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Recreating the tumor microenvironment in a bilayer, hyaluronic acid hydrogel construct for the growth of prostate cancer spheroids

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ABSTRACT

Cancer cells cultured in physiologically relevant, three-dimensional (3D) matrices can recapture many essential features of native tumor tissues. In this study, a hyaluronic acid (HA)-based bilayer hydrogel system that not only supports the tumoroid formation from LNCaP prostate cancer (PCa) cells, but also simulates their reciprocal interactions with the tumor-associated stroma was developed and characterized. HA hydrogels were prepared by mixing solutions of HA precursors functionalized with acrylate groups (HA-AC) and reactive thiols (HA-SH) under physiological conditions. The resultant viscoelastic gels have an average elastic modulus of 234 ± 30 Pa and can be degraded readily by hyaluronidase. The orthogonal and cytocompatible nature of the crosslinking chemistry permits facile incorporation of cytokine-releasing particles and PCa cells. In our bilayer hydrogel construct, the top layer contains heparin (HP)-decorated, HA-based hydrogel particles (HGPs) capable of releasing heparin-binding epidermal growth factor-like growth factor (HB-EGF) in a sustained manner at a rate of 2.5 wt%/day cumulatively. LNCaP cells embedded in the bottom layer receive the growth factor signals from the top, and in response form enlarging tumoroids with an average diameter of 85 μm by day 7. Cells in 3D hydrogels assemble into spherical tumoroids, form close cellular contacts through E-cadherin, and show cortical organization of F-actin, whereas those plated as 2D monolayers adopt a spread-out morphology. Compared to cells cultured on 2D, the engineered tumoroids significantly increased the expression of two pro-angiogenic factors, vascular endothelial growth factor-165 ($VEGF_{165}$) and interleukin-8 (IL-8), both at mRNA and protein levels. Overall, the HA model system provides a useful platform for the study of tumor cell responses to growth factors and for screening of anticancer drugs targeting these pathways.

1. Introduction

For decades, cancer biologists relied on two-dimensional (2D) monolayer cell culture platforms and/or *in vivo* animal models (xenografts) to investigate the complex mechanisms of tumorigenesis, angiogenesis, invasion and metastasis [1,2]. Using these complementary systems, researchers have gained improved understanding of cancer biology and have developed many efficacious anti-cancer treatment methods. However, both monolayer cultures and xenografts have inherent limitations [3]. Because of the lack of spatial guidance cues needed to establish proper cell–cell contacts and cell-matrix interactions, 2D cell cultures are physiologically irrelevant and experimentally unreliable [4–6]. Although studies based on animal models predict more pathologically relevant outcomes, the presence of many uncontrollable variables associated with these models makes it challenging to determine the impact of specific factors on tumor progression or to identify the therapeutic efficacies of novel personalized medicine [3]. Moreover, testing therapeutic agents in animal xenografts is expensive, tedious and time consuming, thus further delaying the translation of new technologies from bench to bedside [4].

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Recently, various three-dimensional (3D) cell culture systems have been developed as alternative *in vitro* platforms for improved understanding of cancer biology [1,2,5–9]. These 3D culture systems have the potential to overcome the limitations associated with traditional 2D monolayer cultures, as well as the complexities of *in vivo* models, by generating a tightly controlled molecular and mechanical microenvironment typical of tumors *in vivo* [6]. Both natural (e.g. basement membrane extract [10,11], type I collagen [3,7,12]) and synthetic materials (e.g. PLGA [6] and PEG [5,13]) have been used to create matrices and scaffolds for tumor engineering purposes. For instance, researchers have cultured breast cancer cells in Matrigel™ to investigate the relationship between colony morphology and gene expression [14]. In other studies, medical grade poly(ε-caprolactone)-tricalcium phosphate composite scaffolds were used to study prostate cancer (PCa) bone metastasis [15]. In general, hydrogels derived from animal tissues suffer from batch-to-batch variations [5], whereas those prepared using synthetic polymers are physiologically irrelevant [16]; therefore these materials have limited applications in *in vitro* tumor engineering.

We are interested in the *in vitro* reconstruction of prostate tumor tissues using hyaluronic acid (HA)-based hydrogels [17]. HA is a ubiquitous non-sulfated glycosaminoglycan (GAG) present in extracellular matrices (ECM) of all vertebrates [18]. HA is not only a structural component in the tumor ECM that contributes to the overall tissue integrity but also a biologically active molecule that promotes tumor progression through cell signaling. Expressed by both motile PCa epithelial cells and associated stromal cells, HA is concentrated within the tumor-associated stromal ECM [19,20]. The entangled, HA-rich matrix can be partially degraded by hyaluronidase (HAase), thereby creating a permissive pathway for the migration of tumor cells [21]. The migration process is mediated by cell surface HA receptors such as CD44 or RHAMM [19]. HA also protects the tumor tissues against immune surveillance and chemotherapeutic agents [19,22]. Finally, the angiogenic properties of HA fragments [23] facilitate the recruitment of new blood vessels into the growing tumor. Therefore, HA-based hydrogels have been used for *in vitro* culture of poorly adherent bone metastatic PCa cells (C4-2B) [17] and in studies of the mechanobiology of malignant brain tumors [8].

Increasing evidence points to the active roles cancer-associated stroma plays in tumor initiation and progression [24]. The crosstalk between the tumor tissue and its surrounding stroma relies on the biological paracrine signaling mediated by soluble factors secreted by cancer cells or neighboring stromal cells. These secreted factors direct the remodeling of the stromal microenvironment and the progression of the disease [25–27], thus profoundly affecting the growth, invasion and metastasis of tumor tissues [26]. Although various *in vitro* tumor models have been created using 3D matrices, the stromal component often has been overlooked. Our studies were designed to address the need to establish 3D culture systems that can recreate the unique tumor-stroma niche. In particular, we sought to introduce the controlled release of ECM bound factors to allow for the *in vitro* engineering of tumor tissues under well-controlled and reproducible conditions that are amenable to clinical testing. Among the various biological cues involved in prostate tumor-stromal interactions, heparin-binding epidermal growth factor-like growth factor (HB-EGF) is widely recognized as a crucial mediator for tumorigenesis and cancer progression [28]. HB-EGF is secreted by a variety of stromal cells, such as fibroblasts [27] and associated inflammatory cells [29], and is mainly localized in smooth muscle compartments within the prostate stroma [28]. HB-EGF serves as a strong mitogen for tumor growth [27,30] and is a potent inducer of angiogenesis [30].

We describe herein a bilayer hydrogel platform that was created using orthogonally functionalized HA derivatives as the building blocks and HA-based hydrogel particles (HGPs) [31] as HB-EGF-releasing devices. To mimic the tumor/stroma interaction, HB-EGF-loaded HGPs were entrapped within the top gel layer while LNCaP PCa cells were encapsulated in the bottom layer (Fig. 1A). The mechanical properties and enzymatic degradation of the hydrogel matrix were evaluated by rheometric and colorimetric analyzes, respectively. The HB-EGF release kinetics were quantified by ELISA. The cytocompatibility of the hydrogel matrix was confirmed both by live/dead staining and PicoGreen assay. Cell adhesion and cell morphology were characterized by standard immunohistochemical analyzes. The expression and production of angiogenic factors were quantified at both the mRNA level and the protein level. Overall, the HA-based bilayer platform supports the growth of prostate tumoroids, models paracrine interactions in the tumor microenvironment, and leads to the production of pro-angiogenic signals in growing tumoroids.

2. Materials and methods

2.1. Materials

HA (sodium salt) was generously donated by Genzyme Corporation (500 kDa, Cambridge, MA) or purchased from Lifecore Biomedical (357 kDa, Chaska, MN). 3,3′-Dithiobis (propanoic acid), hydrazine hydrate, 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC), 1-hydroxybenzotriazole (HOBt), dithiothreitol (DTT), adipic acid dihydrazide (ADH), N-acryloxysuccinimide (NHS-AC), heparin (HP, sodium salt, 18 kDa, 177 USP units mg^{-1}) from porcine intestinal mucosa, dioctyl sulfosuccinate sodium salt (AOT, 98%), 2,2,4-trimethylpentane (isooctane, anhydrous), divinyl sulfone (DVS) and bovine testicular hyaluronidase (HAase, 30,000 U/mg) were obtained from Sigma Aldrich (Milwaukee, WI). Cascade blue hydrazide (CB, sodium salt) was purchased from Molecular Probes (Carlsbad, CA). Recombinant human HB-EGF and the Quantikine ELISA kits for vascular endothelial growth factor-165 ($VEGF_{165}$) and interleukin-8 (IL-8) were purchased from R&D Systems (Minneapolis, MN). The ELISA kit for HB-EGF was purchased from RayBiotech, Inc. (Norcross, GA). Alexa Fluor® 488 Mouse anti-human E-Cadherin was obtained from BD Pharmingen ™ (San Diego, CA). LNCaP PCa cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). NuPAGE® Bis-Tris Gel was purchased from Life Technologies (Grand Island, NY). Rabbit anti-HYAL1 antibody and primary and secondary antibodies for β-actin were purchased from Sigma (Milwaukee, WI). Goat anti-rabbit HRP-conjugated secondary antibody was obtained from Thermo Fisher Scientific (Barrington, IL). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). All other reagents were used as received unless otherwise noted.

2.2. Cell culture

LNCaP PCa cells were maintained in Corning (Lowell, MA) tissue culture flasks (75 cm^2) at 37 °C in 5% (v/v) CO_2 in a RPMI-1640 medium supplemented with 5% (v/v) fetal bovine serum (FBS), 100U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate in 0.085% (v/v) saline (P/S). The medium was changed every other day, and cells were routinely passaged using 0.25% (w/v) trypsin containing ethylenediaminetetraacetic acid (EDTA·4Na).

2.3. Synthesis and characterization of soluble HA precursors

HA with a molecular weight of 500 kDa was used for the synthesis of HA derivatives. Acrylated HA (HA-AC) was synthesized by reacting adipic acid dihydrazide-modified HA (HA-ADH, 35% ADH incorporation determined by ^{1}H NMR [18]) with N-acryloxysuccinimide (NHS-AC), as previously reported [32]. The product was obtained as a freeze-dried solid (yield: 70%). Sulfhydryl groups were incorporated in HA (HA-SH) via the reaction with a disulfide-containing dihydrazide compound, followed by reduction with DTT, according to reported methods [33]. The solid HA-SH was obtained after lyophilization (yield: 80%). The chemically modified HA products, HA-AC and HA-SH, were stored at −20 °C prior to use. The degree of modification and the molecular weight of HA derivatives were characterized by ^{1}H NMR and gel permeation chromatography (GPC), respectively.

2.4. Synthesis and characterization of HA hydrogels

2.4.1. Hydrogel synthesis

HA derivatives were separately dissolved in phosphate buffered saline (PBS, pH 7.4) at a concentration of 20 mg/mL. After the HA-AC and HA-SH solutions were thoroughly mixed (1/1, v/v), the mixture was aliquoted to cell culture inserts (Diameter: 12 mm, pore size: 0.4 μm) and were incubated at 37 °C for 6 h to obtain fully crosslinked hydrogel discs (height: 1.3 mm, diameter: 12 mm).