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Inhibition of Transglutaminase 2 activity increases cisplatin cytotoxicity in a model of human hepatocarcinoma chemotherapy

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ABSTRACT

Transglutaminase 2 (TG2) is a ubiquitous multifunctional enzyme whose expression has been found to be altered in numerous studies of apoptosis and cell survival; its activity has been found to be increased in many types of cancer, where it is often over-expressed.

Cisplatin has long been used as an effective therapeutic drug to treat numerous cancers. Although its activity is based on cross-linking of DNA, cisplatin may also operate *via* other mechanisms that involve modification and alteration in the activity of protein and RNA modulators of the cell cycle and apoptotic processes; these mechanisms are less well characterised.

In this study, we investigated the effects of cisplatin-induced apoptosis on TG2 expression and activity in the human hepatocarcinoma (HepG2) cell line. Through a combination of Western blotting, enzymatic activity assays, flow cytometry and fluorescence microscopy we provide evidence that TG2 is inhibited during initiation of apoptosis by cisplatin, an observation that was reversed by increasing the expression of TG2, by treating cells with retinoic acid. We also report, for the first time, that cisplatin can directly inhibit transglutaminase activity *in vitro*.

Collectively, these studies increase our understanding of the mechanism(s) of action of cisplatin, as cisplatin–mediated reduction in TG2 activity appears to act as an early activator of apoptosis during chemotherapeutic treatment of hepatocarcinoma cells. This observation suggests an explanation as to how increased levels of TG2 activity in cancer cells could contribute to chemotherapeutic resistance to cisplatin, and so has implications for novel approaches to cisplatin therapy.

1. Introduction

Cisplatin (Fig. 1) is one of the most effective drugs in the treatment of several types of cancer. The way cisplatin works in the body is not fully understood, though it is well known that cisplatin cross-links DNA, and in this way inhibits DNA synthesis (Gonzalez et al., 2001; Qin and Ng, 2002; Zhang et al., 2010). Cisplatin thus targets rapidly-dividing cells, and so is preferentially toxic to cancerous cells. However, cisplatin is also toxic to healthy cells, and treatment therefore has side-effects that can cause damage to vital organs (Ishida et al., 2002; Florea and Busselberg, 2011); cisplatin cannot be used at high doses. This problem is further increased during repeated treatments, as cancer cells that are able to evade the cytotoxic effects of low dose treatment of cisplatin become resistant to the concentrations normally used for treatment (Zhou et al., 2010; Lopez-Ayllon et al., 2014; Gumulec et al., 2014). Eventually, the high concentrations of cisplatin that are required to mediate a continued anti-cancer effect become untenable to the patient (Galluzzi et al., 2014).

Transglutaminases are a family of calcium-dependent enzymes that were first discovered almost six decades ago (Clarke et al., 1957). One of the members of this family, TG2, has been found to be variously expressed in several cellular compartments, including the nucleus, cytoplasm, mitochondria, and extracellular matrix (ECM), depending on the cell's physiological and pathological conditions (Nurminskaya and Belkin, 2012; Eckert et al., 2014; Piacentini et al., 2014; Park et al., 2010). TG2 operates by post-translationally modifying exposed glutaminyl residues in protein substrates, and, in the later stages of cell death, cross-links proteins *via* exposed glutaminyl and lysyl residues during apoptotic body formation. Through these activities and associated specific protein-protein interactions, and its G-protein activity, TG2 contributes to multiple cellular processes (Nurminskaya and Belkin, 2012; Eckert et al., 2014). Indeed, its wide range of influence has led to TG2 being described as the molecular equivalent of a “Swiss army knife” (Gundemir et al., 2012). TG2 is a difficult protein to characterise in terms of its particular importance in cell biology, as it appears to be linked to so many processes. However, the fact that TG2 is now implicated in several inflammatory diseases, including coeliac

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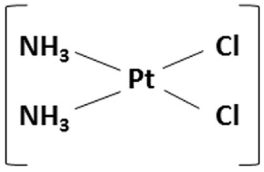

Fig. 1. Chemical Structure of Cisplatin.

disease, diabetes, neurodegenerative diseases, and cancer (Siegel and Khosla, 2007; Odii and Coussons, 2014) is starting to suggest a common mechanistic theme in different cell types, and so beginning to highlight a hierarchy of activities of TG2 that are required for normal cellular health. A further, and possibly revealing, observation is that despite TG2 expression already being high in cancer cells, its levels are even further increased in drug resistant and metastatic cancer (Mehta et al., 2004; Herman et al., 2006; Kumar et al., 2010) - though the precise mechanism of its action remains currently poorly understood.

In order to investigate whether the mechanism of cisplatin cytotoxicity also operates on protein mediators of apoptosis and/or cell survival, we have developed a HepG2 cell line model of cisplatin therapy of human hepatocarcinoma. In this report we show that cisplatin treatment of hepatocarcinoma cells contributes to the induction of apoptosis by down-regulating both TG2 expression and its enzyme activity by approximately 30–50%. Further *in vitro* studies show that although most of the loss of TG2 activity is probably due to the loss of expression of TG2 protein in cells, a contributory inhibitory effect may also be contributed by direct inhibition of TG2 enzyme activity by interaction of cisplatin at the active site of TG2. On the basis of our observations, we speculate that the over-expression of active TG2 in cancer cells may serve to dilute the suppressing effects of cisplatin on TG2 expression and activity, and so contribute to the resistance of cells to cisplatin-induced apoptosis. Such an effect may contribute to one of the major problems in cancer treatment by increasing the drug-resistance of cancer cells in patients undergoing long-term repeated cisplatin-based chemotherapy. The inhibition of TG2 activity may therefore represent a novel combined approach to the clinical treatment of HCC with cisplatin.

2. Materials and methods

All the chemicals used in this study were from Sigma Aldrich, UK, unless otherwise stated.

2.1. Cell line and cell culture preparation

The HepG2 cell line used in our experiments was procured from the European Collection of Cell Cultures (ECCC) (Sigma Aldrich, UK) and was maintained in RPMI 1640 culture medium (Invitrogen, UK) fully supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Aldrich, UK). Cell cultures were maintained at 37 °C in the presence of a humidified 5% CO_2 atmosphere and were kept free of mycoplasma contamination by adding the Plasmocin, a commercially available anti-mycoplasma reagent. Cells were observed on a regular basis and their morphological authentication was confirmed by comparison with online STR profile data (NCBI Biosample database).

2.2. Stock solution preparation

Stock solutions of 2 mM cisplatin were prepared in 18 mΩ ultrapure water under subdued light and stored in the dark at room temperature before use. Freshly-prepared cisplatin solution was used for every experiment, owing to its instability in aqueous solution. Special precautions were taken during preparation of retinoic acid solutions, as it is sensitive to UV light, air and oxidizing agents. Preparation of 10 mM (3 mg/ml) retinoic acid in absolute ethanol was performed under subdued light and in a glove bag under an atmosphere of inert gas and stored at −20 °C. Retinoic acid solutions were diluted with tissue culture medium prior to any treatments and used within two weeks. Freshly prepared stock solutions of 0.5 M cystamine were prepared in ultrapure water and stored at 4 °C for the duration of experiments prior to use.

2.3. Cytotoxicity assay using cell counting kit-8 assay

The cell counting kit-8 (CCK-8) assay is a colorimetric procedure based on the ability of viable cells to reduce a yellow-coloured formazan dye. The CCK-8 uses WST-8 [2-(2-methoxy-4-nitrophenyl) − 3-(4-nitrophenyl) − 5-(2,4-disulfophenyl) − 2H-tetrazolium, monosodium salt] in conjugation with an electron mediator, 1-methoxy-5-methylphenazinium methylsulphate, to assess cell viability.

HepG2 cells were detached by proteolysis using 1% trypsin/0.02% EDTA and were then re-suspended in fresh culture medium at a concentration of 1×10^5 cells/ml. The cells were then seeded into 96-well cell culture plates at 10,000 cells per well in a 100 μl volume of fully-supplemented RPMI 1640 medium. Blank control wells were filled with 100 μl fresh culture medium with omission of the cells. The cells were then grown for 24 h at 37 °C in a humidified 5% CO_2 environment. Following incubation for 24 h, the culture medium was removed and 100 μl of fresh culture medium containing freshly-prepared cisplatin were added to give final concentrations over a range of 0–20 μM. The plates were incubated for 24 h and 48 h time intervals. Following exposure to the drug, 10 μl of the cell counting kit-8 reagent were added directly to the cell cultures and incubated for another 4 h at 37 °C in a humidified 5% CO_2 environment. The WST-8 formazan product was measured at 450 nm using a monochromator-based multi-mode microplate reader equipped with Megalan software (Sunrise, UK). The viable cell numbers were counted and the IC_{50} values, for 24 h and 48 h incubation, were calculated. All samples were prepared in triplicate and the data are derived from the average of five independent experiments.

2.4. Apoptosis analysis by propidium iodide staining with flow cytometry

Briefly, 1×10^6 cells were grown in culture flasks for 24 h, and then treated with cisplatin for different time intervals, at 37 °C in a humidified 5% CO_2 atmosphere. After defined incubation times, floating cells from aspirated medium were collected by centrifugation at 300*g* for 5 min. Adherent cells were washed with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and harvested by trypsinization and the cell pellets were washed once with ice-cold PBS. Cell pellets were re-suspended in 0.5 ml ice-cold PBS and 70% ethanol was added to the cell suspension drop-wise, up to 5 ml, while vortexing. Cells in 70% ethanol were incubated at 4 °C for 2 h, with gentle agitation to avoid clump formation after 1 h. Then, cells were spun at 600*g* for 10 min, and cell pellets were washed twice with ice-cold PBS. 400 μl of propidium iodide (PI) (50 μg/ml) solution were added to cells and the mixtures were incubated at room temperature for 30 min, before analysist by flow cytometry that was carried out on a FACScan equipped with Cell Quest Pro Software (Becton Dickinson, UK).

2.5. Apoptosis analysis by FITC-labelled Annexin-V with flow cytometry

HepG2 cells were harvested by trypsinization. The cell pellets were washed two times in ice-cold PBS. Cells were then diluted with ice-cold binding buffer (0.01 M Hepes/NaOH, 0.14 M NaCl, 2.5 mM $CaCl_2$, pH 7.4) to 1×10^6 cells/ml density. To 100 μl cell suspension (10^5 cells), 5 μl of Fluorescein isothiocyanate (FITC) labelled-Annexin V (5 μg/μl) and 10 μl of PI (20 μg/μl) (BD Biosciences, UK) were added, and mixtures were incubated for 15 min at room temperature in the dark. Then, 300 μl of binding buffer were added, and samples were analysed using a BD Accuri flow cytometer equipped with C6 software (BD Biosciences, UK).

2.6. Nuclear staining with DAPI

HepG2 cells were grown on coverslips in 6-well culture plates in a