Damaged DNA-binding proteins: recognition of *N*-acetoxy-acetylaminofluorene-induced DNA adducts[*][○]

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Proteins which bind to the DNA damaged by genotoxic agents can be detected in all living organisms. Damage-recognition proteins are thought to be generally involved in DNA repair mechanisms. On the other hand, the relevance to DNA repair of some other proteins which show elevated affinity to damaged DNA (e.g. HMG-box containing proteins or histone H1) has not been established. Using the electrophoretic mobility-shift assay we have investigated damage-recognition proteins in nuclei from rat hepatocytes. We detected two different protein complexes which preferentially bound the DNA damaged by *N*-acetoxy-acetylaminofluorene. One of them also recognized the DNA damaged by benzo(a)pyrene diol epoxide (yet with much lower efficiency). The proteins which bind to damaged DNA are permanently present in rat cells and their level does not change after treatment of animals with the carcinogens. Differences in the affinity of the detected damage-recognition proteins to DNA lesion evoked by either carcinogen did not correlate with more efficient removal from hepatic DNA of 2-acetylaminofluorene-induced adducts than benzo(a)pyrene-induced ones.

Genomic DNA of all organisms is under permanent pressure of damaging agents generated as byproducts of cellular metabolism or originating in the environment. All organ-

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Abbreviations: 2-AAF, 2-acetylaminofluorene; AAAF, *N*-acetoxy-acetylaminofluorene; AP-site, apurine/apyrimidine-site; B(a)P, benzo(a)pyrene; BPDE, benzo(a)pyrene diol epoxide; BER, base excision repair; cis-DDP, *cis*-dichlorodiamineplatinum; CPD, cyclobutane pyrimidine dimer; DDB-protein, damaged DNA-binding protein; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; dG-C8-AF, *N*-(deoxyguanosin-8-yl)-2-aminofluorene; dG-C8-AAF, *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene; EMSA, electrophoretic mobility-shift assay; NER, nucleotide excision repair; PARP, poly(ADP-ribose) polymerase; PIK, phosphatidylinositol kinase.

isms have evolved mechanisms of recognition and repair of DNA damage introduced by genotoxic agents. In mammalian cells there are at least six such mechanisms based on different sets of proteins which are responsible for repair of different forms of DNA damage. Among repair mechanisms responsible for repair of modified nucleotides are: direct repair, base excision repair (BER) and nucleotide excision repair (NER). Breaks in phosphodiester backbone of DNA are repaired by homologous recombination or joining of non-homologous ends. In addition, misincorporated nucleotides are corrected by mismatch repair (reviewed in: Wood, 1996; Lindahl *et al.*, 1997).

The mechanisms of BER and NER involve multistep processes in which DNA fragments containing nucleotides with chemically-modified bases are repaired. BER is initiated by one of the specific DNA-glycosylases that recognizes the damage and hydrolyzes the N-glycosidic bond between deoxyribose and modified base. There are two types of glycosylases: (i) glycosylases that cleave the N-glycosidic bond only and (ii) glycosylases showing additionally AP-lyase activity (i.e. enzymes which also cleave the DNA-phosphate backbone). AP-endonucleases are also engaged in initiation of BER. In situations when AP-sites arise independently of glycosylases, such a nuclease becomes the damage-recognition enzyme starting the repair process (reviewed in: Demple & Harrison, 1994; Seeberg *et al.*, 1995; Wilson & Thompson, 1997). The NER pathway starts from recognition of a lesion followed by incision of DNA chain on both sides of the damage. The recognition and incision of a damage depend on the action of a few multiprotein complexes. The first step of initiation-complex formation seems to be binding of heterodimer XPA/RPA to the damaged fragment. Then, other protein complexes — transcription factor TFIIH and excinucleases XPG and XPF/ERCC1 can bind to damaged DNA. The XPA protein recognizes a broad spectrum of

DNA damages and seems to be a general recognition factor which is indispensable for the NER activity. Proteins XPC and XPE (human UV-DDB protein) also seem to play a role in the recognition step, however their role is limited (reviewed in: Sancar, 1996; Friedberg, 1996; Wood, 1997). Double strand breaks (DSB) are induced in DNA mostly by ionizing radiation and seem to be the most disruptive forms of DNA damage. Cells repair this kind of DNA damage by either homologous recombination or non-homologous end joining. Mammalian cells repair DSB preferentially by non-homologous end joining. This process engages DNA-dependent protein kinase (DNA-PK) which is an abundant nuclear serine/threonine kinase that is activated by DNA double strand ends. The catalytic subunit of DNA-PK (DNA-PK$_{CS}$) belongs to the PIK-related kinase superfamily. The binding of DNA-PK to DNA is mediated through the Ku70/Ku86 heterodimer which shows high affinity to free ends, the stem-loop and bubble structures of DNA. It is postulated that DNA-PK functions as an alignment factor which juxtaposes the two DNA ends to be joined and that phosphorylation of DNA-PK subunits plays a regulatory role in this process (reviewed in: Anderson & Lees-Miller, 1992; Jackson & Jeggo, 1995).

A large number of DNA-binding proteins show elevated affinity to damaged DNA but their relation to repair mechanisms has not been established. Some of these proteins show preferential binding to DNA damaged by the anticancer drug *cis*-dichlorodiaminoplatinum (cis-DDP). Cis-DDP induces intrastrand d(GpG) and d(ApG) crosslinks which cause bending and unwinding of the DNA at the lesion site. Such structures are recognized by HMG 1 protein (Pil & Lippard, 1992) and other proteins which contain the "HMG-box" motif (Bruhn *et al.*, 1992; Treiber *et al.*, 1994), and also by histone H1 (Yaneva *et al.*, 1997). HMG-box proteins interact with cis-DDP adducts but not with adducts induced by trans-DDP (it was shown that trans-DDP

had no therapeutic value). It seems possible that HMG proteins can compete with repair proteins in some kinds of lesions, making repair more difficult and facilitating the apoptosis process (Chu, 1994).

Another DDB-protein whose relations with repair mechanisms have not been established is poly(ADP-ribose) polymerase (PARP). PARP binds with high affinity to single and double strand ends which induces enzyme activity. The activation causes rapid attachment of long branched chains of poly(ADP-ribose) to enzyme itself and (with lower efficiency) to other nuclear proteins (e.g. histones). The automodification results in dissociation of PARP from DNA, and then the poly(ADP-ribose) polymer is degraded (Lindahl et al., 1995). Tumor suppressor protein p53, which mediates cell cycle arrest and activates apoptosis, can also bind to damaged DNA. 14 kDa C-terminal domain of p53 protein recognizes insertion/deletion mismatches consisting of a few extra bases, but its affinity to classical mismatches is very low (Lee et al., 1995). It was also reported that the presence of benzo(a)pyrene-induced damage could result in binding of Sp1 transcription factor to non-targeted sites in DNA (MacLeod et al., 1995).

DNA adducts induced by N-acetoxy-acetylaminoflurene (AAAF), benzo(a)pyrene diol epoxide (BPDE) and UV-radiation are repaired by the nucleotide excision repair pathway. We have studied nuclear proteins from rat liver cells which recognized damage induced by these genotoxic agents and attempted to evaluate their importance for DNA repair.

MATERIALS AND METHODS

DNA probes. Synthetic double strand 36-bp long oligonucleotides (5'-AATTCGTAGG CCTAAGAGCA ATCGCACCTG TGCGCG-3', with blunt ends) were used as molecular probes. Oligonucleotides (at 10 μM concentration) were incubated for 4 h at 37°C with 40

μM AAAF or BPDE (Midwest Research Institute, U.S.A.), then purified by phenol/chloroform extraction and ethanol precipitation to remove the carcinogens. Alternatively, oligonucleotides were UV-irradiated at a 5 kJ/m^2 dose using 254 nm UV-crosslinker (Stratagene). Oligonucleotides were end-labeled with (γ-^{32}P)ATP using T4 polynucleotide kinase, and purified by polyacrylamide gel electrophoresis.

Animal treatment and nuclear extract preparation. Adult male WAG rats (3 animals in each group) were i.p. injected with 2-acetylaminofluorene (2-AAF) or benzo(a)pyrene (B(a)P) dissolved in dimethyl sulfoxide (DMSO), at a 50 mg/kg of body weight dose, then livers were collected at different time intervals after treatment. Nuclear extracts were prepared from either control (untreated or solvent-treated) and carcinogen-treated animals. Tissues were homogenized and nuclei were purified. Nuclear proteins were extracted for 30 min at 4°C with 0.1 M NaCl (low salt extract) or 1.0 M NaCl (high salt extract) in buffer consisting of: 10 mM Hepes/NaOH, pH 7.9; 1.5 mM MgCl$_2$; 0.1 mM EGTA; 0.5 mM dithiothreitol; 5% glycerol and inhibitors of proteases. The remaining nuclei were pelleted by centrifugation for 30 min at 16000 r.p.m. at 4°C.

Assay of DNA adducts. Oligonucleotides treated with genotoxic agents and hepatic DNA isolated from carcinogen-treated animals were assayed for the presence of adducts by the ^{32}P-postlabeling method. UV-induced adducts were analyzed according to Widłak et al. (1995). Adducts induced by 2-AAF derivatives were enriched by butanol extraction, while adducts induced by B(a)P derivatives were enriched by nuclease P1 treatment. ^{32}P-Labeled nucleotides were resolved by multi-dimensional thin-layer chromatography. Adduct spots were visualized by autoradiography and quantitated by scintillation counting (Widłak et al., 1996).

Electrophoretic mobility-shift assay (EMSA): Radioactive DNA probes (25 ng)

were incubated with nuclear proteins (5 μg) for 30 min at 4°C. The binding buffer consisted of: 20 mM Tris/HCl, pH 7.6; 5 mM MgCl$_2$; 0.5 mM EDTA; 1 mM dithiothreitol; 5% glycerol and 150 mM NaCl (final concentration). The formation of complexes was studied in the presence of either a non-specific (alternating poly(dI-dC), 2 μg) or specific (damaged or undamaged oligonucleotides, 1 μg) non-radioactive competitor, in a final volume of 20 μl. Complexes were resolved under non-denaturing conditions by 6% polyacrylamide gel electrophoresis (in 0.5 × Tris/borate/EDTA running buffer) and detected by autoradiography.

RESULTS AND DISCUSSION

Preparation of DNA probes containing damaged nucleotides to be used in EMSA method

Identification of proteins which might interact with damaged parts of the genome, their level, inducibility and specificity seem to be crucial for understanding the differences in sensitivity to genotoxic agents observed among individuals and different types of cells. Various methodologies for detection of proteins exhibiting elevated affinity to damaged DNA have been developed. One of them is based on the electrophoretic mobility-shift assay. In this method, DNA modified by chemicals or UV light is complexed *in vitro* with protein extracts in the presence of competitor DNA. The proteins with high affinity to damaged DNA are detected by gel electrophoresis in the form of retarded band, containing protein–DNA complexes (Protic & Levine, 1993).

In our experiments, 36-bp long oligonucleotides damaged *in vitro* by AAAF, BPDE or UV-radiation (254 nm) were used as radioactive molecular probes. The level of lesion in DNA probes was assayed using ^{32}P-postlabeling. DNA adducts present in the probes are visual-ized in Fig. 1. Treatment of the oligonucleotide with three different genotoxic agents resulted in similar levels of DNA damage. After treatment with AAAF about 10% of DNA molecules contained a single lesion (on average), with dG-C8-AAF as a major lesion. After treatment with BPDE about 20% of DNA molecules contained a lesion, almost exclusively dG-BPDE. After UV-irradiation about 15% of DNA molecules contained a lesion, mostly cyclobutane pyrimidine dimers.

Rat liver cells contain nuclear proteins which strongly bind to DNA damaged by AAAF

Using an *in vitro* assay (EMSA) we found that nuclear extracts from rat liver cells contained proteins which preferentially bound to damaged DNA. Proteins that recognize lesions induced by AAAF, BPDE or UV-radiation have been detected in such nuclear extracts (Fig. 2). Among these proteins two classes might be distinguished. The first class consists of proteins which are not attached to nuclear structures and can be eluted from the nucleus with low salt buffers ("low salt extract"). The complex formed between these proteins and DNA is denoted as complex "II" in Fig. 2A. DNA damaged by AAAF bound to these proteins with the highest affinity, however even the complex formed with non-damaged DNA could also be detected (yet with an efficiency lower by one order of magnitude as compared to DNA damaged by AAAF). These complexes were visible when excess of poly(dI-dC) but not of undamaged oligonucleotide (or *E. coli* DNA) was used as a competitor (not shown). This suggested some specificity for a structure which is not present in poly(dI-dC) polymer.

The second class of DDB proteins was found in extracts eluted from nuclei with 1 M NaCl ("high salt extract"). However, these proteins began to be eluted from nuclei with 0.4 M NaCl (not shown). The high salt extract proteins formed strong complexes with the DNA
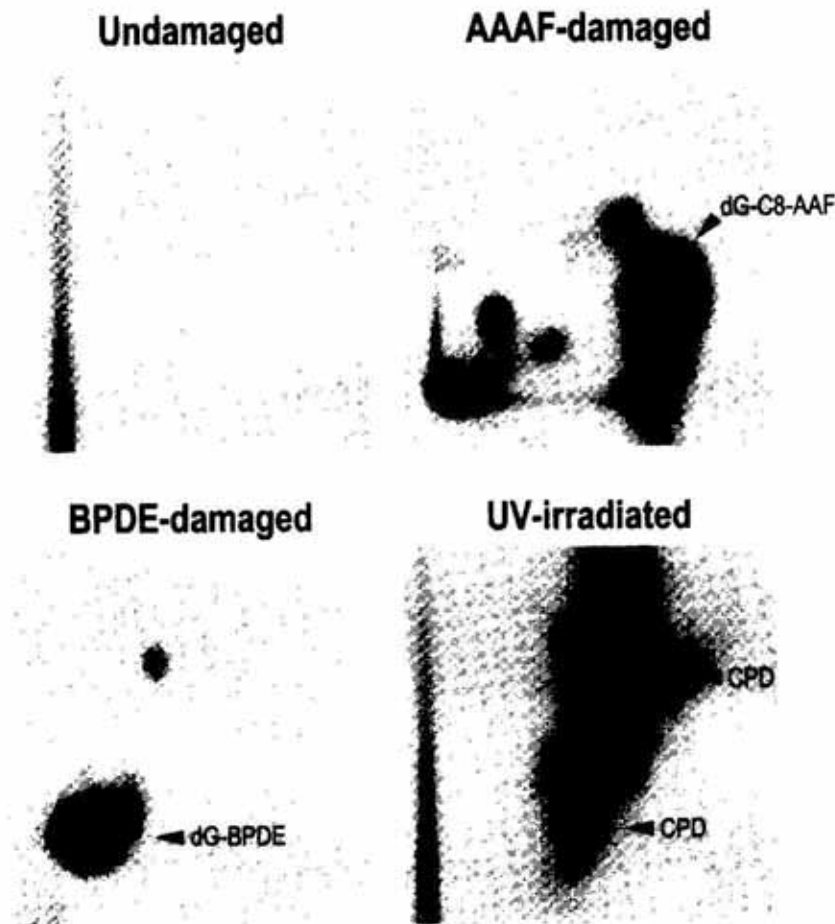
Undamaged   **AAAF-damaged**

BPDE-damaged   **UV-irradiated**

Figure 1. Analysis of DNA damage induced in DNA probe.

Chromatograms of ^{32}P-labeled nucleotides from either undamaged DNA or DNA damaged by AAAF, BPDE and UV-radiation. Major forms of DNA damage are indicated.

damaged by AAAF (denoted as complex "I" in Fig. 2). They possessed higher electrophoretic mobility as compared to complex "II". UV-irradiated DNA formed specific complexes with high salt extract proteins (denoted as complex "III" in Fig. 2) which migrated with much lower electrophoretic mobility. To elucidate the affinity of different DNA lesions to proteins in complex "I", the complexes were formed in the presence of specific competitors. High salt extract proteins were incubated with the radioactive oligonucleotide damaged by AAAF in the presence of excess of undamaged or damaged non-radioactive oligonucleotides (Fig. 2B). The complex visible in the presence of undamaged competitor was absent when the oligonucleotide damaged with AAAF was used as a competitor. The oligonucleotide damaged by BPDE was a

weaker competitor while the oligonucleotide damaged by UV does not seem to affect the formation of complexes with radioactive AAAF-damaged probe. This experiment shows that proteins present in complex "I" specifically bind DNA damaged by BPDE (yet with much lower efficiency as compared to AAAF-damaged DNA) but not UV-irradiated DNA. A similar experiment, in which UV-irradiated DNA was used as a radioactive probe, proved that neither AAAF-damaged DNA nor BPDE-damaged DNA bound to UV-DDB protein in complex "III" (not shown).

The detected AAAF–DDB proteins are probably not involved in DNA repair

The presence of proteins which have higher affinity to damaged than to undamaged DNA
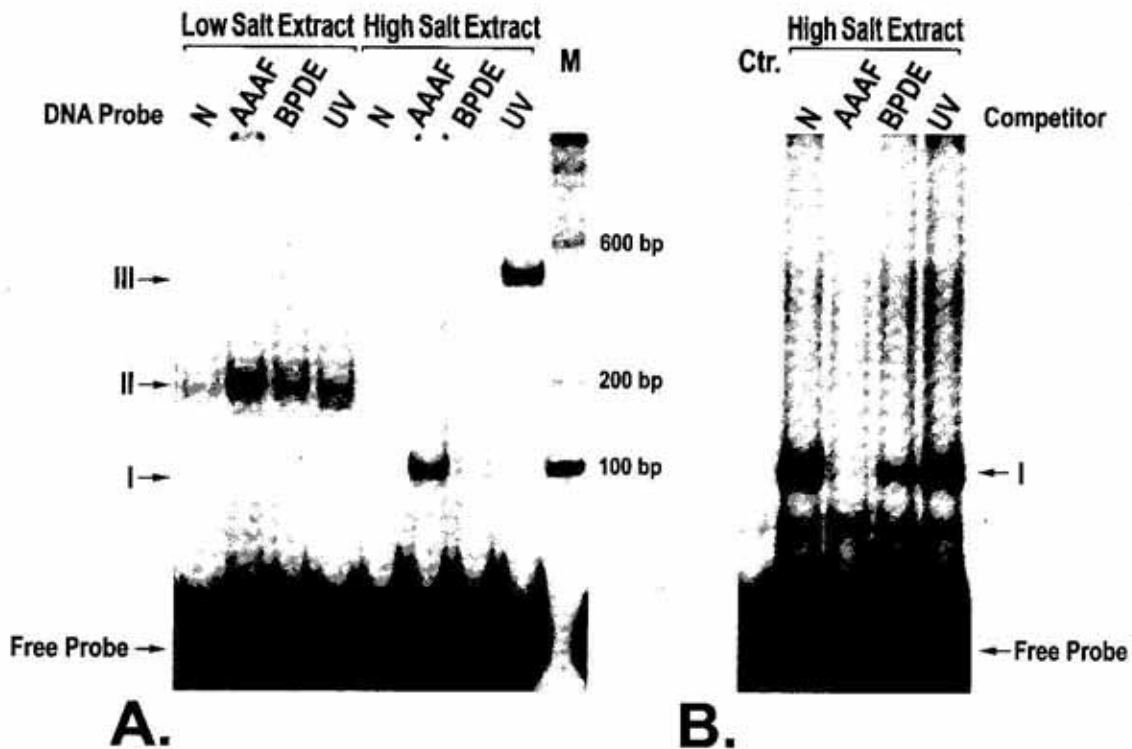
Figure 2. Analysis of DNA damage–recognition proteins from rat liver nuclei.

Panel A. The *in vitro* complexes were formed between radioactive DNA probes (non-damaged (N) or damaged by AAAF, BPDE or UV) and **Low Salt Extract** or **High Salt Extract** proteins in the presence of poly(dI-dC). Complexes were then analyzed by native polyacrylamide gel electrophoresis. Denoted are positions of free probe and retarded complexes (**I, II, III**). Lane M represents a DNA-size marker (100-bp ladder).

Panel **B.** **High Salt Extract** proteins were incubated with radioactive oligonucleotide damaged by AAAF in the presence of non-radioactive specific competitors, non-damaged (N) or damaged by AAAF, BPDE or UV. Lane **Ctr.** represents radioactive DNA probe alone.

raises the question whether these proteins are engaged in the DNA repair process. To address this question, we analyzed whether treatment of animals with carcinogens affected the level of DDB-proteins. Rats were injected with either 2-AAF or B(a)P, then the levels of DNA adducts and DDB-proteins were assayed in samples collected after different time intervals. We found that DDB-proteins (which form either a "I" or "II" complex) were permanently present in cells from rat tissues and their levels were unchanged after treatment of animals with the carcinogens (not shown).

One can suppose that, if DDB-proteins are engaged in the damage recognition step of the repair process, lesions differing in affinity to DDB-proteins may differ also in kinetics of

their removal from DNA. Damages induced by either 2-AAF or B(a)P are removed from DNA by the nucleotide excision repair pathway. The comparison of adduct levels detected in rat liver DNA 24 and 48 h after injection of B(a)P or 2-AAF is shown in Fig. 3. Both carcinogens induced similar levels of DNA adducts in liver cells 24 h after injection. At 48 h after treatment the level of B(a)P-induced adducts decreased significantly (to about 20% of 24 h level), while at the same time the level of 2-AAF-induced adducts was unchanged. On the other hand, DNA molecules damaged by the 2-AAF derivative exhibited much higher affinity to DDB-proteins (in both detected complexes) as compared to DNA damaged by the B(a)P derivative (Fig. 2). Thus, the high affinity of damage-
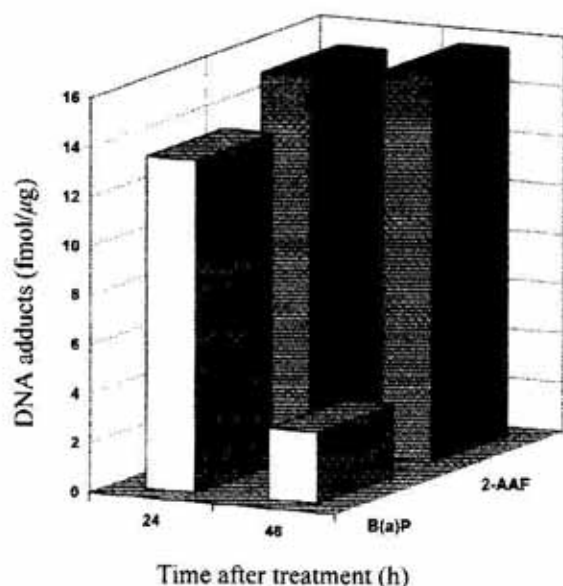
Figure 3. The removal of B(a)P- and 2-AAF-induced damage from rat liver DNA.

Hepatic DNAs were isolated 24 and 48 h after treatment with 2-AAF or B(a)P, then the level of DNA adducts was analyzed using the ^{32}P-postlabeling method. Values are the means from 3 animals.

recognition proteins to AAAF-damaged DNA did not correlate with more efficient removal of the damage from DNA.

The data presented in this communication suggest that DNA damage–recognition proteins from rat liver nuclei which bind with high affinity to AAAF-induced adducts are not involved in the DNA repair pathway. Instead, it seems possible that these proteins may mask the damage, making its repair slower. In fact, the major DNA adduct induced by 2-AAF (i.e. dG-C8-AF) shows slow repair kinetics (Gupta & Dighe, 1984). The nature and function of AAAF-DDB proteins detected in rat liver will be further studied in our laboratory.

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