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Mass spectrometric detection of 27-hydroxycholesterol in breast cancer exosomes

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ABSTRACT

Exosomes from cancer cells are rich sources of biomarkers and may contain elevated levels of lipids of diagnostic value. 27-Hydroxycholesterol (27-OHC) is associated with proliferation and metastasis in estrogen receptor positive (ER+) breast cancer. In this study, we investigated the levels of 27-OHC, and other sidechain-hydroxylated oxysterols in exosomes.

To study both cytoplasmic and exosomal oxysterol samples of limited size, we have developed a capillary liquid chromatography-mass spectrometry platform that outperforms our previously published systems regarding chromatographic resolution, analysis time and sensitivity. In the analyzed samples, the quantified level of cytoplasmic 27-OHC using this platform fitted with mRNA levels of 27-OHC's corresponding enzyme, CYP27A1.

We find clearly increased levels of 27-OHC in exosomes (i.e., enrichment) from an ER+ breast cancer cell line (MCF-7) compared to exosomes derived from an estrogen receptor (ER-) breast cancer cell line (MDA-MB-231) and other control exosomes (non-cancerous cell line (HEK293) and human pooled serum).

The exosomal oxysterol profile did not reflect cytoplasmic oxysterol profiles in the cells of origin; cytoplasmic 27-OHC was low in ER+ MCF-7 cells while high in MDA-MB-231 cells. Other control cancer cells showed varied cytoplasmic oxysterol levels. Hence, exosome profiling in cancer cells might provide complementary information with the possibility of diagnostic value.

1. Introduction

The enzyme CYP27A1 interacts with cholesterol to form 27-hydroxycholesterol (27-OHC, or more precisely, (25R)-26-hydroxycholesterol [1]). 27-OHC promotes proliferation and metastasis in estrogen receptor (ER+)-dependent breast cancers via the ER receptor and liver X receptor (LXR), respectively [2–5]. Moreover, in breast cancer CYP27A1 expression levels correlate with tumor grade [2,3]. It is therefore reasonable to investigate intra and extracellular biomarker traits of 27-OHC in ER+ and ER- breast cancers [4]. Exosomes have recently caught attention as valuable components of extracellular communication. Exosomes are small (typically 30–100 nm) extracellular vesicles released by exocytosis [6–8]. Initially exosomes were thought to be of little clinical relevance, however, evidence accumulates that exosomes are involved in a plethora of functions including tumor promotion and metastasis [9,10] and priming selected organs for metastasis [9]. Exosomes can cargo a multitude of cellular molecules including oncogenic metabolites, RNA and proteins [6,7,10–12]. Cancer cells typically release greater quantities of exosomes compared to healthy cells [6]. Exosomes can contain abundant amounts of lipids [12]. However, the presence of oxysterols in exosomes has not been reported.

A key challenge in exosome analysis is their availability; extraction from biosamples will only result in limited amounts (e.g., 0.78 μg exosome protein/1×10^6 cell [12]). Therefore, it is imperative to have access to highly sensitive analytical platforms. In addition, the platforms must be robust enough to legitimize the handling of scarce biosamples from e.g., patients or blood banks. Since oxysterols are neutral compounds when measured with electrospray ionization-mass spectrometry (ESI-MS) [13–15], derivatization into charged species (e.g., using Girard reagents) is required [15–19] to enable sensitive measurements [17,20]. We have previously reported highly automated, robust and sensitive systems for ESI-MS based determination of "charge-tagged"

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side-chained hydroxycholesterols, employing microbore liquid chromatography (LC) and nano LC [17,18]. These systems efficiently remove excess reagents in an on-line and automated manner. Here, we describe a novel capillary LC-MS (cap LC-MS) based system, which outperforms our previous systems regarding chromatographic resolution, speed, sensitivity and precision. We have employed this system for initial explorations on the presence and levels of 27-OHC (and other side-chained hydroxycholesterols) in exosomes from various cells including ER+ and ER- breast cancer cells.

2. Materials and methods

2.1. Reagents

27-OHC (cholest-25(*R*)-5-ene-3β,26-diol, also known as (25*R*), 26-hydroxycholesterol [1]) and 24S-hydroxycholesterol (24S-OHC, cholest-5-ene-3β,24(*S*)-diol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA), while 25-hydroxycholesterol (25-OHC, cholest-5-ene-3β,25-diol) and 22R-hydroxycholesterol (22R-OHC, cholest-5-ene-3β,22(*R*)-diol) together with Girard T, KH2PO4, cholesterol oxidase from *Streptomyces* sp. and formic acid (FA) were all purchased from Sigma-Aldrich (St. Louis, MA, USA). 25-Hydroxycholesterol-26,26,26-27,27,27-d6 (cholest-5-ene-3β,25-diol-d6) was obtained from CDN isotopes (Quebec, Canada) and used as an internal standard. Glacial acetic acid was from Merck (Merck KGaA, Darmstadt, Germany). All stock solutions of oxysterols or internal standard (100–500 μM) were prepared in 2-propanol (Rathburn Chemicals Ltd., Walkerburn, Scotland, UK) and stored at 4 °C. These were used to prepare working solutions (1 nM of each oxysterol and 1.5 nM internal standard in 2-propanol). Cholesterol-25, 26, 27 ^{13}C (Sigma-Aldrich) was used as an autoxidation monitoring standard [17].

Methanol (MeOH, Hipersolv) was from Prolab, (VWR, Radnor, PA, USA) and type 1 water from a Millipore integral water purification system (Millipore, Billerica, MA, USA).

2.2. Standard solutions for calibration and validation

Appropriate aliquots (10–80 μL) of 1 nM 24S-OHC, 22R-OHC, 25-OHC and 27-OHC solutions were mixed with 25 μL of 1.5 nM 25d$_6$-OHC (internal standard, IS) and evaporated into dryness before reconstituted in 20 μL 2-propanol to make calibration and validation solutions. Analytes were derivatized with Girard T reagent as described in [17].

For recovery (or more correctly apparent recovery [21]), lysates of 100,000 cells (n = 3) were spiked with 10, 40 or 80 μL 1 nM standard solution and 25 μL internal standard solution before evaporated into dryness and derivatized with Girard T reagent. Recovery was calculated by the formula:

$$recovery = \frac{a}{b} \times 100\%$$

where a and b is the regression line slope of A_{Ox}/A_{IS} vs. C_{Ox}/C_{IS} for the spiked cell lysate sample and the standard solutions, respectively, Ox is analyte and IS is internal standard.

2.3. Cell lines and cell culture

The pancreatic adenocarcinoma cell line BxPC-3 (ATCC® CRL-1687 ™S, VA, USA) and the embryonic kidney cell line HEK293 (ATCC®CRL-1537™) were grown in RPMI medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltman, MA, USA), and penicillin/streptomycin (P/S, BioWhittaker, Walkersville, MD, USA). BxPC-3 was also supplemented with 500 μL Insulin-Transferrin-Selenium (Life Technologies, Carlsbad, CA, USA). The human colon carcinoma cell line RKO (ATCC® CRL-2577™) was grown in EMEM medium (ATCC®™) supplemented with 10% FBS and P/S. The mammary epithelial adenocarcinoma cell lines MCF7 (ATCC® HTB-22™) and MDA-MB-231 (ATCC® HTB-26™) were grown in RPMI medium without phenol red (11835-030, Gibco, Thermo Fisher Scientific) but supplemented with 10% FBS and P/S. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. All cell lines were mycoplasma negative.

2.4. Cell extraction and protein measurements

Cells were detached using trypsin EDTA solution (Sigma-Aldrich) and washed in PBS, before they were counted using an automated cell counter (TC20, Bio-Rad, Hercules, CA, USA).

Cell extracts were made by adding cold RIPA buffer (Thermo Fisher Scientific) containing protease inhibitors (Protease Inhibitor Coctail Tablets, Roche, Basel, Switzerland) and phosphatase inhibitors (PhosStop Tablets, Sigma-Aldrich) to cells, following manufacturer's protocol for preparation of cell extracts from adherent cells. Protein concentration was determined using Pierce™ BCA protein Assay Kit (Thermo Fisher Scientific). 10 μg of protein was loaded on to gels (Novex™ Bis-Tris gels, Life Technologies) with PageRuler pre-stained protein ladder (26616, Fermentas) and analyzed with a Novex electrophoresis chambers (Life technologies). Proteins were transferred to 0.2 μm PVDF membranes (Immobilon®-P^{SQ}, ISEQ00010, Merck), blocked with 5% milk (A0830,0500, AppliChem), 0.05% tween-20 in TBS (09-7510-100, Medicago) for 1 h, and stained with primary (4 °C overnight with rocking in 1% milk, 0.05% tween-20 in TBS) and secondary antibodies (1 h at room temperature with rocking in 5% milk, 0.05% tween-20 in TBS). Primary antibodies used were polyclonal rabbit anti-CYP27a1 (1:500 dilution, AP06773PU-N, Acris, Herford, Germany), polyclonal rabbit anti-Flag (1:500 dilution, F7425, Sigma-Aldrich), and rabbit anti-actin (1:2000 dilution, A2066, Sigma-Aldrich). Secondary antibody used was donkey anti-rabbit (1:5000 dilution, SC, Santa Cruz biotech, Dallas, TX, USA). Bands were visualized using Pierce™ ECL western blotting substrate (Thermo Fisher Scientific) in a ChemiDoc™ Touch Imaging system (Bio-rad) developer. Image Lab™ Software (Bio-rad) was used to quantify bands (normalized against loading controls. For MDA-MB-231 the top visible bond, with correct mass was used for quantification. The identity if the protein causing the second visible bond is unknown).

2.5. Real-time quantitative PCR (qRT-PCR)

Total RNA was isolated using the GeneElute™ Mammalian Total RNA miniprep kit (Sigma-Aldrich) following the manufacturer's instructions. cDNA was synthesized using the SuperScript VILO™ cDNA Synthesis kit (Life Technologies), and qRT-PCR was carried out using TaqMan gene expression master mix (Life Technologies) and the CYP27A1 TaqMan probe (Hs01026016_m1, Life Technologies) according to the manufacturer's instructions on a StepOnePlus cycler (Life Technologies). TaqMan probe GAPDH (Hs02758991_g1, Life Technologies) was used to normalize the amount of cDNA in each sample, and to guarantee the comparability of the calculated mRNA expression in all samples analyzed. Data was analyzed by the comparative Ct (ΔΔCt) method. RNA expressions from the various cell lines were calculated relative to that of MCF-7, which was set to 1.

2.6. Exosome samples

Purified human exosomes from MCF-7 (human breast cancer, ER+), MDA-MB-231 (human breast cancer, ER-), Human embryonic