Original Article

Inhibition of proliferation and migration of stricture fibroblasts by epithelial cell-conditioned media

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ABSTRACT

Introduction: Urethral stricture is characterized by urethral lumen narrowing due to fibrosis. Urethroplasty of the urethral stricture involves excision of scar, and may be followed by reconstruction of the urethra using split-thickness skin, buccal mucosa, urethral mucosa or, more recently, tissue-engineered grafts. The stricture wound healing process after urethroplasty is known to be mediated by an interaction between keratinocyte and fibroblasts; however, the underlying mechanisms are not studied in detail yet. We investigated the influence of epithelial cell-conditioned medium (ECCM) (obtained from confluent penile skin, buccal mucosa and urethral cell cultures) on the proliferation and migration of stricture fibroblasts using an *in vitro* scratch assay.

Materials and Methods: ECCM was collected from confluent primary epithelial cell cultures of three different human biopsies (penile skin, buccal mucosa and urethral mucosa), whereas stricture fibroblasts were isolated from human urethral stricture biopsies. The effect of ECCM on stricture fibroblasts' proliferation and migration into the scratch was observed using a standard *in vitro* scratch assay over a period of 3 days. Four experiments were performed independently using four stricture fibroblasts from four patients and ECCM was collected from 12 different patients' primary cell cultures.

Results: ECCM from primary epithelial cells cultures obtained from penile skin, buccal mucosa and urethra inhibited stricture fibroblasts' proliferation and migration in the *in vitro* scratch assay.

Conclusion: These results demonstrate the ability of ECCM to inhibit the proliferation and migration of stricture fibroblasts and present it as an effective adjunct in urethroplasty, which may influence stricture wound healing and inhibit the recurrence of stricture.

Key words: Epithelial cell-conditioned medium, scratch assay, tissue-engineered epithelial cell graft, urethral stricture, urethroplasty, wound healing

INTRODUCTION

Urethral stricture is a urologic disorder characterized by narrowing of the urethra due to inflammation, injury, scar tissue formation from surgeries, catheterization or dilations.[1] Narrowing of the urethral lumen is a result of fibrosis or increased extracellular matrix collagen in the connective tissue beneath the urethral mucosa.[2] Histologically, a stricture is composed of elongated myofibroblast and multinucleated giant cells that are responsible for excess collagen deposition and hence formation of stricture.[3] Urethroplasty is the surgical repair of urethral strictures; the surgical procedure involves either excision of scar or excision of scar followed by urethral reconstruction using autologous tissue such as genital skin, bladder mucosa, buccal mucosa or colonic mucosa.[4] Although autologous buccal mucosal grafts are widely used for repairing urethral strictures, complications such as limitation of patient's own donor tissue and donor site morbidity pose a major concern.[5] To overcome these challenges, tissue-engineered grafts comprising of epithelial cells seeded on scaffolds have been explored for urethroplasty.[6] Epithelial cells from various tissues such as urinary bladder, urethra and buccal mucosa have been used in tissue-engineered grafts for urethroplasty as well as the urethral reconstructive surgeries.[7-9]

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Graft transplantation at the site of stricture follows the same processes as the normal wound healing procedure of minimal inflammation phase, re-epithelialization, new connective tissue formation and scar re-modeling.[10] Epithelial–mesenchymal interactions play a crucial role in regulating tissue homeostasis and wound repair.[11] Researchers have developed co-culture model systems to investigate epithelial cell–fibroblast interaction using normal epithelial cells and fibroblasts. It has been suggested that epithelial cells downregulate the expression of connective tissue growth factor (CTGF) in fibroblasts via soluble factors, which in turn results in decreased extra-cellular matrix production and hence fibrosis.[12] It has also been shown that keratinocyte-conditioned medium (KCM) contains anti-fibrotic cytokines that inhibit collagen I synthesis in fibroblasts.[13] Harrison and colleagues demonstrated that KCM from fully confluent epithelial cell cultures inhibited fibroblast proliferation.[14] Moreover, it has been reported that KCM may also reduce contraction of reconstructed skin grafts *in vitro*.[15]

Treatment of urethral stricture using tissue-engineered epithelial cell graft has evoked keen interest among urologists because of the ample availability of tissue-engineered graft and minimal donor site morbidity.[16] Nonetheless, the basic underlying mechanism by which cellular graft helps in stricture inhibition has not been studied in detail yet. We hypothesize that epithelial cell-conditioned medium (ECCM) from various confluent epithelial cell cultures may inhibit stricture fibroblasts' proliferation and migration. To test our hypothesis, we isolated primary stricture fibroblasts from human urethral stricture biopsies and ECCMs were obtained from isolated primary epithelial cells from three different widely used human tissue biopsies for urethroplasty (penile skin, buccal mucosa and urethra). The influence of ECCM on stricture fibroblasts was investigated using an *in vitro* scratch assay.

MATERIALS AND METHODS

This study was approved by the Rangadore Memorial Hospital's ethics committee and informed consent was obtained from patients or legal guardians. Urethral stricture (one from each patient) and normal urethral mucosa biopsies (one from each patient) were obtained from 4 patients (aged 24–76 years) undergoing urethroplasty surgery. The biopsy from the stricture area included fibrosis from the epithelium and spongiosum. Stricture tissue biopsy was obtained without resorting to excision and end-to-end anastomosis. Buccal mucosa biopsies (one from each patient) were obtained from 4 patients (aged 44–76 years) undergoing urethroplasty surgery. Penile skin (one from each patient) was obtained from 4 patients (aged 2–76 years) undergoing circumcision. These patients did not show any evidence of Lichen sclerosus or balanitis.

Tissue histology and Masson trichrome staining

Hematoxylin and eosin staining was performed to characterize urethral stricture tissue histology. Collagen deposition in urethral stricture tissue was assessed by Masson trichrome staining according to the supplier's guidelines. Normal urethra was used as the control.

Primary cell culture

Fibroblasts were isolated from human urethral stricture biopsy. Epithelial cells were isolated from human penile skin, buccal mucosa and urethra biopsies.[17,18] Briefly, biopsies were digested in a series of recombinant enzymes; Dispase, Collagenase type IV and TrypLE to release epithelial cells; primary cells were isolated and cultured in complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Epithelial cells were cultured in complete Epilife media with S7 supplement on collagen-coated plates. The cells were incubated at 37°C in a humidified 5% CO_2 incubator.

Reagents

DMEM, FBS, Epilife Medium, S7 Supplement, Dulbecco's Phosphate-Buffered saline (DPBS), Antibiotic–Antimycotic, TrypLE Express, Collagenase Type IV, Dispase and Recombinant Collagen Type-I Coating Matrix were obtained from GIBCO/Invitrogen (Life Technologies, Grand Island, NY, USA).

Collection of ECCM

Epithelial cells (Passage 2 or 3) were grown in complete Epilife media up to 50–60% culture confluency. Fresh complete Epilife media was added to the proliferating culture. After 24 h, when the culture was 70–80% confluent, the ECCM was collected and centrifuged at 200 g for 10 min at room temperature. ECCM was filtered using a 0.45 micron syringe filter and stored at -80°C.

In vitro scratch assay

An *in vitro* scratch assay was performed as described earlier.[19] In brief, 12,000 fibroblasts/cm^2 (Passage 3 or 4) were seeded in complete DMEM in a 24-well plate. Cells were grown up to 100% culture confluency to form a monolayer. Fibroblasts were deprived of serum in the basal DMEM medium for 16–17 h (overnight). A "scratch" was created using a p100 pipet tip. Cells were washed with DPBS and 1 mL ECCM from penile skin, buccal mucosa and urethra was added in the respective wells. Complete DMEM medium was used as a positive control and basal DMEM was used as the negative control. Fibroblast proliferation and migration into the scratch area was observed for 3 days. Four experiments were performed independently using four stricture fibroblasts and 12 ECCMs from different patients.

Image acquisition

Marks (marker pen) were created as reference points close to the scratch and images were captured under the phase

contrast inverted microscope (Olympus INV). Images were captured on Days 0, 1, 2 and 3. All images were taken at 280X magnification. The experiment was repeated four times using four different stricture fibroblasts and ECCMs.

RESULTS

Urethral stricture tissue histology

Hematoxylin and eosin-stained slides showed the histology of normal urethra and stricture urethral tissue [Figure 1a and b]. Masson trichrome staining showed excess collagen deposition (blue color) in the urethral stricture biopsy [Figure 1d] as compared with the normal urethra [Figure 1c]. Nuclei are stained black and the cytoplasm, muscle and erythrocytes are stained red.

In vitro scratch assay using different ECCMs and stricture fibroblasts

To evaluate the effect of ECCM on the proliferation and migration of stricture fibroblasts, an *in vitro* scratch assay model was used. A scratch was created on the 100% confluent monolayer of stricture fibroblasts and the effect of ECCM on proliferation and migration of stricture fibroblast into the scratch was observed over a period of 3 days. The experiment was performed in duplicate. The entire experiment was repeated four times using four different stricture fibroblasts and 12 ECCMs.

As shown in Figure 2, on Day 0, the scratch area created was devoid of any cells or cellular debris [Figure 2a–e]. On Day 1, wells containing ECCMs showed inhibition of stricture fibroblast migration in the scratch area [Figure 2f–h], whereas in the positive control well (complete DMEM) showed about 5% fibroblasts migration from the edges of the scratch [Figure 2i]. The negative control well showed empty scratch area [Figure 2j].

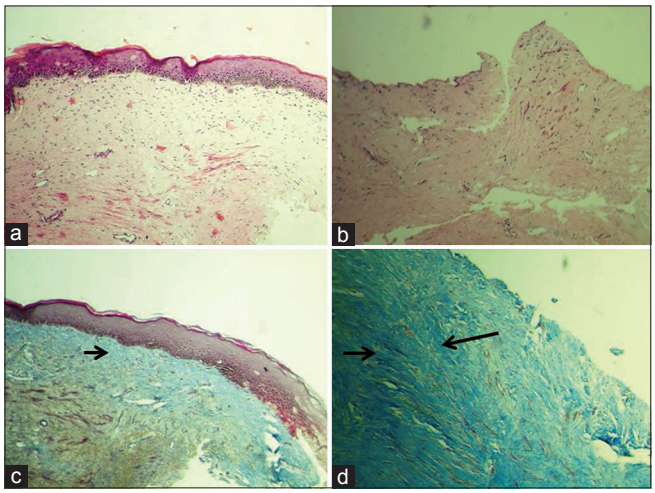

Figure 1: Hematoxylin and eosin-stained (a) normal (b) stricture urethra. Masson Trichrome-stained normal (c) and stricture urethra (d). Collagen deposition (blue) is indicated by black arrows. Nuclei are stained black while the cytoplasm, muscle and erythrocytes are stained red. Magnification, X280

On Day 2, we observed ECCM wells with clear scratch area [Figure 3a–c], whereas the positive control well showed about 40–50% fibroblast migration into the scratch area [Figure 3d]. The negative control well showed empty scratch area [Figure 3e].

On Day 3, the ECCM wells showed empty scratch area [Figure 3f–h], whereas the positive control well showed closure of the scratch area with fully confluent fibroblasts [Figure 3i]. Negative control showed empty scratch area, as expected [Figure 3j]. In conclusion, ECCMs from the buccal mucosa, penile skin and urethral mucosal epithelial cell cultures inhibited the proliferation and migration of stricture fibroblasts in the *in vitro* scratch assay, and complete inhibition was obtained by Day 3.

DISCUSSION

Urethral stricture is a chronic wound characterized by increased fibrous tissue formation in the urethra. In the wound healing process, paracrine communication between keratinocytes and fibroblasts is essential as these cells together regulate cell proliferation, differentiation and extracellular matrix production.[10,11] It has been shown that a delay in re-epithelialization results in hypertrophic scar formation

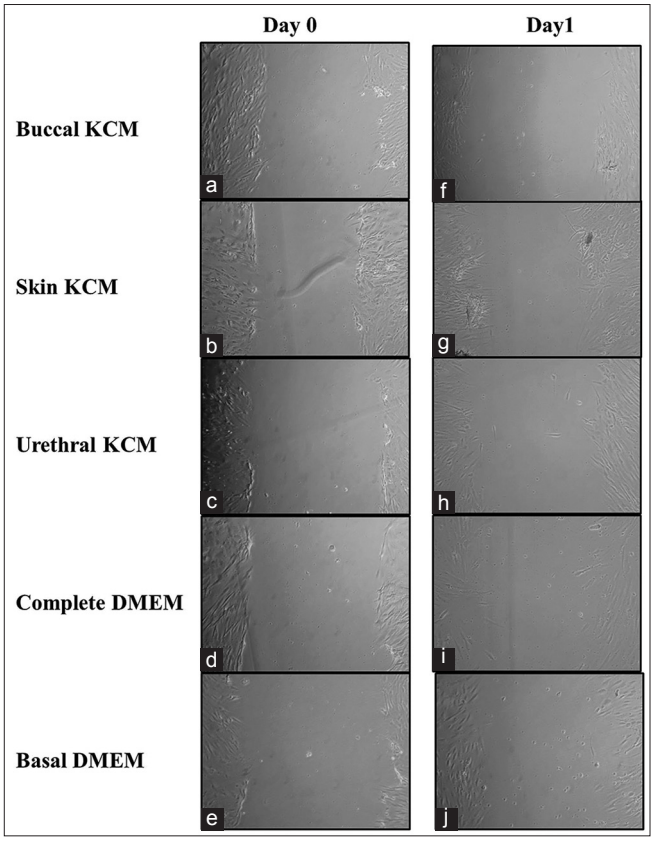

Figure 2: *In vitro* scratch assay using epithelial cell-conditioned medium from the buccal mucosa (a, f), penile skin (b, g), urethral mucosa (c, h), complete Dulbecco's modified Eagle's medium (DMEM) medium (d, i) and basal DMEM (g, j). (a–e) denote Day 0. (f–j) denote Day 1. Magnification, X280

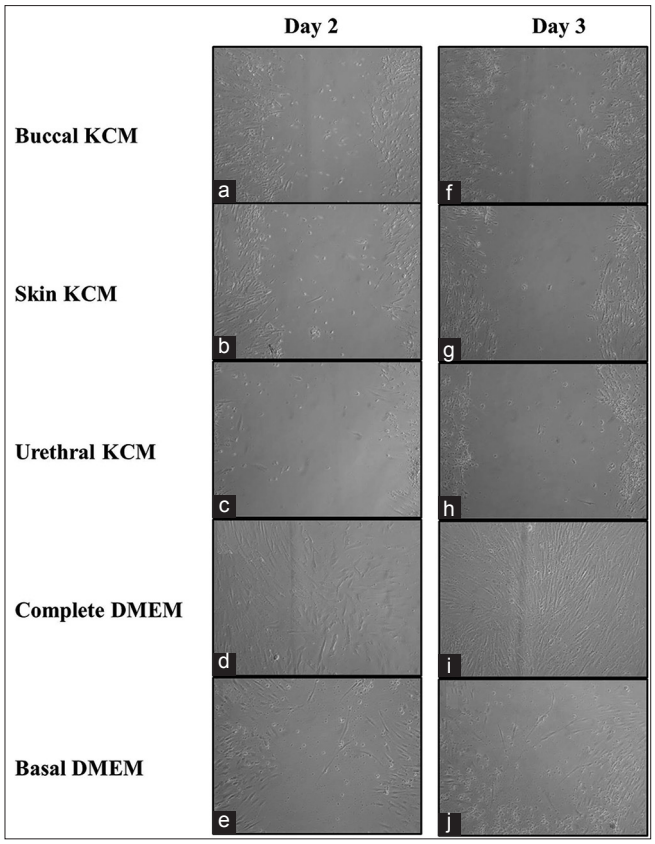

Figure 3: *In vitro* scratch assay using epithelial cell-conditioned medium from buccal mucosa (a, f), penile skin (b, g), urethral mucosa (c, h), complete Dulbecco's modified Eagle's medium (DMEM) medium (d, i) and basal DMEM (g, j). (a–e) denote Day 2. (f–j) denote Day 3. Magnification, X280

or fibrotic tissue.[20] Cytokines secreted by keratinocytes have been reported to downregulate the expression of extracellular matrix proteins in the fibroblast.[12,21] Moreover, urethral stricture, which is composed of dense collagen and fibroblasts, shows an increased expression of extracellular matrix, CTGF.[22] Urethroplasty is the gold standard treatment for stricture patients as it has a lower stricture rate and lower complication rate, and success is maintained long term.[23] The surgery involves excision of scar tissue followed by urethral reconstruction with an autologous tissue graft or tissue-engineered epithelial cell graft. Yet, the mechanisms by which cellular grafts help in stricture wound healing after urethroplasty is not fully elucidated.

To this end, we have investigated the effect of ECCM on stricture fibroblasts using a simple *in vitro* scratch assay model. We utilized primary stricture fibroblasts isolated from urethral stricture patients undergoing urethroplasty. We used primary human epithelial cells from three different tissues (penile skin, buccal mucosa and urethral mucosa) that are most commonly used for the development of tissue-engineered cell graft for urethroplasty. We observed that ECCMs from all three different primary epithelial cell cultures showed the inhibition of stricture fibroblast proliferation and migration in the scratch area. These results suggest that ECCM may contain soluble biomolecules, which inhibits the growth of stricture fibroblasts. Hence, we speculate in urethroplasty surgeries, autologous tissue grafts or tissue-engineered epithelial cell grafts may help in re-epithelialization and soluble cytokines secreted by epithelial cells may suppress the proliferation and migration of stricture fibroblasts. One may be constrained to apply these laboratory data, where the tests have been done for 3 days, to clinical situations of mitigation of stricture and recurrence of stricture, which manifests usually 5–6 months later, etc. To address this, one would have to conduct a statistically significant Phase 2 clinical trial, where strictures are grafted with laboratory expanded keratinocytes from both allogeneic and autologous sources and followed-up for about 9 months after intervention. Further investigation to understand the interplay between epithelial cells and stricture fibroblasts and the underlying molecular mechanisms are underway as an extension of this study. In summary, our results underscore the inhibitory function of ECCM on stricture fibroblasts' proliferation and migration.

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