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Tumor stroma-containing 3D spheroid arrays: A tool to study nanoparticle penetration

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ABSTRACT

Nanoparticle penetration through tumor tissue after extravasation is considered as a key issue for tumor distribution and therapeutic effects. Most tumors possess abundant stroma, a fibrotic tissue composed of cancer-associated fibroblasts (CAFs) and extracellular matrix (ECM), which acts as a barrier for nanoparticle penetration. There is however a lack of suitable *in vitro* systems to study the tumor stroma penetration of nanoparticles. In the present study, we developed and thoroughly characterized a 3D co-culture spheroidal array to mimic tumor stroma and investigated the penetration of silica and PLGA nanoparticles in these spheroids. First, we examined human breast tumor patient biopsies to characterize the content and organization of stroma and found a high expression of alpha-smooth muscle actin (α-SMA; 40% positive area) and collagen-1 (50% positive area). Next, we prepared homospheroids of 4T1 mouse breast cancer cells or 3T3 mouse fibroblasts alone as well as heterospheroids combining 3T3 and 4T1 cells in different ratios (1:1 and 5:1) using a microwell array platform. Confocal live imaging revealed that fibroblasts distributed and reorganized within 48 h in heterospheroids. Furthermore, immunohistochemical staining and gene expression analysis showed a proportional increase of α-SMA and collagen in heterospheroids with higher fibroblast ratios attaining 35% and 45% positive area at 5:1 (3T3:4T1) ratio, in a good match with the clinical breast tumor stroma. Subsequently, we studied the penetration of high and low negatively charged fluorescent silica nanoparticles (30 nm; red and 100 or 70 nm; green; zeta potential: −40 mV and −20 mV) and as well as Cy5-conjugated pegylated PLGA nanoparticles (200 nm, −7 mV) in both homo- and heterospheroid models. Fluorescent microscopy on spheroid cryosections after incubation with silica nanoparticles showed that 4T1 homospheroids allowed a high penetration of about 75–80% within 24 h, with higher penetration in case of the 30 nm nanoparticles. In contrast, spheroids with increasing fibroblast amounts significantly inhibited NP penetration. Silica nanoparticles with a less negative zeta potential exhibited lesser penetration compared to highly negative charged nanoparticles. Subsequently, similar experiments were conducted using Cy5-conjugated pegylated PLGA nanoparticles and confocal laser scanning microscopy; an increased nanoparticle penetration was found in 4T1 homospheroids until 48 h, but significantly lower penetration in heterospheroids. Furthermore, we also developed human homospheroids (MDA-MB-231 or Panc-1 tumor cells) and heterospheroids (MDA-MB-231/BJ-hTert and Panc-1/pancreatic stellate cells) and performed silica nanoparticle (30 and 100 nm) penetration studies. As a result, heterospheroids had significantly a lesser penetration of the nanoparticles compared to homospheroids. In conclusion, our data demonstrate that tumor stroma acts as a strong barrier for nanoparticle penetration. The 30-nm nanoparticles with low zeta potential favor deeper penetration. Furthermore, the herein proposed 3D co-culture platform that mimics the tumor stroma, is ideally suited to systematically investigate the factors influencing the penetration characteristics of newly developed nanomedicines to allow the design of nanoparticles with optimal penetration characteristics.

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1. Introduction

Nanomedicines are designed to target anti-cancer agents to the dynamic tumor microenvironment sparing healthy tissues from severe side effects and thereby enhancing therapeutic index of anti-cancer

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therapies. Nanoparticles extravasate into tumors through leaky and tortuous blood vasculature and are retained intratumorally for prolonged periods as the lymphatic system is absent or only poorly developed, a phenomenon referred to as Enhanced Permeability and Retention (EPR) effect [1–5]. The physicochemical properties of nanoparticles such as size, shape, charge, and the nature of material they are made of play a major role in determining their fate in the body as well as within tumors [6–9]. Despite an overwhelming therapeutic success of nanomedicines in preclinical tumor models, only a limited therapeutic benefit has been achieved in the clinical situation. One major concern in the clinic is the limited penetration of nanoparticles into tumors [10,11]. In contrast to commonly used preclinical subcutaneous tumor models, clinical tumors are highly different due to the presence of fibrotic tumor stroma [12]. Recent studies have acknowledged that penetration of nanoparticles in the target tissue and their accumulation at the tumor site is affected by many factors such as the characteristics of nanoparticles (size, charge, and shape), as well as the tumor microenvironment and intracellular signaling networks [13–16].

Evidence is increasing that tumor growth is not solely dependent on cumulative gene mutations, but also significantly influenced by the surrounding tumor stroma [17]. Specifically, cancer cells co-exist in the tumor microenvironment with stromal components which is mostly comprised of fibroblasts, endothelial cells, inflammatory immune cells, adipocytes, and extracellular matrix (ECM) [18]. Complex interactions between tumor cells and the stroma govern tumorigenesis, tumor progression, and metastasis. Fibroblasts, as the most abundant component in certain tumor types such as breast and pancreas cancer, are protumorigenic, and can transform into cancer associated-fibroblasts (CAFs) [17,19]. CAFs secrete an enormous amount of ECM which develops fibrotic tissue within the tumor stroma. Furthermore, CAFs provide resistance to tumor cells by secreting growth factors, which ultimately leads to treatment failure [17,19]. Thus, reflecting these key-characteristics of the tumor stroma in an *in vitro* culture model is of high interest for drug and/or nanomedicine screening.

Three-dimensional (3D) culture models such as spheroids better resemble the *in vivo* situation compared to 2D models, and more realistically recapitulate the tumor microenvironment offering advantages of resembling *in vivo* tumor microenvironment, enabling thereby a better understanding of molecular and cellular mechanisms [20,21] and cell-matrix interactions [22–24]. Furthermore, they can facilitate better screening of nanomedicines [25,26]. 3D *in vitro* models also yield more predictive *in vitro* data and support the reduction of animal studies which are costly and suffering from high failures rates; for all these reasons, 3D *in vitro* models are particularly attractive for screening of clinically relevant properties of nanomedicines [27].

Various platforms have been proposed for generating 3D cell models and for 3D cell cultures, using scaffolds based on different polymers [28], hydrogels [29], microwell arrays [30], hanging drop method [31], and microfluidic devices [32], or combinations thereof [33]. In particular, Sridhar et al. [34] reported a hot-embossed polystyrene-based microwell array in a conventional Petri dish for production of homogenously-sized spheroids, which is rapid and easy to handle while being suitable for *in situ* microscopic examination.

In this study, we developed a 3D spheroid array by co-culturing tumor cells and fibroblasts to mimic tumor stroma *in vitro*, which we subsequently applied for studying nanoparticle penetration. Spheroid arrays were generated in a microwell array which are hot embossed in a polystyrene dish, as reported earlier [34]. Homospheroids (single cell type) and heterospheroids (tumor cells and fibroblasts) were prepared, subsequently characterized for cellular re-organization using confocal laser scanning microscopy as well as for the expression of tumor stromal biomarkers at the transcription and protein levels, and compared to tumor biopsies from patients. To investigate the effect of stroma on nanoparticle penetration in tumors, we incubated homo- and hetero-spheroids with silica nanoparticles of different sizes and surface charges for up to 48 h and examined/quantified their distribution in the spheroids. Finally, we prepared pegylated poly(D,L-lactic-*co*-glycolic acid) (PLGA) nanoparticles which are of high clinical relevance [35], and similarly studied their penetration into homo- and heterospheroids using confocal laser scanning microscopy.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) 4.5 g/l with L-glutamine and RPMI-1640 without L-glutamine, L-glutamine were purchased from PAA/GE Healthcare (Eindhoven, The Netherlands). penicillin/streptomycin, hematoxylin, β-mercaptoethanol and polyvinyl alcohol (PVA, Mw 30,000–70,000) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium was purchased from Lonza Benelux BV (Breda, The Netherlands). Trypsin-EDTA 0.5% and fetal bovine serum (FBS) were purchased from Life Technologies (Bleiswijk, The Netherlands), together with Cell Trace® Calcein Red-Orange AM and Calcein AM. Pluronic®F-127 was from BASF (USA). Cryomatrix™ was purchased from Thermo Scientific (Cheshire, UK). Target Retrieval Solution at pH 9 was obtained from Dako Agilent (Heverlee, Belgium). VectaMount™ Permanent Mounting Medium was purchased from Vector Laboratories (Peterborough, UK). 3-Amino-9-ethyl-carbazole (AEC Red) was from Invitrogen (Breda, The Netherlands). Aquatex® aqueous mounting medium was purchased from Millipore (USA). iScript™ cDNA Synthesis Kit was purchased from BioRad (Hercules, CA). 2× SensiMix SYBR and Fluorescein Kit was purchased from Bioline (Luckenwalde, Germany). Silica nanoparticles (sicastar®-redF-COOH or sicastar®-redF-NH_2 (size of 30 nm, excitation: 569 nm, emission: 585 nm) and sicastar®-greenF-COOH (size 100 nm, excitation: 485 nm, emission: 510 nm) or sicastar®-redF-NH_2 (size of 70 nm, excitation: 485 nm, emission: 510 nm) were obtained from Micromod Partikeltechnologie GmbH (Rostock, Germany). Uncapped PLGA (lactide/glycolide molar ratio 50:50, IV = 0.4 dl/g) were obtained from Corbion Purac (Amsterdam, The Netherlands). $mPEG_{2000}$-PLGA was synthesized by ring opening polymerization [36]. Ethyl acetate was from VWR chemicals (Amsterdam, The Netherlands). Cyanine-5 amine was from Lumiprobe (Hannover, Germany). MilliQ water was obtained using Millipore Advantage A10 (USA).

2.2. Tissue microarrays (TMAs)

The TMAs of human breast cancer patients were prepared at the pathology lab of LabPON (Hengelo, The Netherlands) which were constructed from biopsies isolated from 11 patients of invasive adenocarcinoma grade 2 to 3 (4 different spots of 2 mm in diameter from each patient). The TMAs were stored at room temperature and subjected to immunohistochemistry staining for activated fibroblast marker (α-SMA and collagen-1α1). Immunohistochemical staining was carried out including standard deparaffinization, through heating to 80 °C in Target Retrieval Solution at pH 9.0 overnight before use, incubation with primary, secondary, and tertiary antibodies and development with DAB (di-aminobenzidine). The TMAs were subsequently counterstained with hematoxylin and mounted with VectaMount™ Permanent Mounting Medium. The TMA slides were scanned using Nanozoomer-RS (Hamamatsu Photonics, Japan). The tissue microarray cores were individually analyzed using NIH Image J software to quantitate the intensity of brown staining from DAB color development for each patient core.

2.3. Cell culture

Mouse 4T1 breast cancer cells, murine NIH3T3 fibroblasts, human pancreatic cancer cell line (Panc-1), and human breast tumor cell line (MDA-MB-231) were obtained from American Type Culture Collection

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