Characterization of a Duocarmycin-DNA Adduct-recognizing Protein in Cancer Cells

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Abstract

Duocarmycins have been reported to derive their potent antitumor activity through a sequence-selective minor groove alkylation of N3 adenine in double-stranded DNA. We have used gel mobility shift assays to detect proteins that bind to DNA treated in vitro with duocarmycin SA and identified a protein, named duocarmycin-DNA adduct recognizing protein (DARP), which binds with increased affinity to duocarmycin-modified DNA. Examination with partially purified DARP revealed that the protein recognized not only the DNA adduct of structurally related drug, CC-1065, but unexpectedly, the protein also recognized the DNA adduct of another chemotype of minor groove binder, anthramycin. These results demonstrate that DARP recognizes the structural alteration of DNA induced by these potent DNA-alkylating drugs, suggesting the possibility that the protein might modulate the antitumor activity of these drugs.

Introduction

DUMs belong to a new class of antitumor antibiotics containing the unique spirocycloporylhexadienone moiety that is responsible for DNA alkylation (1). A water-soluble derivative, KW-2189, exhibits broad spectrum antitumor activity in a series of experimental tumor models and is presently in Phase II clinical trials (2, 3). DUMs have been reported to derive their antitumor activity through a sequence-selective minor groove alkylation at N3 adenine in double-stranded DNA (1). This kind of lesion of DNA could induce the conformational alteration of DNA double helix, presumably triggering a cellular response leading to apoptosis (4). Intracellular interactions with the DUM-DNA adduct are likely to be of central importance in explaining the toxicity of the drug toward rapidly dividing tumor cells. Considerable information is now available on the binding of DUMs to DNA (1). Accordingly, attention has turned to the study of cellular proteins that interact in vivo with DUM-modified DNA. Clinically useful antitumor drug CDDP forms bifunctional adducts on DNA (5). In particular, d(GpG) and d(ApG) 1,2-intrastrand cross-links are the main adducts induced by CDDP, and these adducts have been reported to be specifically recognized by some proteins containing the HMG-domain DNA binding motif (6, 7). HMG-domain proteins do not bind to DNA adduct of the inactive trans isomer, trans-diaminomedi-chloroplatinum(II). Furthermore, HMG-domain proteins are thought to modulate CDDP cytotoxicity in vivo (8). More recent investigations revealed that the DNA adduct of Et743, a DNA minor groove alkylation agent, was recognized by a nuclear protein, topoisomerase I (9, 10). We presume that the DNA adducts of other DNA alkylating agents could be recognized by cellular proteins that might modulate the cytotoxicity of these drugs. To investigate this possibility, we have attempted to identify cellular proteins that recognize DUM lesions with the ultimate aim of understanding how such interactions might lead to the apoptosis of tumor cells. In the present study, we described the detection of the DARP in nuclear extract from HeLa S3 cells by the gel mobility shift assay. Examination with partially purified DARP revealed that the protein recognized not only DUM-modified DNA but also the DNA adducts of other MGBs, such as CC-1065 and anthramycin. These MGBs are among the most potent antitumor antibiotics available. The selective interaction of DARP with DNA adduct induced by these MGBs might contribute to their potent biological activity.

Materials and Methods

Materials. DUMs were isolated from the culture broth of their respective producing organisms. CC-1065 was a kind gift from Upjohn Co., Ltd. CDDP, Adriamycin, distamycin A, calf thymus DNA, and poly(deoxyinosinic-deoxycytidylic acid) were purchased from Sigma Chemical Co. T4 polynucleotide kinase was purchased from Takara. [γ-32P]ATP was purchased from Amersham. The oligonucleotides used in the gel mobility shift assay were synthesized. The oligonucleotide containing the 5'-ATTAG-3' sequence recognized by DUMSA (5'-GATGCGGTAGTACGCTGGGATCCGATTACGCCACCT-3'), its complementary one, DUM modified and unmodified duplex oligonucleotides are referred to as DO1, DO2, DO2D, and DO12, respectively. The drug solution was prepared with N,N-dimethylformamide as a 10 mM stock solution. Radioactivity of the electrophoretic bands was detected with a Bio imaging analyzer (BA2000; Fuji Film).

Purification of DARP. DARP was detected by its ability to bind to DUMSA-DNA adduct in gel shift assays. For preparation of DUMSA-DNA adduct-cellulose, calf thymus DNA immobilized cellulose (Sigma) was suspended in 10 mM potassium phosphate buffer (pH 7), and the stock solution of DUMSA was added to the suspension (final concentration, 1 mM), followed by rotation at 4°C for 3 days. After washing with potassium phosphate buffer (pH 7), the resin was stored at 4°C. Nuclear and cytoplasmic extracts from HeLa S3 cells (American Type Culture Collection) were prepared according to procedures published previously (11). The extracts were then dialyzed against 0.1 M KCl HEDG [20 mM HEPES, 0.2 mM EDTA, 0.5 mM DTT, and 10% glycerol (pH 7.9)] with 0.5 mM phenylmethylsulfonyl fluoride and 0.1 mM of pepstatin A) and stored at −70°C. DARPs were precipitated by the addition of ammonium sulfate (final concentration, 25%). The resulting pellet was dissolved in 0.1 M KCl HEDG. After removal of ammonium sulfate by dialysis against 0.1 M KCl HEDG, the material was applied to DEAE-Sephacel column (Pharmacia). After washing with 0.1 M KCl HEDG, DARPs were eluted with 0.2 M KCl HEDG. KCl was added to the eluted material (final concentration, 2 M), and the material was applied to aminoethyl-agarose column (Sigma). The flow-through fraction was dialyzed against 50 mM KCl HEDG and was applied to (calf thymus DNA)-cellulose (Sigma). After washing with 0.1 M KCl HEDG, DARPs were eluted with 0.2 M KCl HEDG. After dialysis in 50 mM HEDG, the material was applied to (DUMSA-DNA adduct)-cellulose and washed with 0.1 M KCl HEDG. The material eluted with 0.5 M KCl was dialyzed against 50 mM KCl and 0.02% NP40 HEDG and was reapplied to (DUMSA-DNA adduct)-cellulose. After washing with 0.1 M KCl, 0.02% NP40...
HEDG and DARP were eluted with 0.3 M KCl and 0.02% NP40 HEDG. Dialysis of the eluted material against 0.1 M KCl and 0.02% NP40 HEDG gave highly active material in gel shift assay. Protein concentrations were estimated using Bio-Rad protein assay, and the quality of the each fraction was checked by Coomassie Brilliant Blue or silver staining of SDS-polyacrylamide gels.

Drug Treatment of Calf Thymus DNA. The drug-modified calf thymus DNA was prepared as follows. Each drug (78 μM), except MMC, was incubated with calf thymus DNA (1.6 mM/bp) in 1% N,N-dimethylformamide, 10 mM potassium phosphate buffer (pH 7) at 37°C for 24 h. As for MMC, drug was incubated with DNA in the presence of Na₂S₂O₄ in anaerobic conditions at 37°C for 24 h (12). These adducts were used without further purification.

DUM Treatment of Oligonucleotides. Complimentary single-strand oligonucleotides, DO1 and DO2, were ³²P-end-labeled with T4 polynucleotide kinase and annealed. Annealed oligonucleotides at 10 ng/μl were incubated with 0.2 mM DUMSA at 4°C for 24 h when DOD12 was prepared. The reaction mixtures were extracted with 1:1 phenol:chloroform, followed by ethanol precipitation of the oligonucleotides. The oligonucleotides were gel purified prior to using.

Gel Mobility Shift Assay. Labeled oligonucleotide (1 ng) was incubated with cell extract (final protein concentration, 20 ng/μl) at 30°C for 30 min in the presence of 2 μg of poly(deoxyinosinic-deoxyctydilic acid) and 1 μg of BSA, except where stated, in a final volume of 15 μl of 0.1 M KCl HEDG. Where indicated, drug modified or unmodified calf thymus DNA was added to the reactions at the concentrations stated. Samples were electrophoresed in 6% polyacrylamide gel, dried, and scanned.

Results

Detection and Purification of DARP. DUMSA (Fig. 1), one of the DUMs, shows potent cytotoxic activity against HeLa S3 (IC₅₀ was 0.2 mM at 72-h exposure). An oligonucleotide, DO1, containing two preferred alkylation sites for DUMSA with other nonspecific sequences in a molecule, was designed as a 40 mer and synthesized with its complementary oligonucleotide, DO2. Thermally induced strand breakage assay (13) clearly showed that DUMSA bound to both expected substrate sites on DO12 (data not shown). When DUMSA-treated duplex DOD12 was incubated with HeLa S3 nuclear extract, a retardation complex was observed that was not observed in the experiments using the unmodified duplex DO12. The binding of the complex was proteinnase K sensitive and RNase resistant and did not appear in the presence of cytoplasmic extract, indicating that the shifted band represented the complex of the nuclear protein(s) with DOD12 (data not shown). We carried out purification of the protein(s) from the nuclear extract of HeLa S3 cells to characterize the binding entity because the initial crude extract was not enough to give clear and reproducible results. The purification procedure described in “Materials and Methods” gave highly active and reproducible material. Silver staining analysis of SDS-polyacrylamide gels showed that the affinity purified material contained two main proteins M₄ ~60,000 with several minor ones as shown in Fig. 2A, Lane 3. We used this partially purified material of Lane 3 in all of the experiments described below. Further purification efforts with anion exchange column chromatography gave a single band of M₄ 60,000 corresponding to the indicated band in Fig. 2A (data not shown). Thus, we identified DARP as a nuclear protein of M₄ 60,000. Large-scale preparation of the purified DARP is under way for amino acid sequencing (full details of isolation and characterization of DARP will be reported elsewhere). The affinity-purified fraction used in this study showed about 100 times more activity than the initial crude preparation at equivalent protein concentrations (Fig. 2B, compare Lanes 8 and 2). Coincubation of an excess amount of DUMSA-treated calf thymus DNA with the labeled DOD12 and the partially purified DARP clearly

Fig. 1. Structures of DNA-alkylating MGBs that induce DNA-lesions recognized by DARP.

Fig. 2. Partial purification of DARP from HeLa S3 nuclear extract. A, SDS-PAGE analysis with silver staining of nuclear extract (Lane 1), first adduct-cellulose affinity purified material (Lane 2), and second adduct-cellulose affinity purified material (Lane 3). B, gel shift assay under conditions of similar protein concentration (20 ng/μl). Lane 1, buffer control; Lane 2, nuclear extract from HeLa S3; Lanes 3–8, each material purified with the treatments mentioned at the top of autoradiogram. Lanes 9–12, second adduct-cellulose affinity purified material. Lanes 9 and 10, competition experiment in the presence of 30 and 300 ng of calf thymus DNA-DUMSA adduct; Lanes 11 and 12, competition experiment in the presence of 30 and 300 ng of calf thymus DNA.
resulted in a reduction in the intensity of the retarded band in the gel mobility shift assay (Fig. 2B, Lanes 9–12). Untreated calf thymus DNA at the same concentration showed a smaller effect on the band signal (Lanes 7–9, 11). These results demonstrate that the protein binds selectively to DNA modified with DUMSA.

To confirm whether DARP might recognize the skeleton of DUMSA associated with DNA, we examined the binding of DARP to free DUMSA or DUMSA-N3 adenine adduct. Free DUMSA-N3 adenine adduct was obtained from its DNA adduct as described previously (14). Coincubation of a molar excess of intact free DUMSA or the DUMSA-N3 adenine adduct with the labeled DOD12 and DARP resulted in no reduction of the retarded band (Fig. 3A). These results show that DARP recognizes the damaged structure on DNA induced by DUMSA and does not recognize the drug itself.

**Binding of DARP to DNA Treated with Other Antitumor Drugs.** We next examined the binding of DARP to DNA treated with other DUMs or other antitumor drugs. When the labeled DOD12 was incubated with the partially purified HeLa nuclear extract in the presence of the calf thymus DNA treated with DUMA or an active metabolite of KW-2189, DUB6 (15), the shifted band disappeared as expected (data not shown). Examination of the structurally related antitumor antibiotic, CC-1065, resulted in a reduction of the binding activity as shown in Fig. 3B. Distamycin A is known to be a nonalkylating MGB that binds to similar AT-rich sequences as DUM and CC-1065. However, DARP did not bind to distamycin A-DNA adduct. In contrast, coincubation with anthramycin-DNA adduct resulted in a decrease of the shifted band (Fig. 3B). Anthramycin is a known potent MGB that has preference for GC-rich sequences and alkylates the N2 guanine. DARP did not recognize the DNA adducts of other DNA binding drugs, such as bifunctional DNA-alkylating agents CDDP and MMC, and a DNA intercalator, Adriamycin. These results showed that DARP recognized the DNA damage induced by DNA-alkylating MGBs and that this recognition was not dependent on the DNA sequence modified by MGB.

**Discussion**

We reported previously that DUMs bind to DNA mediating N3 adenine covalent adduct formation in vitro (14) and in vivo (15), indicating that cellular DNA is the primary target of DUMs. In the course of our investigation, we have identified a nuclear protein that was able to recognize DNA modified by DUM. The protein, DARP, preferentially bound to the DNA damage induced by DNA-alkylating MGBs such as DUMs and CC-1065, and unexpectedly, anthramycin. DARP did not recognize the skeleton of the drug, and its binding was not dependent on the DNA sequence. From these observations, it appears that DARP recognizes the areas of distortion where the drug forms covalent DNA adducts, in a manner similar to HMG proteins. CC-1065 was reported to induce DNA bending and winding of the helix (16). Hurley et al. (17) reported that the spirocyclopropylhexadienone moiety, which is the conserved subunit in DUMs and CC-1065, is responsible for the alteration of high dimensional structure of DNA (17), strongly suggesting that DUMs could also induce the bending and winding of DNA. The magnitude of DNA bending induced by CC-1065 and its analogues was reported to be approximately 14–19° toward the minor groove (17). Anthramycin is known to be a MGB that alkylates N2 guanine residue in the minor groove of DNA. This kind of MGBs have also been reported to induce DNA bending toward the minor groove with similar bending magnitude (6.3–14.4°) to DUMs (18). On the basis of these structural analyses, we would like to suggest that DARP might recognize the altered structure of DNA, especially the bend toward the minor groove with 5–20° bending angle, which is induced by these DNA alkylating MGBs. Although distamycin A is also a MGB (19), the DNA complex was not recognized by DARP, presumably because of its inability to induce sufficient distortion of DNA. Recently, a M₉, 100,000 protein has been identified that recognized DNA damaged by MGBs such as distamycin A, Hoechst 33258, and CC-1065 (20). This protein appears to be different from DARP.

A clinically useful antitumor drug, CDDP, forms bifunctional adducts with DNA, in particular, (dGpG) and (dApG) 1,2-intrastrand cross-links, which comprise 90% of the CDDP adducts formed with DNA (5). These adducts bend the helix by 34° in the direction of major groove and unwind it by 13° (21). Several reports in the literature have used the gel mobility shift assay to detect proteins that specifically recognized CDDP-damaged DNA. Indeed, several proteins have been identified that recognize and specifically bind to
CDDP adducts. These proteins contain the homologous DNA binding region, termed HMG box, which occurs in many DNA binding proteins including transcription factors and regulators. It has been suggested that the HMG box recognizes specific structural motifs in DNA rather than having particular sequence requirements (6). Because our competition experiments showed that DARP did not bind to CDDP-DNA adducts (Fig. 3B), DARP seems to be a distinct protein from HMG family proteins. Gel mobility shift assays using a UV-damaged DNA probe identified a nuclear protein, XPE binding factor, that recognized many DNA lesions including those induced by UV radiation and CDDP (22). Because there is evidence that CC-1065 is recognized by nucleotide excision repair (23), DARP might be one of the components involved in the nucleotide excision repair pathway. Another possible function of DARP may be to inhibit binding of the repair enzymes. Ixr1 is a yeast HMG-domain protein that binds specifically to DNA adducts formed by CDDP. Interruption of the Ixr1 gene in yeast desensitizes cells to CDDP (8). Thus, HMG proteins may potentiate the cytotoxicity of CDDP in cells. Examinations by Lippard et al. indicate that HMG protein binding may shield excision repair of CDDP-DNA adducts (24). Our studies in vitro indicate that DARP could bind to DNA lesions induced by some MGBs in cells. Because this class of drugs possesses potent cytotoxicity, DARP may modulate the cytotoxicity against tumor cells. Large scale preparation of purified DARP for further characterization, including amino acid sequencing and expression pattern in other tumor cell lines, is under way.

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References


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