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CROSSLINKING OF CHROMOSOMAL PROTEINS TO DNA IN HeLa CELLS BY UV, GAMMA RADIATION AND SOME ANTITUMOR DRUGS

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SUMMARY: Immunochemical techniques were used to investigate the protein-DNA crosslinking by ultraviolet (UV) and gamma radiation as well as 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) or cis- and trans-diamminedichloroplatinum II (cis-DDP and trans-DDP). Antisera to 0.35 M NaCl extract and 0.35 M NaCl residue of HeLa nuclei were employed. Both gamma and UV irradiation, exposure to cis- or trans-DDP and, to a lesser extent, BCNU, resulted in crosslinking of various antigens to the DNA. Although several antigens were crosslinked by all the employed agents, other exhibited agent-specific crosslinking patterns.

DNA in the nuclei of eukaryotic cells is packaged as chromatin. Chromatin represents a large complex of DNA with histones and a considerable number of chromosomal nonhistone proteins whose biological significance remains mostly unknown. Since Smith (1) and Alexander et al (2) independently published results indicating that ultraviolet light could induce crosslinking of proteins to DNA in living cells, there has been increasing interest in crosslinking of proteins to nucleic acids. Several investigators have reported that ionizing radiation, e.g., gamma, could also induce protein-DNA as well as DNA-DNA crosslinks. Various chemical agents have also been tested for their possible protein-DNA crosslinking effects. Among them aldehydes, carbodiimides, nitrous acid, alkylating agents and heavy metals, were all shown to induce protein-DNA crosslinks. DNA is believed to be the principal target for the bifunctional anticancer drug cis-DDP and its inactive isomer trans-DDP (3). Recent literature

Abbreviations used: BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; cis-DDP, cis-diamminedichloroplatinum; trans-DDP, trans-diamminedichloroplatinum; PMSF, phenylmethylsulfonylfluoride; UV, ultra violet; SDS, sodium dodecyl sulfate; PAP, peroxidase-antiperoxidase.

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indicates that in addition to intra- and inter-strand crosslinking of DNA, the platinum coordination complexes could also form covalent protein-DNA crosslinks (4,5).

A variety of physical methods has been used to detect the presence of protein-DNA crosslinking adducts. However, the nature of the crosslinked proteins is difficult to identify. Sensitive immunological techniques, combined with the high resolution power of polyacrylamide gel electrophoresis provide a new experimental approach to the detection of the nature of DNA-crosslinked proteins. In this communication we use this approach to investigate the protein-DNA crosslinking by UV and gamma radiation as well as BCNU, cis- and trans-DDP. Our results show that several antigens became crosslinked to DNA by all the employed agents and that some exhibited agent-specific crosslinking patterns. Thus, radiation and chemical crosslinking of antigenic proteins to DNA followed by their immunological detection can be used to probe chromatin structure and function.

MATERIALS AND METHODS

Isolation of nuclei: HeLa cells (S3) grown in Joklik-modified Eagle's minimum essential medium (6) supplemented with 3.5% each of calf and fetal calf serum were used to isolate nuclei by homogenizing them in a loose-fitting Teflon pestle homogenizer in 10 volumes of 0.25 M sucrose:0.01 M Tris-HCl:0.1 mM PMSF, pH 7.5. The nuclei were collected at 660 x g for 10 minutes. The 660 x g nuclear pellets were further purified by homogenization in 2.2 M sucrose:5 mM $MgCl_2$:10 mM Tris-HCl:0.1 mM PMSF, pH 7.5, and centrifugation at 110,000 x g for 60 minutes. The purified nuclei were homogenized in 0.25 M sucrose:10 mM Tris-HCl:0.5% (v/v) Triton X-100:0.1 mM PMSF, pH 7.5 until dispersed, then centrifuged at 1000 x g for 10 minutes. Triton X-100 was removed by washing the nuclei with 0.25 M sucrose:10 mM Tris-HCl:0.1 mM PMSF, pH 7.5, followed by centrifugation at 1000 x g for 10 minutes.

Nuclei (5×10^7/ml in 1 mM potassium phosphate:5 mM $MgCl_2$:150 mM NaCl:0.1 mM PMSF, pH 7.5) were irradiated at 0°C with a short wave UV source R-52 (Ultraviolet Products, Inc., San Gabriel, CA) with the dose of 4.3×10^4 Erg/mm at the primary wavelength of 2540 A following the densitometry described by Spelsberg et al (7). Gamma irradiation was performed using 137-Cesium unit with a dose of 100 Krad.

Drug treatment: BCNU (Experimental Drug Branch of the National Cancer Institute, Bethesda, MD) was dissolved immediately before use in 95% ethanol. The stock solutions were made such that the final ethanol concentration in the incubation mixture did not exceed 0.4%. Cis- or trans-DDP (Sigma) were dissolved with gentle heating and stirring in water and used within two hours. Cells in culture were collected by centrifugation at 1200 rpm for 10 minutes. Suspension of cells (1×10^8 cells/ml in fresh serum-free medium) or nuclei (5×10^7/ml in 1 mM potassium phosphate:5 mM $MgCl_2$:150 mM NaCl:0.1 mM PMSF, pH 7.5) were incubated with the above drugs at a final concentration of 2 mM at 37°C for two hours. The reaction was quenched by raising the chloride concentration to 0.2 M and making the incubation mixture 5 mM thiourea (8) by the addition of a concentrated NaCl and thiourea solutions. Controls were treated with the same solvents without drugs. After incubation, non-reacted drugs were removed by centrifugation at 3000 rpm for 15 minutes.

Isolation of proteins crosslinked to DNA: Control and treated cells or nuclei were dissociated in 2% SDS:50 mM Tris-HCl:5 mM thiourea:0.1 mM PMSF, pH 8. The noncrosslinked proteins were removed by centrifugation at 110,000 x g for 18 hours. The resulting DNA pellets were then hydrated, sonicated and/or digested (1 hour, 4°C) with

40 µg of DNase I (Worthington Biochemical Corp., Freehold, N.J.) per mg DNA. The released proteins were solubilized for gel electrophoresis.

SDS-polyacrylamide gel electrophoresis and immunodetection of crosslinked antigens: Samples were mixed with 0.9 volume of solubilizing solution (0.125 M Tris:4% SDS:20% (w/v) glycerol:pyronin Y (2.5 mg/ml)), and with 0.1 volume of 2-mercapto-ethanol. The samples were boiled for 5 minutes, and electrophoresed under conditions described by Laemmli (9) using a 3% stacking gel and a 7.5% resolving gel. The gels were stained with Coomassie blue.

Identification of immunoreactive proteins crosslinked to DNA was accomplished by the method described by Glass et al (10). Proteins separated by SDS-gel electrophoresis were electrophoretically transferred to nitrocellulose sheets (11), incubated with the appropriate antiserum and the antigen-antibody complexes were visualized using the peroxidase-antiperoxidase (PAP) procedure (12).

Antisera: Two antisera, raised in New Zealand White rabbits following the previously described schedule (13), were used in this study. One antiserum was raised against the 0.35 M NaCl extract of HeLa nuclei and the other one to the residual, 0.35 M NaCl washed nuclei.

RESULTS AND DISCUSSION

Antibodies reacting with chromosomal proteins as well as their complexes with DNA in HeLa cells can be elicited by immunization with dehistonized HeLa chromatin preparations. We have shown that some of these antibodies recognize specific complexes of nonhistone proteins and DNA (14,15). Previous studies from this laboratory, using complement fixation assays, showed that treatment of HeLa chromatin or nuclei with UV light, gamma radiation or BCNU significantly increased their immunological reactivity with specific antisera (16,17). This suggested that these agents crosslinked the antigenic nonhistone proteins to DNA and thus stabilized the antigenic nonhistone protein-DNA complexes and possibly caused their increased reactivity. In an attempt to identify the proteins involved in such complexes, we have applied a sensitive immunological method, combined with the resolution power of polyacrylamide gel electrophoresis, to investigate the protein-DNA crosslinking by those agents. Antiserum to 0.35 M NaCl extract of HeLa nuclei and another one to the residual, 0.35 M NaCl washed nuclei were used. DNAs from the control and treated nuclei were deproteinized by dissociation in 2% SDS and the unbound proteins were removed by extensive ultracentrifugation. The crosslinked proteins were then analyzed by SDS-gel electrophoresis and by the immunotransfer method (see Materials and Methods). Several but not all of the nuclear proteins were crosslinked (since they resist extraction by 2% SDS) to DNA when purified nuclei were irradiated with gamma radiation (Figure 1, lane 4); UV light, at the described dose, was rather ineffective in crosslinking the nuclear proteins to DNA (lane 3). Similarly, no crosslinked proteins could be detected in the Coomassie blue stained gel

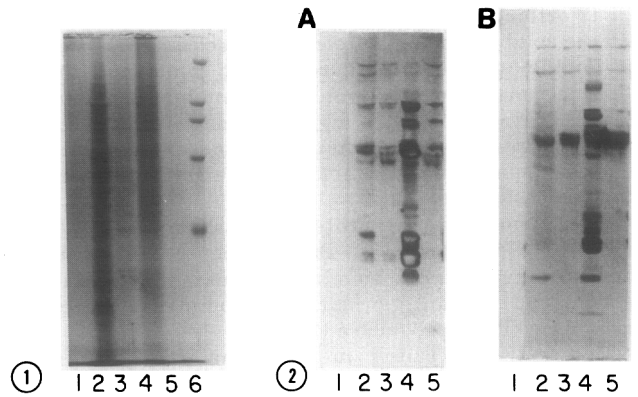

FIGURE 1. SDS-polyacrylamide gel electrophoresis of: lane 1, DNA from nuclei treated with BCNU; lane 2, total proteins of HeLa nuclei (untreated and undissociated); lane 3, DNA from nuclei irradiated with UV light; lane 4, DNA from nuclei irradiated with gamma radiation; lane 5, DNA from untreated nuclei; and lane 6, high-molecular weight standards (Bio-Rad) (myosin, M_r 200,000; β-galactosidase, M_r 116,000; phosphorylase β, M_r 94,000; bovine serum albumin, M_r 68,000; ovalbumin, M_r 43,000). Proteins were stained with Coomassie Brilliant Blue R-250.

FIGURE 2. Immunochemical localization of electrophoretically separated proteins transferred to nitrocellulose sheets with antiserum to: (A) the 0.35 M NaCl extract of HeLa nuclei; (B) the residual, 0.35 M NaCl washed nuclei. Lane 1, DNA from untreated nuclei; lane 2, DNA from nuclei irradiated with gamma radiation; lane 3, DNA from nuclei irradiated with UV light; lane 4, total proteins of HeLa nuclei (untreated and undissociated); lane 5, DNA from nuclei treated with BCNU.

when isolated nuclei were treated with BCNU (lane 1). When the proteins were transferred to nitrocellulose sheets and the antigens were visualized by incubation with appropriate antisera, followed by the PAP staining, several antigens were found to be crosslinked by all the agents employed (Figure 2). Not all the antigens that can be detected with both antisera were crosslinked by the different agents. There were differences between crosslinked proteins detectable by the two antisera. However, the extent of the crosslinking suggests that the 0.35 M NaCl insoluble chromosomal proteins as well as the 0.35 M NaCl soluble are closely associated with the DNA.

It has been reported that UV radiation can form stable covalent crosslinks between a variety of proteins and nucleic acids. These include bovine serum albumin (18,19), DNA and RNA polymerases (20,21) or the Lac repressor (22). Similarly, ionizing radiation was reported to crosslink not only DNA to DNA but also proteins to DNA (1,23). Protein-DNA crosslinking was also reported when chromatin was treated with BCNU (24), nitrogen mustard (25) and other alkylating agents.

To test the protein-DNA crosslinking by cis-DDP and trans-DDP, purified nuclei or live HeLa cells were incubated with the above drugs and the presence of protein-DNA

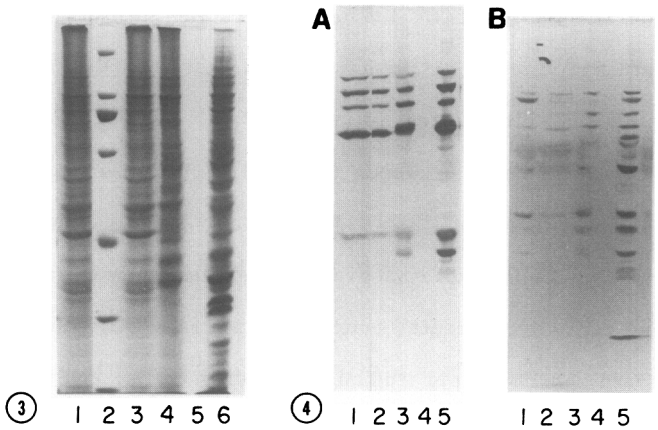

FIGURE 3. SDS-polyacrylamide gel electrophoresis of: lane 1, DNA from cells treated with cis-DDP; lane 2, high and low molecular weight standards (Bio-Rad) (myosin: β-galactosidase: phosphorylase β: bovine serum albumin and carbonic anhydrase, M 32,000); lane 3, DNA from cells treated with trans-DDP; lane 4, DNA from nuclei treated with cis-DDP; lane 5, DNA from untreated cells; and lane 6, total proteins of HeLa nuclei. Proteins were stained with Coomassie Brilliant Blue R-250.

FIGURE 4. Immunochemical localization of electrophoretically separated proteins transferred to nitrocellulose sheets with antiserum to: (A) the 0.35 M NaCl extract of HeLa nuclei; (B) the residual, 0.35 M NaCl washed nuclei. Lane 1, DNA from cells treated with cis-DDP; lane 2, DNA from cells treated with trans-DDP; lane 3, DNA from nuclei treated with cis-DDP; lane 4, DNA from untreated cells; and lane 5, total proteins of HeLa nuclei.

crosslinks were analyzed. Figure 3 shows that numerous chromosomal proteins were extensively crosslinked to DNA. There was little difference between the protein patterns crosslinked by either of the isomers. This similarity was also confirmed by the analysis of proteins transferred to nitrocellulose sheets (Figure 4). Many, but not all the antigens that can be detected with both antisera became crosslinked to DNA, indicating some selectivity of the crosslinking process. There were qualitative and quantitative differences between crosslinked proteins detectable by the two antisera indicating again that many of the 0.35 M NaCl soluble chromosomal proteins seem to associate with DNA. The Coomassie blue staining of the crosslinked proteins as well as their antigenic profiles showed little difference in crosslinking performed by incubation of intact cells or isolated nuclei, indicating that the isolation procedure of nuclei did not change significantly their protein-DNA relationships. Our data also show that cis-DDP is more efficient chromosomal nonhistone protein-DNA crosslinker than trans-DDP, BCNU, UV or gamma radiation.

The high affinities of bifunctional platinum compounds for sulfur atoms within proteins and amino acids (26,27) as well as their ability to inhibit enzymes (28,29),

suggest that the DNA-protein crosslinking involves the sulfhydryl groups of proteins. However, Lippard and Hoeschele (30) have shown that the DNA crosslinking can also involve histones which, with the exception of H3, do not contain sulfhydryl residues. DNA-proteins crosslinks have also been detected in bacteriophage treated with cis-dichloroethylenediamine platinum II (31). Reduction in alkaline elution rates, which were reversed by incubation of the cell lysates with proteinase K, were reported when L1210 mouse leukemia cells were treated with cis- or trans-DDP (32). As was already mentioned, Lippard and Hoeschele studied the binding of cis- and trans-DDP to isolate nucleosome cores (30). Cis-DDP reacted mainly with DNA and also produced histone-DNA crosslinks but only at higher doses. On the other hand, trans-DDP produced extensive histone-histone and histone-DNA crosslinks. Presently, we can not explain the opposite situation seen in our experiments. Except for the histones, the identity of proteins crosslinked to the DNA by the different crosslinking agents is unknown. The immunological detection of crosslinked proteins used in our experiments can not identify the individual antigenic species, but it does suggest that identical antigens are crosslinked under different experimental conditions, and with different crosslinking agents.

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