Poly(ADP-ribose) Polymerase Can Bind Melphalan Damaged DNA


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Abstract

As a means of identifying damage recognition proteins involved in repair of nitrogen mustard lesions in chronic lymphocytic leukemia, we performed Southwestern analysis using a probe damaged with melphalan and protein extracts from chronic lymphocytic leukemia patients. We detected proteins with molecular weights of 116,000, 66,000, and 64,000 which bound the damaged probe with a higher specificity than the undamaged probe. The M, 66,000 and 64,000 proteins were determined to be degradation products of the M, 116,000 protein. The M, 116,000 protein was identified as poly(ADP-ribose) polymerase. The use of methoxyamine, an inhibitor of DNA strand breakage following depurination, significantly reduced binding of the melphalan damaged probe to poly(ADP-ribose) polymerase. Following depletion of poly(ADP-ribose) polymerase from the cell extracts, no other binding activity was discovered. Thus, poly(ADP-ribose) polymerase is the only demonstrable protein in chronic lymphocytic leukemia cells which can bind to a DNA probe damaged with melphalan.

Introduction

The nitrogen mustards represent a group of chemotherapeutic drugs with activity against many human tumors. The cytotoxicity of these agents has been correlated to their ability to form interstrand cross-links in the DNA. We have been using nitrogen mustard resistant CLL to study acquired resistance in vivo. CLL lymphocytes obtained from the peripheral blood of resistant patients exhibit resistance to nitrogen mustards in vitro which correlates with their clinical status (1). In studying CLL, we found no significant differences in transport kinetics, accumulation, or metabolism of the nitrogen mustard MLN in vitro. We did, however, find a decrease in the accumulation of cross-links in resistant patients’ lymphocytes following in vitro incubations with MLN (2). Further studies demonstrated that lymphocytes from resistant CLL patients possess an increased capacity to remove cross-links as compared to lymphocytes from sensitive patients (3). Thus, we have been studying the role of enhanced DNA repair in CLL as a potential mechanism of drug resistance.

The complexity of the repair process makes analysis of the pathway of MLN damage repair quite difficult. In order to understand the mechanisms of enhanced cross-link removal in CLL, we must first develop a model for normal repair. The first step probably involves recognition of the MLN lesion. Over the past several years many groups have identified proteins which bind to sites of DNA damage. These include proteins which bind to cis-Pt damaged and UV light damaged DNA (4, 5). While the exact role of the DNA binding proteins is unknown, they may serve as a signal for repair of the damaged site. Using cis-Pt resistant HeLa cells, one group identified a binding activity which was increased in the resistant cell line (4). However, others were unable to demonstrate correlation between the binding proteins and resistance levels in a panel of ovarian carcinoma cell lines (5). We have detected a binding protein in CLL lymphocytes which recognizes a DNA probe damaged with MLN. In this paper we present initial characterization of this binding protein. We have identified this protein as poly(ADP-ribose) polymerase.

Materials and Methods

Protein Extraction. B-lymphocytes were obtained from the peripheral blood of CLL patients by centrifugation on Ficoll-Paque (Pharmacia) as described (2). Nuclear extracts were prepared by the method of Dignam et al. (6).

Preparation of Fl96 Probe. A 180-base pair PvuIl-Accl fragment was cut out of pSV2cat, spanning base pairs 340 to 520 of the plasmid. The fragment was subcloned into pUC19 and released as a Smal/HincII fragment of 196 base pairs with blunt ends (f196). The fragment contains multiple GAC sequences, which serve as sites for MLN alkylation and cross-link formation. Following release of the f196 from the pUC19 vector, the fragment was purified on PAGE (8% polyacrylamide) and the band containing the f196 was extracted overnight at 37°C in elution buffer (0.5 mM ammonium acetate and 1 mM EDTA). The fragment was end-labeled with [y-32P]dATP by the T4 polynucleotide kinase (Pharmacia). Following the labeling reaction, the fragment was purified on Sephadex G-50 (Pharmacia) and ethanol precipitated. The radiolabeled f196 was resuspended in 50 mM HEPES-1 mM EDTA, pH 7.4, and treated with MLN at various concentrations for 4 and 24 h. In the indicated experiments, MLN treatment was performed in the presence of 5 mM methoxyamine to prevent nick formation at apurinic sites (7). The final products have been demonstrated to be a single band upon PAGE analysis (the MLN treated fragment migrates with slower mobility as described for cis-Pt treated probes). Using 160 μM [chloroethyl-14C]MLN in Tris-EDTA (pH 7.4) at 37°C for 4 h, there were 22.8 ± 5(SD) [14C]MLN adducts/f196 (0.065 MLN adduct/nucleotide).

PARP Purification and Depletion. PARP was purified as described (8). Depletion was performed as described (9). Briefly, 2 mg of nuclear extract were diluted in Buffer A (50 mM Tris-HCl, pH 8.0-10 mM β-mercaptoethanol-10% glycerol) to a final volume of 1 ml. The sample was then applied to a double stranded DNA cellulose column (Sigma), and the flow through was collected as fraction 1. Elution with 1 ml of Buffer A containing 0.3 mM NaCl followed by 1 ml of Buffer A containing 0.4 mM NaCl yielded fractions 2 and 3, respectively. These two fractions were then pooled with fraction 1 to constitute the PARP depleted extract. The pooled fractions were then dialyzed extensively against Buffer D (50 mM HEPES-KOH, pH 7.9-20% glycerol-0.1 mM KCl-0.2 mM EDTA-0.5 mM dithiothreitol-0.5 mM phenylmethylsulfonyl fluoride) to remove excess salt. The dialyzed extract was concentrated on Centricron-3 filters (Amicon) at 7500 rpm in an SS34 rotor (Sorvall) for 2.5 h at 4°C.

Southwestern and Western Analysis. Nuclear extracts were separated by sodium dodecyl sulfate-PAGE (10% polyacrylamide) without prior boiling of the samples. The separated proteins were electrophoretically transferred onto a nitrocellulose filter at 4°C. For Southwestern analysis, all manipulations were carried out at 4°C. The filter was soaked in HEPES-salt buffer (20 mM HEPES-KOH, pH 7.9-5 mM MgCl2-50 mM NaCl-1 mM dithiothreitol) for 15 min followed by Blotto-5% (HEPES-salt buffer with 5% Carnation powdered skim milk) for 1 h. Subsequently, the filter was soaked overnight in Blotto-0.5% with the radio-labeled probe (1 x 106 cpm/20 ml). The filter was washed in Blotto-0.5% once
The filter was dried at room temperature and exposed to Kodak XAR-5 with increasing concentrations of melphalan (0-160 µM) for 4 or 24 h and electrophoresed through an 8% acrylamide gel. The DNA was electrophoresed through an 8% polyacrylamide gel containing 8 M urea at 750 V for approximately 1.5 h. The running buffer used for the electrophoresis was 1 X 0.090 M Tris-borate. Following electrophoresis the gel was dried and exposed for autoradiography.

For Western analysis, the filter was blocked for 6 h in TSM and then washed quickly with 4 changes of TS. The mouse monoclonal antibody C-2-10 was used for PARP detection at a dilution of 1:1000 in TSM (10). The filter was incubated in the presence of the antibody overnight at 4°C, followed by 3 washes (15 min each) in TS. The blot was then incubated in the presence of a horseradish peroxidase conjugated anti-mouse antibody (diluted 1:500 in TSM) for 90 min. The blot was washed 4 times in TS and PARP was detected using 4-chloronaphthol.

Denaturing Gel Analysis. An aliquot of 1200 cpm of radiolabeled DNA was resuspended in 5 volumes of formamide dye (96% formamide-0.05% bromophenol blue-0.05% xylene cyanol). The DNA was electrophoresed through an 8% polyacrylamide gel containing 8 M urea at 750 V for approximately 1.5 h. The running buffer used for the electrophoresis was 1 X 0.090 M Tris-borate. Following electrophoresis the gel was dried and exposed for autoradiography.

Results

In order to investigate the potential role of DNA damage recognition proteins in the repair of MLN lesions in CLL lymphocytes, we performed Southwestern analysis using nuclear protein extracts from CLL patients' lymphocytes and a DNA probe treated with MLN. Southwestern analysis has been used previously for the detection of proteins which bind to cis-Pt damaged DNA (5). MLN alkylation of the DNA probe caused a mobility shift upon PAGE analysis as seen in Fig. 1. We have incubated the probe in the presence of MLN for both 4 and 24 h. The 24-h incubation was done to allow for reaction of all free chloroethyl groups. Initial studies with gel shift analysis failed to detect any specific binding activity (data not shown). Using Southwestern analysis, several proteins were detected at M, 116,000, 66,000, and 64,000, the M, 116,000 protein being predominant. The lower molecular weight proteins were not apparent in each sample and the signal at M, 116,000 was always diminished in those samples where binding to the lower species did appear (data not shown). Thus, the lower molecular weight binding activities would seem to be degradation products of the higher one. While a certain amount of binding is observed when the untreated probe was used, binding increased as MLN treatment increased (Fig. 2). Thus the binding appeared to be specific for the MLN damage.

PARP, a well characterized protein implicated in DNA repair, has an apparent molecular weight of 116,000 (10), similar to the binding activity we have identified. In order to investigate the possibility that the binding protein is PARP, we performed Western and Southwestern analysis using purified PARP, samples from 2 CLL patients (P1 and P2), and a HeLa cell protein extract. The proteins were separated on sodium dodecyl sulfate-PAGE and transferred to nitrocellulose in duplicate. One blot was probed with a radiolabeled DNA probe and the other with a monoclonal antibody raised against PARP (Fig. 3). It is clear that not only can PARP bind to the MLN damaged probe but also the PARP antibody recognized the same proteins which bound to the MLN damaged DNA probe. The antibody also detected the M, 66,000 binding protein in the extract from P1 (Fig. 3B, Lane 3) confirming this protein as a degradation product of PARP. Therefore it would appear that the activity observed in CLL lymphocytes is PARP.

The binding spectrum of PARP includes single and double DNA strand breaks as well as single stranded DNA (11). The binding to the MLN treated probe may represent a novel binding activity or may be a result of structural changes induced by MLN alkylation. It is well known that alkylation on the N-7 position of guanine results in spontaneous depurination. The depurination is then followed by ß- and δ-elimination reactions, which can occur spontaneously, breaking the phosphoribosyl backbone (12). We sought to determine if MLN induced nicks were the cause of PARP binding. Denaturing gel analysis of the probe was used to identify nicks induced by MLN alkylation of the f196. There is significant nick formation following 24-h treatment with MLN. Concomitant treatment with methoxyamine, which has been demonstrated to protect apurinic sites from spontaneous nicking (7), eliminates breakdown of the MLN treated probe (data not shown). Southwestern analysis also demonstrates reduced binding of the probe...
to PARP when alkylation was carried out in the presence of methoxyamine (Fig. 4, Lanes 1 and 4). Thus, binding seems to be largely a function of nicks induced by MLN treatment, although a minor component of the increased binding may be secondary to MLN alkylation of DNA.

Finally in order to identify other proteins which may also bind to MLN damaged DNA, the extracts were depleted of PARP prior to Southwestern analysis. Western analysis of these samples indicated complete depletion of PARP (data not shown). As can be seen in Fig. 4, binding is reduced by the addition of methoxyamine, but it is eliminated in PARP depleted samples. However, no other protein was detected. Therefore PARP seems to be the only demonstrable DNA binding protein in CLL cells which can recognize MLN lesions.

Discussion

Resistance to the nitrogen mustards in CLL is associated with increased DNA repair activity in B-lymphocytes from these patients. Previous studies have detected proteins which bind to a variety of DNA lesions including cis-Pt and UV damage. The exact function of these binding proteins is unknown, but they may be involved in the DNA repair process. In order to investigate the possible role of such a binding protein in the repair pathway associated with nitrogen mustard resistance in CLL, we have performed Southwestern analysis utilizing protein extracts from CLL patients' B-lymphocytes and a 196-base pair DNA probe which is treated with MLN. These experiments revealed the presence of several proteins which recognized the damaged probe. The lower molecular weight bands are believed to be degradation products of the upper band inasmuch as they were detected by Western analysis using a monoclonal antibody against PARP. The binding was clearly dependent upon MLN damage since the binding increased as the MLN treatment increased.

We postulated that the binding protein might be PARP. Using purified protein and a monoclonal antibody, we demonstrated quite clearly that the activity present in the CLL cells is PARP. Methoxyamine treatment, which protects apurinic sites from subsequent breakdown, reduced binding of the MLN damaged probe. This indicated that the majority of the binding to PARP may result from MLN induced nicks in the DNA probe. Finally in order to identify other proteins which may bind the MLN damaged probe, we depleted the extracts of PARP but found no other binding. Thus PARP appears to bind the f196 DNA probe largely as a result of nicks created by MLN alkylation, although a minor component of the binding may be due to other MLN induced alterations in the f196 DNA probe.

The significance of this finding is unclear. Recently Satoh et al. (9) demonstrated that PARP activity is involved in the base excision repair process. 3-Methyladenine DNA glycosylase, a base excision
PARP can bind melfalan damaged DNA

repair enzyme, has been implicated in resistance to both nitrogen mustards and chloroethylnitrosoureas (13, 14). N-7-Alkylguanine lesions are very labile and subject to spontaneous depurination. The subsequent β- and δ-eliminations that result in strand “nicking” also occur spontaneously and can be enhanced by the presence of polyamines like histones (12). These breaks are very likely to occur in vivo and may play a role in toxicity and repair. The binding of MLN damaged DNA should activate PARP, resulting in automodification which leads to a loss of DNA binding affinity. This may account for the fact that we did not find PARP binding in the gel shift assay where the in vitro incubation may lead to such automodification. PARP activation probably causes nucleosomal unfolding via histone shuttling off and back onto the DNA (15). In DNA excision repair, the tight association of histones with DNA is locally disrupted. Thus, the binding of PARP to DNA damaged by MLN may be the initial step in repair of at least part of this damage. More specifically, the nicks in MLN damaged DNA may activate PARP which in turn allows DNA repair enzymes access to the DNA with consequential repair of the nicks and other DNA lesions incurred by MLN. We have acquired highly specific PARP inhibitors to analyze the effect of PARP inhibition on MLN toxicity (16).

References

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