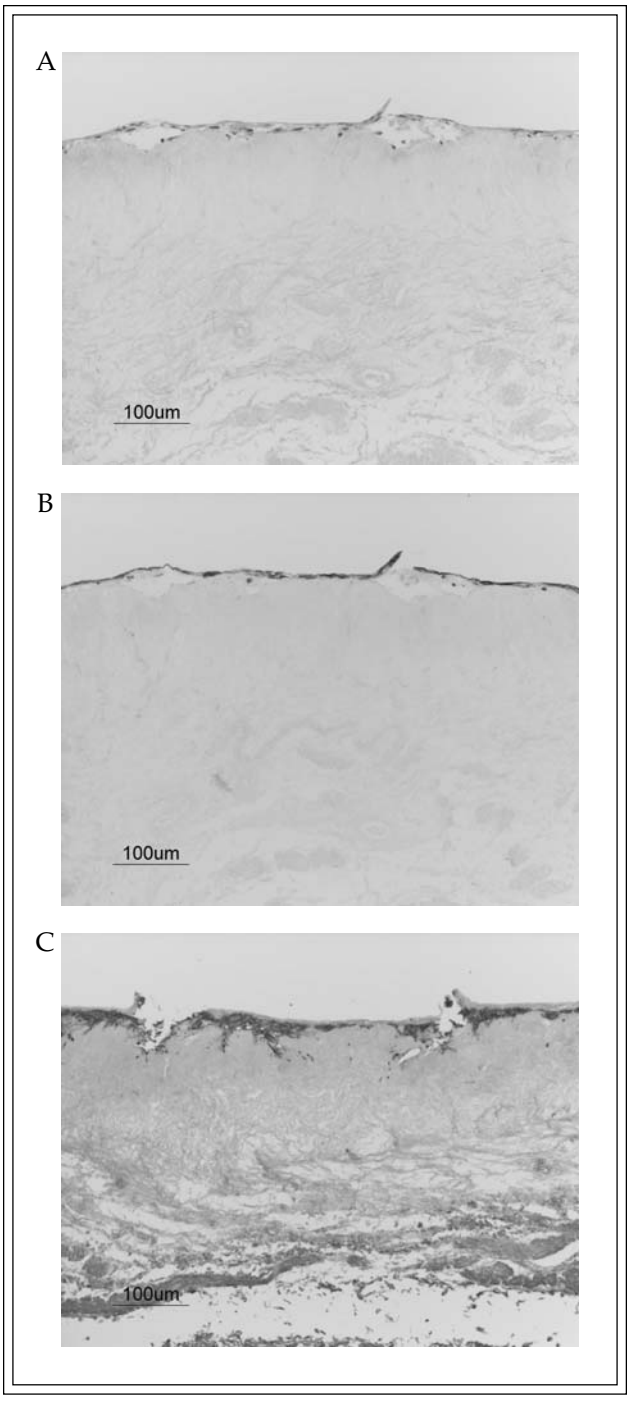

Figure 3. Although matrix penetration by SMC was not noted at any time point, in 10 days sprayed co-cultures. there was distinct cell sorting with SMC multilayered on luminal surface and stratified UC on top of SMC. However, cell coverage of the ACM was still patchy after 10 days of incubation.
A. H& E staining
B. CK 7 immunostaining
C. alpha-actin immunostaining

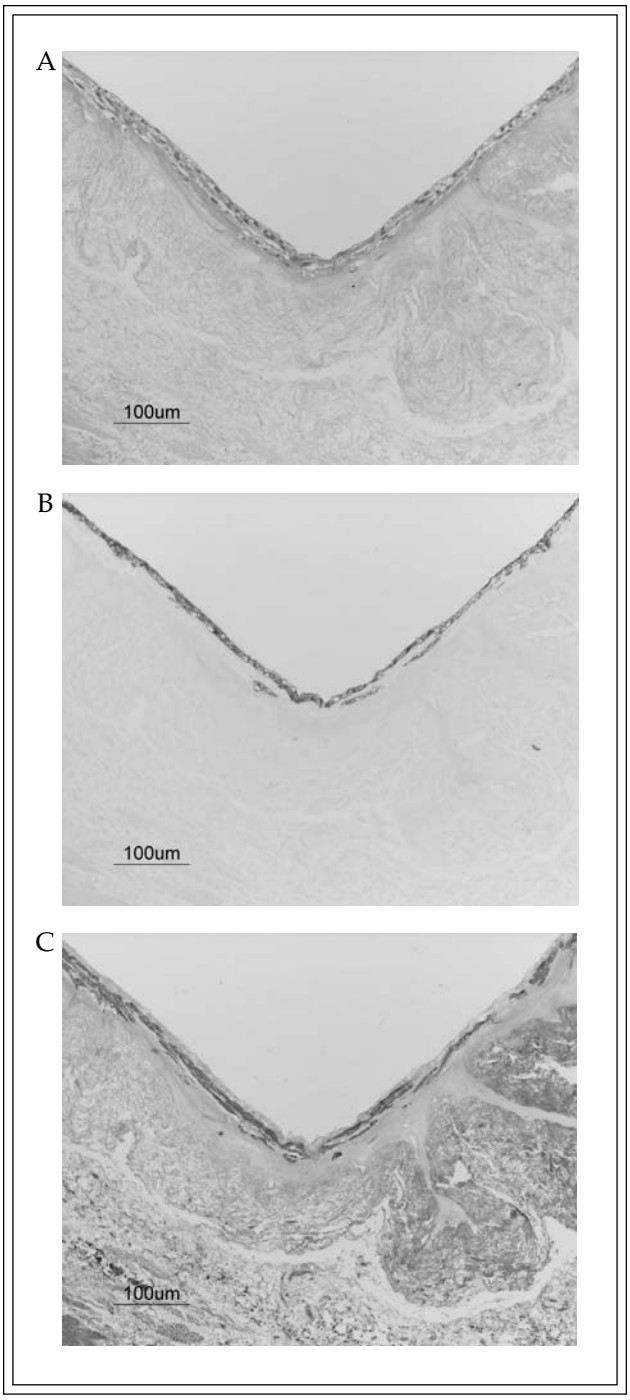

Figure 4. A mixture of UC and SMC suspended in fibrin glue and sprayed on ACM and cultured for 10 days. There was evidence of cell sorting with UC growing on top of SMC, confirmed by imunohistochemistry. Although there was no evidence of ACM penetration, note the uniform and continuous multilayered cell coverage.
A. H& E staining
B. CK 7 immunostaining
C. alpha-actin immunostaining

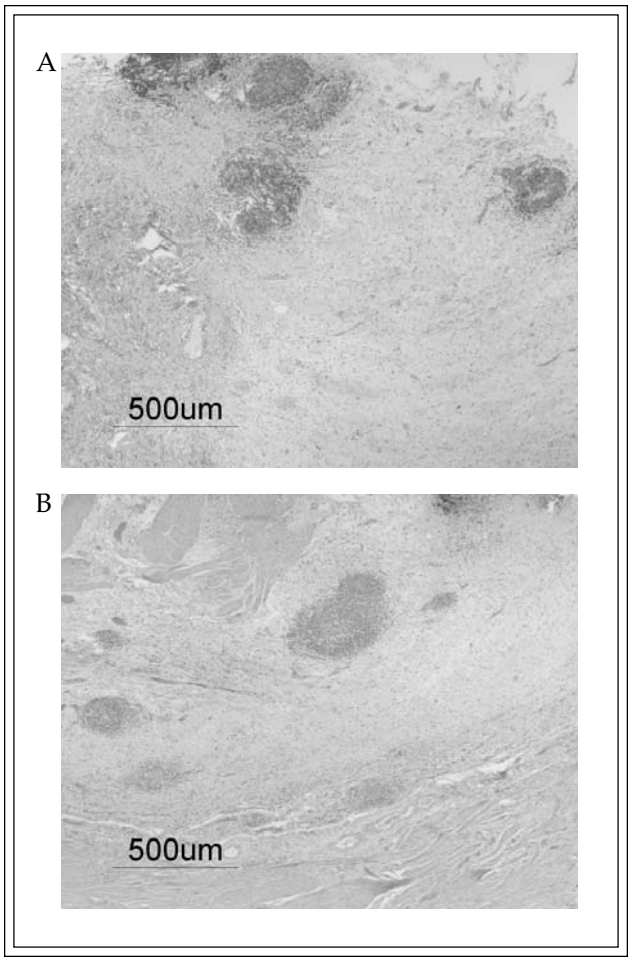

Figure 5. Inflammatory cellular infiltration in cell seeded grafts with (A) and without (B) fibrin glue at one week *in vivo*.

periphery with fibrin glue than without, and there was minimal or no cellularization in the center of the graft in either group. Furthermore, in both groups there were similar degrees of graft fibrosis and contraction, particularly in the graft center.

Discussion

We have initiated development of a porcine bladder acellular matrix (ACM) graft using a patented detergent-based acellularization technique[2,13] in order to retain the biocompatible properties of bladder extracellular matrix. Studies have shown that cell seeded scaffolds can improve *in vivo* biocompatibility of grafts for bladder substitution,[14] thereby reducing contraction and fibrosis. Whether the presence of autologous cells on the scaffold alters the implant degradation process and preserves its structural integrity until neovascularization and definitive

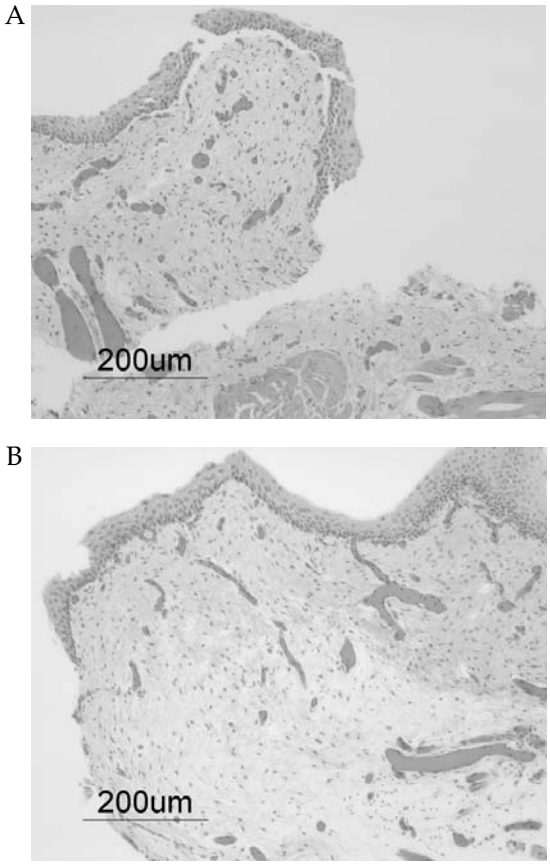

Figure 6. At 6 weeks in vivo, acute inflammation subsided with improved cellular repopulation of the fibrin glue group (B) compared to the control group (A) with minimal central cellular repopulation in both groups.

scaffold incorporation into the surrounding tissue occur is unknown. The presence of urologically specific cell components such as urothelial and smooth muscle cells is important for this technology; on the other hand, the challenge is in generating a sufficient number of expanded cells for seeding from an abnormal bladder.

Fibrin glue possesses several important features as an ideal cell delivery vehicle in that it is of human serum origin, biocompatible, biodegradable, and has a high affinity to adhere to biological surfaces. Additionally, it aids cellular adherence to biomaterials, acts as a nutrient, may enhance growth factor diffusion, and has been extensively investigated in urological application.[4,5,15] These properties may be important for the interim support of grafted cells until revascularization and definitive incorporation occurs.

Furthermore, uniform seeding of ACM with cells has been a challenge. To enhance even cellular distribution, we utilized an aerosol technique[7] which had been used previously for successful coverage of a 25 cm^2 de-epithelialized surface with epithelial cells from only 1 cm^2 biopsy of skin in pigs, representing a 25-fold initial expansion of coverage, and a logical extension of the principle of meshing skin grafts. The combination of fibrin glue and aerosol delivery provided homogeneous distribution of cells and a biocarrier for cell deployment over large demucosalized porcine segments previously[8] and on the ACM in our current study.

With respect to bladder, there is evidence that the cellular interactions between urothelium and mesenchyme will in time positively change the urothelium phenotype.[11,16] In our study, the SMC and UC co-cultures had a growth promoting reciprocal effect, which was more pronounced in the presence of fibrin glue. This finding was also reported by Zhang et al[16] when small intestinal submucosa (SIS) was used as a substratum for *in vitro* cell cultures. This cross talk between bladder cell types could be due to the influence of secreted soluble factors stimulated by contact between UC and SMC. *In vivo*, these cell types are separated by a definitive basement membrane and it would be interesting to determine if in our model UC produce a basement membrane separating them from underlying SMC.

It is evident from this study that the ACM acts as a biocompatible three-dimensional cell culture substratum that supports bladder cell growth. This biocompatibility could be related to the fact that the resulting ACM may already contain the necessary extracellular matrix components for facilitating cell adhesion and proliferation. By providing a naturally occurring extracellular matrix such as the ACM, it is plausible that the pattern or mode of cell growth will more closely mimic that observed *in vivo*.[17] On the other hand, a major difference between the *in vitro* microenvironment and the *in vivo* environment is the source of nutrition. While the *in vitro* nutritional environment may be closely controlled and artificially maintained, *in vivo* nutritional support relies on a vascular supply or imbibition. As the ACM itself is avascular, angiogenesis is essential to maintain cell growth and proliferation. The proximity of host tissue vasculature to the graft periphery, and absence in the graft centre likely are significant factors contributing to the differences between the central and peripheral graft cellularity with time *in vivo*. While it is plausible that cellular repopulation from the edges of the defect contributed to the overall recellularization in both

groups, it is clear that the fibrin glue group showed better cellular proliferation than that without fibrin glue. The mechanisms behind this enhanced cellular proliferative pattern need to be further investigated by tagging the seeded cells to further identify the source of the cellularization pattern.

It is believed that newly formed blood vessels participate in provisional granulation tissue formation and provide nutrients and oxygen to growing tissues or cell seeded scaffolds. Although we were successful in organizing cell combinations into complex three-dimensional functional structures using fibrin glue, the seeded matrix lacked the ability to rapidly induce angiogenesis, crucial for the survival of the seeded cells. In constructs that are thicker than a few millimeters, the rapid development of a vascular network from the surrounding host tissue is necessary for a sufficient supply of nutrients, however this process usually requires weeks.[17,18] In urothelial autologous implantation models, it has been shown that on longer term follow-up, the survival rate of implanted cells on collagen gels drops markedly.[19] This finding may be explained by the lack of hastening neovascularization, which is the critical challenge in engineering neo-organs. Furthermore, in this study, we believe that fibrin glue alone did not support cell survival in areas of the grafts more distant from the normal bladder vascularity as evidenced by fibrosis and a paucity of cells in the central graft. Incorporation of the bladder ACM scaffold involves two processes of cellular ingrowth, one from the edge of the defect and the other from islands of cells in the midst of the defect. Cellularity was different between the center and periphery of the scaffold with or without fibrin glue, again suggesting that survival of matrix seeded cells likely relies on fast vascularization as occurs at the bladder ACM interface, accelerating cellular recruitment into the ACM. On the other hand, central seeded cells did not survive, resulting in contracture, the natural history of the ACM in the absence of cellular repopulation.

Subsequently, there are some limitations to this study; for instance labeling of the cultured cells with membrane bound markers or transfected reporter genes may be necessary to differentiate them from the host cells. Furthermore, the number of animals used per time point/group (n=2) may not be sufficient to draw concrete conclusions, however, we believe that further studies using a larger sample and sophisticated cell labeling technology might be needed to study the cell-matrix interaction and enhance in vivo cellular grafting.

Conclusion

When the cell number is limited, fibrin glue admixed with a combination of urinary bladder cells, applied to ACM with a spray method, may provide uniform cell coverage of ACM and may support reconstruction of bladder cell phenotypic behavior and interaction. Our method allowed for the creation of a three-dimensional cell seeded construct *in vitro*, with co-cultured cell types producing the best proliferative results. On implantation of grafts seeded with mixed cell types, those constructs which utilized fibrin glue demonstrated better ACM biograft recellularization patterns than those without fibrin glue. However, the means to supply cells embedded within these scaffolds with sufficient oxygen and nutrients to sustain their survival and proliferation during the integration of the scaffold with the surrounding tissue is still unknown. Rapid neovascularization may be the key to minimize the time required for the seeded cells to survive by diffusion alone prior to the establishment of an appropriate vascular network to support graft survival and functional preservation. Studies in our laboratory are underway to investigate whether methods such as growth factors incorporation into the matrix[20] or a third significant cell type, such as the bladder vascular endothelium, may accelerate vasculogenesis. □

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