Biomaterials 35 (2014) 3319–3330

Contents lists available at ScienceDirect

Biomaterials

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

A hydrogel-based tumor model for the evaluation of nanoparticle-based cancer therapeutics

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ARTICLE INFO

Article history:
Received 11 December 2013
Accepted 23 December 2013
Available online 18 January 2014

Keywords:
Hyaluronic acid
Hydrogel
3D tumor model
Nanoparticles
Cancer therapeutics
Drug resistance

ABSTRACT

Three-dimensional (3D) tissue-engineered tumor models have the potential to bridge the gap between monolayer cultures and patient-derived xenografts for the testing of nanoparticle (NP)-based cancer therapeutics. In this study, a hydrogel-derived prostate cancer (PCa) model was developed for the *in vitro* evaluation of doxorubicin (Dox)-loaded polymer NPs (Dox-NPs). The hydrogels were synthesized using chemically modified hyaluronic acid (HA) carrying acrylate groups (HA-AC) or reactive thiols (HA-SH). The crosslinked hydrogel networks exhibited an estimated pore size of 70–100 nm, similar to the spacing of the extracellular matrices (ECM) surrounding tumor tissues. LNCaP PCa cells entrapped in the HA matrices formed distinct tumor-like multicellular aggregates with an average diameter of 50 μm after 7 days of culture. Compared to cells grown on two-dimensional (2D) tissue culture plates, cells from the engineered tumoroids expressed significantly higher levels of multidrug resistance (MDR) proteins, including multidrug resistance protein 1 (MRP1) and lung resistance-related protein (LRP), both at the mRNA and the protein levels. Separately, Dox-NPs with an average diameter of 54 ± 1 nm were prepared from amphiphilic block copolymers based on poly(ethylene glycol) (PEG) and poly(ε-caprolactone) (PCL) bearing pendant cyclic ketals. Dox-NPs were able to diffuse through the hydrogel matrices, penetrate into the tumoroid and be internalized by LNCaP PCa cells through caveolae-mediated endocytosis and macropinocytosis pathways. Compared to 2D cultures, LNCaP PCa cells cultured as multicellular aggregates in HA hydrogel were more resistant to Dox and Dox-NPs treatments. Moreover, the NP-based Dox formulation could bypass the drug efflux function of MRP1, thereby partially reversing the resistance to free Dox in 3D cultures. Overall, the engineered tumor model has the potential to provide predictable results on the efficacy of NP-based cancer therapeutics.

1. Introduction

Over the past few decades, a concerted effort has been made to develop nanotechnology-based cancer diagnosis and therapy, with the premise of potentially prolonging patients' life expectancy, at the same time reducing treatment-related side effects [1,2]. Encapsulating anti-cancer drugs in polymeric nanoparticles (NPs) are proposed, and in some cases have been shown, to improve the pharmacological properties of drugs [3]. The NP-based drug delivery systems also have the potential to increase the therapeutic efficacy through the enhanced permeability and retention (EPR) effect or the active targeting strategies [4]. To date, the promise of nanomedicine in patients has not been fully realized owing to the numerous biological barriers to the delivery of cancer therapeutics [5,6].

Currently, preliminary screening of NP-based cancer therapeutics is usually performed on two-dimensional (2D), monolayer cell culture systems. While straightforward and easy to perform, 2D cultures identify promising drug formulations that do not translate similarly *in vivo* in animal models or in patients [7], and many limitations associated with NP formulations are not discovered until a later stage of product development. The inconsistency in therapeutic outcomes can be attributed, in part, to the inability of

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http://dx.doi.org/10.1016/j.biomaterials.2013.12.080

monolayer cultures to accurately account for the extracellular barriers [8]. While NPs delivered to a monolayer cell culture typically reach cells without any physical restriction, the diffusion of NPs administrated *in vivo* would be hindered by the complex, tumor-associated extracellular matrix (ECM) [8,9]. The 3D organization of a tumor mess also fundamentally alters the diffusion profile for drugs, both through the cell–cell contacts and cell–matrix interactions [8].

In addition to altered cell organizations and extracellular environments, 2D monolayer cultures promote cells to adopt a non-natural phenotype, thereby influencing cellular responses to the delivered drugs [8]. Whereas cells in 2D cultures are exposed to a uniform environment with sufficient oxygen and nutrients, cells in the solid tumor tissues are exposed to gradients of critical chemical and biological signals [10]. Such a unique microenvironment can exert both stimulatory and inhibitory effects on tumor progression [10]. Moreover, tumor cells from cancer patients are frequently found to be resistant to a broad spectrum of chemotherapeutic drugs without previous exposure to those cytotoxic agents [11–13]. The intrinsic drug resistance can be attributed, in part to the overexpression of the multidrug resistance (MDR) proteins by tumor cells [12–14]. The tumor microenvironments, namely hypoxic conditions [12,15], low nutrients supply [12] and low pH [16], all have been suggested to upregulate the expression of MDR proteins through specific cellular signaling pathways. Obviously, these essential environmental conditions cannot be recapitulated in traditional 2D monolayer cultures.

To overcome the limitations associated with traditional 2D monolayer cultures, various 3D culture systems aiming to recreate the tightly controlled molecular and mechanical microenvironment typical of tumors *in vivo* have been developed and characterized [17]. These systems may bridge the gap between 2D experiments and animal studies, providing physiologically relevant platforms for optimizing the drug formulations prior to the *in vivo* assessment [8]. Both natural (e.g. type I collagen [18–20] and basement membrane extract [21,22]) and synthetic materials (e.g. poly(ε-caprolactone) (PCL) [23], poly(lactic-co-glycolic acid) (PLGA) [24] and poly(ethylene glycol) (PEG) [25]) have been used as the scaffolding materials for the engineering of 3D tumor models. While natural materials derived from animal tissues are chemically ill-defined and suffer from batch-to-batch variations, most synthetic polymers are mechanically inappropriate and physiologically irrelevant [17]. These drawbacks limit their utility as artificial matrices for the construction of physiologically relevant tumor models.

We are interested in the *in vitro* engineering of 3D models of prostate cancer (PCa) using hyaluronic acid (HA)-based hydrogel systems [17,26,27]. Produced by PCa cells and the stromal cells, HA is found to be enriched in the tumor-associated stroma [28,29]. In addition to providing the structural support to the tumor tissues [17], HA interacts with its cell surface receptors, such as CD44 or RHAMM [26], to alter cell adhesion, migration and proliferation [26]. HA's biodegradation by hyaluronidase (HAase) helps the cancer cells to escape from the primary tumor mass [26], and degraded HA fragments promote angiogenesis to allow further tumor expansion [30]. Finally, HA protects the tumor tissues to evade the immune surveillance [28]. Using HA derivatives carrying orthogonal functionalities, we have developed HA-based hydrogel systems that not only support the tumoroid formation from PCa cells [27], but also simulate the reciprocal interactions with the tumor-associated stroma [17]. The resultant tumoroids closely mimic the morphology of the native tumor tissues and exhibit strong angiogenic potentials [17]. The engineered tumor models have also been used successfully to test the efficacy of anti-cancer drugs [27].

Herein, we report the physical and biological characterization of the hydrogel-based PCa model, and explore the utility of the engineered model in the evaluation of an anti-cancer drug, doxorubicin (Dox), both in its free form and encapsulated in polymeric NPs. To this end, HA-based hydrogels were prepared using HA derivatives carrying complementary reactive groups via a Michael-type addition reaction. The resultant hydrogel matrices were used for the 3D culture of LNCaP PCa cells *in vitro*. Separately, Dox was formulated into NPs derived from an amphiphilic block copolymer. The goal of this investigation was to repurpose the nanoformulations readily available in our laboratories rather than to design polymeric nanocarriers. Using the engineered tumor model, the transport properties of the NPs and the apoptotic properties of the nanoformulation were evaluated.

2. Materials and methods

2.1. Chemicals and reagents

HA (500 kDa, sodium salt) was generously donated by Genzyme Corporation (Cambridge, MA). All chemicals necessary for the synthesis of HA derivatives and the amphiphilic block copolymers were purchased from Sigma–Aldrich (St. Louis, MO). Doxorubicin hydrochloride (Dox-HCl) was obtained from BIOTANG Inc. (Waltham, MA). Nile red (NR), bovine testicular HAase (30,000 U/mg), bovine serum albumin (BSA), sucrose, chlorpromazine hydrochloride, methyl-β-cyclodextrin (M-β-CD), genistein, colchicine and quercetin were purchased from Sigma–Aldrich (St. Louis, MO). Cell Titer-Blue® cell viability assay was obtained from Promega (Madison, WI). PEGylated (methoxy-PEG5000-SH) gold nanoparticles (PEG-AuNPs) were purchased from Cytodiagnostics (Burlington, ON). Cell Death Detection ELISA^{PLUS} was obtained from Roche Applied Science (Mannheim, Germany). Mouse anti-multidrug resistance protein 1 (MRP1) mAb (MRPm6) was purchased from Kamiya Biomedical Company (Seattle, WA). Mouse anti-lung resistance-related protein (LRP) mAb (LRP 1014) and goat anti-mouse IgG-FITC were obtained from Santa Cruz Biotechnology (Dallas, TX). All cell culture reagents were purchased from Life Technologies (Grand Island, NY). All other reagents were used as received unless otherwise noted.

2.2. Particle preparation and drug/dye encapsulation

Amphiphilic block copolymers consisting of hydrophilic PEG and hydrophobic polyester bearing pendent cyclic ketals [PEG-*b*-P(CL-*ran*-TSU)] were synthesized by ring-opening co-polymerization of ε-caprolactone (CL) and 1,4,8-trioxaspiro-(4,6)-9-undecanone (TSU), using α-hydroxyl, ω-methoxy PEG as the initiator and stannous octoate as the catalyst [31]. At a 20 wt% TSU in feed, the copolymer (abbreviated as ECT2) was found to contain 14 mol% TSU in the hydrophobic segment. ECT2 with a number average molecular weight (M_n) of 40.6 KDa was used to formulate Dox-loaded NPs. Prior to drug encapsulation, Dox-HCl was neutralized to generate Dox, following reported procedures [32]. NPs were prepared using a nanoprecipitation method. Briefly, an acetone/DMSO (1:1, v/v) solution of ECT2 (10 mg/ml, 1 ml) was slowly added to a stirred (900 rpm) aqueous phase (5 ml DI water). The mixture was allowed to stabilize for 2 h under constant agitation at room temperature to obtain blank-NPs. Dox- or NR-loaded NPs were prepared using an acetone/DMSO (1:1, v/v) solution of ECT2 (10 mg/ml, 1 ml) containing 2 mg/ml Dox or 0.1 mg/ml NR. Centrifugation (4000 rpm for 10 min) was applied to all NP suspensions to remove large polymer aggregates. The supernatant containing NPs was collected, and the free drug or dye and the solvent were removed by filtration using Amicon regenerated cellulose centrifugal filters (MWCO = 30 kDa, EMD Millipore). The collected NPs were thoroughly washed with DI water or phosphate buffered saline (PBS, pH 7.4) three times by centrifugation using the centrifugal filters. The resultant formulations were immediately subjected to physical and biological analyses.

2.3. Characterization of NPs

The hydrodynamic diameters of NPs were measured using a Zetasizer nanoZS (Malvern Instruments, Westborough, MA) by dynamic light scattering (DLS) at a concentration of 0.5 mg/ml in PBS. Transmission electron microscopy (TEM) was used to examine the particle size and morphology. TEM samples were prepared by applying a drop of NP suspension (3 μl) directly onto a carbon-coated copper TEM grid. Samples were allowed to dry under ambient condition prior to imaging using a Tecnai G2 12 Twin TEM (FEI Company, Hillsboro, OR). The mean diameter of the NPs was estimated using ImageJ based on 50 counts of the particles from the TEM image.

2.4. Drug/dye loading and release

Aliquots (1 ml each) of the Dox-NPs and NR-NPs suspension in DI water were collected and lyophilized. The dried powder was weighed accurately before being dissolved in DMSO (1 ml). The fluorescence intensity of the drug or dye was determined using a plate reader (DTX880 Multimode Detector, Beckman Coulter, Fullerton, CA) at 485 nm (excitation)/595 nm (emission) for Dox and at 535 nm