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High hydrostatic pressure affects antigenic pool in tumor cells: Implication for dendritic cell-based cancer immunotherapy

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ABSTRACT

High hydrostatic pressure (HHP) can be used to generate dendritic cell (DC)-based active immunotherapy for prostate, lung and ovarian cancer. We showed here that HHP treatment of selected human cancer cell lines leads to a degradation of tumor antigens which depends on the magnitude of HHP applied and on the cancer cell line origin. Whereas prostate or ovarian cell lines displayed little protein antigen degradation with HHP treatment up to 300 MPa after 2 h, tumor antigens are hardly detected in lung cancer cell line after treatment with HHP 250 MPa at the same time. On the other hand, quick reduction of tumor antigen-coding mRNA was observed at HHP 200 MPa immediately after treatment in all cell lines tested. To optimize the DC-based active cellular therapy protocol for HHP-sensitive cell lines the immunogenicity of HHP-treated lung cancer cells at 150, 200 and 250 MPa was compared. Lung cancer cells treated with HHP 150 MPa display characteristics of immunogenic cell death, however cells are not efficiently phagocytosed by DC. Despite induction of the highest number of antigen-specific CD8$^+$ T cells, 150 MPa-treated lung cancer cells survive in high numbers. This excludes their use in DC vaccine manufacturing. HHP of 200 MPa treatment of lung cancer cells ensures the optimal ratio of efficient immunogenic killing and delivery of protein antigens in DC. These results represent an important pre-clinical data for generation of immunogenic killed lung cancer cells in ongoing NSCLC Phase I/II clinical trial using DC-based active cellular immunotherapy (DCVAC/LuCa).

1. Introduction

High hydrostatic pressure (HHP), which refers to non-physiological pressures > 100 MPa, has been known to affect biological systems for more than 100 years [1]. In biotechnology, HHP up to 800 MPa is used to preserve food [2]. Short-term HHP > 300 MPa exposure has been tested experimentally to inactivate viral vaccines, microorganisms or cancer cells in resected tissues in orthopedic surgery [1,3,4]. HHP as a cancer treatment modality was first described in 1972 for treatment of bladder carcinoma [5]. Later, it has been shown that pressurized murine and human tumor cells exhibit an increased immunogenicity *in vivo* [6–8]. HHP-treated cells can be used for cancer vaccine preparation [9]. In our laboratory, we showed that HHP of 250 MPa induces immunogenic cell death (ICD) in human leukemia, prostate and ovarian cancer cell lines and in primary tumor cells [10]. HHP-induced ICD of cancer cells displays similar molecular characteristics described for ICD induced by anthracyclins [11–13] such as the induction of endoplasmic stress response and reactive oxygen species formation, cell surface exposure of heat shock proteins and calreticulin and the release of ATP and HMGB1. Phagocytosis of HHP-treated tumor cells induced maturation of dendritic cells (DC) which led to stimulation of tumor-antigen specific CD8$^+$ and CD4$^+$ T cells *in vitro* [10]. Immunization with HHP-treated TC-1 cells, which represent a murine model for human papilloma virus-associated tumors, confer prophylactic immunity in mice [14]. Moreover, DC-based vaccine pulsed with HHP-treated tumor TC-1 or prostate tumor cells TRAMP-C2 combined with docetaxel chemotherapy significantly inhibited growth of tumors in mouse models [14]. HHP treatment of tumor cells can be standardized according to GMP requirements and incorporated into manufacturing protocols for DC-based active cellular cancer immunotherapy. According to our standard operating procedure, we use commercially available allogeneic cancer cell lines that are exposed to HHP 250 MPa for 10 min, incubated for 2 h at 37 °C, then frozen in the preservation medium at −80 °C and stored in liquid nitrogen prepared for loading of patient's DC. Several clinical trials Phase II for ovarian and prostate cancer and one Phase III for prostate using HHP 250 MPa are now in

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progress aiming at evaluation the potential of DC-based cancer vaccine loaded with immunogenic HHP-killed tumor cells to modulate the clinical course of the disease [15].

HHP treatment of tumor cells not only affects their viability in dependence on its magnitude but also induces changes in biomolecules [16]. HHP alters phospholipid bilayers from liquid-crystalline to a gel-like phase [17]. HHP inhibits synthesis of cellular proteins and their enzymatic functions [18,19]. While HHP > 400 MPa readily denature proteins, exposure to HHP ~200 MPa often only affects their tertiary and quaternary structures [20]. On the other hand, DNA seems to be resistant to HHP induced damage up to 1000 MPa [21]. As we observed disappearance of some cellular proteins after 24 h during our previous studies we were interested in the stability of protein tumor antigens in HHP-treated tumor cells. This might impact on the capacity of DC-based vaccine pulsed with HHP-treated cells to induce tumor-antigen specific T cell responses. We tested the antigen content in selected prostate, ovarian and lung cancer cell lines after HHP treatments with 150–350 MPa. Importantly, we assessed an optimal HHP value 200 MPa to prepare HHP-sensitive cell lines such as lung cancer cell lines for antigen pulsing of DC-based vaccines to preserve an optimal ratio between immunogenicity, antigen content and viability.

2. Materials and methods

2.1. Human cancer cell lines

Prostate cancer (LNCap), ovarian cancer (OV90 and SK-OV-3) and lung cancer cell lines (H522 and A549) were obtained from American Type Culture Collection (Manassas, USA). LNCaP, OV90, SK-OV-3 and H522 were cultured in RPMI-1640 complete medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Aldrich), 2 mM GlutaMAX I CTS (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco). A549 cells and A549 stably expressing an influenza matrix protein 1 (A549-MP1) [22] were grown in F12 medium (Gibco) supplemented as other cell lines. Cells were incubated at 37 °C, 5% CO_2.

2.2. HHP treatment of tumor cells

Tumor cell lines in cell culture medium in 1.5 ml tube were treated with HHP of 150, 200, 250 or 300 MPa for 10 min (LNCaP, H522, OV90, A549, A549-MP1) or for 15 min with HHP 150 up to 350 MPa (SK-OV-3) in custom-made device (Resato International BV) located in the GMP manufacturing facility of Sotio, Prague, Czech Republic. After HHP treatment cells were incubated at 37 °C, 5% CO_2 and collected at different time points. For most experiments, cells were collected after 2 h, resuspended in CryoStor™ CS-10 (BioLife Solution) and stored at −80 °C for 24 h before storage in liquid nitrogen for at least 24 h as described in our standard operation procedure for DC vaccine manufacturing.

2.3. Detection of protein tumor antigens by western blot and flow cytometry

Non-treated and HHP-treated tumor cells were collected at the indicated time points, washed with PBS and lysed on ice for 20 min in RIPA buffer (10 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100) with a protease inhibitor cocktail (Roche Diagnostics) and 1 mM phenylmethylsulfonyl fluoride. For separation of plasma membrane and cytosol, Plasma Membrane Protein Extraction Kit (Abcam) was used according to manufacturer's instructions. Lysates were diluted to the same protein concentration using BCA Protein Assay Kit (Thermo Scientific). Proteins were then separated by 12% SDS-PAGE and transferred to nitrocellulose membranes by Wet/Tank Blotting Sysems (Bio-Rad). Equal protein loading (2 µg – plasma membrane isolation; 8 µg – whole cell lysates) was verified by Ponceau-S staining. Membranes were further blocked with 5% non-fat dry milk in TBST buffer containing 50 mM Tris, 150 mM NaCl and 0.05% Tween 20 for 1 h at room temperature and incubated with primary antibodies to PSA, Her2 (both Cell Signaling Technology, 1:500), PSMA (Abcam, 1:800), CEA (1:500), MAGE-A3 (1:250), PRAME (1:500), Influenza A MP1 (1:500) (all from Thermo Scientific). overnight at 4 °C. After a washing step with TBST, membranes were incubated with secondary anti-mouse (1:10 000) or anti-rabbit (1:10 000) antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories) for 1 h at room temperature and washed again with TBST. Membranes were incubated in Restore Stripping Buffer (Thermo Scientific Pierce) and protein loading verified with Na/K ATPase (Cell Signaling Technology, 1:1000), actin (Sigma Aldrich, 1:1000), GAPDH (GeneTex, 1:1000) or actin (Sigma Aldrich, 1:1000) antibodies. Chemiluminescence was detected using G-box (Tecan) after incubating membranes with SuperSignal™ West Dura Extended Duration Substrate (Thermo Scientific).

For time-course analyses of the antigen degradation, 3×10^5 cells/well non-treated or HHP-treated were stained extracellularly with primary antibodies EpCAM-PerCP-Cy5.5 and Her2-APC (Biolegend) (2 µl/sample) or their isotype IgG2bκ-PerCP-Cy5.5 (Biolegend) and IgG1-APC (Exbio) at corresponding concentrations. Then cells were fixed for 30 min, 4 °C, permeabilized (Fix/Perm Solution, eBioscience) and stained intracellulalry with the same antibodies for 20 min at 4 °C. Cells were washed with PBS and analysed by LSRFortessa (BD Biosciences). Data were analyzed with FlowJo software (Tree Star). The mean fluorescence intensity (MFI) of each sample was determined and the MFI of the appropriate isotype control was deducted.

2.4. Immunofluorescence

2×10^5 cell/well non-treated cells or HHP-treated were washed with PBS and fixed with 4% paraformaldehyde for 30 min, 4 °C. After the washing with PBS, cells were incubated in 1% BSA in PBS for 30 min and subsequently incubated with Her2 (Cell Signalling, 1:200) primary antibody in PBS overnight at 4 °C. Cells were washed with PBS and incubated with AlexaFluor488 goat anti-mouse IgG1 secondary antibody (Life Technologies, diluted 1:200) for 1 h at 4 °C. After a washing step with PBS cells were incubated with Wheat Germ Agglutinin, Alexa Fluor® 594 Conjugate (Invitrogen, 5 µg/ml), for 10 min, washed with PBS, diluted in 4% paraformaldehyde and mounted on slides with ProLong Gold antifade reagent with DAPI (Molecular Probes) using StatSpin Cytofuge. Cells were analysed under a DMI 6000 inverted Leica TCS AOBS SP5 tandem scanning confocal microscope and a 63x oil immersion objective.

2.5. RNA isolation, reverse transcription and qPCR of cancer cell lines

Total RNA was isolated using an RNeasy Mini Kit (Qiagen). Each sample containing RLT buffer and cell lysate was quickly thawed and processed in accordance with the manufacturer's protocol which included a DNase I digestion step. The RNA concentration and purity were determined using a NanoDrop 2000c (Thermo Scientific), and the RNA integrity was assessed using an Agilent 2000 Bioanalyzer (Agilent). Purified RNA samples were stored at −80 °C until further use. cDNA was synthesized from 100 ng of total RNA using an iScript cDNA Synthesis Kit (BioRad). Expression of the selected genes was determined by qPCR on CFX96 Touch™ Real-Time PCR Detection System (BioRad). The each 10 µl reaction contained 5 µl of KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems), 0.5 µl of each forward and reverse primers (500 nM each; TIB Molbiol,), 0.5 µl of TaqMan probe (200 nM; TIB Molbiol), 1.5 µl of RNase-free water and 2 µl of 5x diluted cDNA. Each reaction was done in triplicate. The temperature cycling protocol was following: 3 min at 95 °C followed by 45 cycles (95 °C for 15 s and 60 °C for 60 s). The formation of PCR products of the expected lengths was confirmed by agarose gel electrophoresis. The Cq values were determined using CFX Manager