Minireview

Chlorambucil Drug Resistance in Chronic Lymphocytic Leukemia: The Emerging Role of DNA Repair

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Abstract

Various mechanisms have been implicated in nitrogen mustard drug resistance. The role of these mechanisms in the development of chlorambucil drug resistance in chronic lymphocytic leukemia (CLL) is discussed. We review these mechanisms with emphasis on the emerging role of DNA repair, and specifically, recombinational repair. Inhibition of these repair processes may lead to new therapies, not only in CLL, but in other malignancies as well.

CLL

A model of drug resistance with direct relevance to clinical practice is a malignancy with easy access to a homogeneous population of malignant cells that represents the clinical status of the patients. CLL is characterized by the proliferation and accumulation of B lymphocytes that appear to be mature but are biologically immature. In some patients, CLL has an indolent course and does not require treatment for many years. When treatment is necessary, single-agent chemotherapy with a NM, usually chlorambucil, is the standard initial therapy, although fludarabine, a new exciting agent, may be incorporated in frontline treatment. At least 60–80% of patients respond to NM therapy, often for years, but eventually all patients become resistant to these agents (1). Furthermore, many patients with CLL respond well to low-dose chlorambucil treatment, which indicates that this disease is initially often very sensitive (hypersensitive) to these anticancer agents, to a greater extent than virtually all epithelial malignancies. A homogeneous monocellular population of malignant B lymphocytes is easily obtained from CLL patients, thus providing a relatively unique opportunity to study clinically derived cells. We and others have previously demonstrated that there is a strong correlation between in vitro cytotoxicity of chlorambucil (measured by the MTT assay) and in vivo response in CLL patients (2–4). Therefore, CLL is an excellent malignancy for in vitro studies that should have direct clinical applicability.

Resistance to the NMs

The NMs are an important group of alkylating agents with activity against several human tumors (5–8). Many NM analogues are transported by carrier-mediated systems into cells and alkylate DNA, RNA, and proteins (9–11). Alkylation of DNA, and more specifically, the formation of DNA ICLs has been considered to be responsible for the cytotoxicity of NMs (12–14). Resistance to the NMs in murine and human tumor cells has been reported to be secondary to: (a) alterations in the transport of these agents (15); (b) alterations in the kinetics of DNA ICL formation and removal (see Fig. 1; Ref. 23). There have been previous reports of alterations in the kinetics of DNA ICL formation and removal associated with resistance to the NMs (13, 14, 16), whereas others have found no differences in the ability of sensitive or resistant cells to remove NM-induced cross-links (24, 25). In this review of NM drug resistance in CLL, preference will be given to investigations that describe protein levels and/or activity rather than mRNA expression. Investigations that describe mRNA expression will be discussed when studies of activity and/or protein levels are not available.

NM Drug Resistance in CLL

Transport and Metabolism. The precise mechanisms responsible for the development of resistance to the NMs in the clinical setting are not known. However, there may be multiple mechanisms that could differ as a function of the type of cancer. Using CLL as an interesting and easily available clinical model, we have investigated the transport, metabolism, and DNA interaction of melphalan in malignant B-lymphocytes obtained from CLL patients (26). We have determined that lymphocytes from chlorambucil-resistant CLL patients are cross-resistant to melphalan as compared with untreated CLL lymphocytes, and that there is an excellent correlation between chlorambucil sensitivity and melphalan sensitivity in these pa-
In general, chlorambucil-resistant CLL lymphocytes are 5- to 6-fold resistant in vitro as compared with sensitive lymphocytes using the MTT assay (2). We found no significant difference between untreated and treated resistant patients' lymphocytes in either the kinetic parameters of melphalan transport or the intracellular levels of intact melphalan. There was no evidence of a difference in melphalan metabolism in untreated patients' lymphocytes versus resistant patients' lymphocytes using TLC (26). In a separate study, chlorambucil uptake and metabolism was investigated in 17 CLL patients.
including 3 patients who were clinically resistant to chlorambucil. The pattern of drug uptake by CLL lymphocytes of a resistant patient was similar to that obtained from untreated patients. Chlorambucil metabolism, as measured by a HPLC assay, suggested that drug decomposition occurred by a simple chemical breakdown and that chlorambucil resistance was not explained by differences in intracellular metabolism (28). Thus, it is unlikely that alterations of drug uptake or metabolism are responsible for NM resistance in CLL.

**GSH and GST.** Alterations in GSH and GST have been correlated with NM drug resistance. NMs are a good substrate for GST-α (29). However, transfection of GST (α or µ classes) in MCF-7 cells did not result in NM drug resistance (30, 31). Transfection of γ-glutamylcysteine synthetase subunits into COS-7 cells resulted in a 2.6-fold increase in GSH levels and a 2-fold increase in melphalan resistance at the LD₉₀ concentration (32). Expression of a rat GST cDNA in rat mammary carcinoma cells resulted in a 6- to 30-fold increase in NM drug resistance (33).

GST activity was analyzed in a group of CLL patients who were either untreated or clinically resistant to chlorambucil. There was a 2-fold increase in GST activity in the resistant patients’ lymphocytes, the predominant isozyme being GSTp1 (34). However, it has been suggested that GSTp1 overexpression is probably an accompaniment rather than a cause of resistance (29). DiSimone et al. (35) found that GST activity was increased in treated patients and was probably not related to alkylating agent resistance. In contrast, others found no difference in GST activity or GSH levels between untreated and treated CLL patients (36, 37). We found no differences in GST activity and GSH levels between sensitive CLL lymphocytes and chlorambucil-resistant CLL lymphocytes as defined by both the MTT assay and the clinical parameters (2). Thus, there is somewhat conflicting data as concerns the role of GSH/GST in NM drug resistance in CLL. However, the lack of evidence for altered intracellular metabolism of melphalan or chlorambucil in resistant CLL lymphocytes along with the conflicting evidence of GSH/GST involvement suggest that this detoxification system is unlikely to be the predominant mechanism of NM drug resistance in CLL.

Another potential detoxification system that may play a role in NM resistance involves metallothionein. We found no differences in metallothionein mRNA expression between sensitive and resistant CLL lymphocytes (27).

**Apoptosis vis-à-vis NM Drug Resistance in CLL.** CLL is a malignancy of B-lymphocytes that are, at least at early stages, largely (>95%) mature cells in the G₀-G₁ phase of the cycle. The malignant B-lymphocytes accumulate in the body because they survive an abnormally long time because of a dysregulation in the programmed cell death process. Programmed cell death results in apoptosis with its characteristic changes. The Bcl-2 family has a critical role in this process. There are three groups of Bcl-2 proteins. There is a dynamic relationship between the group I, pro-apoptotic family (Bax and Bak), the group II antiapoptotic proteins (Bcl-2, Bcl-Xᵢ, Mcl-1, A1, and Bel-W), and the group III proapoptotic proteins (Bad, Bcl-Xₛ, and Bik). The cell death pathway involves cell death signals from various agents, including chemotherapeutic drugs, that initiate apoptosis. Resistance to chlorambucil and cross-resistance to glucocorticoids and nucleoside analogues has been correlated with altered apoptosis. This initiation leads to various biological effects that ultimately converge on the Bcl-2 family pathway. At this point, a cell can be protected from apoptosis or be committed to the process. Activation of the cysteine proteases known as caspases results in DNA degradation. Bcl-X and the Bcl-2:Bax ratio are important factors in the chemoresistance to apoptosis (38).

Expression of apoptosis-regulating proteins in CLL was examined in a prospective fashion and correlated with in vitro chemosensitivity using the TUNEL assay and clinical response (39). The CLL patients were previously untreated and enrolled on an Eastern Cooperative Oncology Group (ECOG) trial according to which they were randomized to receive fludarabine, chlorambucil, or both agents. The combined arm was discontinued because of unacceptable toxicity. Also, chlorambucil in vitro sensitivity could not be determined using the TUNEL assay because of technical problems. However, analysis of DNA integrity has been correlated with chemotherapy response and high risk/refractory disease in CLL (40, 41). In the ECOG trial, in vitro chemosensitivity of fludarabine did not correlate with the levels of any of the apoptosis-regulatory proteins (Bcl-2, Bax, Mcl-1, Bak, Bag-1, and caspase-3). The in vitro chemosensitivity data did not correlate with response. However, there was a significant (P < 0.01) association between higher Mcl-1 levels and failure to achieve complete response, as compared with partial response or no response, in a group of 37 patients. Also, a weaker (P < 0.04) association between poor clinical response and higher Bag-1 protein was observed. These results were not biased by the type of chemotherapy. Neither Bcl-2 levels nor the Bcl-2:Bax ratio correlated with clinical response (39).

Pepper et al. (42, 43) reported a correlation between a greater Bcl-2:Bax ratio and resistance to chlorambucil-induced apoptosis. However in 25 CLL patients, Johnston et al. (44) found no correlation between chlorambucil or fludarabine sensitivity, as measured by the MTT assay and the levels of the Bcl-2 protein or Bcl-2:Bax ratio. We also found no significant correlation between Bcl-2 protein levels or the Bcl-2:Bax ratio and the in vitro (MTT assay) or the in vivo response to chlorambucil or melphalan in 28 patients (14 untreated and 14 treated resistant patients; Ref. 45). Morabito et al. (46) found no correlation between Bcl-2 protein levels in previously untreated CLL lymphocytes and in vitro chlorambucil cytotoxicity determined by the MTT assay.

The design of these studies was different. Kitada et al. (39) looked prospectively to identify innate drug resistance, whereas others examined previously treated resistant CLL patients and thus, examined acquired drug resistance. Therefore, the role of the various proteins involved in apoptosis, such as Bcl-2, Bax, Bag, and Mcl-1, in chlorambucil drug resistance in CLL is not clear, and additional investigations in acquired and innate drug resistance should be done. Also, the role of the MTT assay vis-à-vis assays measuring apoptosis in predicting chlorambucil response in CLL needs to be examined prospectively.

**p53 Mutations vis-à-vis Chlorambucil Drug Resistance.** There is a remarkable concordance in the literature as concerns p53 mutations in CLL. Only 10–15% of CLL patients have p53 mutations but in the vast majority of cases, they are associated
with innate chlorambucil resistance (44–49). The overall frequency of p53 immunohistochemical positivity in CLL was also 15% and correlated with a poorer response to therapy (50). p53 mutations are not a major factor in acquired drug resistance (45). The mechanism by which a p53 mutation results in innate chlorambucil resistance is not known.

**DNA Cross-Links vis-à-vis NM Drug Resistance in CLL.** Chlorambucil-induced alkylation of CLL lymphocyte DNA results predominantly in the development of purine-drug complexes (28). The NMs, including chlorambucil, may also form intrastand and/or ICLs at N-7 guanines (51). The ICLs are considered to be important in the cytotoxicity of these drugs (12–14).

There are technical problems involved in quantitating NM-induced ICLs. NMs produce thermolabile glycosyl bonds (N-7-guanine adducts), which yield apurinic sites and which, in turn, can cause strand breaks and/or breaks of cross-links (reviewed in Ref. 52). Strand breaks can interfere with molecular size-based assays. The ethidium bromide fluorescence assay has the advantage that strand breaks are less likely to influence the quantification of cross-links (53). A widely used assay to determine DNA cross-links is the alkaline elution assay (54–56). This technique involves molecular size differences. However, the strand breaks induced by NMs may complicate interpretation of repair of ICLs when using this assay.

Using the ethidium bromide fluorescence assay, we originally reported (26) that DNA ICL formation at 4 h post-melphalan incubation (a time point believed to be associated with maximal cross-link formation) was decreased in malignant B lymphocytes from resistant CLL patients. However, when we examined cross-link formation and removal at 0, 4, and 24 h after a 35-min melphalan incubation, there was evidence of a greater amount of cross-links at time 0 in malignant B lymphocytes from resistant CLL patients as compared with those from untreated CLL patients. Moreover, the untreated patients’ lymphocytes developed a greater amount of cross-links at 4 h without evidence of removal at 24 h, whereas there was evidence of progressive removal of DNA cross-links at 4 and 24 h in lymphocytes from resistant CLL patients. This suggests that enhanced DNA repair is implicated in this process (57). In another study, a patient with advanced CLL was treated with i.v. cyclophosphamide, and DNA ICLs in the lymphocytes were measured by the alkaline elution technique. Maximal DNA ICL formation occurred 12 h after injection. However, the level of cross-links was just above the sensitivity of the assay at 12 and 24 h after drug administration (58). Also, using the alkaline elution technique, Johnston et al. (37) examined DNA cross-link formation in CLL lymphocytes at 6 h after an in vitro incubation with chlorambucil. They found that the lymphocytes from two resistant CLL patients had as many DNA cross-links as the lymphocytes from patients sensitive to chlorambucil.

**DNA Repair of NM DNA Cross-Links in CLL.** The mechanism of removal of DNA ICLs in mammalian cells is poorly understood. There are several different DNA repair systems that could be involved in the repair of NM-induced DNA ICLs, including base excision repair, nucleotide excision repair, and recombinational repair. The mammalian base excision repair enzyme, alkyl-N-purine DNA glycosylase (3-methyladenine-DNA-glycosylase), can excise damaged guanine bases from DNA treated with chlorambucil (59). We measured 3-methyladenine-DNA-glycosylase activity in CLL extracts and found a significantly (~1.7-fold) higher activity in lymphocytes from resistant CLL patients as compared with those from untreated CLL patients. Because this activity may vary with cell proliferation, it was corrected for differences in DNA synthesis using [3H]thymidine incorporation (there were differences in DNA synthesis between the two groups although the vast majority of malignant B lymphocytes are nonproliferative), and this resulted in no significant difference in enzyme activity between the two groups (60). Moreover, overexpression of the human alkyl-N-purine DNA glycosylase in CHO cells did not result in NM resistance, which suggested that alkyl-N-purine DNA glycosylase was not a rate limiting enzyme in NM drug resistance (61). Furthermore, mouse embryonic stem cells bearing null mutations in this enzyme are not hypersensitive to the NMs (62).

Possible insights into mechanism(s) of ICL repair are gained by examining NM hypersensitivity in DNA repair mutants. Significant NM and/or mitomycin C hypersensitivity (varying from moderate to severe) is found in several DNA repair mutants including ERCC-1, ERCC-4 (XPF), XRCC-2, XRCC-3, Rad54, Ku70, Ku86, and DNA-PKcs (63–67).

This analysis, along with information gained from studying cross-link removal in both bacteria and Saccharomyces cerevisiae, and the possibility that DNA DSBs are repaired in a similar fashion to ICLs, has resulted in the proposed model that nucleotide excision repair, via the ERCC-1/ERCC-4 endonuclease, results in an incision 5’ to the ICL and that recombinational repair is involved in further processing of the lesion (reviewed in Refs. 68, 69). As concerns repair of DSBs, nonhomologous DNA end-joining uses no, or very limited, sequence homology to rejoin ends directly, whereas homologous recombination requires extensive regions of DNA homology. HR would be necessary for error-free repair of ICLs, whereas an illegitimate or NHEJ mechanism of repair could result in deletional repair of ICLs. It is also conceivable that all three types of repair (nucleotide excision, HR, and NHEJ) are implicated in the repair simultaneously or are involved, depending on the phase of the cell cycle, in the processing of ICL. The various genes implicated in NHEJ include the components of DNA-PK, XRCC-4, and ATM. NHEJ is a major mechanism of DSB repair in mammalian cells (reviewed in Refs. 70, 71). HR in human cells implicates the HsRad51 family of proteins including HsRad51, HsRad52, Rad51B, Rad51C, Rad51D, HsRad54, XRCC-2, and XRCC-3. Rad51 binding to DNA requires the precedent binding of Rad52. In addition, other Rad51 protein members may be involved in the assembly of Rad51 complex. Interactions of Rad51 with BRCA2, c-Abl kinase, and p53 have also been detected (reviewed in Refs. 69, 72).

To gain insight into possible mechanisms of DNA cross-link removal in NM-resistant CLL, Bramson et al. analyzed in vitro cross-resistance in CLL (2). Chlorambucil-resistant CLL lymphocytes were completely cross-resistant to melphalan and mitomycin C; partially cross-resistant to cis-platinum and not cross-resistant to UV light nor methylmethane sulfonate. Because UV radiation damage is repaired by nucleotide excision repair and methylmethane sulfonate is repaired by base excision repair, it appears that these repair systems are not up-regulated in NM drug resistance in CLL (2). Also, ERCC-1 protein levels...
were not increased in NM-drug-resistant CLL lymphocytes (27). Nucleotide excision repair activity was very low in most CLL lymphocytes, including the majority of those obtained from previously treated CLL patients (73).

**NHEJ.** Because ionizing radiation results in DSBs that are largely repaired by NHEJ (70) and because DSBs are probably repaired in a similar fashion to ICLs, cross-resistance studies between chlorambucil and ionizing radiation may be informative. Indeed, it appears that there is evidence of cross-resistance between ionizing radiation and chlorambucil in CLL lymphocytes (74).

DNA-PK, a nuclear serine/threonine kinase, is a protein complex that includes a catalytic subunit of M$_r$ 460,000, DNA-PKcs, and a DNA binding subunit, the Ku autoantigen (a dimer of the Ku70 and Ku86 proteins). Ku binds to DSBs and other discontinuities in the DNA and recruits DNA-PKcs to the damaged site (70, 71). The active DNA-PK complex can then phosphorlyate many DNA-bound proteins in the vicinity (75). Because mutations in DNA-PK result in X-ray and alkylating agent sensitivity (64, 65), and because X-ray resistance develops in parallel with chlorambucil resistance in CLL (74), determination of DNA-PK activity in CLL should be informative. In a preliminary report with a small sample of CLL patients, an increase in DNA-PK activity was found in resistant samples (76). In collaboration with Catherine Muller and Bernard Salles (Institute of Pharmacology and Structural Biology, Toulouse, France), our laboratory examined DNA-PK activity in a group of 34 patients (18 patients resistant to chlorambucil both in vitro and in vivo). There was an excellent linear correlation between DNA-PK activity and in vitro chlorambucil cytotoxicity (r = 0.875; P = 0.00001). The increased DNA-PK activity was independent of other clinical and biological factors. The regulation of DNA-PK activity was associated with increased DNA-binding activity of its regulatory subunit, Ku, and increased Ku protein levels. Interestingly, most untreated CLL patients have very low levels of DNA-PK activity, which suggests that, initially, resistance in CLL may be simply a state in which tumor cells lose an abnormal sensitivity to alkylating agents. In ~25% of the samples from untreated CLL patients, a variant (truncated) form of the Ku86 protein was associated with very low DNA-PK activity and hypersensitivity to chlorambucil (77). Wortmannin, a nonspecific inhibitor of DNA-PK, which also inhibits other phosphatidylinositol 3-kinases, sensitized CLL lymphocytes to the effects of chlorambucil. Moreover, there was a significant correlation between the synergistic sensitization and fold decrease in DNA-PK activity, but because Wortmannin also inhibits other phosphatidylinositol 3-kinases, these results must be interpreted with caution (78).

The immunohistochemical expression of Ku autoantigen and DNA-PKcs was examined in various human tissues. There was a large variation in expression depending on the specific tissue type (79). This supports our observations that there is a variation in DNA-PK expression in human tissues. Although it appears reasonable that increased DNA-PK activity is associated with increased repair of NM-induced ICLs and thus, increased drug resistance, it is possible that other mechanisms are involved, including a role for DNA-PK with respect to apoptosis (80, 81).

DNA-PKcs is a member of the phosphatidylinositol 3-kinase superfamily. Other members include ATM and the cell cycle checkpoint protein, ATR (71). Recently, loss of heterozygosity or mutations of the ATM gene and a decrease in ATM protein levels have been found in ~30–40% of B-cell CLL patients. These factors appear to be associated with a shorter survival, at least in younger patients (82–85). The association of ATM with chlorambucil drug resistance in CLL has not been investigated to date.

**HR.** The involvement of nucleotide excision repair and HR in repair of ICLs is inferred from the fact that the mutant cell lines with the greatest sensitivity (10- to 100-fold) to alkylating agents that produce ICLs are those that are deficient in, or lacking, XRCC2, XRCC3, ERCC1, and ERCC4/XPF (63, 67, 69). The nucleotide excision repair complex (ERCC-1/ERCC-4) in mammalian cells makes dual incisions 22–28 bp apart on the same strand, 5' to the ICL (86). This would then be followed by HR.

Several human genes implicated in HR have been characterized including HsRad52, HsRad51, Rad51B, Rad51C, Rad51D, HsRad54, XRCC2, and XRCC3 (reviewed in Refs. 69, 72). A recent model of interaction in yeast proposes that Rad52 interacts with RPA, followed by Rad52 association with Rad51. This leads to the assembly of Rad51 and associated proteins onto ss-DNA which then initiate recombinational DSB repair (87). XRCC3 is necessary for the assembly of Rad51 foci, and these proteins physically interact (67, 88). In fact, if all of the interactions described occur in one complex, then HsRad51, XRCC3, Rad51C, Rad51B, Rad51D, and XRCC2 are complexed together (reviewed in Ref. 69). Rad54 appears to be required after the association of the above mentioned proteins and Rad54 may assist Rad51 in interacting with damaged DNA (89).

The percentage of Rad51 foci-positive cells and the number of foci per cell increased after treatment of human fibroblast and lymphoblast cells with methylmethane sulfonate, γ radiation, and UV radiation (90). In view of the critical role of the Rad51 protein in HR and its probable involvement in repair of ICL, we investigated HsRad51 foci formation after in vitro chlorambucil treatment of CLL lymphocytes. In vitro chlorambucil treatment induced HsRad51 expression as measured by increased immunopositive staining in all of the CLL samples. In the chlorambucil-resistant CLL lymphocytes, there was a linear correlation between induction of HsRad51 foci at 5.4 μM chlorambucil and the in vitro LD$_{50}$ concentration of chlorambucil. Interestingly, there were no differences in HsRad51 protein levels between sensitive and resistant CLL lymphocytes (91).

**Summary of Results with Recombinational Genes**

The regulation of DNA-PK activity appears to be tightly associated with the development of chlorambucil drug resistance in CLL. In particular, low DNA-PK activity is associated with hypersensitivity to chlorambucil. Furthermore, increased levels of DNA-PK activity are associated with chlorambucil resistance in CLL. Moreover, in resistant samples, stimulation of HsRad51 foci formation by chlorambucil correlates with chlorambucil drug resistance (i.e., the more resistant CLL lymphocytes demonstrate a greater HsRad51 foci formation). Because HsRad51 protein levels were not different between sen-
sitive and resistant CLL lymphocytes, the increased Rad51 foci formation after chlorambucil treatment in resistant CLL samples suggests that these foci represent an active DNA repair process involving other Rad-51 related proteins. A plausible hypothesis to explain these results is that low DNA-PK activity defines a hypersensitive state, whereas high DNA-PK activity, along with increased homologous recombination as determined by HsRad51 foci formation, contributes to the resistant state in CLL.

Future Studies

The role of apoptosis in chlorambucil drug resistance should be examined in more detail. In particular, the role of various proteins involved in apoptosis need to be further defined in both prospective and retrospective studies of innate and acquired drug resistance. Also, a comparative prospective trial of the MTT assay versus assays of DNA integrity in predicting chlorambucil response in CLL would be informative.

The mechanism of low DNA-PK activity in sensitive CLL lymphocytes involves decreased Ku protein levels and a variant form of Ku86, as compared with resistant samples. The regulation of this process needs to be further investigated at both the translational and transcriptional levels. Inhibition of DNA-PK activity by small molecules and/or antisense technology in combination with NM chemotherapy may improve the therapeutic index of the latter compounds. As concerns HR, the implication of other proteins such as Rad54, XRCC-2, and XRCC-3 needs to be examined. Recently, a model of repair of DSB has been proposed in which either Ku or HsRad52 binds DSBs, thus directing entry into NHEJ or HR, respectively (92). The relationship between Ku and HsRad52 with respect to repair of DNA cross-links should also be examined. Experiments designed to alter the expression of genes involved in NHEJ and/or HR vis-à-vis chlorambucil drug resistance in CLL should help to clarify their respective roles in this process. Again, inhibition of HR (for example Rad51 inhibition) may result in sensitization of tumors that are resistant to the NMs. Lastly, the possible implication of DNA repair in NM drug resistance in other types of cancer needs to be examined.

References


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