Defective DNA endonuclease activities in Fanconi’s anemia cells, complementation groups A and B

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Summary

Cells from patients with the inherited disorder, Fanconi’s anemia (FA), were analyzed for endonucleases which recognize DNA interstrand cross-links and monoadducts produced by psoralen plus UVA irradiation. Two chromatin-associated DNA endonuclease activities, defective in their ability to incise DNA-containing adducts produced by psoralen plus UVA light, have been identified and isolated in nuclei of FA cells. In FA complementation group A (FA-A) cells, one endonuclease activity, pI 4.6, which recognizes psoralen intercalation and interstrand cross-links, has 25% of the activity of the normal human endonuclease, pI 4.6, on 8-methoxypsoralen (8-MOP) plus UVA-damaged DNA. In FA complementation group B (FA-B) cells, a second endonuclease activity, pI 7.6, which recognizes psoralen monoadducts, has 50% and 55% of the activity, respectively, of the corresponding normal endonuclease on 8-MOP or angelicin plus UVA-damaged DNA. Kinetic analysis reveals that both the FA-A endonuclease activity, pI 4.6, and the FA-B endonuclease activity, pI 7.6, have decreased affinity for psoralen plus UVA-damaged DNA. Both the normal and FA endonucleases showed approximately a 2.5-fold increase in activity on psoralen plus UVA-damaged reconstituted nucleosomal DNA compared to damaged non-nucleosomal DNA, indicating that interaction of these FA endonucleases with nucleosomal DNA is not impaired. These deficiencies in two nuclear DNA endonuclease activities from FA-A and FA-B cells correlate with decreased levels of unscheduled DNA synthesis (UDS), in response to 8-MOP or angelicin plus UVA irradiation, in these cells in culture.
and a predisposition to develop cancer (Fanconi, 1967; German, 1982; Schroeder, 1982; Glanz and Fraser, 1982). Studies have shown that cells derived from patients with FA have a hypersensitivity to DNA interstrand cross-linking agents (Sasaki and Tonomura, 1973; Auerbach and Wolman, 1976; Fujiwara et al., 1977; Weksberg et al., 1979; Ishida and Buchwald, 1982; Papadopoulo et al., 1987; Digweed et al., 1988, Auerbach et al., 1989). They display reduced cell survival and increased chromosome damage when treated with such agents as mitomycin C, nitrogen mustard and psoralen plus UVA irradiation (Sasaki and Tonomura, 1973; Lalt et al., 1975; Auerbach and Wolman, 1976; Fujiwara et al., 1977; Weksberg et al., 1979; Kano and Fujiwara, 1982; Wunder and Fleischer-Reischman, 1983; Papadopoulo et al., 1987; Digweed et al., 1988). They are thought to have a defect in repair of lesions in their DNA produced by these various cross-linking agents (Fujiwara, 1982; Gruenert and Cleaver, 1985; Plooy et al., 1985; Papadopoulo et al., 1987; Averbeck et al., 1988; Moustacchi et al., 1989). Patients with FA have been grouped into two complementation groups, A and B (FA-A and FA-B), based on their cellular response to DNA interstrand cross-linking agents (Duckworth-Rysiecki et al., 1985; Moustacchi et al., 1987). FA-A cells show greater growth inhibition by mitomycin C (Duckworth-Rysiecki et al., 1985), and much greater reduction in rate of semi-conservative DNA synthesis after 8-MOP plus UVA treatment than FA-B cells (Moustacchi et al., 1987). Although both FA-A and FA-B cells display reduced capacity to incise DNA interstrand cross-links and monoadducts produced by selected cross-linking agents (Papadopoulo et al., 1987; Averbeck et al., 1988; Moustacchi et al., 1989), FA-A cells show a greater reduction in ability to incise DNA containing interstrand cross-links (Papadopoulo et al., 1987; Moustacchi et al., 1989), whereas FA-B cells are more sensitive to psoralen monoadducts (Averbeck et al., 1988; Moustacchi et al., 1989).

Although evidence indicates that there is a defect in the ability of FA cells to repair DNA damage produced by cross-linking agents, to date no DNA endonuclease(s) has been identified in FA cells which specifically incises DNA containing interstrand cross-links or monoadducts and which is defective in its ability to recognize these forms of damage. We have recently isolated two chromatin-associated DNA endonuclease activities from normal human lymphoblastoid cell nuclei which recognize and incise DNA containing adducts produced by psoralen plus long wavelength (366 nm) ultraviolet radiation (UVA) (Lambert et al., 1988). One of these endonuclease activities, pI 4.6, recognizes the intercalation of the psoralen molecule into DNA and also the interstrand cross-link, against which it shows the greater activity; the other endonuclease activity, pI 7.6, recognizes the psoralen monoadduct (Lambert et al., 1988; Lambert and Parrish, 1989; Parrish and Lambert, 1990).

The present study examines FA-A and FA-B cells for endonucleases active on psoralen plus UVA-treated DNA. We report here on the isolation of two nuclear, chromatin-associated DNA endonuclease activities, pI's 4.6 and 7.6, from FA cells which are active on DNA-containing monoadducts and interstrand cross-links produced by 8-MOP plus UVA irradiation or monoadducts produced by angelicin plus UVA irradiation. These FA-A and FA-B endonucleases show differences in activity compared to each other and to endonucleases from cells from normal individuals. In addition, we have examined the influence of nucleosome structure on the activity of these endonucleases.

Materials and methods

Cell culture

Normal human lymphoblastoid cell lines (GM 1989 and GM 3299) (transformed with Epstein-Barr virus) were obtained from the Coriell Institute for Medical Research, Camden, NJ. Lymphoblastoid cell lines (transformed with Epstein-Barr virus), obtained from FA patients complementation group A (HSC 72) and complementation group B (HSC 62), were a gift from Dr. Manuel Buchwald. The cells were grown in suspension culture in RPMI 1640 medium, supplemented with 12.5% fetal calf serum (Grand Island Biological Co.) and harvested under conditions of maximal proliferation as previously described (Okorodudu et al., 1982). Cell cultures...
were routinely tested for mycoplasma (Okorodudu et al., 1982).

**Measurement of unscheduled DNA synthesis**

Cells in culture were treated with 1 μg/ml 8-MOP (Sigma Chemical Company) in phosphate-buffered saline (PBS) (0.15 M) for 20 min at room temperature in the dark. They were then irradiated with UVA light (principally 366 nm) (10 W/m² for 10 min). The cells were washed with PBS and exposed to a second dose of UVA irradiation (10 W/m² for 10 min) (Tsongalis et al., 1990). Cells in culture were also treated with 5 μg/ml angelicin (Elder Company) in PBS for 20 min followed by irradiation with UVA light (10 W/m² for 5 min). The cells were then suspended in media containing 10 μCi/ml [3H] methylthymidine (specific activity 61 Ci/m mole) (ICN Radiochemicals) and incubated for 2 h at 37°C (Tsongalis et al., 1990). Unincorporated thymidine was removed by several washes with cold PBS. Cells were smeared onto glass slides and the slides were dipped in Kodak NTB3 nuclear emulsion and exposed for 7 days at 4°C (Tsongalis et al., 1990). The slides were then treated with Kodak D19 developer and stained with Giemsa. Grains per nucleus were counted in random fields. Cells with 40–100 grains per nucleus were classified as undergoing UDS. For each individual experiment 300–500 cells were counted.

**DNA endonuclease extraction**

Cell nuclei were isolated and the chromatin-associated proteins separated from the nucleoplasmic proteins in a series of steps, passed through a CM sephadex column and electrophoresed on an isoelectric focusing column as previously described (Lambert et al., 1982, 1988). Fractions collected from the column were assayed for DNA endonuclease and exonuclease activity (Lambert et al., 1982). Peaks of endonuclease activity were pooled, dialyzed into 50 mM potassium phosphate (pH 7.1), 1 mM β-mercaptoethanol, 1 mM NaEDTA, 0.25 mM phenylmethylsulfonylfluoride (PMSF), and 40% ethylene glycol and stored unfrozen at −20°C (Lambert et al., 1982, 1988). Protein concentrations were determined by the BioRad protein assay (BioRad Laboratories).

**Histone isolation**

Nuclei were isolated from normal lymphoblastoid cells and histones were extracted and separated as previously described (Kaysen et al., 1986). Histone H1 was removed by precipitation with 5% perchloric acid (Kaysen et al., 1986). The purity of core (H2A, H2B, H3, and H4) and total (core plus H1) histones was monitored electrophoretically (Kaysen et al., 1986; Amari et al., 1986). Calf-thymus histones (Boehringer-Mannheim Biochemicals) were used as standards for comparison.

**Plasmid growth and purification**

*Escherichia coli* strain HB101 containing plasmid pWT830/pBR322 (a clone of the entire SV40 and pBR322 genomes) were grown, harvested and lysed as previously described (Lambert et al., 1988). The DNA was extracted with phenol, treated with ribonuclease I and electrophoresed on 0.9% agarose gels. The uncleaved, circular, Form I DNA band was cut from the gel, eluted and recovered by ethanol precipitation (Parrish and Lambert, 1990). The DNA was further purified on a NACS 37 (Bethesda Research Laboratory) column. DNA was eluted from the column with a 0.5 M to 0.7 M NaCl gradient (Parrish and Lambert, 1990). DNA eluting from the column which was greater than 90% Form I was recovered by ethanol precipitation and resuspended in 10 mM Tris–HCl, pH 8.0.

**Nucleosome reconstitution**

Nucleosomal DNA was prepared by reconstituting the plasmid DNA with normal human or XPA histones (+histone H1), at a histone : DNA weight ratio of 1.0, in a buffer containing 2 M NaCl, 50 mM Tris–HCl (pH 8.0), 0.1 M EDTA and 0.25 mM PMSF (Kaysen et al., 1986). The NaCl was progressively decreased by stepwise dialysis at 4°C over a 28-h period to 50 mM (Kaysen et al., 1986; Parrish and Lambert, 1990).

**Reaction of psoralen with DNA**

8-MOP was recrystallized and its purity checked by thin-layer chromatography (Lambert
et al., 1988). Non-nucleosomal and nucleosomal DNA was photoreacted with 8-MOP (7 μg/ml) utilizing a protocol which involved exposing the DNA to two doses of UVA light, an initial one (10 W/m² for 10 min) after the 8-MOP had intercalated into the DNA and a second one (10 W/m² for 10 min) after the unbound 8-MOP was removed by dialysis (Lambert et al., 1988). This procedure has been shown to increase the number of DNA interstrand cross-links (Ben-Hur and Elkind, 1973; Bredberg, 1982) and produced cross-links in 99% of the non-nucleosomal DNA molecules (Lambert et al., 1988). Non-nucleosomal and nucleosomal DNA was reacted with angelicin (25 μg/ml) for 20 min and then exposed to UVA light (10 W/m²) for 5 min (Lambert et al., 1988). Cross-linking of psoralen to DNA was determined by an alkaline gel electrophoretic method (Lambert et al., 1988). Control DNA for the psoralen treated DNAs was exposed to UVA irradiation only.

**DNA endonuclease assay**

DNA endonuclease activity on non-nucleosomal and nucleosomal DNA was assayed using a gel electrophoretic assay as previously described (Lambert et al., 1988). This assay measures conversion of circular, supercoiled uncleaved Form I DNA by the endonuclease to nicked, relaxed circular Form II DNA (Lambert et al., 1988; Parrish and Lambert, 1990). The endonucleases were incubated with 0.10 μg DNA in 10 mM MgCl₂, and 10 mM Tris–maleate (pH 7.5) at 37°C for 2 h (Lambert et al., 1988; Parrish and Lambert, 1990). The concentration of each DNA endonuclease activity, from both the normal and FA cell lines, was adjusted, at similar levels of protein, to produce 0.05 ± 0.01 breaks per DNA molecule on non-damaged DNA. The enzymatic reaction was terminated with 0.1 M EDTA and the DNA samples were treated with 0.4% Sarkosyl (Ciba-Geigy) and 50 μg/ml Proteinase K (Sigma Chemical Co.) for 1 h at 37°C (Kaysen et al., 1986; Parrish and Lambert, 1990). Samples were electrophoresed on 1.0% agarose gels, subsequently stained with 0.5 μg/ml ethidium bromide, photographed, and the negatives of the gels scanned. Endonuclease activity, expressed as the number of enzyme induced breaks per DNA molecule, was determined as previously described (Lambert et al., 1988; Parrish and Lambert, 1990). Depending on the concentration, ethidium bromide may bind differentially to different forms of DNA (Freifelder, 1983). However, in the low concentration used here, we determined that binding of ethidium bromide to superhelical versus non-superhelical forms of DNA was completely equivalent and that calculation of the various forms of DNA was not influenced by differential binding of ethidium bromide to any one of them (Kaysen, 1984). Also, in the exposure ranges used here, the response of the film used in photographing gels was tested and determined to be linear.

**Kinetic analysis**

For kinetic analysis of these endonuclease reactions, assays were performed on undamaged and damaged non-nucleosomal DNA as described above but with graduated reductions in substrate concentration (0.1–0.025 μg). These assays were carried out over a range encompassing at least a 4-fold decrease in substrate concentration. Over this range all components of the assay system remained linear. Results were plotted according to Lineweaver and Burke as [S]⁻¹ versus 𝜈⁻¹, and also as 𝜈 versus 𝜈/[S] according to Eadie and Hofstee, where [S] and 𝜈 represent initial molar substrate concentration and velocity of the enzymatic reaction, respectively. Values of 𝑉_max (maximum velocity) and 𝐾_m (Michaelis constant) were determined from linear extrapolations on these graphic representations using standard methods based on the Michaelis–Menten equation. Values of 𝐾_cat, turnover number, were computed from these values for 𝑉_max.

**Results**

**UDS in FA cells treated with psoralen plus UVA**

UDS was observed in both FA-A and FA-B cells in culture exposed to 8-MOP plus two doses of UVA light, however, the levels were lower than those found in normal cells (Fig. 1A). UDS in FA-A cells was approximately 40% of that found in normal cells and in FA-B cells it was approximately 46% of normal levels. UDS was also examined in these cells exposed to angelicin
Fig. 1. UDS in FA cells, complementation groups A and B, treated with (A) 8-MOP (1 μg/ml) plus two doses of UVA irradiation and (B) angelicin (5 μg/ml) plus UVA irradiation. Results are expressed as percent of normal UDS (100%) ± S.E.M. for 8–10 separate experiments with a total of 2.4×10^3–5.0×10^3 cells counted.

plus UVA light. In FA-A and FA-B cells UDS was 87% and 33% of normal levels, respectively (Fig. 1B). The effect of these psoralens on UDS was concentration-dependent.

**Endonuclease activity on 8-MOP plus UVA-treated non-nucleosomal DNA**

9 chromatin-associated DNA endonuclease activities were isolated from the nuclei of normal human and of FA cells, complementation groups A and B, and examined for ability to incise plasmid DNA treated with 8-MOP plus UVA light. As we have previously reported, selective activity against 8-MOP plus UVA-treated DNA was present in two normal endonuclease activities, pIs 4.6 and 7.6, which showed similar levels of activity on this substrate (Fig. 2) (Lambert et al., 1988). Selective activity against 8-MOP plus UVA-treated DNA was also found in these same two endonuclease activities from FA-A and FA-B cells (Figs. 2, 3). However, the levels of activity of both FA-A endonucleases and of the FA-B endonuclease, pI 7.6, were decreased compared to those of

Fig. 2. Action of chromatin-associated DNA endonuclease activities from FA cells on DNA treated with 8-MOP plus UVA light. DNA endonuclease activities from normal human cells and FA cells, complementation groups A and B, were examined for activity on plasmid DNA treated with 8-MOP (7 μg/ml) plus two doses of UVA light. These values have had subtracted from them the enzyme activity on undamaged DNA (0.05 ± 0.01 breaks). Vertical lines represent ± S.E.M.
the corresponding normal endonucleases (Fig. 2). A relative decrease was observed in activity of the FA-A endonuclease, pI 4.6, compared with activity of the FA-A endonuclease, pI 7.6, as well as in activity of the FA-B endonuclease, pI 7.6, compared with activity of the FA-B endonuclease, pI 4.6 (Fig. 2). The FA-A endonuclease, pI 4.6, was approximately 25% and 30% as active on 8-MOP plus UVA-treated DNA as the corresponding normal and FA-B endonuclease, respectively (p < 0.001), and 35% as active on this substrate as the FA-A endonuclease, pI 7.6 (p < 0.001). The FA-A endonuclease, pI 7.6, was approximately 75% as active as the corresponding normal endonuclease on this substrate (p < 0.001). The FA-B endonuclease, pI 7.6, was approximately 50% as active on this substrate as the corresponding normal endonuclease and 55% as active as the FA-B endonuclease, pI 4.6 (p < 0.001). The activity of the FA-B endonuclease, pI 4.6, on this substrate was also slightly reduced compared to the activity of the corresponding normal endonuclease, but this reduction was not statistically significant (Fig. 2).

**Endonuclease activity on 8-MOP plus UVA-treated nucleosomal DNA**

The reconstituted nucleosomal system, which we have previously characterized, utilized a plasmid containing the entire SV40 genome. It gave standard patterns of digestion with micrococcal nuclease and DNAase I and showed positioning of nucleosomes in a region near the SV40 origin of replication (Kaysen et al., 1986, 1987; Amari et al., 1986). Fig. 4 shows the electrophoretic separation of normal histones used in the reconstitution experiments on polyacrylamide gels.

The activity of the two endonucleases, pI's 4.6 and 7.6, from both FA-A and FA-B cells on reconstituted core (minus histone H1) nucleosomal DNA treated with 8-MOP plus UVA was increased approximately 2.5-fold compared to their activity on damaged non-nucleosomal DNA. This increase was not observed on undamaged nucleosomal DNA. When histone H1 was added to the reconstituted system, this increase in activity of all of these enzymes was reduced approximately 36% (Fig. 5A and 5B). As we have previously reported, results similar to this were also seen with the normal endonucleases (Fig. 5A and 5B) (Parrish and Lambert, 1990). The decrease in activity observed in the FA-A endonuclease, pI 4.6, and the FA-B endonuclease, pI 7.6, on 8-MOP plus UVA non-nucleosomal DNA was still present when the endonucleases were assayed on nucleosomal (± histone H1) DNA (Fig. 5A and 5B). Thus, the relative activities of all of the
normal and FA endonucleases on nucleosomal DNA remained similar to those on non-nucleosomal DNA.

**endonuclease activity on angelicin plus UVA-damaged non-nucleosomal and nucleosomal DNA**

These two endonuclease activities from normal and FA cells were also examined for ability to incise DNA treated with angelicin plus UVA radiation, which produced monoadducts rather than cross-links in DNA. In both normal and FA cells, the endonuclease activity, pl 7.6, had the greater activity on angelicin-treated DNA (Fig. 6). In FA-A and FA-B cells the activity of this endonuclease on damaged non-nucleosomal DNA was 72% and 55%, respectively, of that of the normal endonuclease, pl 7.6. Both in normal and in FA-A and FA-B cells the activity of the endonuclease, pl 4.6, was present but markedly reduced (Fig. 6A), compared to its activity on 8-MOP plus UVA-treated DNA (Fig. 5A) or to the activity of the endonuclease, pl 7.6, on angelicin plus UVA-treated DNA (Fig. 6B). In normal cells the activity of the endonuclease, pl 4.6, was 23% of the activity of the normal endonuclease, pl 7.6, on this substrate (Fig. 6A and 6B). The FA-A and FA-B endonucleases, pl 7.6, had 10% and 60%, respectively, of the activity of the corresponding FA-A and FA-B endonucleases, pl 7.6, and 30% and 120%, respectively, of the activity of the normal endonuclease, pl 4.6, on angelicin plus UVA-treated DNA. Again, the activity of both of the normal and of both of the FA-A and of the FA-B endonucleases increased approximately 2.5-fold when angelicin plus UVA-treated nucleosomal DNA (minus histone H1) was used as substrate (Fig. 6A and 6B), and this increase was reduced when histone H1 was added to the system. The same differences in endonuclease activity that were seen on the dam-

![Fig. 5. Influence of nucleosome structure on FA endonuclease activity on 8-MOP plus UVA light damaged DNA. The action of the DNA endonuclease activities, pl 4.6 and 7.6, from normal human, FA-A and FA-B cells was examined on 8-MOP plus UVA treated naked, core (histones H2A, H2B, H3, H4) and total (core + histone H1) nucleosomal DNA. Endonuclease activities (A) pl 4.6 and (B) pl 7.6 (0.34 ± 0.04 μg) were incubated with non-nucleosomal and nucleosomal plasmid DNA (0.1 μg) which had been treated with 8-MOP (7 μg/ml) plus UVA. These values have had subtracted from them the enzyme activity on undamaged DNA. Vertical lines represent ± S.E.M. of 4–6 Expts.](image-url)
Fig. 6. Influence of nucleosome structure on FA endonuclease activity on angelicin-treated DNA. The DNA endonuclease activities, pI 4.6 and 7.6, from normal human, FA-A and FA-B cells were examined for activity on angelicin plus UVA-treated DNA. Endonuclease activities (A) pI 4.6 and (B) pI 7.6, (0.34 ± 0.04 µg) were incubated with non-nucleosomal and nucleosomal plasmid DNA (0.1 µg) which had been treated with angelicin (25 µg/ml) plus UVA. These values have had subtracted from them the enzyme activity on undamaged DNA. Vertical lines represent ± S.E.M. of 3 or 4 Expts.

aged non-nucleosomal DNA were observed on angelicin plus UVA-damaged nucleosomal DNA (± histone H1).

Kinetic analysis of the activities of the FA endonucleases

Kinetic analysis of the endonuclease activities, pI's 4.6 and 7.6, from normal, FA-A and FA-B cells on DNA treated with 8-MOP plus UVA produced linear, highly reproducible plots using both the Lineweaver and Burke and the Eadie and Hofstee methods, with coefficients of correlation of at least 0.99 and 0.97, respectively (Fig. 7). The turnover number (K_{cat}) of both of the normal, both of the FA-A, and both of the FA-B endonuclease activities were similar to each other on control DNA (DNA treated with UVA but without 8-MOP) and on DNA treated with 8-MOP plus UVA (Fig. 7). All 6 enzymes showed slightly reduced turnover numbers on the latter substrate, however.

In contrast to these results, reductions were observed in the K_{m}'s of these endonuclease activities on 8-MOP plus UVA-treated DNA versus control DNA. However, these reductions were not uniform (Fig. 8). K_{m}'s of the endonuclease activity, pI 4.6, from normal, FA-A and FA-B cells on control DNA did not differ significantly (Fig. 8). The K_{m}'s of all three endonuclease activities, pI 4.6, were reduced on 8-MOP plus UVA-treated DNA (p < 0.001), but the K_{m} of the FA-A endonuclease was much higher than that of either the normal endonuclease (p < 0.001) or the FA-B endonuclease (p < 0.001), which did not differ significantly from each other (Fig. 8). Since there is little difference in the K_{cat}'s, this indicates that all three endonuclease activities, pI 4.6, show greater affinity for or rate of association with 8-MOP plus UVA-treated DNA than control DNA, but that this increase is much less for the FA-A endonuclease than for either the corresponding normal or FA-B endonuclease.
Fig. 7. Kinetics ($V_{max}$) of the interaction of FA endonuclease activities with 8-MOP plus UVA-treated DNA. Maximum velocities ($V_{max}$) and turnover numbers ($K_{cat}$) were determined for the normal human, FA-A and FA-B endonuclease activities, $pI$ 4.6 and 7.6, (0.34 ± 0.04 μg) assayed on non-nucleosomal DNA irradiated with UVA light and treated either with 8-MOP or without (control DNA). Graduated reductions in DNA concentration (0.1-0.025 μg) were used. Values shown represent the mean of those obtained from at least 3 Expts. Vertical bars represent ± S.E.M. These values were obtained from linear plots of data producing correlation coefficients, $R$, as shown (numbers within bars), for Lineweaver and Burke (L-B) and Eadic and Hofstee (E-H) plots. Mean $R$ values of each are shown ± S.E.M.

The $K_m$'s of the endonuclease activities, $pI$ 7.6, from normal, FA-A and FA-B cells on control DNA were not significantly different (Fig. 8). All three endonuclease activities, $pI$ 7.6, showed a much lower $K_m$ on 8-MOP plus UVA-damaged DNA than on control DNA. The $K_m$'s of both the FA-A and the FA-B endonucleases, $pI$ 7.6, were less reduced than for the corresponding normal endonuclease, however ($p < 0.01$), with the $K_m$ of the FA-B endonuclease higher than that of the FA-A endonuclease ($p < 0.05$). Since there was little difference in the turnover numbers of either the normal or the FA-A or FA-B endonuclease activities, $pI$ 7.6, on 8-MOP plus UVA-treated DNA, these decreases in $K_m$'s are again due primarily to increases in affinity for or rates of association with the damaged DNA.

Discussion

Various lines of investigation have related the hypersensitivity of FA cells to DNA interstrand cross-linking agents to a defect in repair of DNA damage produced by these agents (Fujiwara et al., 1977; Papadopoulo et al., 1987; Gruenert and Cleaver, 1985; Plooy et al., 1985; Averbeck et al., 1988; Matsumoto et al., 1989). This defect has been proposed to occur in the initial incision step of the repair process in certain FA cell lines.
(Fujiwara et al., 1977; Fujiwara, 1982; Gruenert and Cleaver, 1985). However, in other studies a defect in repair of interstrand cross-links has not been detected (Fornace et al., 1979; Kaye et al., 1980; Poll et al., 1984). Some of this discrepancy has been attributed to the genetic heterogeneity of the FA cell lines (Duckworth et al., 1985; Papadopoulo et al., 1987; Moustacchi et al., 1987) and to different culture ages of the cell lines used (Sognier and Hittleman, 1983). The presence of at least two FA complementation groups has been demonstrated (Duckworth et al., 1985; Moustacchi et al., 1987). However, to date, a defective repair enzyme has not been identified in FA cells.

The present study analyzes FA cells from both complementation groups A and B, in culture, for their ability to repair damage produced by psoralen plus UVA light. In addition, it also reports on the identification and partial purification of two chromatin-associated DNA endonuclease activities, pI's 4.6 and 7.6, in FA-A and FA-B cells which recognize adducts produced by psoralen plus UVA light but which are defective in their ability to incise DNA containing these adducts.

**Reduced UDS in FA cells**

In the present study, analysis of DNA repair in FA cells in culture demonstrates that in response to monoadducts and interstrand cross-links pro-
duced by 8-MOP plus UVA light, both FA-A and FA-B cells show reduced levels of UDS as compared to normal cells with levels of UDS in FA-A cells slightly lower than those in FA-B cells. In response to monoadducts produced by angelicin plus UVA light, FA-B cells show much lower levels of UDS than either FA-A or normal cells. Plooy et al. (1985) examined UDS in one FA fibroblast cell line exposed to mitomycin C and did not detect any UDS in these cells; however, they also did not detect UDS in similarly damaged normal cells. Fujiwara and Tatsumi (1977) also measured UDS in normal and in FA fibroblasts exposed to mitomycin C; they found similar but extremely low levels of UDS in both groups of cells. However, in both of these studies FA cells showed decreased cell survival and decreased rates of removal of cross-links when exposed to mitomycin C (Fujiwara and Tatsumi, 1977; Plooy et al., 1985). On the other hand, Gruenert and Cleaver (1985) showed decreased repair replication, measured using isopyknic gradient centrifugation, in a SV40 transformed FA cell line containing cross-links produced by 8-MOP plus UVA compared to normal cells. This same FA cell line showed levels of repair replication in response to angelicin which were similar to those of normal cells (Gruenert and Cleaver, 1985). All of these studies predated the establishment of complementation groups in FA cells. The present results are mainly in agreement with the studies on repair replication carried out by Gruenert and Cleaver (1985). Differences between our results and the above studies could be due to the genetic heterogeneity of the cell lines used or to the different types of DNA interstrand cross-links formed [i.e., pyrimidine-pyrimidine (8-MOP plus UVA) versus purine-purine (mitomycin C)].

Defect in ability of the FA endonuclease activities to incise psoralen plus UVA-damaged DNA

The present study shows that FA-A cells have an endonuclease activity, pI 4.6, and that FA-B cells have an endonuclease activity, pI 7.6, which are defective in their ability to incise DNA interstrand cross-links and monoadducts, respectively, produced by 8-MOP or angelicin plus UVA light. These results correlate with our studies of reduced levels of UDS in FA-A and FA-B cells in culture exposed to these agents. In addition, we have found that the FA-A but not the FA-B endonuclease activity, pI 4.6, has significantly reduced ability, compared to the normal endonuclease, pI 4.6, to incise a shuttle vector containing a single, site-directed nitrogen mustard interstrand cross-link (obtained from Dr. Edward Loechler) (unpublished observation). The FA-A, FA-B and normal endonuclease, pI 7.6, had little activity on this cross-linked substrate. This further indicates that FA-A but not FA-B cells have an endonuclease activity which is defective in its ability to incise DNA interstrand cross-links.

These studies are in agreement with those of Papadopoulo et al. (1987), and Averbeck et al. (1988), respectively, which suggest that FA-A cells show a greater reduction in ability to incise DNA-containing interstrand cross-links and FA-B cells are less efficient at incising DNA containing monoadducts produced by 8-MOP plus UVA. In both of these studies, the FA-B cell line used was derived from the same patient as the one used in the present study. The studies of Papadopoulo et al. (1987) also indicate that the repair defect in FA-A cells appears to be more pronounced than that in FA-B cells, which is in agreement with our findings on endonuclease activity in these cells.

Previous studies examining the viability of FA cells, where the complementation group (A versus B) is known, are also in agreement with our findings. Both Papadopoulo et al. (1987), treating fibroblasts with 8-MOP plus 365-nm UVA light, and Matsumoto et al. (1989), who administered mitomycin C to cells from the same lines examined in the present study, found viability of FA-A and FA-B cells to be approximately 40% and 66%, respectively, of that of similarly treated normal cells. When treated with 4,5',8-trimethylpsoralen plus 405-nm light so as to induce only monoadducts, however, FA-B cells were more sensitive, showing 20% of normal survival versus 33% for FA-A cells (Averbeck et al., 1988). Adjustment of treatment conditions so as to induce both monoadducts and cross-links produced between 17% and 33% of normal survival in the FA cells of the two complementation groups, depending on the ratio of the two types of adducts induced in cellular DNA (Averbeck et al., 1988).
The slightly greater reductions in enzyme activity which we have found, compared to the above reductions in cell survival, may be due to the complex mechanisms involved in the latter and the proportion of monoadducts to cross-links present in the DNA.

Dean (1989) recently reported that a FA-A cell line was able to repair 8-MOP plus UVA-induced cross-links in the dihydrofolate reductase (DHFR) gene. However, he also reported formation of 2–3 times as many adducts in FA-A cells as in normal cells. Since the number of adducts produced in his system was small, it is possible that significant repair of adducts occurred in normal cells during his treatment protocol with 8-MOP plus UVA, and that this repair was defective in FA-A cells. In contrast, Matsumoto et al. (1989) have shown that a FA-A cell line exhibits a deficiency in the removal of cross-links induced by mitomycin C in ribosomal RNA genes. Thus, the preponderance of evidence from several laboratories clearly indicates that there is a deficiency in cross-link removal in FA cells.

We have introduced by electroporation the normal endonuclease activities, p/4.6 and 7.6, into 8-MOP plus UVA-treated FA-A and FA-B cells, respectively, in culture and have been able to correct the repair defect in these cells (in preparation). The repair deficiencies in FA cells, therefore, appear to reside in the endonuclease activities we have isolated. The FA repair defect has also been corrected in other laboratories by transfection of FA-A and FA-B cells, exposed to various cross-linking agents, with plasmid and human DNA (Buchwald et al., 1987; Shaham et al., 1987; Moustacchi et al., 1989) and HeLa mRNA (Digweed and Sperling, 1989).

Decreased affinity, or rate of association, of the FA endonuclease activities for 8-MOP plus UVA-irradiated DNA

Kinetic analysis indicates that the defect in the FA-A endonuclease activity, p/4.6, and the FA-B endonuclease activity, p/7.6, lies in their ability to associate with damaged substrate. Both normal endonuclease activities show a decrease in $K_m$, with little or no change in $K_{cat}$, indicating an increase in affinity for and/or rate of association with psoralen plus UVA-treated non-nucleosomal DNA. However, the FA-A endonuclease activity, p/4.6, and the FA-B endonuclease activity, p/7.6, fail to show as great a decrease in $K_m$ as do the corresponding normal endonucleases, indicating that their affinity for and/or rate of association with this damaged DNA is less than that of the corresponding normal endonuclease activities. The results correlate with our studies on decreased activity of these endonucleases on 8-MOP plus UVA-treated DNA. The fact that there were decreases in affinity of these FA endonucleases for damaged DNA and that the normal, FA-A and FA-B endonuclease samples assayed contained similar amounts of protein further suggests that it is very unlikely that the decreases we observed in the activity of these FA endonucleases were due to quantitative differences in the amount of endonuclease obtained from similar numbers of cells from the respective cell lines in culture.

Nucleosome structure enhances FA and normal endonuclease activity on psoralen plus UVA-damaged DNA

The present study indicates that the FA endonuclease activities were not defective in their ability to interact with damaged nucleosomal DNA. Both of the FA-A and FA-B endonuclease activities, p/4.6 and 7.6, showed approximately the same 2.5-fold increase in activity on core nucleosomal (minus histone H1) DNA treated with 8-MOP or angelicin plus UVA light as was observed with the two normal endonucleases. The presence of histone H1 reduced this increase, which is consistent with the proposed role of histone H1 in the condensation of chromatin (McGhee and Felsenfeld, 1980; Igo-Kemenes et al., 1982; Klingholz and Stratling, 1982; Watanabe, 1984), making it less accessible to endonucleolytic attack. This increase in endonuclease activity, which was dependent upon nucleosome assembly, did not correlate with a change in the number of psoralen adducts, since the number of adducts was reduced approximately 50% and 60% on core and total nucleosomal DNA, respectively (Parrish and Lambert, 1990). Our studies indicate that associated with each endonuclease activity is an accessibility factor, which is a protein which makes the psoralen adducts on the nucleosomal
DNA more accessible to endonucleolytic attack (Parrish and Lambert, 1990). Our studies also indicate that the endonuclease and chromatin-interacting protein form part of a complex which is involved in the initial damage recognition/incision step in the repair process (Lambert et al., in preparation).

We have examined cells from another genetic disease, xeroderma pigmentosum, complementation group A (XPA), (Parrish and Lambert, 1990) which are also defective in ability to repair damage produced by 8-MOP plus UVA (Kraemer et al., 1987; Lambert and Lambert, 1987, Cleaver and Kraemer, 1989). In XPA cells neither of the endonuclease activities, pIs 4.6 and 7.6, showed a defect in activity on psoralen plus UVA-damaged naked DNA (Lambert et al., 1988). However, both showed a defect in their ability to interact with damaged nucleosomal DNA (Lambert and Parrish, 1989; Parrish and Lambert, 1990). These studies indicate that XPA cells are defective in a protein associated with the endonuclease, which is necessary for interaction of this enzyme with damaged nucleosomal DNA (Parrish and Lambert, 1990). In contrast, the present results indicate that the FA-A and FA-B endonuclease activities are not defective in their interaction with psoralen plus UVA-damaged nucleosomal DNA but rather are defective in their ability to incise damaged naked DNA. Associated with this defect is a reduced affinity and/or rate of association of these endonuclease activities with 8-MOP plus UVA-treated naked DNA. The defect in FA-A cells is greatest in the endonuclease activity, pI 4.6, which recognizes DNA interstrand cross-links, and, in FA-B cells it is greater in the endonuclease activity, pI 7.6, which recognizes the psoralen monoadduct. Whether this defect lies in the recognition or incision step, or both, is currently under investigation.

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