Neuroscience Letters 541 (2013) 248–252

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Prostate carcinoma cell growth-inhibiting hydrogel supports axonal regeneration in vitro

K. Franke[a], M. Baur[a], L. Daum[b], M. Vaegler[b], K.-D. Sievert[b], B. Schlosshauer[a,*]

[a] NMI Naturwissenschaftliches und Medizinisches Institut an der Universität Tübingen, Markwiesenstr. 55, D-72770 Reutlingen, Germany
[b] Department of Urology, Eberhard Karls University Tuebingen, Hoppe-Seyler-Str. 3, D-72076 Tuebingen, Germany

HIGHLIGHTS

- Novel implant concept of cell type-specific regeneration matrix after prostatectomy.
- Injectable two components: collagen fragments and crosslinking transglutaminase.
- Resulting hydrogel dotted with cytostatics.
- Axonal and Schwann cell growth but apoptosis of cancer cells on hydrogel.
- Eight different physical and biological assays for characterization.

ARTICLE INFO

Article history:
Received 17 December 2012
Received in revised form 18 January 2013
Accepted 29 January 2013

Keywords:
Axon growth
Cytosine arabinoside
Gelatin hydrogel
Nerve regeneration
Prostatectomy
Transglutaminase

ABSTRACT

Prostate cancer is the most common malignant tumor in men. Radical prostatectomy, the most common surgical therapy, is typically accompanied by erectile dysfunction and incontinence due to severing of the axons of the plexus prostaticus. To date, no reconstructive therapy is available as the delicate network of severed nerve fibers preclude the transplantation of autologous nerves or synthetic tube implants. Here, we present an injectable hydrogel as a regenerative matrix that polymerizes in situ and thus, adapts to any given tissue topography. The two-component hydrogel was synthesized from a hydrolyzed collagen fraction and stabilized by enzymatic crosslinking with transglutaminase. Physical analysis employing osmolarity measurements and cryosectioning revealed an isotonic, microstructured network that polymerized within 2 min and displayed pronounced adhesion to abdominal tissue. Cell culturing demonstrated the biocompatibility of the gel and a general permissiveness for various neuronal and non-neuronal cell types. No effect on cell adhesion, survival and proliferation of cells was observed. A chemotherapeutic drug was integrated into the hydrogel to reduce the risk of fibrosis and tumor relapse. Significantly, when the hydrogel was employed as a drug release depot in vitro, aversive fibroblast- and prostate carcinoma cell growth was inhibited, while axonal outgrowth from peripheral nervous system explants remained completely unaffected. Taken together, these results suggest that the gel's adequate viscoelastic properties and porous microstructure, combined with its tissue adhesion and neuritotrophic characteristics in the presence of a cell type-specific cytostatic, may constitute an appropriate hydrogel implant applicable to patients suffering from prostatectomy associated side effects.

1. Introduction

With more than 220,000 new cases per year in the US, prostate carcinoma is the most frequent cancer among the male population. Despite improved diagnostic tools, prostate carcinoma has remained the third major cause of mortality among men [1]. Radical prostatectomy is especially recommended for younger patients with a life expectation longer than 10 years. For the majority of afflicted patients, transient or permanent erectile dysfunction (ED) and impaired micturition (incontinence) have a considerable impact on quality of life. These conditions are caused by intraoperative lesioning of sensory fibers projecting to the sacral spinal cord, and of sympathetic and parasympathetic axons which run from the plexus prostaticus at the dorsolateral prostate to the corpora cavernosi of the penis. In contrast to compact peripheral nerves, these nerve bundles are histologically imposing, comprising dozens of widespread, diffuse microstructures that are extremely difficult to address in microsurgery [17]. Transplantations of autologous Nervus suralis from the lower leg or of synthetic nerve guides, in order to bridge nerve gaps after radical prostatectomy have been

* Corresponding author. Tel.: +49 7121 51530 20; fax: +49 7121 51530 16.
E-mail address: schlosshauer@nmi.de (B. Schlosshauer).

0304-3940/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neulet.2013.01.057

of limited success and have not evolved into an accepted curative approach [16]. We therefore aimed to develop a biocompatible hydrogel that adapts to the variable topography of the operation situs, that provides a neuritotrophic growth substratum into which pharmacological agents can be incorporated to counteract growth-inhibitory fibroblasts and potential carcinoma relapses.

2. Materials and methods

2.1. Human prostate

The indication for the radical prostatectomy in the selected 82-year-old male was given by the histologically confirmed prostate cancer. A radical retropubic prostatectomy was performed via a lower midline incision in bilateral nerve sparing ascending fashion. Besides photographic documentation, no other manipulation of the dissected organ was performed.

2.2. Rats

Sprague Dawley rats were used for the hydrogel adhesion experiments and for the preparation of primary dorsal root ganglia, whilst (for logistic reasons) Lewis rats were employed for the isolation of sciatic nerve fibroblasts and Schwann cells. For hydrogel adhesion tests in vivo, rats were euthanized and the anteriolateral surface of the prostate in the lower abdomen was exposed by medial laparatomy. The mixed hydrogel solution including transglutaminase (see below) was pipetted onto the prostate and photographically documented.

2.3. Hydrogel

For hydrogel production, purified gelatin granules (GELITA AG, Eberbach, Germany) were dissolved in cell culture medium to obtain a 10% gelatin solution and incubated for 30 min at 37 °C before homogenizing for 2 h at 50 °C. For the determination of the osmolarity of stock solutions a VapoPressure Osmometer (Wescor Inc., USA) was used. For production of AraC containing hydrogels, AraC (cytosine arabinoside, Sigma–Aldrich Chemistry GmbH, Steinheim, Germany; in PBS or cell culture medium, filtered and stored at −20 °C) was added to gelatin and homogenized (4 h, 42 °C). For AraC concentrations see "AraC impact". The cross-linking bacterial enzyme transglutaminase (TGs, EC2.3.2.13, N-Zyme BioTec GmbH, Darmstadt, Product No. T014, Lot No. 1007T014) was dissolved (7.5 U/ml PBS; 1 U = 1 μmol reacted substrate/min) and sterile filtered. For microsocpic gel analysis, gels were quick-frozen and cryosectioned (10 μm slices; Cryostat Leica CM3050S, Leica Microsystems, Wetzlar, Germany) and hematoxylin–eosin stained.

2.4. Cells and tissues

Human prostate carcinoma cell line DU-145 and L929 cells (immortalized mouse fibroblasts) from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig Germany); Sciatic fibroblasts were isolated from neonatal Lewis rat nerve explants [15]. Schwann cells from the sciatic nerves of adult Lewis rats were enriched in three consecutive steps as previously described: (a) elimination of proliferating cells, (b) complement-mediated cell lysis of contaminating fibroblasts, and (c) growth factor mediated Schwann cell expansion; dorsal root ganglia (DRG) from Sprague Dawley rat embryos (E17/18) [6]. Cultures were performed as described previously [7].

2.5. AraC impact

Cells (5000 cells/cm^2) were pre-incubated (6 h, 37 °C) before AraC (cytosine arabinoside, Sigma) was added to the medium for 42 h resulting in seven different final concentrations (0.1 μM, 1 μM, 5 μM, 10 μM, 50 μM, 100 μM and 1 mM). Cells without AraC-medium served as positive control, cells with medium/10% DMSO as negative control. To analyze the impact of AraC in hydrogels, cells (5000 cells/cm^2), or DRG explants on PDL/laminin coated coverslips (or directly onto hydrogels) were precultured (6 h) on the bottom of transwell culture dishes (Greiner Bio-One GmbH, Frickenhausen, Germany). Transwell membrane inserts (5 μm pores) were coated with 70 μl of the 75 μM AraC hydrogel, incubated for 1 h at 37 °C and transferred to the cells/explants dishes. The volume of the hydrogel was equal to 1/10th of the total medium volume to achieve a maximal concentration of 7.5 μM AraC in the culture. After 42 h cell viability was determined by a resazurin assay (Sigma–Aldrich, Germany) and by immunocytochemistry.

2.6. Immunocytochemistry

Primary antibodies (SMI31 against neurofilament; Sternberger Monoclonals, USA; 1:1000 in 1% BSA/PBS) were applied for 1–2 h/22 °C, or 16 h/4 °C. Secondary antibody (Cy3- or Alexa488-labeled goat anti-mouse; Dianova, Hamburg, Germany) were used 1:250 in 1% BSA/PBS, 1–2 h, 22 °C. Cell nuclei (5 μg/ml DAPI (4′,6-diamidine-2-phenylindol) in PBS, (10 min, 22 °C); phalloidin oregon green (1:50/PBS, 3 h, 22 °C; Invitrogen, Darmstadt, Germany).

2.7. Statistics

Statistical analysis was based on at least three independent series of experiments with duplicates per series, using one-way ANOVA followed by a Tukey HSD posttest comparison. Data (mean ± SD) were considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Physical properties of the gelatin hydrogel

The initial effort was to synthesize an implantable neuritotrophic growth substratum that was biocompatible, degradable and adaptable to variable tissue topographies as encountered during surgical interventions in humans. The basic principle was to employ a soluble matrix that would rapidly coagulate in situ after addition of a crosslinker. We chose hydrolyzed collagen type I (i.e. a specific gelatin fraction from pig skin) as matrix component, because it met the relevant characteristics (see Section 4 for more details). To counteract the solubility of hydrolyzed collagen at physiological temperature and avoid cytotoxicity of aldehyde-crosslinking (typically used to stabilize gelatine matrices), we instead employed purified transglutaminase which is used in food processing and approved for human consumption. With a final concentration of 8% gelatin supplemented with 1.5 units/ml transglutaminase, a translucent hydrogel formed at 22 °C within 2 min as qualitatively judged by its cohesive consistency (Fig. 1A). For the characterization of the native microstructure, enzymatically crosslinked hydrogels were exposed to rapid freezing without prior aldehyde-fixation, cryosectioned and stained. As evident from Fig. 1B the macroscopically homogeneous appearance of the hydrogel was based on a (probably liquid-filled) meshwork of collagen hydrolysate fibers. Such a highly heterogeneous, non-compact microarchitecture is likely to facilitate subsequent resorption in vivo.