An altered apurinic DNA endonuclease activity in group A and group D xeroderma pigmentosum fibroblasts

(xeroderma pigmentosum/de Sanctis-Cacchione syndrome/endonuclease/depurinated DNA/DNA repair)

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ABSTRACT Enzyme activity upon depurinated DNA was measured in extracts of cultured fibroblasts from xeroderma pigmentosum patients. Cell lines from complementation groups A, B, C, E, and the XP-variant had slightly reduced levels of activity, but cell lines from complementation group D had one-sixth of the normal activity. An altered pH dependence and a higher apparent Km for substrate in D-cell lines indicate that the remaining activity is also qualitatively different from the activity found in normal cells. A higher Km was also found in cell lines from the A-complementation group but not in cell lines from the C-complementation group. These defects in apurinic DNase might account for the neurological disorders in patients from the D- and the A-complementation groups.

Xeroderma pigmentosum (XP) is a genetic disease which renders the afflicted individual sensitive to sunlight. Exposed areas of the skin develop various forms of skin tumors and metastatic epitheliomatosis often causes death at an early age. A more serious form of the disease, the de Sanctis-Cacchione syndrome, is accompanied by severe neurological disorders (1).

Cell lines developed from skin biopsies of these individuals have been reported to be defective in excision repair of DNA damage induced by ultraviolet light or by various chemical agents (2, 3). This defect is inferred from a reduction in the overall rate of DNA repair synthesis, and it remains unclear at which step the repair pathway is affected. A few XP-cell lines have been tested for defects in early steps of excision repair: two were shown to be unable to excise pyrimidine dimers from DNA (4), and another one was reported to be defective in the first step of repair, the excisionolytic cleavage presumably near UV lesions of the DNA (5). Somatic cell hybridization studies have recently shown that mutations which lead to the XP phenotype fall into five or six complementation groups (6, 7). This number exceeds the number of steps postulated for excision repair, suggesting that the repair pathway is regulated in a very complex manner or that there exist multiple, or branched pathways of repair.

Several enzyme activities believed to be involved in excision repair have been measured in extracts of XP-cell lines. Pedrini et al. (8) found no significant differences in the levels of polynucleotide ligase activity, DNA polymerase, single-strand exonuclease and double-strand endonuclease. Bacchetti et al. (9) compared excisionolytic activity specific for UV-irradiated DNA in normal and two XP-cell lines and detected no difference. Under their assay conditions the major UV-specific endonuclease activity in fibroblast extracts did not cleave the DNA near pyrimidine dimers, but at other undetermined lesions. In an attempt to identify excision repair endonucleases we have also found that extracts from a normal cell line and from the XP-cell lines CRL 1157 and CRL 1160 (see Materials and Methods) contained equal levels of an endonuclease activity which preferentially cleaves UV-irradiated DNA at sites which are about 100 times less frequent than the number of pyrimidine dimers in the DNA. However, these studies led us also to compare endonuclease activity with depurinated DNA as a substrate in extracts from normal and XP fibroblasts. Such an activity has been previously reported in many microorganisms, plants and mammals (10-13).

MATERIALS AND METHODS

Growth of Cells and Preparation of Extracts. Fibroblast cell lines from skin biopsies were obtained from the American Type Culture collection (ATCC). Strain numbers, complementation groups and relevant properties are summarized in Table 1. Cells were grown at 37° in 32 ounce prescription bottles with 50 ml of Dulbecco’s modified Eagle’s medium (Gibco), supplemented with 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes-HCl) (pH 7.4), 0.4 mM NaHCO3, 2.5 mM NaCl, 10% fetal calf serum (Irvine Scientific), and 1% Antibiotic-Antimycotic solution (Gibco). Confluent cells were “split” 1:2 after treatment with trypsin, and extracts were prepared from cells grown in 0.5 gallon roller bottles with 100 ml of medium.

For extraction, cells were suspended in 10 ml of phosphate-buffered saline (25 mM KPO4, pH 7.0; 0.15 M NaCl; 0.015 M sodium citrate) by scraping from the bottle surface, and washed twice in phosphate-buffered saline by centrifugation and resuspension. Finally, they were resuspended in 1 ml of 25 mM glycyglycine-NaOH buffer (pH 7.0) and stored in liquid nitrogen. For extraction, the cell suspension was thawed and sonicated six times for 5 sec each with a Biosonic sonicator (needle probe, 50 W). The sonicate was centrifuged for 50 min at 50,000 rpm in a Beckman Type 50 rotor and the supernatant was dialyzed for 8 hr against 25 mM glycyglycine buffer (pH 7.0) and stored in liquid nitrogen. One roller bottle gave 2 to 7 × 107 cells and 0.5–1.5 mg of extract protein.

endonuclease Assay. Unless otherwise indicated, reaction mixtures (0.05 ml) contained 25 mM glycyglycine (pH 7.8), 10 mM MgCl2, and 0.1 mM PM2 [3H]DNA nucleotide, a 5-fold dilution of depurination buffer introduced with the depurinated DNA, and 0.05–0.30 μg of protein. The final pH of the reaction mixture was 7.5. After incubation for 10 min at 37°, 0.6 ml of 0.01% sodium dodecyl sulfate, 2.5 × 10−4 M EDTA, at pH 7.0, was added and the DNA was extracted with 0.5 ml of chloroform-octanol (9:1 vol/vol). Two hundred microliters of 0.3 M K2HPO4·KOH, at pH 12.3 (measured at 0.3 M with a GK 2302 C electrode...
from Radiometer, Copenhagen) was added; then, after 7 min at 25°C, 100 µl of 1 M KH₂PO₄·HCl, at pH 4.0 was added. This treatment denatures nicked PM2 DNA, but not covalently closed circular PM2 DNA molecules (14). Two hundred microliters of 5 M NaCl and 5 ml of 1 M NaCl, 50 mM Tris·HCl (pH 8.2) were added successively and the solution was finally filtered through a Schleicher & Schuell type B-6, 0.45 µm pore size filter, which selectively retains denatured DNA. The filters were washed with 5 ml of 0.3 M NaCl, 0.03 M sodium citrate, dried, and counted in a liquid scintillation counter with 4 ml of scintillation fluid.

The number of nicks introduced was calculated by assuming that the distribution of target sites among DNA molecules follows a Poisson distribution and that each site has the same probability of becoming nicked during the enzyme reaction. From the fraction of molecules which contain no nicks (U), the average number of nicks per DNA molecule, μ, can then be calculated from the equation U = e^-μ. DNA was assumed to contain 18,000 nucleotides (15) and total DNA present in a reaction was determined either by measuring the radioactivity of an aliquot of the chloroform-octanol supernatant or by following the procedure for the determination of nicked DNA with 0.3 M KH₂PO₄·KOH, at pH 13.2, instead of 12.3. The higher pH also irreversibly denatures un nicked DNA molecules. Nicking levels were corrected for the nicking which occurs during the preparation of apurinic DNA, and the exposure to alkali during the nick determination. This blank amounted to an average of 0.3 to 0.4 nicks per molecule. A unit of endonuclease activity catalyzes 1 pmol/min of nicks.

Preparation of Apurinic PM2 DNA. Phage PM2 was grown on the Pseudomonas thymidine auxotroph, Bal 31-14, in medium containing [⁵⁷S]thymidine (10 µg/ml, 2 mCi/liter), then purified by CsCl gradient centrifugation as described by Espejo and Canedo (15), except that the dextran-polyethylene glycol step was replaced by centrifugation for 3 hr at 45,000 × g in a Spinco Type 21 rotor to collect the phage. DNA was extracted from the purified phage according to Espejo et al. (16), and depuration was accomplished as described by Lindahl and Anderson (17) by incubating the DNA at 70°C for 15 min at a concentration of 0.5 mM nucleotide in 0.1 M NaCl, 0.01 M sodium citrate, and 0.01 M Tris, at pH 5.2 adjusted with HCl. The number of apurinic sites was determined by exploiting the alkali-lability of the phosphodiester bonds at either side of the depurinated site. An aliquot of apurinic DNA was incubated in 0.15 M KH₂PO₄·KOH, at pH 12.3, at 25°C for at least 2 hr, neutralized with 1 M KH₂PO₄·HCl, then the number of nicks introduced were determined with a nitrocellulose filter as described above. The hydrolysis of apurinic sites under these conditions was complete, whereas normal DNA was not affected by the treatment. DNA used in these studies contained 2.2 alkali-labile sites per molecule.

Other Methods. Nucleotide rendered acid-soluble during a reaction was determined by adding 50 µl of 10 mg/ml of bovine serum albumin and 250 µl of 7% trichloroacetic acid to a 150 µl aliquot of chloroform-octanol supernatant. After 5 min at 0°C, the precipitate was removed by centrifugation and the radioactivity contained in 300 µl of the supernatant was determined.

Phosphatase assays (100 µl) contained 50 mM Tris·HCl, at pH 7.5, 10 mM glycine-NaOH, at pH 7.0, 2 mM of dithiothreitol, 1 mM AMP, and 40 µl of diluted extract containing 8-40 µg of protein. After 30 min at 37°C, inorganic phosphate released was measured by the method of Chen et al. (18). A unit of activity releases 1 nmol/min of inorganic phosphate.

Protein was determined by the procedure of Lowry et al. (19), and PM2 DNA concentration was determined by measuring the absorbance at 260 nm and assuming a molar extinction of 6.5 × 10⁵.

RESULTS

Some properties of the endonuclease activity

Endonuclease activity upon apurinic DNA in fibroblast extracts was assayed with depurinated duplex circular DNA of phage PM2 by the nitrocellulose filter assay described in Materials and Methods. Both the initial rate and limit of the reaction were proportional to the amount of extract present (Fig. 1). No acid-soluble material was released during the reaction, and DNA which had not been depurinated was inert, indicating that nonspecific endonuclease is not detectable in our assay. Similarly, no nicking occurs when depurinated DNA is incubated without extracts. The proportionality between the limit of the reaction and the amount of extract present suggests that the reaction does not terminate because of limiting substrate, but rather because of a limited turnover of the enzyme.

Treatment with saturating amounts of extract removes all of the alkali-labile sites from the DNA. The enzyme activity is maximal with 10 mM MgCl₂⁺; with 5 mM or 20 mM MgCl₂⁺ the activity is reduced by a factor of 5 and 7, respectively. Fifteen percent of optimal activity is observed in the absence of divalent cation. 2-Mercaptoethanol or dithiothreitol does not affect the activity.

Comparisons between normal and XP-fibroblasts

The initial velocities of the endonuclease activity in extracts from skin fibroblasts of the five known XP-complementation groups (6) and of the "XP-variant" [a cell line from a patient who displays the symptoms of the disease but whose cells are...
Table 1. Enzyme levels in cell lines from various XP patients

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Complementation group*</th>
<th>UV-induced DNA repair rate (% of normal)*</th>
<th>Endonuclease activity on depurinated DNA (units/mg)</th>
<th>Average endonuclease activity on depurinated DNA (% of normal)</th>
<th>Phosphatase activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRL 1125</td>
<td>Normal</td>
<td>100</td>
<td>384</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>CRL 1126</td>
<td></td>
<td>100</td>
<td>668</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>CRL 9249</td>
<td></td>
<td>100</td>
<td>420</td>
<td>88</td>
<td>88</td>
</tr>
<tr>
<td>CRL 1223</td>
<td>A</td>
<td>2</td>
<td>366</td>
<td>60</td>
<td>64</td>
</tr>
<tr>
<td>CRL 1261</td>
<td></td>
<td>100</td>
<td>221</td>
<td>81</td>
<td>81</td>
</tr>
<tr>
<td>CRL 1199</td>
<td>B</td>
<td>3-7</td>
<td>242</td>
<td>49</td>
<td>70</td>
</tr>
<tr>
<td>CRL 1167</td>
<td>C-heterozygote</td>
<td>15-25</td>
<td>278</td>
<td>57</td>
<td>52</td>
</tr>
<tr>
<td>CRL 1166</td>
<td></td>
<td>25-55</td>
<td>195</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td>CRL 1161</td>
<td></td>
<td>25-55</td>
<td>206</td>
<td>43</td>
<td>68</td>
</tr>
<tr>
<td>CRL 1158</td>
<td></td>
<td>25-55</td>
<td>227</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>CRL 1200</td>
<td>D</td>
<td>25-55</td>
<td>105</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>CRL 1157</td>
<td></td>
<td>25-55</td>
<td>62</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>CRL 1160</td>
<td></td>
<td>25-55</td>
<td>72</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>CRL 1259</td>
<td>E</td>
<td>100</td>
<td>198</td>
<td>40</td>
<td>52</td>
</tr>
<tr>
<td>CRL 1262</td>
<td>XP-variant</td>
<td>100</td>
<td>204</td>
<td>42</td>
<td>68</td>
</tr>
</tbody>
</table>

Assays were described in Materials and Methods. The concentration of apurinic sites used in these assays is about ten times the $K_m$ of the activity in extracts from normal cells. CRL 1167 was derived from the father of the donors of CRL 1166, CRL 1161, and CRL 1158. The CRL 1161 and CRL 1158 lines are from identical twins. In the D group CRL 1157 and CRL 1160 were derived from sisters, and CRL 1200 from an unrelated individual. CRL 9249 was derived from a foreskin; all others were from skin biopsies. The value for enzyme activity in CRL 1200 is the average from three extracts (passes 8, 9, and 10), whereas that of CRL 1157 is the average of two extracts (passes 3 and 9).

*The values for DNA repair rates and the assignment of complementation groups are from Kraemer et al. (1), Day (21) and de Weerd-Kastelein (7). The XP-variant CRL 1262 is described by Lehmann et al. (19).

not deficient in DNA repair (20)] were compared (Table 1). Normal cell lines had an average specific activity of 490 units/mg of extract protein. Extracts from fibroblasts from the XP patients as well as of a probable heterozygote, CRL 1167 (the father of the three representatives of the C-complementation group), all had somewhat less activity. The decreased activity was especially pronounced in the D-complementation group, which had roughly one-sixth of the average normal value. Appropriate mixing experiments provided no evidence for the presence of active inhibitor in the D-group extracts. Cell lines CRL 1157 and CRL 1160 originate from siblings, whereas line CRL 1200 originates from an unrelated patient.

Phosphatase activity with AMP as substrate was also measured in order to determine if an unrelated enzyme activity also differed between cell lines. In contrast to the endonuclease, the average phosphatase activity in the different complementation groups was normal.

To distinguish whether the lower activity in extracts of some of the XP-cell lines was due to lower amounts of enzyme or to an altered enzymatic activity, we compared some of the properties of the activities. In glycglycine buffer the activity in extracts from normal cells had a very pronounced pH optimum at pH 7.5 with shoulders at pH 8.0 and 8.3, indicating that there may be several enzyme species present in the extract (Fig. 2). In contrast, extracts from cells belonging to the D-complementation group show an approximately constant activity over the pH range tested (Fig. 2). Cell lines from all other complementation groups showed a pH dependence similar to that of the normal cell lines (some data not shown).

For further documentation of an altered endonuclease activity in cell lines from the D-complementation group, we compared the DNA apparent $K_m$ values at the pH 7.5 optimum (Table 2). The apparent $K_m$ values of the activity in D-cell lines were about 6-fold higher than in normal cell lines. Surprisingly, apparent $K_m$ values averaging 9-fold higher than normal were found in the two cell lines belonging to the A-complementation group. The $K_m$ values of the cell lines from the C-complementation group were the same as from normal cells. Patients from complementation groups A and D are similar in that they exhibit severe impairment of the nervous system and low levels of host cell reactivation.

FIG. 2. Dependence of initial velocity upon pH. Reactions were as described in Materials and Methods in glycglycine-NaOH buffer. The pH values are of the final reaction mixture.
Table 2. Phenotypes and apparent $K_m$ values from extracts of cells of individuals of various XP-complementation groups

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Complementation group</th>
<th>Neurological complications*</th>
<th>Host-cell reactivation of UV-damaged DNA (% of normal)†</th>
<th>$K_m$ [nM depurinated sites]</th>
<th>Average $K_m$ of group [nM depurinated sites]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRL 1125</td>
<td>Normal</td>
<td>–</td>
<td>100</td>
<td>1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>CRL 9249</td>
<td></td>
<td></td>
<td></td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>CRL 1261</td>
<td>A</td>
<td>+</td>
<td></td>
<td>6.4</td>
<td>9.4</td>
</tr>
<tr>
<td>CRL 1223</td>
<td></td>
<td></td>
<td>3.4</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>CRL 1166</td>
<td>C</td>
<td>–</td>
<td></td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>CRL 1161</td>
<td></td>
<td></td>
<td>35</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>CRL 1200</td>
<td>D</td>
<td>+</td>
<td></td>
<td>7.3</td>
<td>6.2</td>
</tr>
<tr>
<td>CRL 1157</td>
<td></td>
<td></td>
<td>3.3</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>CRL 1160</td>
<td></td>
<td></td>
<td>3.3</td>
<td>5.5</td>
<td></td>
</tr>
</tbody>
</table>

The reaction velocities were measured at five different substrate concentrations with three different enzyme levels. The apparent $K_m$ values were then calculated by the regression method of Wilkinson (26). In order to obtain the apparent $K_m$ in nM of PM2 DNA molecules, the values should be divided by 2.2, the average number of depurinated sites per molecule.

* From Kraemer et al. (1).
† From data of Day (21) with UV-irradiated adenovirus.

of UV-inactivated adenovirus (21) in addition to the classical XP symptoms.

**DISCUSSION**

Apurinic endonuclease activity is defective in extracts from cells of XP-complementation groups A and D. Extracts from both groups were found to have a significantly higher $K_m$ for substrate than normal cells, and, in the case of the D-cell lines, to have a 6-fold reduction of the enzyme activity. Cell lines from the C-complementation group have the same $K_m$ as normal cells and only a 2-fold lower enzyme level, a reduction which might not be statistically significant. Only slight reductions of the enzyme level were found in cell extracts from the B and E group and the XP-variant, but the $K_m$ has not yet been determined for these cell lines.

A change in $K_m$ is usually attributed to a mutation in the structural gene for a single enzyme, but could also be due to a mutation in a regulatory gene for one of a set of related enzymes, presumably in our case in that member with the lowest $K_m$. Multiple enzyme forms which have apurinic endonuclease activity have been observed in *Escherichia coli* (22), and we have found that human placenta contains several apurinic endonuclease activities which can be separated by column chromatography (S. Linsley, E. E. Penhoet, and S. Linn, unpublished).

The involvement of apurinic endonuclease in DNA repair has been documented in *E. coli*, where mutants were isolated which are partially deficient in apurinic endonuclease (endonuclease II) (13). The most deficient of these mutants, which has a residual activity of 12%, is sensitive to methylmethane sulfonate, mitomycin C and, to a lesser extent, ultraviolet- and γ-irradiation. We are presently determining whether the XP-cell lines with an altered apurinic endonuclease have an increased sensitivity to methylmethane sulfonate and mitomycin C. We found that in the D-cell line, CRL 1160, the rate of methylmethane sulfonate-induced repair synthesis is reduced 2-fold, a value similar to the reduction of UV-induced synthesis (Table 1).

The cell lines of the A and D group which are defective in apurinic endonuclease have a very low host-cell reactivation of UV-irradiated adenovirus, whereas in the C-cell lines, where we find an essentially normal apurinic endonuclease, the host-cell reactivation is still 35% of normal. This correlation seems to suggest that apurinic endonuclease might take part in the repair of UV damages. However, we observe that purified enzyme has very little activity on UV-irradiated DNA versus depurinated or alkylated DNA, indicating that the apurinic activity is required for the excision of only a minority, if any, of the lesions introduced by UV-irradiation.

The existence of such a minor class of UV lesions might be implied by the difference between host-cell reactivation of UV-irradiated adenovirus and the rate of repair replication encountered in the cell lines from the D-complementation group (21). Host-cell reactivation in these cell lines is very low, despite the high residual repair replication. Evidently repair replication levels might not necessarily reflect the efficiency of repair in a biological sense.

A major role of an apurinic DNase is likely to be the repair of DNA depurinated sites that arise spontaneously at a rate of about 10 per min per cell (23). Because of the constant generation of these sites an organism or cell might be expected to be viable with a reduced, but not abolished apurinic repair system. From the maximal velocity of the apurinic DNase in *vitro* of about 400 units/mg we can estimate that the maximal velocity per cell is about 107 cleavages per min. Since the depurination rate is many orders of magnitude lower than the maximal repair velocity, we expect that the steady state concentration of apurinic sites in a cell (the concentration at which the repair rate equals the depurination rate) is also several orders of magnitude lower than the $K_m$ we observed in *vitro*. At this low concentration, the steady state concentration of depurinated sites is expected to be proportional to both the $K_m$ and the maximal velocity of the repair endonuclease. We might therefore expect that the cell lines of the A- and D-complementation groups could contain 10- to 30-fold more apurinic sites in their DNA than do normal or cells from the C-complementation group. The neurological disorders encountered in patients from the A- and D-complementation groups might be a consequence of a higher level of potentially mutagenic apurinic sites contained in the DNA. Indeed, nerve cells which are not regenerated during the life span might be especially sensitive to an increased steady-state level of depurinated nucleotides. Neurological symptoms similar to those of the de Sanctis-Cacchione syndrome have been reported in the genetic disease ataxia telangiectasia (24). Cells from patients with this disease are sensitive to mitomycin C, methylmethane sulfonate and γ-rays, the same agents to which the apur-
The defect in apurinic DNase is likely to be responsible only for some of the symptoms of xeroderma pigmentosum, since other cell lines have been reported to be defective in photoreactivation (25) and in postreplication repair (20). The multiplicity of complementation groups coupled with the wide range of phenotypes and repair deficiencies of the cultured cell lines make it quite possible that the XP groups have multiple repair defects.

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