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Cell cycle control pathways act as conditioning factors for TK/GCV sensitivity in pancreatic cancer cells

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ABSTRACT

The suicide system TK/GCV is an enzyme/prodrug therapy that involves the transfer of the cDNA for the herpes simplex virus thymidine kinase gene (TK) into tumor cells which then sensitizes the cells to the non-toxic antiviral drug ganciclovir. Although extensively characterized, the suicide system TK/GCV conceals the details of its mechanism of action. In order to shed some light on this issue, we conducted experiments designed to identify key features of sensitive cells, as compared to cells that displayed reduced sensitivity to TK/GCV. Cell lines displaying different degrees of sensitivity underwent apoptotic cell death upon treatment with TK/GCV. S-phase delay, however, was almost exclusively restricted to sensitive cells and was impaired in a model of treatment-induced resistance. In this model genes with differential expression associated to induced resistance were identified. Noteworthy, two cell cycle-related genes (CCNE1 and GADD45) were functionally validated as conditioners of cellular sensitivity to TK/GCV. The relevance of cell cycle control was further demonstrated by experiments showing the association of Chk1 activation with greater TK/GCV cytotoxicity. Combination treatment with Chk1 inhibitor UCN-01 induced, in sensitive cells, an antagonistic effect on TK/GCV cytotoxicity highlighting the relevance of Chk1's activity on TK/GCV mechanism of action. These results reveal the relevance of cell cycle control pathways in the cytotoxicity induced by the TK/GCV system identifying candidate genes as conditioners of TK/GCV sensitivity. Moreover it points out, for the first time at Chk1 activation as a key factor to mediate TK/GCV cytotoxicity.

1. Introduction

Therapies based on in situ enzymatic activation of a systemically administered non-toxic prodrug, into its cytotoxic metabolite, hold the promise of presenting a broader therapeutic window than conventional chemotherapeutic agents by achieving high doses of the active compound restricted to specific tissue, thus allowing for more aggressive interventions. This is particularly relevant for the treatment of malignant diseases that are refractory to conventional chemotherapy, such as pancreatic adenocarcinoma.

Among the so-called gene-directed prodrug enzyme-activated therapies or suicide therapies, the one that combines the activating enzyme Herpes simplex virus thymidine kinase (TK) with the prodrug ganciclovir (GCV) remains by far the most extensively studied [1] presenting promising results for the treatment of some malignant conditions [2]. Nevertheless, details regarding its mechanism of induction of cytotoxicity are yet to be disclosed.

Previous studies have documented induction of apoptosis in response to TK/GCV treatment, putatively triggered by the formation of Double Strand Breaks (DSB), induced by the incorporation of phosphorylated GCV, in cellular DNA [3]. Although it had been proposed that a p53-dependent translocation of CD95 was responsible of the induction of the extrinsic pathway of apoptosis [4] later studies demonstrated that TK/GCV induced mitochondrial apoptosis regardless of p53 status or Death receptors [5,6].

S- or S-and G2/M-Phase arrest in cell cycle progression had also been associated with response to TK/GCV in several cellular models [3,5,7]. Nevertheless, whether these changes play a relevant role in TK/GCV mechanism of action or are mere side events remains to be clarified.

In this study we have analyzed, by using a first generation adenoviral vector to express TK under a CMV promoter, the response to TK/GCV treatment of a panel of a p53-deficient cells derived from human pancreatic adenocarcinoma. Our results show that sensitive and resistant cells display differential properties in terms of cell cycle control in a model of intrinsic resistance and in a model of treatment-induced resistance. A comparative transcriptomic analysis has identified a list of differentially expressed genes, some of which have been validated as potential resistance-marker genes and a functional analysis

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of DNA damage response induced by TK/GCV treatment has pointed out Checkpoint Kinase 1 (Chk1) as a relevant mediator of its mechanism of action.

2. Materials and methods

2.1. Constructs

CCNE1 full-length clone (IRAUp969H0878D) and GADD45A EST clone (IMAGp998l1012777Q) were purchased from ImaGenes GmbH (Berlin, Germany). CCNE1 and GADD45A cDNAs were cloned into pLXSN and pLHCx plasmids (Clontech, Saint-Germain-en-Laye, France), to generate the expression vectors pL-CCNE1-SN and pLHC-GADD45A, respectively.

2.2. Cell lines and transfection

Human pancreatic tumour cell lines BxPC-3 and PANC-1 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). NP-9, NP-31 and NP-18 cells have been described elsewhere [8]. MIAPaca-2 and RWP-1 pancreatic cancer cell lines were kindly provided by Dr F.X. Real. Cells lines were cultured as previously described [9,10].

Generation of NP-18AR cells: NP-18 cells were submitted to 4 rounds of transduction with AdTK and subsequent cultured with 10 μg mL^{-1} GCV (Cymevene, Roche, Madrid, Spain). Viral doses were scaled up from 3 vp mL^{-1}, in the first two transductions, to 150 vp mL^{-1} and 350 vp mL^{-1} in the third and fourth transductions, respectively. GCV treatment was applied during 12, 11, 27 and 15 days right after first, second, third and fourth transduction, respectively.

Transient transfection of RWP-1 and MIAPaca-2 cells was performed using Lipofectamine2000 and PlusReagent (Invitrogen, Barcelona, Spain) following manufacturer's instructions.

Retroviral particles were generated by calcium-phosphate DNA precipitation transfection of retroviral vectors pL-CCNE1-SN, pLXSN, pLHC-GADD45A or pLHCx into the Phoenix amphotropic packaging cell line (ATCC; Rockville, MD). After 48 h the viral supernatant was collected, passed through 0.45 μm filters and used for transduction.

BxPC-3 and NP-9 cells overexpressing GADD45A or cyclin E1, respectively, and the corresponding control cells were obtained by three consecutive rounds of retroviral transduction with 2 ml of either RvGADD45A or RvCH or RvCCNE1 or RvCSN virus-containing media in the presence of 8 μg/ml polybrene (Sigma-Aldrich; Saint Louis, MI, USA) for 16 h at 37 °C. Forty-eight hours after the third transduction, cells were selected in either 200 μg/ml of Hygromicin or 800 μg/ml G418 (Sigma; Saint Louis, MI, USA) for 7 days. Resistant clones were pooled and used for subsequent experiments.

2.3. Adenoviral infection and ID50

Serotype 5 adenovirus encoding TK under the control of a CMV promoter (AdTK) has been previously described [11].

Culture viability was measured and dose–response curves were constructed for NP-9, NP-18, NP-31, PANC-1, MIAPaca-1, RWP-1 and BxPC-3 transduced with AdTK and cultured with 10 μg mL^{-1} during 5 days as previously described [9]. ID50 values were estimated from dose–response curves by standard non-linear regression, using an adapted Hill Equation (GraFit v3.0, Erithacus Software, Surrey, UK).

2.4. Apoptosis induction

Induction of programmed cell death was assayed by monitoring PARP cleavage or active caspase-3 in whole cell extracts by Western blotting, using a purified mouse anti human PARP antibody (BD Biosciences, San Jose, CA) or anti-cleaved caspase-3 (Cell Signaling Technology; Danvers, MA, USA).

2.5. Cell cycle analysis

Cells were trypsinized, counted and resuspended in complete medium at a concentration of 1×10^6 cells mL^{-1}. Hoechst 33342 (Sigma-Aldrich, Madrid, Spain) was added to a final concentration of 5 μg mL^{-1} and mixture was incubated 1 h at 37 °C. DNA content was measured in an LSR Flow Cytometer (BD Biosciences, San Jose, CA). Quantification of percentage of cells in each phase was performed using ModFit 3.0 LTTM Software (Verity Software, Topsham, ME).

2.6. RNA extraction and real-time RT-PCR analysis

Total RNA was extracted with TriPure reagent (Roche), and reverse transcribed with Retroscript RT kit (Ambion, Austin, TX), using random decamers, in accordance with the manufacturer's protocols. Real-Time RT-PCR was performed using SYBRgreen (Roche) in a capilaries LightCycler (Roche) thermal cycler. HPLC-purified primers were purchased form Sigma-Aldrich. Primer sequences are described in Supplementary Table I. Statistical analysis of relative expression of genes was performed by pair wise fixed reallocation randomization test using the Relative Expression Software Tool (REST©) [12,13].

2.7. Microarray analysis

Cyanine 3- or Cyanine 5-labeled cRNA probes were synthesized using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent, Las Rozas, Spain), according to manufacturer's recommendations. Probes were mixed in hybridization buffer (In situ Hybridization Kit Plus, Agilent) and two-color competitive hybridizations were performed in 60mer oligonucleotide microarrays (H22K, Las Rozas, Spain) during 18 h at 60 °C. Fluorescent images were obtained using an Agilent G2565BA scanner at 100% PMT 100% laser power settings and quantified through the GenePix 6.0 software (Axon, Molecular Devices, Sunnywale, CA) using the irregular feature finding option.

Extracted raw data were filtered and normalized using the Limma package developed within the Bioconductor project in the R statistical programming environment [14]. Target genes were considered as differentially expressed when above the 95% rank in the B empirical Bayes statistic and above a set fold change of 1.2. All quantitative and statistical analyses were performed using Limma package in the R environment [15], Lowess-normalized log2 ratio representing resistant versus sensitive cells. (Data available in GEO, accession number GSE17137).

2.8. Western blotting

Nuclear extracts were prepared using a protocol adapted from Ref. [16]. Whole-cell extracts for the detection of P-Chk1/2 were prepared by lysing the scrapped cells with a modified RIPA buffer. For the detection of histone gamma-H2AX and active caspase-3 cells were lysed in hot sodium-dodecyl sulfate (SDS) sample buffer as described [17].

All protein extracts were quantified using BCA kit (Promega, Madrid, Spain). SDS-PAGE and Western blot experiments were performed following standard protocols and developed using Enhanced Chemoluminescence Kit (Amersham).

Antibodies against Phospho-Chk1 (Ser 345) (133D3) and Phospho-Chk2 (Thr 68) were purchased from Cell Signaling (Danvers, MA). Antibody against Cdc25A (clone DC5120) was purchased from Upstate (Temecula, CA). Antibodies for the detection of Chk1 (G-4) sc-8408, Chk2 (H-300) sc-9064, GADD45a (H-165, sc-797, rabbit polyclonal), Lamin B1 (H-90, sc-20682, rabbit polyclonal), and Cyclin E (HE12, sc-247, mouse monoclonal) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). As loading controls antibodies anti-α-tubulin (T9026, mouse monoclonal, Sigma-Aldrich, Madrid, Spain) and anti-GAPDH (MAB 374, mouse monoclonal, Millipore, Temecula, CA) were used. Secondary