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Caveolin-1 mediated radioresistance of 3D grown pancreatic cancer cells

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ABSTRACT

Background and purpose: Resistance of pancreatic ductal adenocarcinoma (PDAC) to chemo- and radiotherapy is a major obstacle. The integral membrane protein Caveolin-1 (Cav-1) has been suggested as a potent target in human pancreatic carcinoma cells.
Materials and methods: Human pancreatic tumor cells were examined in a three-dimensional (3D) cell culture model with regard to clonogenic survival, apoptosis, radiogenic DNA-double strand breaks and protein expression and phosphorylation under siRNA-mediated knockdown of Cav-1 without and in combination with irradiation (X-rays, 0–6 Gy). Immunohistochemistry was used to assess Cav-1 expression in biopsies from patients with PDAC.
Results: Tumor cells in PDAC showed significantly higher Cav-1 expression relative to tumor stroma. Cav-1 knockdown significantly reduced β1 integrin expression and Akt phosphorylation, induced Caspase 3- and Caspase 8-dependent apoptosis and enhanced the radiosensitivity of 3D cell cultures. While cell cycling and Cav-1 promoter activity remained stable, Cav-1 knockdown-induced radiosensitization correlated with elevated numbers of residual DNA-double strand breaks.
Conclusions: Our data strongly support the concept of Cav-1 as a potent target in pancreatic carcinoma cells due to radiosensitization and Cav-1 overexpression in tumor cells of PDAC. 3D cell cultures are powerful and useful tools for the testing of novel targeting strategies to optimize conventional radio- and chemotherapy regimes for PDAC.
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Particularly in advanced-stage metastatic tumors, resistance to multimodality treatment and poor prognosis in a variety of human cancers including pancreatic ductal adenocarcinoma (PDAC) [1–3], critically hamper the success of conventional chemo- and radiotherapy. Caveolin-1 (Cav-1) received attention as a multitasking, cancer-associated molecule [4–6] that serves in cellular transformation, tumor growth, cell migration and metastasis, cell death, survival and angiogenesis in a tissue- and stage-dependent manner [7].

Caveolin, essential for caveolae-related endo- and exocytosis transport processes and intracellular signal transduction, is a 22-kDa protein consisting of three isoforms of which Cav-1 and -2 are ubiquitously expressed [5]. In caveolae, i.e. flask-like invaginations of the plasma membrane (<100 nm diameter) present in many vertebrate cell types, Caveolins oligomerize and associate with cholesterol and sphingolipids in distinct areas of the cell membrane. Common to a large set of signaling proteins is their activation by conformational change upon release from Caveolins, which possess a specific inhibitory binding domain called caveolin scaffolding domain (CSD). In addition to Src and MAPK signaling pathways, Cav-1 perpetuates Akt activation possibly via interaction with Cav-1's CSD and inhibition of protein phosphatases 1 and 2A and phosphatidylinositol-3-OH kinase [8,9].

Due to its integral plasma membrane localization, Cav-1 allows spatial regulation of signaling events e.g. associated with the epidermal growth factor receptor, Src, or focal adhesion kinase (FAK) [10,11]. Among the integrin family of cell adhesion receptors, β1 integrin is ubiquitous and dimerizes with different α integrin subunits, for example α3 or α5, to provide attachment to different ECM molecules [12]. Disruption of integrin-ECM binding results in a special form of apoptosis, called anoikis [13]. Execution of this type of apoptosis involves e.g. Procaspase 8 and Procaspase 3 [14]. Besides a partial dependence of Cav-1 on β1 integrin for signal transduction, a co-localization of both proteins was reported in human endothelial cells and human chondrocytes [6,11,15,16].

Abbreviations: Cav-1, Caveolin-1; DNA-DSB, DNA-double strand break; ECM, extracellular matrix; FAK, focal adhesion kinase; GSK3β, glycogen synthase kinase-3β; ILK, integrin-linked kinase; IP, immunoprecipitation; lrECM, laminin-rich extracellular matrix; PINCH-1, particularly interesting new cysteine–histidine-rich protein 1; rDNA-DSB, residual DNA-double strand break.

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Such complex molecular interactions might be responsible for the essential role of Cav-1 in the cellular radiation response [17,18].

Emphasizing the well-known impact of 3D integrin-mediated cell–extracellular matrix (ECM) interactions for cell survival [19–21], this study evaluated Cav-1 as a putative therapeutic cancer target in biopsies of PDAC and in 3D human pancreatic cancer cell cultures.

Materials and methods

Cell culture

MiaPaCa2 and Panc1 human pancreatic tumor cell lines were obtained from ATCC. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; PAA, Cölbe, Germany) containing glutamax-I (L-alanyl-L-glutamine) supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany) and 1% non-essential amino acids (PAA) at 37 °C in a humidified atmosphere containing 7% CO_2.

Radiation exposure

Irradiation was delivered at room temperature using single doses of 200 kV X-rays (Yxlon Y.TU 320; Yxlon, Copenhagen, Denmark) filtered with 0.5 mm copper. The dose-rate was approximately 1.3 Gy/min at 20 mA and applied doses ranged from 0 to 6 Gy. The absorbed dose was measured using a Duplex dosimeter (PTW, Freiburg, Germany).

Caveolin-1 constructs and stable transfection

The Cav-1 expression vector pCav-1 (mouse Cav-1 cDNA (97% homology to human Cav-1) inserted into the EcoRI restriction site of pcDNA3; Invitrogen, Karlsruhe, Germany) was a kind gift from M. Liscovitch (Weizmann Institute of Science, Israel) [18,22]. The transfection procedure is described in Supplementary information. Selection of stable transfectants was performed with G418 (Calbiochem, Bad Soden, Germany) and single clones were isolated. Overexpression of Cav-1 was confirmed by Western blotting or fluorescence microscopy.

siRNA-mediated knockdown of Caveolin-1 and β1 integrin

Cav-1 and β1 integrin siRNAs were obtained from Applied Biosystems (Darmstadt, Germany) besides Cav-1 siRNA 2, which was obtained from MWG (Ebersberg, Germany). Negative control siRNA #1 (Applied Biosystems) was used as a negative control. The target sequences efficiently mediating silencing of Cav-1 or β1 integrin expression are listed out in Supplementary Table 1. The transfection procedure is described in Supplementary information. Twenty-four hours after transfection cells were trypsinized and transferred to lrECM for either colony formation assays, Western blotting, real-time PCR or immunofluorescence staining.

Colony formation assay

Measurement of 3D clonogenic cell survival was performed as published previously [21]. Further details are described in Supplementary information. Where indicated, cells were treated before with dimethyl sulfoxide (DMSO; 1:1000, AppliChem, Darmstadt, Germany), 20 μM z-VAD-fmk (pan-Caspase inhibitor; Sigma, Taufkirchen, Germany) or 2 μM IETD-fmk (Caspase 8 inhibitor; Chemicon, Hampshire, UK). Each point on survival curves represents the mean surviving fraction from at least three independent experiments.

Real-time PCR

Twenty-four hours after transfection with Cav-1-specific and non-specific siRNAs, cells were trypsinized and plated in 3D lrECM. After 24 h, cells were isolated and total RNA was extracted using the NucleoSpin RNA II kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions. Then, RNA was transcribed into cDNA (Superscript III Reverse Transcriptase, Invitrogen). Reactions were performed using the LightCycler FastStart DNA Master PLUS HybProbe kit (Roche, Mannheim, Germany) and specific primers and probes (TIB MOLBIOL, Berlin, Germany) listed out in Supplementary Table 2. The expression levels of Cav-1 and β1 integrin were normalized to the expression of the housekeeping gene TATA-box binding protein (TBP) as published [23].

Protein extraction and Western blotting

Cells were grown in lrECM as indicated above for 24 h with or without DMSO, 20 μM z-VAD-fmk or 2 μM IETD-fmk. Total proteins from non-irradiated or irradiated cells were harvested, stored and subjected to Western blotting as described previously [21]. The following antibodies were used: anti-α3 integrin, anti-β1 integrin, anti-β3 integrin, anti-Caveolin Y14, anti-FAK, anti-GSK3β, anti-ILK (clone 3), anti-PINCH-1 (BD), anti-FAK Y397 (Calbiochem), anti-AKT, anti-AKT S473, anti-Caspase 8, anti-Caspase 9, anti-GSK3β S9, anti-Histone H3, anti-p130Cas Y410, anti-P-p44/p42 MAPK, anti-p44/p42 MAPK, anti-Paxillin Y118, anti-Src Y416 (Cell Signaling, Frankfurt a.M., Germany), anti-Caspase 3 (Invitrogen), anti-Caveolin-1 (Santa Cruz, Heidelberg, Germany), anti-β-actin, anti-Paxillin, anti-Paxillin Y31 (Sigma), anti-p130Cas, anti-Src (Upstate, Hamburg, Germany), anti-α5 integrin (Biomol, Hamburg, Germany), horseradish peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse (Amersham, Freiburg, Germany) secondary antibodies. Densitometric analysis was accomplished with TotalLab TL100 software (Biostep, Jahnsdorf, Germany).

Cell cycle analysis

Knockdown cell cultures were transferred to lrECM after 24 h, grown in 3D for an additional 72 h, and incubated with 10 mM 5-bromo-2-deoxyuridine (BrdU; Serva, Heidelberg, Germany) for 30 min prior to isolation with 5 mM EDTA/PBS and trypsin (PAA) on ice. Cells were prepared for cell cycle analysis as published [24]. Detection of BrdU was accomplished with anti-BrdU (BD) and anti-mouse IgG FITC (Dako, Hamburg, Germany) antibodies and total DNA staining with propidium iodide (PI, Sigma) solution. Acquisition of data for 50,000 events was performed with a CyFlow (Partec, Münster, Germany). The distribution of cells in the different phases of the cell cycle was analyzed from the DNA-dot-blots and -histograms using FloMax software. Experiments were repeated thrice.

DAPI staining for apoptosis

Where indicated, knockdown cell cultures were treated with DMSO, 20 μM z-VAD-fmk or 2 μM IETD-fmk. Cells were then transferred to 0.5 μg/μl lrECM (2×10^4 cells) and after indicated time points, cells were fixed with 80% ethanol and stained with 4′, 6-diamidino-2-phenylindole (DAPI; Alexis, Grünberg, Germany). At least 150 cells were counted from two to three independent experiments. Fluorescence images were obtained using a LSM 510 Meta equipped with Zeiss LSM 510 software (Zeiss).

Immunofluorescence staining

Residual DNA-double strand breaks (rDNA-DSBs) were detected with the phosphorylated H2AX (γH2AX)/p53 binding protein-1 (53BP1) focus assay [25] as described before [26]. Staining for β1 integrin in 3D grown cells was performed with a fluorescence-labeled (Alexa Fluor 568 Protein Labeling Kit, Invitrogen) anti-β1

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