Chlorambucil Induction of HsRad51 in B-Cell Chronic Lymphocytic Leukemia¹

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ABSTRACT

Our previous studies with B-cell chronic lymphocytic leukemia (B-CLL) have suggested that one of the mechanisms of nitrogen mustard (NM) drug resistance is increased repair of drug-induced damage. We have postulated that recombination may play a crucial role in this process. The human homologue of Rad51, (HsRad51), has homology to the RecA protein in Escherichia coli, which is implicated in recombination repair and induction of DNA repair enzymes. In this report, we have examined the expression and distribution of HsRad51 protein in lymphocytes from patients with B-CLL to see whether the expression of HsRad51 is associated with NM damage to the malignant B lymphocytes, specifically chlorambucil (CLB), which is the standard alkylating agent used to treat patients with B-CLL. We have analyzed the intracellular distribution of HsRad51 protein in these lymphocytes before and after treatment with CLB by immunofluorescence. In vitro CLB treatment induces Rad51 expression, as measured by increased immunopositive staining in all CLL samples. In the CLB-resistant CLL lymphocytes, there was a linear correlation between induction of Rad51 protein at 5.4 μM CLB and the in vitro LD₅₀ dose of CLB. Surprisingly, although it has been reported that Rad51 is induced in S phase and only 10% of cells from cell lines expressed positive immunostaining for Rad51, our CLL lymphocytes, which were not subjected to in vitro drug exposure, were 90% positive for Rad51, despite their non-proliferative state, which suggests that there is chronic activation of the protein. Our results suggest that CLB activates HsRad51-directed recombination repair and that this process may be important in NM drug-induced cytotoxicity.

INTRODUCTION

Drug resistance to chemotherapy represents one of the key problems in cancer chemotherapy. Among the mechanisms of drug resistance, it has been suggested that increased repair of drug-induced damage is important. It has been postulated that recombination may play a crucial role in repair of DNA damage (1). Recombination is a fundamental process that is essential for all living cells, as is the repair of damaged DNA. In Escherichia coli, the major pathway for homologous recombination requires the activity of the RecA protein. RecA searches for homologous regions between two double-stranded molecules and promotes strand exchange between them (2). The RecA protein plays an important role in repair of DNA lesions by ionizing radiation and other DNA-damaging agents (3). The Rad51 gene in S. cerevisiae was identified as the homologue of RecA in E. coli (4). Genes homologous to E. coli recA and yeast rad51 have now been isolated from all groups of eukaryotes, including mammals (5, 6). Whereas yeast deficient in rad51 are viable (4), targeted disruption of the rad51 gene in mice results in an embryonic lethal phenotype (7, 8). Mammalian Rad51 has been strongly correlated with meiotic recombination and DNA repair (6, 9). The finding that human Rad51 (HsRad51) promotes homologous pairing and strand exchange reactions in vitro has suggested that Rad51 may also play a role in recombination repair in humans (10).

One study suggested a strong association of HsRad51 protein to repair of damaged DNA. In cultured cell lines, localization of Rad51 changes in response to DNA damage. Immunocytochemistry analysis revealed that the HsRad51 protein exhibits an interesting pattern of nuclear foci, and that the percentage of foci-positive cells and the number of foci per cell increase in response to DNA-damaging agents (11). The number of foci formed in the nucleus was reported to increase after treatment of human fibroblast and lymphoblast cells with methylnitrosurea, gamma ray and UV radiation, suggesting the involvement of Rad51 in repair of DNA damage, but bifunctional alkylating agents were not examined in that investigation (11).

The capacity to repair cytotoxic DNA lesions induced by alkylating agents has been identified as an important mechanism underlying drug resistance in cell lines derived from primary tumors (12, 13). The repair of DNA interstrand cross-links is a complex process involving the coordinated successive action of many proteins. In both bacteria and yeast, cross-link removal depends on both excision repair and a recombination function.
DNA interstrand cross-links and certain DSBs\(^3\) require information for error-free repair that must be supplied by another chromosome. A cross-link poses a major obstacle to DNA replication and will cause a permanent disruption of the chromosome unless there is a bypass mechanism (15). This uncharacterized process in mammalian cells likely involves recombination.

The repair of double-strand breaks produced by ionizing radiation in E. coli or yeast involves homologous recombination (16, 17). Using mutant cell lines hypersensitive to ionizing radiation, a homology-independent mechanism has been implicated in DSB repair in mammalian cells. The 2–6-fold hypersensitivity to ionizing radiation of mutants defective in this process strongly suggests that this mechanism may be the principal pathway for DSB repair (15). Some of these mutants are also hypersensitive to bifunctional alkylating agents; therefore, this pathway may be involved in the repair of cross-links (18).

Many studies have been conducted to elucidate mechanisms of recombination and related processes in mammalian cells. Studies have shown that in yeast, the Rad51 gene is induced by DNA-damaging agents such as ionizing radiation, UV irradiation, and alkylating agents (19, 20). In another study, they examined the formation of Rad51 protein nuclear foci in human peripheral blood lymphocytes after mitogen stimulation and found that formation of the Rad51 foci was induced in S phase (21). The study suggested that the Rad51 protein forms nuclear foci not only in cells undergoing DNA repair but also in cycling cells, especially in S phase cells. The interaction of DNA-PK with Rad51 was investigated by using a mutated DNA-PK. In brief, they used a wild-type hamster parental cell line and a DNA-PK catalytic subunit mutant. Interestingly, the mutant showed no difference from the wild type in Rad51 expression patterns. In both cell lines, Rad51 proteins were expressed as a function of the cell cycle. The study was consistent with Rad51 being involved in a DSB repair pathway that did not activate the end-rejoining pathway undertaken by DNA-PK (22).

A different study suggested that distinctive Rad51 foci are induced by DNA-damaging agents and cell activation and that the response to DNA damage may involve pathways distinct from those associated with B-cell activation and switch combination (23, 24). In a more recent study, they investigated the biological significance of mouse Rad51 protein in cell growth using antisense oligonucleotides (25). Rad51 antisense oligonucleotides markedly inhibited the growth of mouse cells, and the effect was cytotoxic. The suppression of Rad51 not only inhibited cell proliferation but also augmented their sensitivity to radiation.

The fact that Rad51 is required for both repair of damaged DNA and general recombination is of interest and led us to undertake a study of this protein and its expression in B-CLL. CLL represents a model particularly suited to study the development of drug resistance because a homogeneous population of leukemic cells can be easily obtained from the peripheral blood of affected patients, and using the MTT assay, we have shown that the lymphocytes in vitro reflect their clinical status (26).

Patients are commonly treated with single-agent NM therapy, usually CLB, for many years, which eventually results in the development of resistance. Alkylation of DNA, and more specifically the cross-linking of DNA, has been considered to be responsible for the cytotoxicity of NMs such as CLB. Previous reports have shown that alterations in the kinetics of DNA interstrand cross-link formation and removal are associated with resistance to the NMs (27). We have also shown previously that there is enhanced repair of DNA cross-links in NM-resistant CLL lymphocytes (28). Recent studies in our laboratory have shown that nonhomologous DNA end-joining, involving DNA-PK, is a determinant factor in NM drug resistance in B-CLL (29, 30). An intermediate in the repair of DNA interstrand cross-links is believed to be a DSB. Because there is a possibility of redundancy in recombinational DNA repair pathways, we examined the involvement of Rad51 in the repair of DNA damage induced by alkylating agents, such as CLB.

### MATERIALS AND METHODS

#### Preparation of Peripheral Blood Lymphocytes.

Lymphocytes were isolated from the peripheral blood of B-CLL patients by sedimentation centrifugation on Ficoll-Paque (Pharmacia). An aliquot of 3 ml of whole blood was layered onto 4 ml of Ficoll-Paque and centrifuged at 1800 rpm for 40 min. The WBC layer was recovered, washed two times with RPMI 1640 supplemented with 20 mM HEPES, and resuspended in lymphocyte culture medium (RPMI 1640, 10% FCS, 20 mM HEPES, and 10 mg/ml gentamicin) and processed as described below. All patients were diagnosed with B-CLL and separated into two groups according to the following clinical criteria: (a) untreated patients, the vast majority of whom will respond to one of the NMs if and when they are treated; and (b) treated resistant patients, those who were treated with CLB for at least 3 months and failed to have a 25–30% reduction in their peripheral lymphocyte count (31). In addition, when the lymphocytes were tested for in vitro resistance, the LD\(_{50}\) of CLB in the lymphocytes from resistant patients was at least 6-fold greater than the average LD\(_{50}\) of the lymphocytes from untreated patients. The B lymphocytes from 16 patients (8 untreated, 7 treated and resistant to chlorambucil, and 1 sample, which was de novo resistant, were used; Table 1).

#### MTT Assay.

The samples were screened for their sensitivity to CLB using the MTT assay as described previously (32). Briefly, cells were seeded into 96-well microtiter plates using 200 \(\mu\)l/well of cell suspension containing 1.5 \(\times\) 10\(^8\) lymphocytes/ml of RPMI supplemented with 10% FCS. CLB was dissolved in DMSO and further diluted in PBS before it was added to the cells. After 72 h, 50 \(\mu\)l of a solution of 3 mg of MTT/ml of PBS were added to each well, and the plates were incubated at 37°C for 4 h. Thereafter, the plates were centrifuged for 10 min at 1500 rpm. The medium was removed from each well, and 200 \(\mu\)l of DMSO and 25 \(\mu\)l of Sorensen’s glycine buffer were added to each well. The absorbance was read at a wave length of 570 nm.

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\(^3\)The abbreviations used are: DSB, double strand break; DNA-PK, DNA-dependent protein kinase; B-CLL, B-cell chronic lymphocytic leukemia; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NM, nitrogen mustard; CLB, chlorambucil; FTOA, FITC total object area; CTOA, colocatized total object area.
Induction of HsRad51 Protein after \textit{in Vitro} Drug Treatment. To induce cross-links in the DNA of the lymphocytes and activate the repair process, the alkylating agent, CLB (Sigma Chemical Co.) was added to aliquots of the lymphocytes in final concentrations of 5.4 and 30 μM, and these were incubated for 4, 18, and 24 h, after which the cells were washed with PBS and compared to untreated aliquots. (The choices of drug concentration required to reduce the absorbance at 570 nm to 50% of the control was taken to be the LD\textsubscript{50} of the sample.)

**Immunofluorescent Staining Using Anti-HsRad51 Antiserum.** The density of peripheral blood lymphocytes were adjusted to 5 × 10⁶ cells/ml in RPMI with 10% FCS. Aliquots (0.5 ml) of the cell suspension were centrifuged onto clean glass slides at 800 rpm for 4 min in a cytocentrifuge. After cytocentrifugation, the slides were fixed in methanol-acetone (1:1 v/v) for 10 min at −20°C. After three washes with PBS (pH 7.4), the nuclei were stained with propidium iodide at a concentration of 5 μg/ml for 5 min. After three washes with PBS, the slides were immersed into 0.2% Triton X solution for 10 min at room temperature. The slides were then washed with PBS at least four times to remove the Triton X. The slides were immersed into blocking solution [PBS (pH 7.4), 3% BSA; Sigma]. After blocking, the slides were incubated with the primary antibody overnight at 4°C. Rabbit polyclonal anti-human rad51 antibody was generated by immunization with purified recombinant human rad51 protein (11). The dilution of the antibody was made in PBS (pH 7.4) and 1–3% BSA, using a 1:100 and 1:500 dilution for anti-HsRad51 serum and affinity-purified antibodies, respectively. The slides were washed with several changes of PBS for 10 min at room temperature and then incubated for 1 h with FITC-conjugated anti-rabbit immunoglobulin diluted 1:50 with PBS. After four washes with PBS, the preparations were mounted in 50% (v/v) glycerol/PBS.

**Confocal Microscopy.** Confocal microscopy was performed as described previously (33). A ×60 Nikon Plan Apochromat objective of a dual channel Bio-Rad 600 laser scanning confocal microscope equipped with a krypton/argon laser and the corresponding dichroic reflectors were used to distinguish fluorescein and propidium iodide labeling. To generate a composite image of the complete cell depth for the double-labeled cells, optical sections collected at 1 μm steps encompassing the major portion of the immunofluorescent label were projected using Bio-Rad COMOS software in which the most intense value for each pixel was presented. Merging of the fluorescein and the propidium iodide channels generated the green (cytoplasm), red (nucleus), and yellow (nuclear colocalized) immunostaining.

Cells were imaged under conditions of equivalent pinhole and black level settings, and the gain was adjusted for each image to ensure that the pixel values in each section were not maximal and therefore not saturating. The ratio of average pixel intensity relative to the corresponding value for the total cell area is indicative of the proportion of total cellular Rad51 protein. Fluorescence signals were amplified and fed into a multiparameter image analysis program. Image analysis was performed by measuring the total area of colocalized staining and the fluorescence intensity. The relative increase of fluorescent area in the treated lymphocytes was compared with the area of fluorescence of the untreated control cells from the same donor in the same experiment. Confocal images were printed using a Polaroid TX 1500 video printer.

**RESULTS**

In this study, we used immunofluorescence confocal microscopy to investigate whether \textit{in vitro} treatment with CLB could induce alterations in the expression or cellular distribution of Rad51. To study \textit{in situ} localization of Rad51 protein, the anti-HsRad51 antiserum was used to visualize the distribution of HsRad51 protein in B lymphocytes from patients with B-CLL. We examined both CLB-resistant and -sensitive CLL lymphocytes to determine whether there are qualitative and/or quantitative differences in Rad51 protein between these two groups. Using CLB-sensitive and -resistant B-cells from 16 CLL patients (8 sensitive and 8 resistant, as determined by the \textit{in vitro} MTT assay), we examined the spatial distribution of the HsRad51 protein in these cells before and after \textit{in vitro} CLB treatment. Immunofluorescence analysis was performed with anti-HsRad51 antiserum, followed by FITC-conjugated goat-anti-rabbit antiserum to detect the antibody. The use of preimmune serum as well as omission of either the primary or secondary antibody results in the absence of immunopositive cells (data not shown). Immunostaining analysis showed that the Rad51 protein was present in the cells in two forms: in the form of nuclear foci and in a homogeneously dispersed form in the cytoplasm. These immunofluorescent patterns have been described previously (11).

**Induction of HsRad51 by CLB Incubation.** The cells were incubated for 4 h in different concentrations of CLB to allow repair of induced DNA damage to occur, as described previously (28). Clearly, there is activation of the Rad51 protein because the intensity of staining increases with increasing doses of CLB. The representative immunofluorescent photographs of the untreated and resistant B-CLL lymphocyte samples are shown in Fig. 1. Furthermore, the process is observed in both the untreated and resistant CLL lymphocytes. The induction of HsRad51 expression was dose dependent but not time dependent, because prolonged incubation for 18 and 24 h did not

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**Table 1 Drug cytotoxicity profiles of B-CLL samples**

<table>
<thead>
<tr>
<th>Untreated patients</th>
<th>CLB LD\textsubscript{50} (μM)</th>
<th>Resistant patients</th>
<th>CLB LD\textsubscript{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1</td>
<td>3.0</td>
<td>R1</td>
<td>21.0</td>
</tr>
<tr>
<td>U2</td>
<td>6.0</td>
<td>R2</td>
<td>8.9</td>
</tr>
<tr>
<td>U3</td>
<td>8.7</td>
<td>R3\textsuperscript{a}</td>
<td>40.0</td>
</tr>
<tr>
<td>U4</td>
<td>1.5</td>
<td>R4</td>
<td>40.0</td>
</tr>
<tr>
<td>U5</td>
<td>3.5</td>
<td>R5</td>
<td>22.0</td>
</tr>
<tr>
<td>U6</td>
<td>2.6</td>
<td>R6</td>
<td>36.0</td>
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<td>U7</td>
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<td>R7</td>
<td>43.0</td>
</tr>
<tr>
<td>U8</td>
<td>9.0</td>
<td>R8</td>
<td>80.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} R3 is clinically untreated but displayed \textit{in vitro} resistance and thus is classified as B-CLL sample with \textit{de novo} resistance.
Confocal Immunofluorescent Analysis of HsRad51 in B-CLL Cells. The amount of Rad51 expressed by the cells was determined by measuring the FTOA, a value representing the total immunopositive area. When the untreated (U) samples were compared to the resistant (R) samples prior to in vitro CLB treatment, the level of Rad51 was not significantly higher in the resistant group (U = 7845 ± 5521 and R = 10106 ± 6444; P = 0.15). After CLB incubation for 4 h, both the untreated and the resistant groups demonstrated an increase in the level of Rad51 protein. At 5.4 μM CLB, the FTOA value increased by 10% in the untreated samples and by 14% in the resistant samples. At 30 μM, the FTOA value increased by 32% in the untreated samples and by 39% in the resistant samples. The FTOA increased in a dose-dependent manner (Table 2).

The amount of Rad51 localized in the nucleus was expressed as a ratio between the CTOA and the FTOA. Interestingly, in both groups of patient samples, the percentage of nuclear localization of Rad51 relative to the total protein expression changed.

Fig. 1  Representative figures on confocal images taken from one sensitive and one resistant patient’s lymphocytes. Red staining corresponds to propidium iodide in the nucleus. Green fluorescence (FITC) corresponds to cytoplasmic immunopositive staining, and yellow foci correspond to colocalization of HsRad51 in the nucleus.

![Confocal images of HsRad51 in B-CLL cells](image-url)
Table 2  Confocal analysis prior to and after in vitro CLB treatment

The mean pixel values obtained from the confocal image analysis program were quantitated for 8 untreated and 8 resistant B-CLL samples subjected to three different CLB concentrations.

| Samples    | FTOA     | CTOA     | % nuclear localization
|------------|----------|----------|------------------------
|            | (FTOA)   | (CTOA/FTOA) |                        |
| Untreated  |          |          |                        |
| 0 μM CLB   | 7845 ± 5521 | 3349 ± 2312 | 44% ± 16%              |
| 5.4 μM CLB | 8637 ± 6025 | 4511 ± 4178 | 44% ± 21%              |
| 30 μM CLB  | 10394 ± 7034 | 4741 ± 2593 | 47% ± 13%              |
| Resistant  |          |          |                        |
| 0 μM CLB   | 10106 ± 6444 | 4198 ± 2905 | 41% ± 8%               |
| 5.4 μM CLB | 11480 ± 4283 | 4288 ± 2580 | 37% ± 19%              |
| 30 μM CLB  | 14019 ± 5397 | 5397 ± 3716 | 39% ± 9%               |

DISCUSSION

Mammalian cells are thought to repair DNA DSBs through both nonhomologous or end-rejoining and homologous recombination pathways (34, 35). However, the mechanism of homologous recombination in the repair of DNA damage in mammals remains to be elucidated. The DSB end-rejoining pathway has been found to be associated with the DNA-PK complex, but it has not been established as yet that the Rad51 mammalian homologue is required for homologous recombination of DSBs.

In this study, we have examined the expression and spatial distribution of the HsRad51 protein in B-CLL lymphocytes before and after in vitro CLB treatment. Our results with CLB-treated lymphocytes provide evidence that HsRad51 activation is associated with DNA repair of CLB-induced damage in clinical specimens. These results are in agreement with a previous study that reported induction of Rad51 in cultured cell lines in response to DNA damage. The focal sites of Rad51 scattered around the nucleus might represent repair domains, where an underlying structure organizes damaged DNA and enzymes required for repair into functional complexes (11). However, in the absence of stimulation, the previous investigation demonstrated detectable immunolabeling of nuclei in only a small percentage (10% or less) of normal human fibroblast and lymphoblast cell lines, whereas in our studies with clinical specimens, >90% of malignant B lymphocytes showed positive immunostaining, even before treatment with CLB. It is conceivable that this repair process is constantly activated in vivo.

To date, no studies have reported whether HsRad51 protein levels are actually increased in response to DNA damage, and it is unclear whether foci changes represent a true repair response or a nonphysiological effect associated with excess damage. Whether Rad51 induction represents a true DNA repair response or is an early stress response activated by DNA-damaging agents acting as an accessory protein within a far more complicated repair pathway remains to be elucidated.

It is also possible that the induction of Rad51 expression as measured by increased immunopositive staining may be due to a change in protein stability. Unidentified proteins may promote the assembly of or stabilize a higher order Rad51-containing structure required for DNA repair. Identification of proteins that specifically interact or colocalize with Rad51 in B-CLL lymphocytes may provide further insights into this recombination repair process.

Northern analysis was performed to establish whether the increase in Rad51 protein is the result of induction of the rad51 gene. The rad51 mRNA, however, was not expressed at detectable levels (data not shown). This may be a result of the quiescent nature of B-CLL lymphocytes, more specifically the fact that they are in the G0 phase of the cell cycle. Studies have shown that little or no Rad51 protein is detected in G0 human peripheral blood lymphocytes or in quiescent cells (11, 21).

Another study reported that the Rad51 protein was induced in peripheral blood lymphocytes after mitogen stimulation, and that the nuclear foci of the Rad51 protein were observed only in the cells in a specific phase of the S phase (21). Interestingly,
malignant B lymphocytes from CLL patients are, in general, in a nonproliferative state and have a very low level of DNA synthesis (36). Thus, the high percentage of HsRad51 immunostaining that we observed in the CLL lymphocytes samples, that were not subjected to in vitro CLB exposure, is unlikely to be associated with S phase but rather, may represent a high basal level of activation of HsRad51 involved in DNA repair. One possibility is that the HsRad51 protein may participate early in a multistep process before DNA repair actually occurs, and that it might converge at regions where recombination repair is about to occur. Therefore, a constant activation of this protein may serve to prepare the lymphocytes for the occurrence of recombination repair. Alternatively, the chronic Rad51 activation of B-CLL may be another sign of a nonspecific response, possibly related to a proapoptotic stage characterized already in an early phase by the presence of DNA breaks.

Although Rad51 is reported to be a nuclear protein, we also observed Rad51 immunostaining in the cytoplasm. The dispersed form of Rad51 in the cytoplasm may correspond to a function where damaged DNA that underwent repair in the nucleus is being transported to the cytoplasm for further processing. Cells that show cytoplasmic localization of Rad51 may also be in the early stages of cell death. It is also possible that Rad51 in the cytoplasm may have some as yet unidentified function. The cytoplasmic localization may also serve as a mechanism for termination of recombination repair activity in the nucleus. Therefore, the localization of Rad51 may be subject to specific regulation in cells responding to DNA damage. There may also be differences in the composition of Rad51-associated protein complexes that are induced by CLB damage, which may affect the appearance of Rad51 foci.

Both DNA end-joining (DNA-PK) and homologous recombination (Rad51) pathways may contribute to the repair of DSBs. The same end point may be reached by different repair pathways through distinct mechanisms. The two DNA repair pathways might have different properties and, in this respect, are likely to differ in the accuracy of repair. DNA end-joining may not result in accurate repair because nucleotides at the break could be added or lost, and incorrect ends might be joined (37). In contrast, homologous recombination ensures accurate repair because the undamaged homologous chromosome is used as a template. Although accurate repair seems critical for germ and stem cells, inaccurate repair could be more easily tolerated by differentiated somatic cells, given that a large fraction of their genome is no longer functional (37).

In summary, our findings suggest that HsRad51 is activated by CLB and implicate a putative homologous recombination repair process in the cytotoxicity of NMs. Because Rad51 appears to be involved in the repair of DNA damage caused by alkylating agents such as CLB, the inhibition of Rad51 may be a potent way to increase chemosensitivity of CLL lymphocytes. This may open new strategy for the treatment of CLL by combining CLB with Rad51 inhibitors. Our data demonstrate that CLB-treated lymphocytes activate a repair system with altered expression of Rad51. Additional studies will help determine whether inhibition of Rad51 could translate into clinical responsiveness to treatment with alkylating anticancer drugs.

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