Anti-Cancer Drugs 2002, **13,** pp. 211–220

Review paper

The role of DNA repair in nitrogen mustard drug resistance

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The nitrogen mustards are an important class of DNA cross-linking agents, which are utilized in the treatment of many types of cancer. Unfortunately, resistance often develops in the treatment of patients and the tumor either never responds to or becomes refractory to these agents. Resistance to the nitrogen mustards in murine and human tumor cells has been reported to be secondary to alterations in (i) the transport of these agents, (ii) their reactivity, (iii) apoptosis and (iv) altered DNA repair activity. In the present review, we will discuss the role of DNA repair in nitrogen mustard resistance in cancer. The nitrogen mustards' lethality is based on the induction of DNA interstrand cross-links (ICLs). Two DNA repair pathways are known to be involved in removal of ICLs: non-homologous DNA end-joining (NHEJ) and Rad51-related homologous recombinational repair (HRR). The reports discussed here lead us to hypothesize that low NHEJ activity defines a hypersensitive state, while high NHEJ activity, along with increased HRR activity, contributes to the resistant state in chronic lymphocytic leukemia. Studies on human epithelial tumor cell lines suggest that HRR rather than NHEJ plays a role in nitrogen mustard sensitivity. [© 2002 Lippincott Williams & Wilkins.]

Key words: DNA repair, drug resistance, nitrogen mustards.

Resistance to the nitrogen mustards

The nitrogen mustards are an important group of alkylating agents with activity against several human tumors.[1–4] Many nitrogen mustard analogs are transported by carrier-mediated systems into cells, and alkylate DNA, RNA and proteins.[5–7] Alkylation of DNA and, more specifically, the formation of DNA interstrand cross-links has been considered to be responsible for their cytotoxicity.[8–10] Resistance to the nitrogen mustards in murine and human tumor cells has been reported to be secondary to: (a) alterations in the transport of these agents,[11] (b) alterations in the kinetics of the DNA cross-links formed by these agents,[9,10,12] (c) cytoplasmic metabolism of the chloroethyl alkylating moiety to the inactive hydroxyethyl derivative[13] via glutathione (GSH)/glutathione-S-transferase (GST),[14–16] (d) overexpression of metallothionein which confers resistance to cisplatin and cross-resistance to melphalan,[17] (e) changes in apoptosis,[18] and (f) altered DNA repair activity (see Figure 1).[19] There have been previous reports of alterations in the kinetics of DNA interstrand cross-link formation and removal associated with resistance to the nitrogen mustards,[9,10,12] while others have found no differences in the ability of sensitive or resistant cells to remove nitrogen mustard-induced cross-links.[20,21] This review will concentrate on the involvement of DNA repair in nitrogen mustard drug resistance.

The type of DNA repair involved in nitrogen mustard drug resistance may differ as concerns the type of cancer. In this article, investigations done with clinical samples and cancer cell lines will be reviewed.

DNA cross-links vis-à-vis nitrogen mustard drug resistance

Nitrogen mustard-induced alkylation of DNA results predominantly in the development of purine–drug complexes.[22] The nitrogen mustards, including chlorambucil and melphalan, may also form intrastrand and/or interstrand cross-links at N^7 guanines.[23] These interstrand cross-links are considered to be important in the cytotoxicity of these drugs.[8–10]

There are technical problems involved in quantitating nitrogen mustard-induced interstrand cross-links. Nitrogen mustards produce thermolabile

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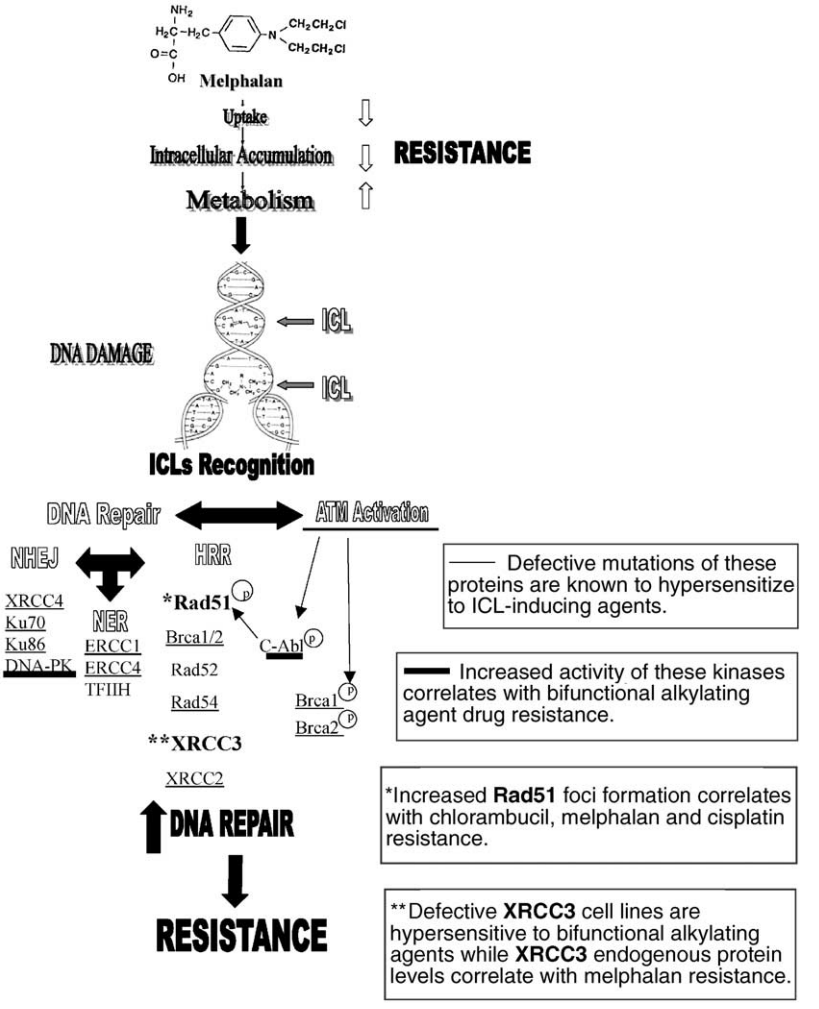

Figure 1. Mechanisms of resistance to DNA-damaging agents. Abbreviations used: ICL, interstrand cross-link; ATM, ataxia talagiectasia mutated kinase; DNA-PK, DNA-dependent protein kinase; NHEJ, non-homologous DNA end-joining; HRR, homologous recombinational repair.

glycosylic 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 Ojwang *et al.*[24]). 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.[25] A widely utilized assay to determine DNA cross-links is the alkaline elution assay.[26–28] This technique involves molecular size differences. However, the strand breaks induced by nitrogen mustards may complicate interpretation of repair of interstrand cross-links when utilizing this assay. More recently, the comet assay has been utilized to quantitate interstrand cross-links, but there are similar problems with the alkaline assay, which may be less with a neutral assay.[29,30]

Nitrogen mustard drug resistance in chronic lymphocytic leukemia

It is difficult to study clinical samples because of the heterogeneity of most tumor samples and the difficulty in obtaining serial samples from the same patient.

A model of drug resistance with direct relevance to clinical practice is a malignancy with easy access to a homogeneous population of malignant cells, which represents the clinical status of the patients. Chronic lymphocytic leukemia is characterized by the proliferation and accumulation of B lymphocytes that appear to be mature but are biologically immature. In some patients, chronic lymphocytic leukemia has an indolent course and does not require treatment for many years. When treatment is necessary,

single-agent chemotherapy with a nitrogen mustard, usually chlorambucil, is the standard initial therapy, although fludarabine, a new exciting agent, may be incorporated in front-line treatment. At least 60–80% of patients respond to nitrogen mustard therapy, often for years but eventually all patients become resistant to these agents.[31] Furthermore, many patients with chronic lymphocytic leukemia respond well to low-dose chlorambucil treatment, indicating 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 chronic lymphocytic leukemia 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 chronic lymphocytic leukemia patients.[32–34] Therefore, chronic lymphocytic leukemia is an excellent malignancy for *in vitro* studies, which should have direct clinical applicability.

Our laboratory, utilizing the ethidium bromide fluorescence assay, originally reported that DNA interstrand cross-link 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 chronic lymphocytic leukemia patients.[35] 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 chronic lymphocytic leukemia patients as compared to those from untreated chronic lymphocytic leukemia patients. Moreover, the untreated patients' lymphocytes developed a greater amount of cross-links at 4 h without evidence of removal at 24 h while there was evidence of progressive removal of DNA cross-links at 4 and 24 h in lymphocytes from resistant chronic lymphocytic leukemia patients. This suggests that enhanced DNA repair is implicated in this process.[36] In another study, a patient with advanced chronic lymphocytic leukemia was treated with i.v cyclophosphamide and DNA interstrand cross-links in the lymphocytes were measured by the alkaline elution technique. Maximal DNA interstrand cross-link 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.[37] Also utilizing the alkaline elution technique, Johnston *et al.* examined DNA cross-link formation in chronic lymphocytic leukemia lymphocytes at 6 h after an *in vitro* incubation with chlorambucil. They found that the lymphocytes from two resistant chronic lymphocytic leukemia patients had as many DNA cross-links as the lymphocytes from patients sensitive to chlorambucil.[38]

Nitrogen mustard drug resistance in epithelial cell lines in other investigations

As concerns epithelial cancer cell lines, DNA repair has been implicated in DNA cross-linking agent drug resistance (enhanced repair of DNA interstrand cross-links in some investigations),[39–44] while in other investigations, drug resistance appears to develop independent of altered DNA repair.[45–47] As previously stated, the assays utilized to quantitate interstrand DNA cross-links have technical problems which may render difficult the interpretation of 'repair' of DNA interstrand cross-links.[25–28] Thus, some of the investigations in which DNA repair is not implicated may be due to these technical problems or may represent alternative mechanisms of drug resistance as initially discussed.

DNA repair of nitrogen mustard DNA cross-links in cancer cells

The mechanism of removal of DNA interstrand cross-links in mammalian cells is poorly understood. There are several different DNA repair systems that could be involved in the repair of nitrogen mustard-induced DNA interstrand cross-links 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.[48] We measured 3-methyladenine-DNA-glycosylase activity in chronic lymphocytic leukemia extracts and found a significantly higher activity (approximately 1.7-fold) in lymphocytes from resistant chronic lymphocytic leukemia patients as compared to those from untreated chronic lymphocytic leukemia patients. Since this activity may vary with cell proliferation, it was corrected for differences in DNA synthesis utilizing [^{3}H]thymidine incorporation (there were differences in DNA synthesis between the two groups even though the vast majority of malignant B lymphocytes are non-proliferative) and this resulted in no significant difference in enzyme activity

between the two groups.[49] Moreover, overexpression of the human alkyl-*N*-purine DNA glycosylase in CHO cells did not result in nitrogen mustard resistance, suggesting that alkyl-*N*-purine DNA glycosylase was not a rate limiting enzyme in nitrogen mustard drug resistance.[50] Furthermore, mouse embryonic stem cells bearing null mutations in this enzyme are not hypersensitive to the nitrogen mustards.[51]

Possible insights into mechanism(s) of interstrand cross-link repair are gained by examining nitrogen mustard hypersensitivity in DNA repair mutants. Significant nitrogen mustard and/or mitomycin C hypersensitivity (varying from moderate to severe) is found in several DNA repair mutants including ERCC-1, ERCC-4 (XPF), XRCC2, XRCC3, Rad54, Ku70, Ku86 and DNA-PK$_{cs}$.[52–56]

This analysis, along with information gained from studying cross-link removal in both bacteria and *Saccharomyces cerevisiae*, and the possibility that DNA double-strand breaks (DSBs) are repaired in a similar fashion to interstrand cross-links, has resulted in the proposed model in mammalian cells that nucleotide excision repair, via the ERCC-1/ERCC-4 endonuclease, results in an incision 5′ to the interstrand cross-link and that recombinational repair is involved in further processing of the lesion (reviewed in Thompson *et al.*[57,58]). As concerns repair of double-strand breaks, non-homologous DNA end-joining (NHEJ) uses no, or very limited, sequence homology to rejoin ends directly, while homologous recombination requires extensive regions of DNA homology. Homologous recombinational repair (HRR) would be necessary for error-free repair of interstrand cross-links while an illegitimate or NHEJ mechanism of repair could result in deletional repair of interstrand cross-links. It is also conceivable that all three types of repair (nucleotide excision, HRR and NHEJ) are implicated in the repair simultaneously or are involved, depending on the phase of the cell cycle, in the processing of interstrand cross-links. The various genes implicated in NHEJ include the components of DNA-PK, XRCC4 and ATM, although ATM may be involved in HRR.[58] NHEJ is a major mechanism of DSB repair in mammalian cells (reviewed in Anderson and Lees-Miller,[59] and Jeggo[60]). HRR in human cells implicates the HsRad51 family of proteins including HsRad51, HsRad52, Rad51B, Rad51C, Rad51D, HsRad54, XRCC2 and XRCC3. 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 (see Figure 1, reviewed in Thompson and Schild[58], and Shinohara and Ogawa[61]).

In order to gain insight into possible mechanisms of DNA cross-link removal in nitrogen mustard-resistant chronic lymphocytic leukemia, Bramson *et al.* analyzed *in vitro* cross-resistance in chronic lymphocytic leukemia.[32] Chlorambucil-resistant chronic lymphocytic leukemia lymphocytes were completely cross-resistant to melphalan and mitomycin C; partially cross-resistant to cisplatin and not cross-resistant to UV light nor methylmethane sulfonate. Since 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 nitrogen mustard drug resistance in chronic lymphocytic leukemia.[32] Also, ERCC-1 protein levels were not increased in nitrogen mustard drug-resistant chronic lymphocytic leukemia lymphocytes.[62] Nucleotide excision repair activity was very low in most chronic lymphocytic leukemia lymphocytes, including the majority of those obtained from previously treated chronic lymphocytic leukemia patients.[63]

NHEJ

Since ionizing radiation results in double-strand breaks that are largely repaired by NHEJ[59] and since double-strand breaks are probably repaired in a similar fashion to interstrand cross-links, 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 chronic lymphocytic leukemia lymphocytes.[64]

DNA-dependent protein kinase, a nuclear serine/threonine kinase is a protein complex including a catalytic subunit of 460 kDa, DNA-PK$_{cs}$, and a DNA binding subunit, the Ku autoantigen (a dimer of the Ku70 and Ku86 proteins). Ku binds to double-strand breaks and other discontinuities in the DNA, and recruits DNA-PK$_{cs}$ to the damaged site.[59,60] The active DNA-dependent protein kinase complex can then phosphorylate many DNA bound proteins in the vicinity.[65] Since mutations in DNA-dependent protein kinase result in X-ray and alkylating agent sensitivity,[53,54] and since X-ray resistance develops in parallel with chlorambucil resistance in chronic lymphocytic leukemia,[64] determination of DNA-dependent protein kinase activity in chronic lymphocytic leukemia should be informative. In a preliminary report with a small sample of chronic

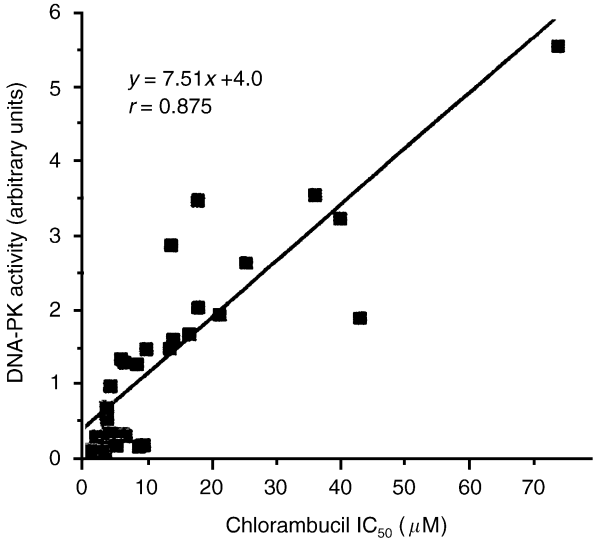

Figure 2. DNA-dependent protein kinase activity versus chlorambucil IC_{50} in chronic lymphocytic leukemia lymphocytes.[67]

lymphocytic leukemia patients, an increase in DNA-dependent protein kinase activity was found in resistant samples.[66] In collaboration with Muller and Salles, our laboratory examined DNA-dependent protein kinase 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-dependent protein kinase activity and *in vitro* chlorambucil cytotoxicity ($r = 0.875$, $p = 0.0001$) (Figure 2).[67] The increased DNA-dependent protein kinase activity was independent of other clinical and biological factors. The regulation of DNA-dependent protein kinase activity was associated with increased DNA-binding activity of its regulatory subunit, Ku, and increased Ku protein levels. Interestingly, most untreated chronic lymphocytic leukemia patients have very low levels of DNA-dependent protein kinase activity suggesting that, initially, resistance in chronic lymphocytic leukemia may be simply a state in which tumor cells lose an abnormal sensitivity to alkylating agents. In approximately 25% of the samples from untreated chronic lymphocytic leukemia patients, a variant (truncated) form of the Ku86 protein was associated with very low DNA-dependent protein kinase activity and hypersensitivity to chlorambucil.[67] Wortmannin, a non-specific inhibitor of DNA-dependent protein kinase, which also inhibits other phosphatidylinositol 3-kinases, sensitized chronic lymphocytic leukemia lymphocytes to the effects of chlorambucil. Moreover, there was a significant correlation between the synergistic sensitization and fold decrease in DNA-dependent protein kinase activity, but since Wortmannin also inhibits other phosphatidylinositol 3-kinases, these results must be interpreted with caution.[68] In contrast, neither Ku protein levels nor DNA-dependent protein kinase activity correlated with melphalan resistance in epithelial tumor cell lines suggesting that DNA-dependent protein kinase is not a rate-limiting step in epithelial cancers.[69]

The immunohistochemical expression of Ku autoantigen and $DNA\text{-}PK_{cs}$ was examined in various human tissues. There was a large variation in expression depending on the specific tissue type.[70] This supports our results that there is a variation in DNA-dependent protein kinase expression in human tissues.

While it appears reasonable that increased DNA-dependent protein kinase activity is associated with increased repair of nitrogen mustard-induced interstrand cross-links in chronic lymphocytic leukemia and thus increased drug resistance, it is possible that other mechanisms are involved, including a role for DNA-dependent protein kinase with respect to apoptosis.[71,72]

$DNA\text{-}PK_{cs}$ is a member of the phosphatidylinositol 3-kinase superfamily. Other members include the gene mutated in ataxia telangiectasia (ATM) and the cell cycle checkpoint protein, ATR.[60] Recently, loss of heterozygosity (LOH) or mutations of the ataxia telangiectasia gene and a decrease in ataxia telangiectasia protein levels have been found in approximately 30–40% of B chronic lymphocytic leukemia patients. These factors appear to be associated with a shorter survival, at least in younger patients.[73–76] The association of ataxia telangiectasia with nitrogen mustard drug resistance in cancer has not been investigated to date.

HRR

The involvement of nucleotide excision repair and HRR in repair of interstrand cross-links is inferred from the fact that the mutant cell lines with the greatest sensitivity (10- to 100-fold) to alkylating agents that produce interstrand cross-links are those that are deficient in or lacking XRCC2, XRCC3, ERCC-1 and ERCC-4/XPF.[52,56,58.] 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 interstrand cross-link.[77] This would then be followed by HRR. Alternatively, it is possible that strand invasion mediated by the Rad51 repairasome including XRCC2 and XRCC3 occurs prior to ERCC-1/XPF endonuclease induced incision.[78]

Several human genes implicated in HRR have been characterized including HSRad52, HsRad51, Rad51B, Rad51C, Rad51D, HsRad54, XRCC2 and XRCC3 (reviewed in Thompson and Schild,[58] and Shinohara and Ogawa[61]). 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 single-stranded DNA which then initiate recombinational DSB repair.[79] XRCC3 is necessary for the assembly of Rad51 foci and these proteins physically interact.[56,80] In fact, if all the interactions described occur in one complex, then HsRad51, XRCC3, Rad51C, Rad51B, Rad51D, and XRCC2 are complexed together (reviewed in Thompson and Schild[58]). Rad54 appears to be required after the association of the above-mentioned proteins and Rad54 may assist Rad51 in interacting with damaged DNA.[81]

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.[82] In view of the critical role of the Rad51 protein in HRR and its probable involvement in repair of interstrand cross-links, our laboratory investigated HsRad51 foci formation after *in vitro* chlorambucil treatment of chronic lymphocytic leukemia lymphocytes. *In vitro* chlorambucil treatment induced HsRad51 expression as measured by increased immunopositive staining in all chronic lymphocytic leukemia samples. In the chlorambucil-resistant chronic lymphocytic leukemia lymphocytes, there was a linear correlation between induction of HsRad51 foci at 5.4 μM chlorambucil and the *in vitro* LD_{50} concent ration of chlorambucil.[83]

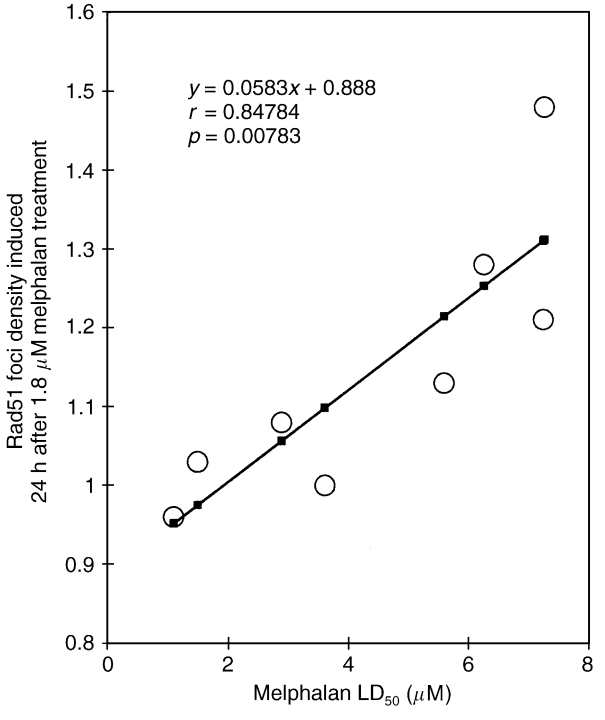

Figure 3. Rad51 foci density versus melphalan LD_{50} in epithelial cell lines.[69]

The involvement of HRR in 14 epithelial tumor cell lines was examined. As previously stated, there were no correlations between Ku protein levels or DNA-dependent protein kinase activity and melphalan cytotoxicity. Instead there was a significant correlation between XRCC3 protein levels and melphalan cytotoxicity. Moreover, there was a highly significant correlation between melphalan induced Rad51 foci density and melphalan cytotoxicity (Figure 3).[69] These results suggest that HsRad51 HRR is implicated in nitrogen mustard drug resistance in chronic lymphocytic leukemia and epithelial cancers.[69]

Summary of results with recombinational genes

The regulation of DNA-dependent protein kinase activity appears to be tightly associated with the development of chlorambucil drug resistance in chronic lymphocytic leukemia. In particular, low DNA-dependent protein kinase activity is associated with hypersensitivity to chlorambucil. Furthermore, increased levels of DNA-dependent protein kinase activity are associated with chlorambucil resistance in chronic lymphocytic leukemia. Moreover, in resistant samples, stimulation of HsRad51 foci formation by chlorambucil correlates with chlorambucil drug resistance (i.e. the more resistant chronic lymphocytic leukemia lymphocytes demonstrate a greater HsRad51 foci formation). The increased HsRad51 foci formation after chlorambucil treatment in resistant chronic lymphocytic leukemia samples may represent an active DNA repair process involving other Rad51-related proteins. A plausible hypothesis to explain these results is that low DNA-dependent protein kinase activity defines a hypersensitive state while high DNA-dependent protein kinase activity along with increased homologous recombination, as determined by HsRad51 foci formation, contribute to the resistant state in chronic lymphocytic leukemia.

As concerns epithelial cancers (as represented by epithelial cancer cell lines), it appears that NHEJ (i.e. DNA-dependent protein kinase) does not correlate with melphalan cytotoxicity but that rather HsRad51-related HRR is implicated in the development of nitrogen mustard drug resistance.

Future studies

The mechanism of low DNA-dependent protein kinase activity in sensitive chronic lymphocytic leukemia lymphocytes involves decreased Ku protein levels and a variant form of Ku86, as compared to resistant samples. The regulation of this process needs to be further investigated at both the translational and transcriptional levels. Inhibition of DNA-dependent protein kinase activity by small molecules in combination with nitrogen mustard chemotherapy may improve the therapeutic index of the latter compounds in chronic lymphocytic leukemia. As concerns HRR, the implication of other proteins such as Rad54, XRCC2 and other Rad51 paralogs needs to be examined. Recently, increased expression of *bcr/abl* in myeloid cells has been shown to result in an associated increase in expression of Rad51 and Rad51 paralogs along with DNA cross-linking agent drug resistance. Phosphorylation of Tyr-315 of Rad51 appears essential for the drug resistance.[84] Some of these proteins may be markers of drug resistance in clinical specimens. A model of repair of double-strand breaks has been proposed in which either Ku or HsRad52 binds double-strand breaks, thus directing entry into NHEJ or HRR, respectively.[85] 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 HRR *vis-à-vis* nitrogen mustard drug resistance should help to clarify their respective roles in this process. Again, inhibition of HRR (e.g. XRCC3 or Rad51 inhibition) may result in sensitization of epithelial tumors and chronic lymphocytic leukemia lymphocytes that are resistant to the nitrogen mustards. Recently, transfection of XPD into a glioma cell line resulted in DNA cross-linking agent drug resistance and XPD co-localizes with Rad51 on immunofluorescence.[86] This suggests that there may be functional cross-talk between an NER protein, XPD and the HsRad51 repairasome. Further investigations of such cross-talk may reveal new and exciting evidence of interactions between different repair systems.

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(Received 5 December 2001; accepted 11 December 2001)