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Non-homologous end joining is the responsible pathway for the repair of fludarabine-induced DNA double strand breaks in mammalian cells

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ABSTRACT

Fludarabine (FLU), an analogue of adenosine, interferes with DNA synthesis and inhibits the chain elongation leading to replication arrest and DNA double strand break (DSB) formation. Mammalian cells use two main pathways of DSB repair to maintain genomic stability: homologous recombination (HR) and non-homologous end joining (NHEJ).

The aim of the present work was to evaluate the repair pathways employed in the restoration of DSB formed following replication arrest induced by FLU in mammalian cells.

Replication inhibition was induced in human lymphocytes and fibroblasts by FLU. DSB occurred in a dose-dependent manner on early/middle S-phase cells, as detected by γH2AX foci formation. To test whether conservative HR participates in FLU-induced DSB repair, we measured the kinetics of Rad51 nuclear foci formation in human fibroblasts. There was no significant induction of Rad51 foci after FLU treatment. To further confirm these results, we analyzed the frequency of sister chromatid exchanges (SCE) in both human cells. We did not find increased frequencies of SCE after FLU treatment.

To assess the participation of NHEJ pathway in the repair of FLU-induced damage, we used two chemical inhibitors of the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), vanillin and wortmannin. Human fibroblasts pretreated with DNA-PKcs inhibitors showed increased levels of chromosome breakages and became more sensitive to cell death. An active role of NHEJ pathway was also suggested from the analysis of Chinese hamster cell lines. XR-C1 (DNA-PKcs-deficient) and XR-V15B (Ku80-deficient) cells showed hypersensitivity to FLU as evidenced by the increased frequency of chromosome aberrations, decreased mitotic index and impaired survival rates. In contrast, CL-V4B (Rad51C-deficient) and V-C8 (Brca2-deficient) cell lines displayed a FLU-resistant phenotype. Together, our results suggest a major role for NHEJ repair in the preservation of genome integrity against FLU-induced DSB in mammalian cells.

1. Introduction

DNA double strand breaks (DSB) are important forms of DNA damage. They occur endogenously as natural and highly regulated processes, such as V(D)J recombination and immunoglobulin G class switch or can be induced exogenously by chemical or physical agents. If left unrepaired, DSB can lead to cell death [1]. If incorrectly repaired, they can result in tumorigenesis through translocations, inversions or deletions [2,3].

Several agents (e.g. arabinosylcytosine and arabinosyladenine monophosphate) have been shown to arrest the replication fork during DNA synthesis [4]. These compounds are metabolized to endogenous nucleosides and nucleotides. Active metabolites interfere with the *de novo* synthesis of nucleosides and nucleotides or inhibit the DNA chain elongation after being incorporated into the DNA strand as terminators [5]. The action of the analogue 9-beta-D-arabinofuranosyl-2-fluoroadenine (FLU, fludarabine) on DNA synthesis has been reported both in vitro and in vivo, and resulted in termination of DNA strand elongation [4]. The underlying mechanisms by which FLU triphosphate affects DNA synthesis include: (a) a competition with the normal substrate dATP to be incorporated into DNA which results in repression of further polymerization; (b) the inhibition of ribonucleotide reductase that results in lowering the normal cellular pool of deoxynucleotides; (c) the inhibition of human DNA polymerases α, β, γ and ε; and (d) the inhibition of DNA ligase I [6].

Replication blocks are known to induce DSB [7]. In response to DSB, cells activate a complex network of cellular processes. These include the regulation of a subset of genes, among which are those

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associated with DNA damage signal transduction, cell cycle regulation, DNA repair, and eventually, cell death [8].

Mammalian cells use at least two distinct pathways to rejoin DSB: homologous recombination (HR) and non-homologous end joining (NHEJ) [9].

HR is a process in which a homologous chromosome or a sister chromatid is used as a template to repair and is essentially error-free [10]. Previous data reported that recombination between sister chromatids participates in mitotic HR at least 100-fold more frequently than the homologous chromosome [11–13] and is one of the principal mechanisms responsible for sister chromatid exchange (SCE) in vertebrate cells [14]. In mammalian cells HR requires, among others, the homologous of the *Saccharomyces cerevisiae* Rad52 epistasis group of proteins, including Rad51, Rad52 and Rad54, and other factors, the breast cancer susceptibility proteins Brca1 and 2. Rad51 is an essential component of HR because it catalyzes the DNA strand exchange reaction [15]. Early reports showed that Rad51 re-localizes in nuclear foci after a genotoxic stress [16].

NHEJ requires the DNA-dependent protein kinase (DNA-PK), which comprises the catalytic subunit DNA-PKcs and the DNA end-binding heterodimer Ku70/Ku80. This complex is involved in DNA end recognition and end joining. The proteins XRCC4 and DNA ligase IV are involved in the final step catalyzing the ends ligation. NHEJ directly ligates severed DNA ends with no apparent requirement for extensive sequence homology. The rejoining process may result in the deletion of short stretches of nucleotides and is therefore potentially mutagenic [17].

Current models for explaining differential activation of both pathways are controversial. A competition between Rad52 and Ku for DNA end binding has been suggested to explain the channeling to a particular pathway [18,19]. However, the work from Ristick et al. suggests that both proteins can bind to different DNA substrates in vitro [20]. On the other hand, there is some evidence for a differential contribution of both NHEJ and HR to DSB repair depending on the moment of the cell cycle when DSB are introduced [21].

The recognition and repair of the replication fork-associated DSB induced by FLU remains unclear. The aim of the present work was to evaluate the main system involved in the repair of DSB resulting from the action of FLU on mammalian cells through the study of different cellular responses. In this way, our data suggest a principal role for NHEJ with no evidence for Rad51-dependent HR activity in the repair of DSB induced by FLU.

2. Materials and methods

2.1. Chemicals

FLU (CAS no. 75607-67-9; Schering Argentina), mitomycin C (MMC; CAS no. 50-07-7; Sigma), hydroxyurea (HU; CAS no. 127-07-1; Microsules Argentina S.A.), bromodeoxyuridine (BrdU; CAS no. 59-14-3; Sigma) and vanillin (VN; CAS no. 121-33-5; Sigma) were dissolved in bidistilled water. Wortmannin (WTM; CAS no. 19545-26-7; Sigma) was dissolved in DMSO.

2.2. Cell cultures and drug treatments

Heparinized peripheral blood samples were obtained from four healthy donors (three women and one man, aged from 32 to 46 years), free of any known exposure to genotoxic agents. Whole blood (0.8 ml) was added to 10 ml of F-10 medium containing 15% fetal bovine serum (FBS) and 2% phytohemagglutinin M (PHA). Low passages of the human foreskin fibroblast cell line PTP were kindly provided by Dr. M.I. Tous (Servicio de Cultivo de Tejidos, Depto Virología, ANLIS "CG Malbrán", Buenos Aires, Argentina). The cell line was grown in Minimum Essential Medium supplemented with 10% FBS and 2 mM L-glutamine. Mutant hamster cell lines deficient in NHEJ (XR-C1 and XR-V15B) and HR (CL-V4B and V-C8) with their correspondent parental cell lines (CHO9, V79B and V79) were used. CHO9, XR-C1 and XR-V15B were grown in F-12 medium supplemented with 10% FBS and 2 mM L-glutamine. V79, V79B, CL-V4B and V-C8 were cultured in Mc Coy's 5A medium (without hypoxanthine and thymidine) supplemented with 10% FBS. The hamster cell lines were kindly provided by Prof. Dr. M.Z. Zdzienicka and Dr. W. Wiegant (Leiden University, Leiden, The Netherlands). All the cultures were incubated at 37 °C under a 5% CO_2 humidified atmosphere. Different doses of FLU, MMC or HU were applied as indicated in the text.

2.3. Cell synchronization, BrdU labelling and γH2AX detection

Human fibroblast cell line was arrested in G1-phase by contact inhibition and maintained under serum starvation conditions for 48 h. Cells were replated at low density on coverslips and released for 24 h in complete medium to enrich the cell population in the S-phase. The percentage of cells in the S-phase obtained with this methodology was checked by flow cytometry with propidium iodide and was higher than 70%. Then, the cultures were exposed for 2 h to FLU 1 μg/ml, HU 1 mM or mock treated. Cells were pulsed with 150 μM BrdU for 10 min before fixation/permeabilization with 2% paraformaldehyde containing 0.5% Triton X-100 in PBS for 20 min. Nuclei were denatured with 0.05 M NaOH for 30 min. Indirect immunofluorescence was performed using primary antibodies against BrdU (1:100; Santa Cruz Biotechnology) and γH2AX (1:300; Cell Signaling), followed by exposure to secondary antibodies (Vector Laboratories). One hundred BrdU-positive nuclei were scored and classified by their BrdU labelling pattern as focal (early S-phase), intermediate and distributed (middle S-phase), and heterochromatic (late S-phase) [22]. The percentage of γH2AX-positive cells in each BrdU labelling pattern was scored. Two independent experiments were carried out for each treatment.

2.4. Cell proliferation and mitotic index

Both human lymphocytes and fibroblasts were cultured for two complete rounds of replication in presence of 10 μg/ml BrdU. Twenty-four hours after the culture onset, lymphocytes were treated with FLU and incubated in darkness for 48 h. Colcemid (0.2 μg/ml) was added 90 min before harvesting. Exponentially growing fibroblasts were treated with FLU and incubated in the dark for 48 h. Colcemid (0.2 μg/ml) was added 4 h before harvesting and then cells were trypsinized. Negative and positive (MMC) control cultures were grown under identical conditions. The cells were then collected by centrifugation, exposed to 0.075 M KCl hypotonic solution and fixed in methanol:acetic acid (3:1). Air-dried chromosome preparations were made and a modification of the fluorescence-plus-Giemsa method [23] was applied to obtain harlequin chromosomes. For cell cycle analysis, each metaphase was classified as being in the first (M_1), second (M_2), or third and further (M_{3+}) division and 200 metaphases per culture were examined. The replication index (RI) was calculated as follows: $RI = 1 \times (\%\text{ of cells in } M_1) + 2 \times (\%\text{ of cells in } M_2) + 3 \times (\%\text{ of cells in } M_{3+})/100$. The MI was calculated as the number of metaphases among 2000 nuclei and expressed as a percentage. Three to five independent experiments were carried out for each end point.

2.5. Analysis of SCE

Human lymphocytes and fibroblasts were incubated in presence of 10 μg/ml BrdU and FLU for two complete rounds of replication (48 h). The SCE average was taken from the analysis of 30 metaphases during the second cycle of division in three to five independent experiments.

2.6. γH2AX fluorescent immunostaining

Exponentially growing human fibroblasts were grown on coverslips for at least 24 h before a 16 h treatment with different doses of FLU. The cells were fixed with methanol:acetone (1:1) for 2 min at room temperature. After washing with PBS, the cells were exposed to blocking solution (3% BSA and 0.25% Tween 20 in PBS) for 1 h and then incubated with rabbit anti-γH2AX (1:300; Cell Signaling) or rabbit anti-Rad51 (1:200; Santa Cruz Biotechnology) antibodies for 2 h. The secondary fluorescein isothiocyanate-conjugated anti-rabbit antibody (1:250; Vector Laboratories) was incubated for 1 h and the slides were mounted in 4′,6′-diamidino-2-phenylindole containing antifade solution (DAPI; Vector Laboratories). Two hundred nuclei were scored per slide in three independent experiments.

2.7. Assessment of chromosome alterations

Exponentially growing human fibroblasts were pretreated with VN 300 μM or WTM 8 μM for 1 h, and then cells were exposed to FLU 1 μg/ml during 48 h in presence of BrdU 10 μg/ml. Both VN and WTM were present in the culture medium during the whole time of treatment. Colcemid (0.2 μg/ml) was added 4 h before harvesting and then cells were trypsinized, exposed to hypotonic solution and fixed. The slides were processed according to Perry and Wolff [23]. Thirty metaphases in second division (bifilarly stained) were analyzed for SCE and 50 metaphases in first division (unifilarly stained) were evaluated for chromosome breakages (CB) in two or three independent experiments. Chromatid and chromosome breaks were scored as one break and chromatid exchange configurations, dicentric and ring chromosomes were scored as two breaks. Gaps were excluded in the result of CB frequencies.