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Homologous recombinational repair vis-à-vis chlorambucil resistance in chronic lymphocytic leukemia

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Abstract

The objective of this study was to further define the role of homologous recombinational repair (HRR) in resistance to the nitrogen mustards in B-cell chronic lymphocytic leukemia (B-CLL). We have demonstrated previously that increased chlorambucil (CLB)-induced HsRad51 nuclear foci formation correlated with a CLB-resistant phenotype in B-CLL lymphocytes. In this report, we measured the protein levels of HsRad51 and Xrcc3 (an HsRad51 paralog) and correlated them with the *in vitro* CLB cytotoxicity (LD_{50}) in lymphocytes from seventeen B-CLL patients. Both HsRad51 ($r = 0.75$, $P = 0.0005$) and Xrcc3 ($r = 0.52$, $P = 0.03$) protein levels correlated with the *in vitro* CLB LD_{50}. In addition, multiple linear regression analysis showed a significant correlation between Xrcc3 and Rad51 protein levels versus the CLB LD_{50} ($r = 0.78$, $P = 0.0014$), suggesting that both proteins influence CLB cytotoxicity. Moreover, since HsRad51 expression varies in cell lines during the cell cycle, we determined proliferating cell nuclear antigen (PCNA) protein levels to assess possible differences in cell cycle progression. There was no correlation between PCNA protein levels and the CLB LD_{50} ($r = 0.042$, $P = 0.87$) or with HsRad51/Xrcc3 protein levels. Our data suggest that HsRad51 and Xrcc3 protein expression may be predictive of the response in B-CLL patients to treatment with nitrogen mustards. © 2002 Published by Elsevier Science Inc.

Keywords: DNA repair; Drug resistance; Nitrogen mustards; Homologous recombinational repair; Chlorambucil; Chronic lymphocytic leukemia

1. Introduction

The nitrogen mustards, such as CLB, comprise agents commonly used to treat B-CLL. The cytotoxicity of these drugs is correlated with the formation of DNA ICLs, which prevent efficient DNA replication and transcription [1]. However, resistance to these agents eventually develops and has been associated with alterations in the DNA repair capabilities of the cell [2]. In mammalian cells, ICL repair is possibly mediated by the coordinated activity of the ERCC-1/XPF endonuclease, which makes an incision 5′ to the ICL [3], and HsRad51 HRR including Xrcc2 and Xrcc3 proteins [4]. Mutations in ERCC-1, XPF, Xrcc2, or Xrcc3 lead to hypersensitivity to ICL-inducing agents [4].

Our previous findings demonstrated that accelerated ICL repair, increased DNA-PK activity (Ku protein levels), and CLB-induced HsRad51 nuclear foci formation are associated with a CLB-resistant phenotype in B-CLL lymphocytes [5–7]. Recently, we determined that increased HRR correlates with melphalan and cisplatin drug resistance in a variety of human tumor cell lines [8]. In an attempt to further define the role of HsRad51 and Xrcc3 in the repair of alkylating agent-induced DNA damage in B-CLL, we have measured the protein levels of HsRad51 and Xrcc3 and correlated them with the *in vitro* CLB cytotoxicity in malignant B-lymphocytes from CLL patients.

2. Materials and methods

Seventeen patients previously diagnosed with B-CLL, either clinically treated (N = 10) or untreated (N = 7),

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Abbreviations: CLB, chlorambucil; B-CLL, B-cell chronic lymphocytic leukemia; ICL, interstrand crosslink; HRR, homologous recombinational repair; DNA-PK, DNA-dependent protein kinase; LD_{50}, lethal dose that kills 50% of control; PCNA, proliferating cell nuclear antigen; NER, nucleotide excision repair; and NHEJ, non-homologous end-joining.

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were included in this study. All the CLL patients had CD5 positive malignant B-lymphocytes (B-CLL). Moreover, we utilized samples that, in general, were obtained from CLL patients with lymphocyte counts $\geq$30,000/μL and with a low percentage of T-lymphocytes (mean value $5.76 \pm 2.11\%$) as previously described [6,7]. B-lymphocytes were isolated from the peripheral blood of patients by sedimentation centrifugation on Ficoll-Hypaque (Pharmacia) as previously described [7], seeded in 96-well plates at 3×10^5 cells/well, and treated with CLB for 72 hr (20 mM in DMSO stock diluted 1:10 in PBS Phenol red 0.01%) using 0–60 μM final concentrations/well. The 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide (MTT) assay was performed as previously described [6] to determine the *in vitro* CLB LD_{50}.The concentration of CLB required to reduce the absorbance at 570 nm to 50% of the control was considered the LD_{50} of the sample.

HsRad51, Ku86, PCNA, α-tubulin, and Xrcc3 protein levels were determined by western blot analysis as previously described [8]. HsRad51, a rabbit polyclonal antibody, was used at a 1:1000 dilution (Santa Cruz Biotechnology); Ku86, a mouse monoclonal antibody, was used at a 1:2000 dilution (Medicorp); PCNA, a mouse monoclonal antibody, was used at a 1:1000 dilution (Novo Castra Laboratories); and α-tubulin, a mouse monoclonal antibody, was used at a 1:5000 dilution (Amersham/Pharmacia Biotech). The Xrcc3 antibody (a gift from Dr. P. Sung at the University of Texas in San Antonio) is a rabbit polyclonal anti-Xrcc3 antiserum that was used at a 1:2500 dilution.

The *r* and *P* values for the linear correlation curves were obtained using the Microsoft-Excel 2000 linear regression analysis tool. The *r* and *P* values for the multilinear regression analysis were obtained using the Analyse-it General and Clinical Laboratory statistics software vsn 1.62 for Microsoft-Excel.

3. Results and discussion

Nitrogen mustards are effective anticancer agents used to treat B-CLL. Approximately 60–80% of B-CLL patients initially respond to treatment but eventually develop resistance to these agents [9]. The precise mechanisms responsible for nitrogen mustard-drug resistance in the clinical setting are unknown. Alterations in the transport, intracellular accumulation, and metabolic inactivation of nitrogen mustards have not been associated with drug resistance in B-CLL [10]. The accelerated removal of DNA cross-links in nitrogen mustard-resistant B-CLL suggests that DNA repair likely plays a role in resistance [11].

Using lymphocytes of B-CLL patients as a model, it appears that the NER pathway is not rate-limiting in CLB resistance in B-CLL [5,12]. In contrast, we have demonstrated that basal DNA-PK activity and *in vitro* HsRad51 foci formation following CLB treatment correlate with the *in vitro* CLB cytotoxicity (LD_{50}) [6,7]. Moreover, we recently reported that in a panel of fourteen human epithelial cell lines Xrcc3 proteins levels also correlated with melphalan resistance [8].

Table 1
HsRad51/Xrcc3 protein levels and CLB LD_{50} in B-CLL lymphocytes

CLB LD_{50} (μM)	HsRad51	Xrcc3
1.90	0.120 ± 0.021	0.020 ± 0.007
3.00	0.130 ± 0.000	0.010 ± 0.004
3.50	0.660 ± 0.007	0.280 ± 0.098
4.00	0.530 ± 0.032	0.360 ± 0.126
5.10	0.440 ± 0.105	0.270 ± 0.095
5.90	0.580 ± 0.077	0.160 ± 0.056
6.02	0.810 ± 0.154	0.200 ± 0.070
8.70	0.630 ± 0.133	0.270 ± 0.095
8.90	0.450 ± 0.007	0.515 ± 0.182
10.70	0.215 ± 0.042	0.360 ± 0.126
12.93	0.790 ± 0.119	0.180 ± 0.063
18.42	0.900 ± 0.203	0.290 ± 0.102
20.90	0.780 ± 0.000	0.810 ± 0.284
21.00	0.910 ± 0.165	0.200 ± 0.070
22.00	0.780 ± 0.000	0.230 ± 0.081
22.48	1.040 ± 0.252	0.830 ± 0.291
27.86	0.980 ± 0.203	0.390 ± 0.134

Lymphocytes from seventeen B-CLL patients were purified by Ficoll-Hypaque and immediately utilized to determine their corresponding chlorambucil LD_{50} by MTT assay or frozen in liquid nitrogen for protein extraction and western blot analysis. Briefly, the proteins were extracted and separated by SDS–PAGE, transferred to a nitrocellulose membrane and probed with specific antibodies against HsRad5l and Xrcc3, and developed by ECL. The films were scanned, and the optical density values were analyzed using the Scion Image software (optical density/area) after calibration with the maximal and minimal reading values for each protein. Previous to the analysis, different amounts of proteins (20–100 μg) were analyzed to determine the working linear range. The HsRad51 and Xrcc3 OD values were normalized by the corresponding α-tubulin OD values. The mean of six different experiments (N = 6) ± SEM was calculated.

To better elucidate the potential role of HRR in nitrogen mustard-drug resistance in B-CLL, we presently determined the basal expression levels of HsRad51 and Xrcc3 proteins and the *in vitro* CLB cytotoxicity (LD_{50}) in lymphocytes from B-CLL patients (Table 1). Linear regression analyses revealed an association between these HRR proteins and nitrogen mustard resistance, as both HsRad51 ($r = 0.75$, $P = 0.0005$) and Xrcc3 ($r = 0.52$, $P = 0.03$) protein levels correlated with the *in vitro* CLB LD_{50} (Fig. 1). Multiple linear regression analysis of Xrcc3 and Rad51 protein levels versus CLB LD_{50} showed a significant correlation ($r = 0.78$, $P = 0.0014$), suggesting that both proteins influence CLB cytotoxicity. This is consistent with the fact that Xrcc3 and Rad51 interact together in the Rad51-repairasome [4]. Also, in agreement with our previous studies, Ku86 (NHEJ) protein levels correlated with CLB *in vitro* cytotoxicity ($r = 0.52$, $P = 0.049$, data not shown).

It has been reported that overexpression of HsRad51, a key factor in HRR, enhances resistance of human tumor cells to radiation, whereas down-regulation of HsRad51 protein levels induces tumor cell radiosensitivity [13]. Recently, we reported that basal HsRad51 protein levels did not correlate

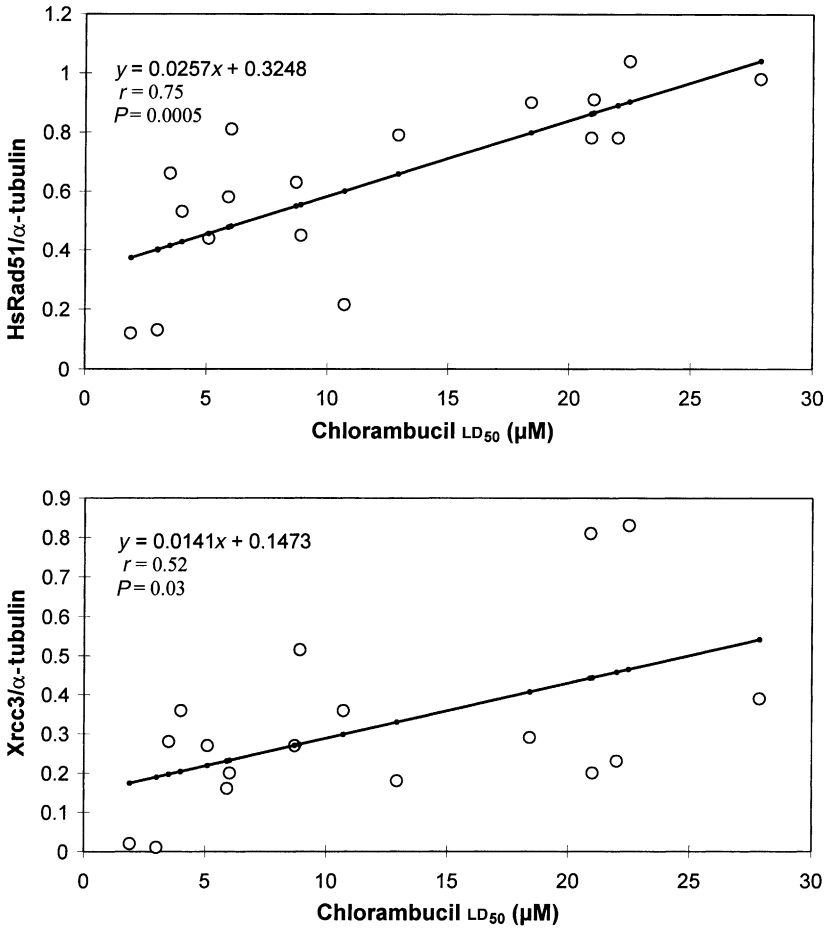

Fig. 1. Correlation between HsRad51 and Xrcc3 protein levels and chlorambucil LD_{50}. Using the data in Table 1, linear regression analysis was assessed as described in "Materials and methods", using the Microsoft-Excel regression analysis tool.

with alkylating agent drug resistance in a variety of human tumor cell lines [8]. In contrast, overexpression of wild-type HsRad51 has been found in 66% of pancreatic adenocarcinomas [14]. The apparent discrepancy between these findings may be due to the experimental cellular environment. HsRad51 accumulates to high levels in three-dimensional cell culture models as well as in orthotopic xenotransplants [14], but not in conventional monolayer cell systems. Thus, it is possible that clinical specimens differ from cell lines in HsRad51 expression levels.

HsRad51 expression in cell lines varies during the cell cycle, being minimal at G_0/G_1 phases and maximal during the S-phase [15]. B-CLL lymphocytes are largely (>95%) biologically immature cells stalled in the G_0/G_1 phases. We therefore determined PCNA protein levels (data not shown) to assess possible differences in cell cycle progression in lymphocytes from B-CLL patients. We found no correlation between PCNA levels and CLB LD_{50} ($r = 0.042$, $P = 0.87$) or with HsRad51/Xrcc3 protein levels (data not shown).

Our data suggest that HsRad51 and Xrcc3 protein expression may be predictive of nitrogen mustard treatment response and may play a role in the development of resistance to these agents in B-CLL patients. The fact that multilinear regression analysis of Xrcc3 and Rad51 protein levels with CLB LD_{50} demonstrated a slightly better correlation (r value) suggests that these two proteins may interact in the determination of CLB cytotoxicity. This is consistent with the fact that Xrcc3 binds directly to Rad51 [4].

Acknowledgments

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