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Comparative proteome analysis of monolayer and spheroid culture of canine osteosarcoma cells

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ABSTRACT

Osteosarcoma is an aggressive bone tumor with high metastasis rate in the lungs and affects both humans and dogs in a similar way. Three-dimensional tumor cell cultures mimic the in vivo situation of micro-tumors and metastases and are therefore better experimental in vitro models than the often applied two-dimensional monolayer cultures. The aim of the present study was to perform comparative proteomics of standard monolayer cultures of canine osteosarcoma cells (D17) and three-dimensional spheroid cultures, to better characterize the 3D model before starting with experiments like migration assays. Using DIGE in combination with MALDI-TOF/TOF we found 27 unique canine proteins differently represented between these two culture systems, most of them being part of a functional network including mainly chaperones, structural proteins, stress-related proteins, proteins of the glycolysis/gluconeogenesis pathway and oxidoreductases. In monolayer cells, a noticeable shift to more acidic pI values was noticed for several proteins of medium to high abundance; two proteins (protein disulfide isomerase A3, stress-induced-phosphoprotein 1) showed an increase of phosphorylated protein species. Protein distribution within the cells, as detected by immunohistochemistry, displayed a switch of stress-induced-phosphoprotein 1 from the cytoplasm (in monolayer cultures) to the nucleus (in spheroid cultures). Additionally, Western blot testing revealed upregulated concentrations of metastasin (S100A4), triosephosphate isomerase 1 and septin 2 in spheroid cultures, in contrast to decreased concentrations of CCT2, a subunit of the T-complex. Results indicate regulation of stress proteins in the process of three-dimensional organization characterized by a hypoxic and nutrient-deficient environment comparable to tumor micro-metastases.

Significance: Osteosarcoma is an aggressive bone tumor that early spreads to the lungs. Three-dimensional tumor cell cultures represent the avascular stage of micro-tumors and metastases, and should therefore represent a better experimental in vitro model compared to two-dimensional monolayer cultures. Significant differences have been reported in response to drug and radiation treatment between these two culture systems. A gel-based proteomic investigation was performed to compare protein patterns of a canine osteosarcoma cell line cultivated under those two conditions, to learn more about altered cell composition and its impact on cell behaviour. Due to the fact that the canine osteosarcoma is an accepted model for the human disease, results will be relevant for the human species as well.

1. Introduction

Osteosarcoma is a life-threatening bone tumor that affects dogs [1] and cats [2]. In the dog, the tumor spreads early to the lungs via the haematogenous way and starts to develop micro-metastases [3, 4] comparable to those of the human osteosarcoma [5].

Abbreviations: 2D, 3D cell culture: two-, three-dimensional cell culture (monolayer vs. spheroids); CCT2, T- complex protein 1 subunit beta; ECM, extracellular matrix; HSP, heat shock protein; PDIA3 (ERp75), protein disulfide isomerase A3; RuBPS, ruthenium(II)tris(bathophenanthroline disulfonate); Sept2, septin 2; STIP1, stress-induced-phosphoprotein 1; TCP, T-complex protein; TPI, triosephosphate isomerase

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The mechanisms of tumor progression and the metastatic process including migratory and colonization events are not fully understood, however, this information is a prerequisite to develop strategies for novel therapeutic interventions.

In vitro systems are valuable tools to study cell adhesion, the cytoskeleton, and cell signaling as response to drugs and other cellular processes. The majority of the in vitro studies on tumor cells are still performed on classical two-dimensional (2D) monolayer cultures although it has been well documented that three-dimensional (3D) cell culture models mimic the natural microenvironment much better than 2D systems and should therefore be favored for experimental approaches [6, 7]. In particular, drug delivery, chemotherapy resistance, and radiation response are known to change significantly from 2D to 3D culture [8–14]. It is clear that, in accordance with the morphological changes from a flat cell monolayer to a 3D organized structure such as a spheroid consisting of multiple layers, also molecular changes will take place. Depending on the size of the generated spheroids, a nutritional-, pH-, and oxygen gradient is a common condition in these multicellular aggregates [15]. This is, however, comparable to the initially growing tumor or metastasis while still avascular. Moreover, production of the characteristic endogenous extracellular matrix is often induced in 3D cultures [16], which is also known to change cell signaling responses and modify gene expression patterns [17].

For investigation of proteins or cell-signaling, scaffold-free 3D cultures such as spheroids are preferable from the technical point of view as it might be difficult to separate scaffold proteins or effects thereof from endogenously produced matrix proteins before analysis. Moreover, certain biomaterials have the ability to bind drugs [18] and could therefore influence the results. Numerous studies emphasized the importance of interactions of cells with matrix proteins and its meaning for the regulation of cell functions [19–22]. Therefore, spheroids are a valuable model to study early tumor growth events including signals regulating angiogenesis.

Untargeted proteomic approaches have been found useful to determine the supposedly wide spectrum of changes between 2D and 3D cultured cells. Comparative studies for 2D versus 3D cultured cells have been performed already for several different cell types (for review see [14]), for instance for neuroblastoma cells by 2D-DIGE and Western blot [23], for glioma and adenocarcinoma by protein arrays [24], and for colon cancer cells by 2-DE [25] or LC–MS/MS [26]. Osteosarcoma cells have been subject of several targeted experimental studies, such as investigations of chemotherapy and radiation response, angiogenesis factors or miRNAs [10, 27, 28]. One study focused on investigating human osteosarcoma cell lines by DIGE with saturation dyes to find proteins responsible for resistance to doxorubicin [12]; apart from this, proteomic investigations concerning basic differences of differently cultured osteosarcoma cell lines are rare. Before starting with planned future hypoxia and angiogenesis studies on the canine osteosarcoma cell line D17, we found it desirable to investigate basic proteome differences between cells grown in 2D monolayer and 3D culture systems, in order to better interpret future results of testing in either system. For this study, we applied an integrative approach with 2D-DIGE in combination with MALDI-TOF/TOF, supported by further testing by LC-QTOF for PTM determination, immunoblotting and immunohistochemistry.

2. Material and methods

2.1. Cell culture 2D/3D

Canine osteosarcoma cells (D17, ATCC CCL-183) were cultivated in non-coated plastic tissue flasks (25 cm^2, Sarstedt, Nümbrecht, Germany) as conventional monolayers in standard medium composed of DMEM high glucose (Lonza, Basel, Switzerland) supplemented with 1% antibiotic-antimycotic solution (PAA, Pasching, Austria), 1% amphotericin B (Sigma-Aldrich, Steinheim, Germany), 1% L-glutamine (Sigma-Aldrich) and 10% fetal bovine serum solution (Biochrom, Berlin, Germany). 3D spheroids were generated by seeding canine osteosarcoma cells (4.0×10^3 cells/well) in non-adherent round bottom 96 well plates (Greiner bio-one, Frickenhausen, Germany) in standard medium as described before [16]. Cells from the 2D system were harvested after 3 days; 3D spheroids were obtained after 14 d of culture. This ensured that both systems established typical and optimal cell features. Longer cultivation of this cell type in a cell culture flask results in a culture with several layers as these cells do not have a contact inhibition; this does no longer represent a classical monolayer culture system. During the longer culture time for spheroids cells are able to organize, differentiate and produce extracellular matrix (ECM) according to the in vivo situation.

The harvested cells from 2D and 3D systems were washed twice in phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+}) and once with 0.9% NaCl solution (Mayrhofer Pharmazeutika, Linz, Austria). A total of four biological replicates were generated for the main DIGE experiment. Cell pellets were either frozen for protein extraction or fixed in 4% neutral buffered formaldehyde and paraffin embedded for the preparation of histological sections. Phase contrast micrographs were generated in using a live cell imaging system (EVOS FL, Thermo Fisher Scientific, MA, USA).

2.2. Immunohistochemistry

Immunohistochemistry was done on paraffin sections of 2D and 3D cultures prepared as above. After rehydration, endogenous peroxidase was blocked by incubation with 0.6% H_2O_2 in methanol for 15 min. After epitope retrieval by heating sections in 0.01 M citrate buffer (pH 6) for 30 min, normal goat serum (1.5%, Dako, Glostrup, Denmark) for 30 min was used as protein block. Sections were incubated overnight at 4 °C with the diluted primary antibody. Antibodies used were against stress-induced-phosphoprotein 1 (STIP1) (rabbit polyclonal, dilution 1:1000, HPA039291, Sigma Prestige, St. Louis, MO, USA) and protein disulfide isomerase A3 (PDIA3 or ERp57) (rabbit polyclonal, dilution 1:100, Enzo Life Sciences, Lausen, Switzerland) and S100A4 (rabbit polyclonal, dilution 1:150, Thermo Fisher Scientific). After washing in PBS, sections were incubated for 30 min at RT with the secondary antibody (BrightVision Poly-HRP-anti-rabbit, ImmunoLogic, Duiven, Netherlands). After another washing step in PBS, the staining was developed with 10 mg DAB (3′3-diaminobenzidine, Sigma) and 0.03% H_2O_2 in 50 ml Tris-HCl buffer (pH 7.4) for 10 min at RT. Nuclei were counterstained with hematoxylin.

2.3. Proteomic analyses

2.3.1. Sample preparation for protein analysis

For protein extraction, cell pellets from 2D and 3D systems were lysed in 150–200 µl Tris-triton X buffer (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium-deoxycolate) for 30–40 min with occasional vortexing for 2–3 s. After centrifugation (10.000 × *g*, 15 min, 4 °C) the supernatant was collected and total protein concentrations determined with a colorimetric protein assay system (Bio-Rad, Hercules, CA, USA) based on Bradford protocol [29]. Samples were frozen at − 20 °C until further use.

2.3.2. DIGE

Cell lysates (prepared according to 2.3.1) were dialysed by using the Plus One Mini Dialysis Kit system (molecular mass cut off 1 kDa, GE Healthcare Life Sciences, Amersham, UK) against DIGE labelling buffer (8 M urea, 4% CHAPS, 30 mM Tris-HCl pH 8.5) for 4 h. Minimal labelling with CyDyes (GE Healthcare) was performed according to published protocols [30], based on manufacturer recommendations (labelling ratio: 8 nmol dye/mg protein). Cy2 was used for the internal standard (a pool of all samples in the set), Cy3 and Cy5 for the single