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Penetration in 3D tumor spheroids and explants: Adding a further dimension to the structure-activity relationship of cell-penetrating peptides

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ABSTRACT

Drug delivery into tumors and metastases is a major challenge in the eradication of cancers such as epithelial ovarian carcinoma. Cationic cell-penetrating peptides (CPPs) are a promising group of delivery vehicles to mediate cellular entry of molecules that otherwise poorly enter cells. However, little is known about their penetration behavior in tissues. Here, we investigated penetration of cationic CPPs in 3D ovarian cancer spheroids and patient-derived 3D tumor explants. Penetration kinetics and distribution after long-term incubation were imaged by confocal microscopy. In addition, spheroids and tumor explants were dissociated and cell-associated fluorescence determined by flow cytometry. CPPs with high uptake activity showed enhanced sequestration in the periphery of the spheroid, whereas less active CPPs were able to penetrate deeper into the tissue. CPPs consisting of D-amino acids were advantageous over L-amino acid CPPs as they showed less but long lasting cellular uptake activity, which benefitted penetration and retention over time. In primary tumor cultures, in contrast to nonaarginine, the amphipathic CPP penetratin was strongly sequestered by cell debris and matrix components pointing towards arginine-rich CPPs as a preferred choice. Overall, the data show that testing in 3D models leads to a different choice of the preferred peptide in comparison to a standard 2D cell culture.

1. Introduction

In oncology, there is still an urgent need for effective therapies with minimal side effects. Epithelial ovarian cancer (EOC) is the most lethal gynaecological malignancy and the sixth deadliest cancer in women in developed countries [1]. Standard therapy consists of (interval) debulking surgery and (neo)adjuvant chemotherapeutic treatment. Complete tumor removal is, however, often impossible and small (micro) metastases remain in the abdominal cavity. These metastases cause tumor recurrence and will eventually be fatal in most cases. Novel treatment options that target these metastases are therefore required. Oligonucleotides, peptides, and proteins have enormous potential to interfere with cancer-related pathways, also inside cells. However, their application critically depends on delivery vectors since they cannot cross the plasma membrane by themselves.

Cell-penetrating peptides (CPPs) induce cellular uptake of molecules that otherwise cannot pass the plasma membrane. CPPs are typically 8–30 amino acids long and are mostly cationic or cationic amphipathic [2]. Depending on the type of cargo, CPPs can either be covalently coupled to other molecules or, due to their cationic nature, complexed *via* electrostatic interaction. While the first mode of conjugation has been employed for small molecule drugs, therapeutic peptides, proteins, and uncharged oligonucleotide analogs, the second one is being employed for charged oligonucleotides such as phosphorothioate antisense oligonucleotides and siRNA [3,4].

Even though most CPPs share positive charge as a common characteristic, differences in uptake mechanism exist. For the amphipathic CPP penetratin and for the arginine-rich CPPs nonaarginine and Tat, direct membrane permeation and endocytic uptake have been reported [5–7]. However, only for nonaarginine and Tat, but not for penetratin, a specific form of rapid cytoplasmic uptake is observed at higher concentrations [8]. This mode of uptake is, however, restricted to small cargo molecules [9]. Remarkably, peptides consisting of D-amino acids are less active in inducing endocytosis than their L-amino acid counterparts [10]. Over longer incubation times, D-analogs may nevertheless outperform the L-peptides because of their higher stability against proteolytic degradation.

At this point, no information is available about the requirements for efficient delivery in a three-dimensional environment as presented by tumors. So far, with the exception of *in vivo* studies, the functional

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characterization of CPPs has relied on two-dimensional tissue cultures. This geometric arrangement fundamentally differs from the three-dimensional situation in which peptides need to penetrate different cell layers in the interstitial space and through cell-produced extracellular matrix before reaching cells in the deeper tumor regions [11,12]. Culturing cells in a multicellular tumor cell spheroid restores some of these physiological features [13]. Therefore, analyzing uptake kinetics of CPPs in this 3D model will give a better understanding of the behavior that a CPP will show *in vivo*.

In addition, CPPs were classically thought to induce cellular uptake non-specificallyin all cells. However, the capacity to internalize molecules by endocytosis is strongly dependent on cellular differentiation [14], and strong preferences for certain organs exists *in vivo* [15]. These results indicate that uptake may also vary strongly for the different cell types present in a tumor. This information is crucial for the further development of CPPs as drug delivery vehicles. At this point, the development of delivery strategies is mostly directed towards cancer cells. However, if for example tumor-associated macrophages, which contribute to tumor progression [16], showed the most efficient uptake, this should result in a rather different choice of cargo.

Ascites derived tumor cell spheroids can be used as a primary cell derived 3D model for the study of novel therapies in ovarian cancer. However, these models still lack the 3D tumor microenvironment that is present in an *in vivo* tumor. To mimic the tumor microenvironment as close as possible, advanced primary tumor explants can be used [17].

To understand how the characteristics of a CPP affect tumor penetration, we first explored to which degree uptake characteristics of a panel of cationic CPPs in the three-dimensional situation differed from those in the two-dimensional situation. For this purpose, we used tumor cell spheroids grown from the SKOV-3 ovarian cancer cell line. Well characterized CPPs with different uptake characteristics in two-dimensional tissue cultures were selected. Namely, nonaarginine (R9) and penetratin, and their D-amino acid counterparts (r9, D-penetratin), as well as a chimeric nonaarginine consisting of L-heptaarginine flanked by D-arginine residues on either side (rR7r) [8,10]. Penetratin is more amphipathic than nonaarginine and therefore has a stronger tendency to interact with cell membranes. Secondly, we investigated CPP uptake in 3D tumor explants derived from patients undergoing a primary debulking for a stage IIIC high-grade serous adenocarcinoma, distinguishing different cell types by multi-color flow cytometry.

2. Materials and methods

2.1. Peptides

All peptides were purchased from EMC microcollections (Tübingen, Germany), N-terminally labeled with carboxyfluorescein and C-terminally amidated. Purity was determined by reverse-phase high-performance liquid chromatography and identity was confirmed by mass spectrometry. Peptides were dissolved in ultrapure milli-Q water and stock concentrations were determined by diluting the stock solution 1:1000 in 100 mM TRIS/HCl buffer pH 8.8 and measuring of absorbance at 492 nm, while assuming an extinction coefficient of $75{,}000\,L \times mol^{-1} \times cm^{-1}$. Stock solutions were made at concentrations > 1 mM and stored at −20 °C. Frozen peptides were warmed to room temperature prior to the uptake experiments and subsequently pre-diluted to 90–100 μM in DMEM without fetal calf serum (FCS). These solutions were diluted further to a concentration of 5 μM at the start of each experiment. Experiment specific uptake protocols are given in their respective paragraphs.

2.2. Cell culture

SKOV-3 cells were purchased from the American Type Culture Collection (Bethesda, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with Glutamax (Life Technologies) and 10% FCS (PAN-biotech, Aidenbach, Germany). SKOV-3-GFP/Luc cells, a kind gift of Harry Dolstra (Dept. of Hematology, Radboudumc) [18]. Cells were cultured in T75 Tissue Culture Flasks (Greiner Bio-One) in a humidified incubator at 37 °C supplemented with 5% CO_2, and medium was refreshed three times per week. When 80% confluency was reached, cells were detached with 1× trypsin/EDTA solution (PAN-biotech) and either passaged or used for experiments. Cells with a passage number lower than 30 were used for experiments.

Tumor cell spheroids were grown by the hanging drop method as described earlier [19]. In short, cells were detached, counted with a hemocytometer and suspended in full culture medium containing 1.2 mg/ml methylcellulose solution (M6385, Sigma-Aldrich), and 200 U/ml Penicillin/Streptomycin (Sigma-Aldrich). 30 μl droplets containing 10,000 cells were pipetted onto an inverted lid of a petri dish (VWR international, 140 × 20.6 mm) and were cultured for 48 h until spheroids formed.

2.3. Tumor explant culture

Peritoneal metastases of ovarian tumors were obtained from three patients (samples 1 to 3) undergoing surgical debulking in the Radboudumc, Nijmegen, The Netherlands. Ethical approval for this study was provided by the Radboudumc Ethical Committee (dossier number 2016–2636), which granted the use of peritoneal tumor deposits that were regarded as 'left over material' in accordance with the code of proper secondary use of human tissue in The Netherlands, as established by the Dutch Federation of Medical Scientific Societies.

Tumor tissue was prepared as described earlier by Naipal et al. [17], with some adjustments. In short, peritoneal depositions were obtained at the operating theater shortly after excision and kept on ice until incubation. The specimens were sliced into 300 μm thick sections using a Leica VT1000 S Vibratome with a vibration frequency of 100 Hz, vibration amplitude of 0.6 mm, and slicing speed at ~0.05 mm/s. Slicing was performed under semi-sterile conditions, with the vibratome outside the sterile flow hood, and the sections were therefore incubated in DMEM supplemented with 10% FCS, GlutaMAX, 200 U/ml Penicillin/Streptomycin, 50 μg/ml gentamycin, and 2.5 μg/ml amphothericin B. Within 3 h after excision, the tumor slices were incubated in a 24-well plate on a rotary shaker in the incubator overnight at 37 °C with 5% CO_2 enriched air. The following day, r9 and D-penetratin were added in a final concentration of 5 μM and incubated for 18 h before being analyzed by flow cytometry.

2.4. Flow cytometry

For uptake experiments in a monolayer, 60,000 SKOV-3 cells per well were seeded one day in advance in a 24-well plate, which corresponded to roughly 80% confluency at the start of the experiment. Spheroids were used 48 h after seeding of cells. Peptide, pre-diluted in DMEM was added into either a well containing cells seeded the day before or, for uptake in spheroids, CPP was added directly in 6 different hanging drops containing a spheroid. After 1 or 24 h incubation, cells were washed twice and incubated for 3–5 min with 1× trypsin/EDTA until a single cell suspension was obtained. Full culture medium was added and the cells were centrifuged for 3 min at 250*g*. The medium was removed and the cell pellet was resuspended in PBS and transferred to a FACS tube. Between 10,000 and 20,000 cells per condition were measured on a FACSCalibur flow cytometer (BD Bioscience, Erembodegem, Belgium).

Uptake experiments in tumor explants were performed by incubating the samples with 5 μM peptide for 18 h. After incubation, the samples were washed twice with PBS and incubated for two hours with 2 mg/ml collagenase (Sigma-Aldrich) in DMEM without FCS to digest collagen, creating a single cell suspension. The suspension was hereafter passed through a 70 μm cell strainer (Corning, New York, USA),