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Histone H2AX phosphorylation as a molecular pharmacological marker for DNA interstrand crosslink cancer chemotherapy

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ABSTRACT

The aims of this study were to investigate mechanisms of action involved in H2AX phosphorylation by DNA interstrand crosslinking (ICL) agents and determine whether γH2AX could be a suitable pharmacological marker for identifying potential ICL cellular chemosensitivity. In normal human fibroblasts, after treatment with nitrogen mustard (HN2) or cisplatin, the peak γH2AX response was detected 2–3 h after the peak of DNA ICLs measured using the comet assay, a validated method for detecting ICLs *in vitro* or in clinical samples. Detection of γH2AX foci by immunofluorescence microscopy could be routinely detected with 6–10 times lower concentrations of both drugs compared to detection of ICLs using the comet assay. A major pathway for repairing DNA ICLs is the initial unhooking of the ICL by the ERCC1-XPF endonuclease followed by homologous recombination. HN2 or cisplatin-induced γH2AX foci persisted significantly longer in both, ERCC1 or XRCC3 (homologous recombination) defective Chinese hamster cells that are highly sensitive to cell killing by ICL agents compared to wild type or ionising radiation sensitive XRCC5 cells. An advantage of using γH2AX immunofluorescence over the comet assay is that it appears to detect ICL chemosensitivity in both ERCC1 and HR defective cells. With HN2 and cisplatin, γH2AX foci also persisted in chemosensitive human ovarian cancer cells (A2780) compared to chemoresistant (A2780cisR) cells. These results show that γH2AX can act as a highly sensitive and general marker of DNA damage induced by HN2 or cisplatin and shows promise for predicting potential cellular chemosensitivity to ICL agents.

1. Introduction

There is currently much interest in the role of DNA repair proteins as predictive, prognostic and therapeutic targets in a wide variety of cancer therapies [1–3]. Chemotherapy based on interstrand crosslinking (ICL) agents such as cisplatin, carboplatin, mitomycin C (MMC) or nitrogen mustard derivatives is used extensively in the clinic and novel ICL cancer chemotherapeutics continue to be developed [4–6]. A major determinant of resistance to cell killing by ICL agents in mammalian cells is the ability to repair DNA ICLs. In dividing cells, the major DNA repair pathway for ICLs requires the ERCC1-XPF endonuclease to initiate dual incisions on either side of one arm of the crosslink, releasing the covalent linkage between the two DNA strands.

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The resected gap produced by this unhooking reaction then provides a suitable substrate for homologous recombination (HR) [7–9]. ICLs can also obstruct DNA replication fork progression in dividing cells resulting in the formation of DNA double strand breaks (DSBs). Unlike the direct DSBs induced by ionising radiation which are predominately repaired by non-homologous end joining, these ICL and replication associated DSBs require HR for their repair [8,9]. Since cells defective in ERCC1, XPF or components of HR are sensitive to cell killing by ICL chemotherapeutic agents it would appear that both unrepaired ICLs and their associated DSBs constitute the major cytotoxic lesions induced by ICL agents [9–12].

For detecting ICLs *in vitro* or in clinical samples a modified comet assay has been validated. Increased repair of DNA ICLs as measured using the comet assay contributes to clinical acquired resistance to melphalan in multiple myeloma and in ovarian tumour cells following platinum therapy [13,14]. In terms of DNA repair, it appears that the modified comet assay measures the initial rate of ICL unhooking by ERCC1-XPF since cells with defective ERCC1 or XPF show decreased unhooking and increased cellular sensitivity [9–11]. Furthermore, expression of ERCC1 can be a predictive and prognostic marker for resistance, normal tissue tolerance and patient outcome in platinum based chemotherapy [15]. However, neither the comet assay nor ERCC1 expression is able to identify ICL chemosensitive cells arising as a result of a HR repair defect. Since there is substantial evidence that defective HR contributes significantly to tumour development in a wide variety of cancers [16,17] it is important that assays relating to ICL induced DNA damage can also identify HR chemosensitivity or resistance.

In response to ionising radiation, the histone protein H2AX is phosphorylated to form γH2AX [18]. Thousands of γH2AX molecules accumulate at sites of DNA double strand breaks (DSBs) to form discrete nuclear foci, which can be visualised by immunocytochemistry [19]. Persistence of γH2AX foci is thought to be a clinical indicator of tumor cell sensitivity after radiotherapy or treatment with radiomimetic chemotherapeutics [20–22] and has been proposed as an effective target for improving radiation therapy [23]. The role of γH2AX is to recruit DNA repair and cell cycle checkpoint proteins required for the efficient processing of DNA double strand breaks. DNA damage response proteins that colocalize with γH2AX foci include the MRE11/RAD50/NBS1 (MRN) complex, BRCA1, RAD51, MDC1 and FANCD2, all major components of HR DNA repair [24,25]. DNA interstrand crosslinking induced by cisplatin, MMC and psoralen plus UV have also been reported to induce γH2AX foci [7,26–28]. This raises the possibility that persistence of γH2AX foci after treatment with ICL agents could reflect a defective HR system either as a direct inability to repair ICLs or replication associated DNA DSBs.

The role of H2AX phosphorylation in sensing ICL DNA damage or repair remains poorly understood. Based on the detection of γH2AX foci, there are conflicting reports regarding the role of ERCC1 and XPF for ICL-associated DSB induction and repair [7,27,28]. It is also unclear whether the γH2AX response is dependent on ICL-associated DSBs or whether ICLs are also important. Consequently, one aim of this study was to determine the types of DNA damage and repair pathways that contribute to the γH2AX response induced by ICL agents. A second aim was to establish if γH2AX could be a potential marker of ICL chemotherapeutic cellular sensitivity or resistance.

To assess the suitability of γH2AX as a marker of DNA damage associated with ICL agents, the γH2AX response was initially compared to interstrand crosslinking as determined using the comet assay in normal human fibroblasts after treatment with the crosslinking agents mechlorethamine (HN2) or cisplatin. In addition to ICLs, HN2 has been shown to rapidly induce ICL-associated DNA DSBs in dividing human and Chinese hamster ovary (CHO) cells while no frank DNA DSBs have been detected after treatment with cisplatin [9–11,26]. To assess the types of DNA damage or DNA repair pathways that contribute towards the formation of γH2AX foci and to establish whether it is feasible to use γH2AX as an assay for predicting chemosensitivity to ICL agents, the γH2AX response was also investigated in a panel of CHO cell lines with specific DNA repair defects. Finally, the potential of the γH2AX assay for identifying chemosensitivity or chemoresistance in tumour cells was established using a pair of cisplatin sensitive (A2780) and cisplatin resistant (A2780cisR) human ovarian cancer cell lines as a model of required resistance involving increased ICL repair [29].

2. Materials and methods

2.1. Cell culture

The normal human fibroblast cell line AGO1522B was grown in alpha minimum essential medium (α-MEM, Sigma, Poole, UK) containing 20% foetal calf serum (FCS, Helena Biosciences Europe, Sunderland, UK), 2 mM L-glutamine, 1% 100× non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (all Sigma).

CHO cell lines were grown in F-12 Ham HEPES medium supplemented with 2 mM L-glutamine and 10% FCS. AA8 and UV96 were obtained from Dr. M. Stefanini (Istituto di Genetica Biochimica et Evoluzionistics, Pavia, Italy). CHO-K1, irs1SF, and xrs5 cell lines were provided by Prof J. Thacker (MRC Radiation and Genome Stability Unit, Harwell, UK). UV96 cells are defective in the NER repair factor ERCC1, irs1SF cells are defective in the RAD51 paralogue XRCC3 involved in homologous recombination (HR) and xrs5 cells are defective in XRCC5 (Ku80) involved in non-homologous end joining (NHEJ) [9,10].

The human ovarian cancer cell line A2780 was established from tumour tissue from an untreated patient [29]. Growing A2780 cells in cisplatin and selecting for cisplatin resistance generated the stably resistant A2780cisR cell line. Both cell lines were grown in RPMI 1640 (Autogenbioclear, Wiltshire, UK) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Cell lines were maintained at 37 °C in dry incubators with 5% CO_2, harvested with trypsin EDTA 1x solution (Autogenbioclear) and shown to be free of mycoplasma.

2.2. ICL agents

Analytical grade mechlorethamine (nitrogen mustard, HN2) was obtained from Sigma. Cisplatin was obtained as a clinical 3.3 mM aqueous solution (David Bull Laboratories, Warwick,