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Guiding spontaneous tissue regeneration for urethral reconstruction: Long-term studies in the rabbit

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Abstract We designed long-term in vivo experiments to study rabbit urethral regeneration and remodelling over a hyaluronan biodegradable prosthesis. Seven months after the resection of a 1.5-cm-long tract of the urethra and its substitution with the prosthesis, radiological analysis showed the disappearance of the implant and the re-establishment of urethral continuity along the transmural defect. The regenerated tissue remodelled around the implant and exhibited good distensibility under pressure. Histological evaluation showed that the neo-urethra was lined with transitional epithelium and the stroma contained abundant elastic fibres. An examination of the pattern of the major cytoskeletal and cytocontractile proteins of smooth muscle cells and fibroblasts was able to distinguish fibroblasts from smooth muscle cells and myofibroblasts in the neo-urethra. These experiments provide evidence for the potential, successful use of biocompatible/bioresorbable devices for reconstructive surgery of the urethra.

Key words Hyaluronan · Urethra · Guided tissue regeneration · Rabbit

Introduction

Urethral reconstruction remains one of the most difficult urological problems. Numerous procedures for the surgical management of strictures and traumatic defects of the urethra have been proposed, including resection of the stricture, total alloplastic replacement of the urethra and the use of autologous tissue grafts [1, 4, 6, 8, 9, 11]. Results of internal urethrotomy are controversial, with risks for fistula formation and stenosis. Reflecting the difficulties involved in the surgical treatment, many patients respond poorly to the treatment itself. Furthermore, most urethroplasties are carried out using skin for the replacement of the urethra, implying consumption of tissue and time.

A novel, alternative approach to creating a new urethra has recently been attempted, based on the process of spontaneous regeneration. Previous experimental evidence has demonstrated the feasibility of this concept, for when a tract of the urethra undergoes total or partial resection and temporary substitution with biodegradable, biocompatible guides, the urethral stumps regenerate and remodel over the prosthesis [2, 5, 10]. Features of the regenerated urethral tissue have not been investigated sufficiently. Our group demonstrated the presence of elastic fibres and fibroblasts in the regenerated urethral tissue, over an observation period of 1 month [5]. The aims of the experiments described here were twofold. First, experiments were designed to investigate the long-term outcome of urethral regeneration over a bioresorbable prosthesis. Second, experiments were carried out to better characterize the regenerated urethra. We took advantage of immunohistochemical methods, employing a panel of monoclonal antibodies specific for the major cytoskeletal and cytocontractile proteins of smooth muscle cells and fibroblasts, namely smooth-muscle myosin and α-actin, desmin and vimentin.

Materials and methods

Animals

A total of 8 male New Zealand rabbits weighing about 3 kg entered the study. Four animals underwent surgery for urethral reconstruction. Four unoperated age-matched rabbits were used as controls. Experiments were performed according to the Federation of European Laboratory Animal Science Association guidelines on the care and use of laboratory animals.

Surgery

Rabbits were anaesthetized with sodium pentobarbital (35 mg/kg i.v.). Firstly, the abdomen was opened by a midline, suprapubic

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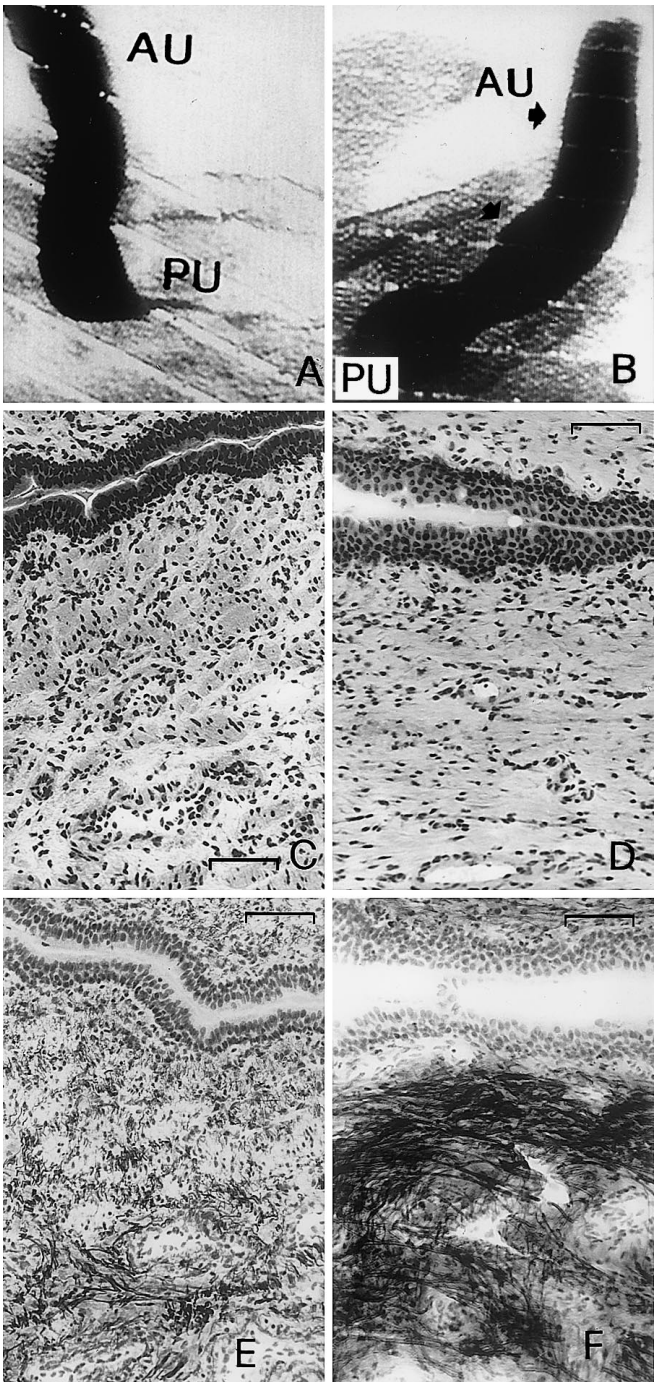

Fig. 1A–F Urethrographic and histological findings. Urethrography of a control (**A**) and of an operated animal (**B**). Grafted area is indicated by *arrows*. *AU* anterior urethra, *PU* posterior urethra. Scale bar represents 5.6 mm (**A**) and 4.0 mm (**B**). Cross-sections of the normal (**C**) and of the regenerated urethra (**D**), stained with haematoxylin and eosin. Scale bar represents 70 μm. Cross-sections of the normal (**E**) and of the regenerated urethra (**F**), stained by Weigert's method. The elastic component is represented by dark fibres. Scale bar represents 75 μm (**E**) and 65 μm (**F**)

incision to expose the bladder, which was transvesically catheterized to allow postoperative diversion of bladder drainage from the urethra. A PE-240 4-cm-long cannula was inserted into the bladder through the apex and secured with a 4–0 silk ligature. The abdomen was then closed using standard surgical technique and the cannula exit was made over the abdomen midline. Secondly, the cavernosal urethra was exposed and mobilized from the corpora cavernosa. A 1.5-cm segment (corresponding to the length of the pendulous urethra) was totally excised and simple interrupted 5–0 catgut sutures used to anastomose the guide, bridging the urethral defect in an end-to-end fashion. The guide consisted of a 2-cm-long 7-Fr hyaluronan–benzyl ester fibre mesh tube, with an external and internal diameter of 2.4 and 1.5 mm respectively (Fidia Advanced Biopolymers, Abano Terme, Padua, Italy). Following anastomosis, the wound and skin were closed in layers with 5–0 catgut and 4–0 silk sutures.

At the end of the intervention, rabbits were housed with a protective collar. Four weeks later, the vesical cannula was surgically removed and the bladder and the abdomen closed using standard surgical technique. Enrofloxacin was given 12 h before surgery and for 5 days postoperatively.

Urethrographic analysis

All rabbits, both operated animals and controls, underwent retrograde urethrography prior to surgery and sacrifice. This was done to ensure that normal anatomy was present. Rabbits were transported to the X-ray room and placed in a prone position. A 10-ml syringe filled with contrast material was connected to a 12-Fr Foley catheter that was temporarily housed in the urethral meatus and fixed with a 2–0 silk ligature. The anterior urethra was filled with contrast material and a complete film of the pelvis was taken. Distensibility of the urethra was observed at 50 mmHg of intraurethral pressure.

Histology

After a rabbit had been killed the urethra was excised, washed several times in phosphate-buffered saline (PBS), frozen in liquid nitrogen and stored at −80°C. Eight-micrometre thick sections were taken serially using a cryostat (Reichert-Jung, Nussloch, Germany). The tissue was used separately for histological examination and immunofluorescence studies (see below). Specimens for histological analysis were stained with the standard haematoxylin and eosin method and by Weigert's method for identification of elastic fibres before light microscopic examination.

Immunofluorescence

Cryosections (8 μm thick) were processed for indirect immunofluorescence using the following primary monoclonal antibodies: anti-desmin and anti-vimentin clone Vim 3B4 (Boehringer Mannheim, Mannheim, Germany), anti-smooth-muscle α-actin (Sigma, St. Louis, Mo.), SM-E7 monoclonal anti-smooth-muscle myosin antibody (previously characterized [17]: a kind gift from Prof. S. Sartore). The secondary antibody was anti-mouse IgG conjugated with fluorescein isothiocyanate (Serotec, Kidlington, Oxford, UK). Unfixed cryosections with the appropriate dilutions of the specific primary antibody in PBS–1% bovine serum albumin were incubated at 37°C in a humidified chamber for 30 min. After washing with PBS, sections were incubated with the secondary antibody under the conditions described above, fixed with 1.5% *p*-formaldehyde in PBS for 10 min and mounted in glycerol–polyvinyl alcohol aqueous. Specimens were observed with a Zeiss epifluorescence microscope (Zeiss, Oberkochen, Germany).

Results

Controls

Radiological examinations confirmed that controls had a normal anatomy of the urethra (Fig. 1A). The urethral lumen was lined with typical transitional epithelium

(Fig. 1C). Elastic fibres were not abundant in the subepithelium as they were in the peripheral connective tissue (Fig. 1E). The stroma stained heavily for smooth muscle myosin and α-actin, desmin and vimentin (Fig. 2A, C, E, G).

Operated animals

Urethral surgery was successful, and operated animals displayed normal voiding function through the reconstructed urethra when the vesical cannula was removed. Urethrographies obtained before the animals were killed documented the total regeneration of the urethral mucosa over the guide, along the transmural defect, and showed no fistula, obstructions or stenosis (Fig. 1B). When placed under 50 mmHg pressure by the radiopaque fluid, the regenerated urethral tract was found to be highly distensible, with a somewhat wider urethra than normal.

Histological examination showed the lumen of the neo-urethra was covered with transitional epithelium (Fig. 1D), similar to the normal lining of the urethra. There were no macroscopic histological differences between the haematoxylin-eosin stained sections of urethral tissue from operated animals and controls.

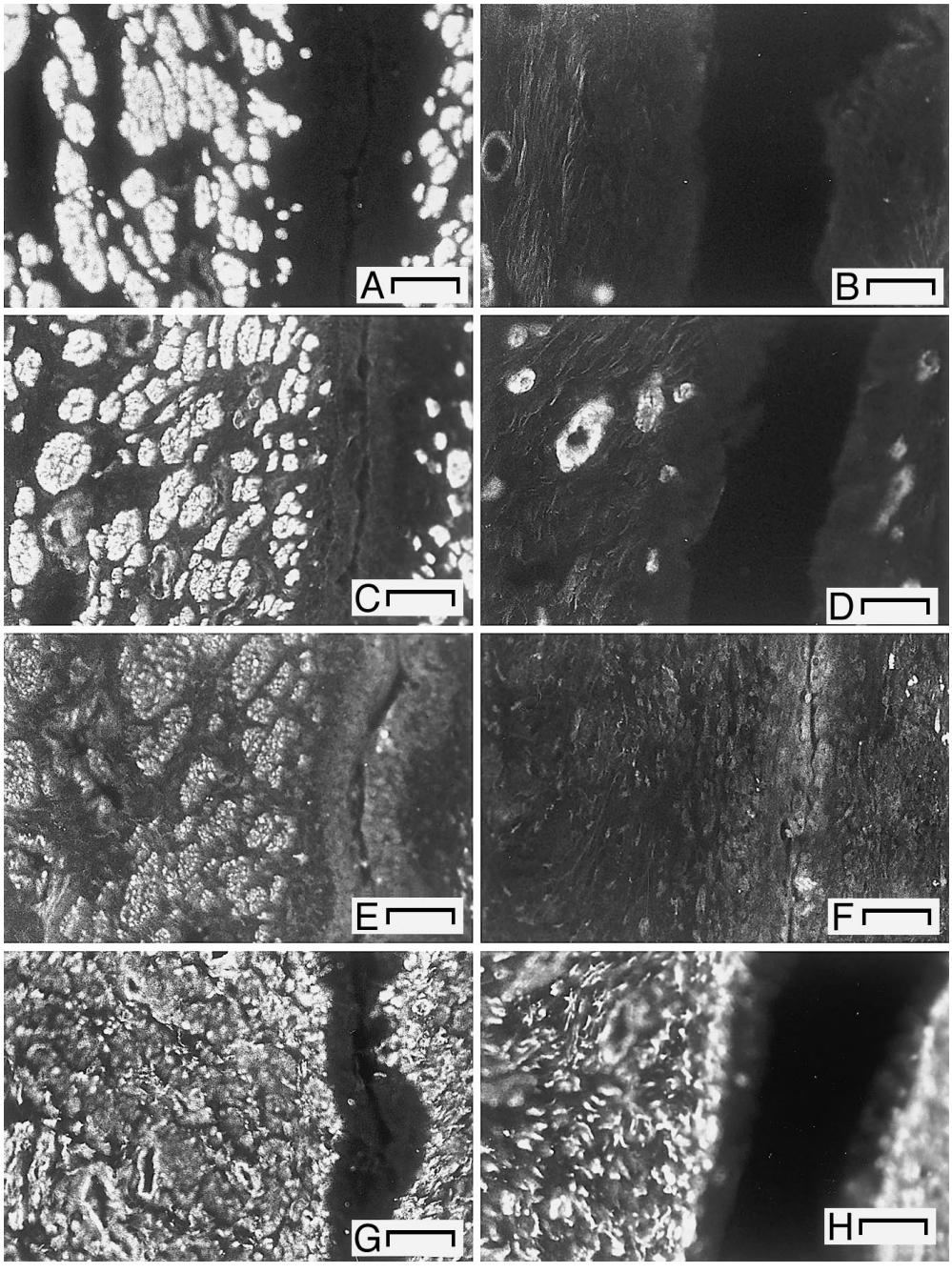

Fig. 2A–H Photomicrographs showing indirect immunofluorescence staining on cross-sections obtained from controls (**A**, **C**, **E**, **G**) and from operated animals (**B**, **D**, **F**, **H**) treated with smooth muscle myosin (**A**, **B**), smooth muscle α-actin (**C**, **D**), desmin (**E**, **F**) and vimentin (**G**, **H**) antibodies. Note that cells of the neo-urethra are stained with vimentin but negative for smooth muscle myosin, α-actin and desmin. Scale bar represents 55 μm

The regenerated connective tissue contained abundant elastic fibres, as did the sub-epithelial layer also (Fig. 1F). Immunostaining with anti-smooth muscle myosin and α-actin and with desmin antibodies was negative, and delineated the vessels alone (Fig. 2B, D, F). The neo-urethra was found to express vimentin (Fig. 2H).

Discussion

In a previous study [5] we assayed the potential advantage of hyaluronan–benzyl ester guide as a temporary urethral prosthesis, and found that urethral continuity over the hyaluronan prosthesis had been re-established within the first postoperative week. By this time eosinophilic cells, strongly suggestive of fibroblasts, rapidly proliferated around the prosthesis and effectively caused remodelling of newly formed tissue. Since fibroblasts are not stable cell phenotypes and may undergo processes of differentiation, in the present study we wanted to characterize the long-term immunospecificity of the neo-urethra for the major cytoskeletal and cytocontractile proteins.

According to some authors, the immunofluorescence pattern depends on the nature and origin of cells. Thus, undifferentiated fibroblasts display a strong positive reaction to the cytoskeletal protein vimentin but not to the cytoskeletal protein desmin or to the cytocontractile proteins myosin and α-actin, typically localized in the smooth muscle cells. Myofibroblasts derive from differentiation of fibroblasts and have morphological characteristics of both fibroblastic and smooth muscle cells. Certain types of myofibroblasts express a marker of smooth muscle differentiation, α-actin [3, 12, 14]. Immunoidentification of cells involved in the regenerative process can be of primary interest to distinguish between tissues that are in the physiological or in the pathological condition. For instance, it is well known that smooth muscle myosin is a marker for terminal smooth muscle differentiation, whilst α-actin is permanently expressed in tissue remodelling and fibrosis [6, 7, 16]. Moreover, smooth muscle and/or myofibroblasts are evident in urethral stricture [13–16].

In the regenerated connective tissue we did not find cellular elements having the known immunofluorescence features of myofibroblasts or smooth muscle cells (positivity to smooth muscle myosin and/or α-actin and/or desmin). The absence of cells containing smooth muscle myosin and/or α-actin in their cytoplasm suggests that the neo-urethra does not have contractile capacity (but the potential presence of a population of muscle or non-muscle cells with minor cytocontractile proteins cannot be excluded). The loss of cytocontractile proteins was not accompanied by marked changes in the mechanical and functional properties of the neo-urethra. It possesses a subepithelial network with plentiful elastic fibres, which make the regenerated tissue distensible and well-functioning, as confirmed by radiological observation. On the other hand, both immunofluorescence and radiological findings indicate that the regenerated urethra does not possess features of pathological conditions. Overall, these findings are very similar to those reported at 1 month following implantation of the hyaluronan guide [5] and provide evidence that the process of spontaneous regeneration may constitute a practicable route for the surgical management of defects of the urethra.

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