Apurinic DNA endonuclease activities in repair-deficient human cell lines

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Received 28 October 1977

ABSTRACT

Several autosomal recessive diseases are associated with apparent DNA repair defects in cell culture. It seemed likely that a defect in excision repair reported for ataxia telangiectasia cells might reflect a lack of apurinic endonuclease activity. We report here normal levels of apurinic endonuclease activity in extracts of cell lines derived from patients with ataxia telangiectasia, xeroderma pigmentosum (complementation group D), Cockayne dwarfism, Fanconi anemia and Bloom syndrome.

INTRODUCTION

A number of human cell lines have been demonstrated to have DNA repair defects in tissue culture. These lines have been derived from patients with autosomal recessive diseases frequently associated with photosensitivity, chromosomal aberrations and increased frequency of cancer. In a report on one such disease, ataxia telangiectasia (AT), Paterson and co-workers (1) noted that fibroblasts from patients demonstrated deficiency in repair of γ-radiation-damaged DNA. This study arose from observations of decreased colony formation in AT fibroblasts following γ-radiation (2). The conclusion of these studies was that AT fibroblasts are deficient in a step or steps in excision repair, based on endonuclease-susceptible sites remaining in the DNA of AT fibroblasts to a greater extent than normal fibroblasts following γ-radiation. However, AT cells have normal capacity to repair single-stranded breaks (2,3). Remsen and Cerutti (4) have recently reported normal excision of γ-radiation products of thymine by extracts from AT cells. AT fibroblasts also have been shown to be more sensitive to actinomycin D, mitomycin C and alkylation than normal cells (2,5). These observations led us to speculate that a possible defect in AT cells might be failure of an incision step in the excision repair process. Since AT cells are sensitive to γ-radiation and alkylating agents, both capable of producing apurinic/apyrimidinic sites...
in DNA, it seemed that an apurinic endonuclease defect was possible. Such a
defect was indicated in another study on xeroderma pigmentosum complementation
group D (XP-D) cells (6).

Endonucleases specific for apurinic sites have been purified from a vari-
ety of sources (7-15). For E. coli it appears that more than one such apur-
inic endonuclease is present. The major activity appears to be associated
with exonuclease III activity (16-18). Because of this and because no mutants
deficient in additional activities have been described it is uncertain what
role apurinic nucleases play in cell survival, although some mutants deficient
in the major activity show an increased sensitivity to alkylation and mitomycin
C. Interpretation of the action of this class of enzymes is further com-
plicated since it appears that they frequently may act in concert with N-
glycosidase activities capable of removing altered or damaged bases and may
coopurify with such enzymes (19-21). It seems reasonable to assume that these
two categories of enzymes participate in a concerted action to remove damaged
or mis-paired bases from DNA. This appears to be demonstrated best in the
case of DNA containing uracil.

Recently there was a report that one complementation group of xeroderma
pigmentosum (XP) fibroblasts has a markedly lowered apurinic endonuclease
activity (6). Because of obvious possibilities relating to the pathogenesis
of the disease state in repair-defective cell lines we have investigated the
levels of apurinic endonuclease, using the assay method of Kuhnlein et al.
(6), in AT and XP cell lines. In addition we have investigated other human
cell lines manifesting DNA repair deficiencies. Fibroblasts cultured from
patients with Bloom syndrome (BS) show an increased rate of sister chromatid
exchange (22). Fibroblasts from patients with Fanconia anemia (FA) demonstrate
increased sensitivity to mitomycin C and other cross-linking reagents, but
show normal levels of post-ultraviolet (UV) DNA repair synthesis (23-25).
Cockayne syndrome (CS) is a severe dwarfism characterized by mental retar-
dation and photosensitivity. Fibroblast cultures derived from such patients
show increased UV radiation sensitivity. Such cultures demonstrate a normal
rate of pyrimidine dimer removal and normal X-ray radiation sensitivity (26).
In all cases the apparent defects in DNA repair have been postulated to be at
least partially responsible for pathogenesis.

We report here an evaluation of apurinic endonuclease activity measured
in cells cultured from patients with the above diseases. We find normal lev-
els of apurinic endonuclease activity in all cases, including the D comple-
mentation group of XP. We have also monitored DNA polymerase levels in the
extracts and find normal levels in all cases, in agreement with the observations of others (27).

MATERIALS AND METHODS

Cell Culture: AT, FA, BS, CS, XP, and control fibroblast and lymphoblast cell lines were from the American Type Culture Collection (designations CRL); the Human Genetic Mutant Cell Repository, Camden, NJ (designations GM); and healthy adult controls (designations by initials) as shown in Table I. All cell lines are from unrelated patients, and fibroblasts and lymphoblasts were not tested on the same patient, except that CRL1160 and CRL1157 are from siblings. Fibroblasts were grown in Dulbecco modified Eagle medium with 10% fetal calf serum and lymphoblasts in RPMI-1640 with 20% fetal calf serum. All cell lines were grown without antibiotics and were tested monthly for mycoplasma contamination by the method of Hayflick (28). Fibroblasts were grown in 150 mm plates and harvested routinely 9 to 11 days after confluence was achieved. Each plate was washed three times with 10 ml of 0.15 M sodium chloride, 15 mM sodium citrate and 25 mM potassium phosphate (pH 7.0), and scraped with a Teflon policeman into 10 ml of the same solution. Lymphoblast suspension cultures of 50 ml were harvested by centrifugation during a phase of rapid growth at a cell density of 9-12 x 10^5 cells/ml and cell viability determined by nigrosin (29) to be 90-95% for the data shown. The cell pellets were washed twice in the above saline solution. Cell pellets for fibroblasts and lymphoblasts were suspended in 1 ml of 25 mM glycylglycine buffer (pH 7.0) and frozen in liquid nitrogen. For extraction the suspension was thawed, disrupted with sonic irradiation 6 times for 5 sec each, and then centrifuged for 50 min at 50,000 rpm in the 50 Ti rotor at 4°C. The supernatant fluid was used before or after dialysis against 25 mM glycylglycine (pH 7.0) for 8 hr at 4°C. Protein concentration was determined by the method of Lowry et al. (30). Fibroblasts were harvested from sub-confluence to 10 days post-confluence with no difference in specific activity of the endonuclease extract. No change in activity of the extract was noted on storage for 4 months at -80°C.

endonuclease Assays: Assays were performed according to the method of Kuhnein et al. (6) with the substitution of 3H-ØX174 RFI DNA, prepared according to Schekman et al. (31), for PM2 DNA. Host cells and Eam3, lysis-deficient ØX174 were a gift from Dr. David Denhardt, McGill University. To determine nicks introduced we assumed that all sites were available equally and distributed among the DNA molecules according to a Poisson distribution. The DNA was assumed to contain 11,000 nucleotides. Depurination of the DNA
was done according to the method of Hadi and Goldthwait (8) by incubating approximately 0.45 mM DNA in 50 mM sodium citrate (pH 3.5) for 30 min at 60°. At the end of the depurination the DNA was added directly to reaction mixtures. The total number of sites introduced was determined by a 2 hr incubation of a DNA sample at pH 12.3 as described (6). In this way the DNA containing apurinic sites was determined for each experiment. In these studies the DNA contained an average of 4.8 alkali-labile sites per molecule. The assay allows supercoiled DNA to renature and pass through the filter. Assays were corrected for nicks occurring in the DNA preparation and generated during depurination. This blank was on the average 0.3 to 0.4 nicks per molecule. We found no significant difference between the blank and a sample incubated 10 min in the reaction mixture without extract.

Reaction mixtures (0.05 ml) contained 25 mM glycylglycine (pH 7.8), 10 mM MgCl₂, 0.07-0.1 mM ØX174 RFI DNA and 0.05-0.15 µg of protein. At the end of the reaction (5 to 30 min at 37°) 0.6 ml of 0.01% sodium dodecyl sulfate - 0.25 mM EDTA (pH 7.0) was added and the DNA was extracted with 0.5 ml of chloroform: octanol (9:1). To the top phase was added 0.2 ml of 0.3 M potassium phosphate at pH 12.3. After 7 min at 25°, 0.1 ml of 1 M potassium phosphate at pH 4.0 was added for neutralization. Then 0.2 ml of 5 M NaCl and 5 ml of 1 M NaCl - 50 mM Tris (pH 8.2) were added successively and the solution was filtered through a Schleicher and Schuell BA-85 filter at 10 ml/min with washing of the filter by 5 ml of 0.3 M NaCl - 0.03 M sodium citrate. The filters were dried and counted in Liquifluor (New England Nuclear). A unit of endonuclease activity catalyzes 1 pmol/min of nicks (6).

DNA Polymerase Assays: Assays were performed with the extracts by incubation for 30 min at 37° in a 0.3 ml volume containing: 30 mM potassium phosphate (pH 7.4), 10 mM MgCl₂, 1 mM β-mercaptoethanol, 30 µM 5'-deoxyribonucleoside triphosphates (3H-TTP at 200 cpm/pmol), 30 µg of activated salmon sperm DNA (32) and 1 to 25 µg of cell extract. The reactions were stopped by addition of 3 ml of ice cold 10% trichloroacetic acid - 0.1 mM sodium pyrophosphate. After 5 min at 4° the precipitate was collected by washing over a glass-fiber filter (Whatman GF/C) with cold 10% trichloroacetic acid - 0.1 M sodium pyrophosphate, followed by 10 ml of cold 0.01 M HCl. The filters were dried and counted in Liquifluor (New England Nuclear).

Isokinetic Gradient Analysis: Evaluation of cleavage of ØX174 RFI DNA was also performed by neutral velocity sedimentation. The reaction was terminated with 60 µl of 0.1% sodium dodecyl sulfate - 2.5 mM EDTA (pH 7.0). The combined sample was loaded onto a 5 ml, 5-20% sucrose gradient containing
1 M NaCl, 100 mM EDTA and 50 mM Tris (pH 8.1). Centrifugation was performed in a SW 50.1 rotor at 20° for 16 min at 48,000 rpm. The gradients were collected and assayed for acid-precipitable DNA by the paper strip method (33). Sedimentation was from right to left as plotted.

RESULTS

We found that all of the cell lines tested had normal levels of apurinic endonuclease activity against ΦX174 RFI DNA in extracts (Fig. 1 and 2 and Table I). We determined relative rates by comparison to extracts from normal fibroblast or lymphoblast lines in all cases. Since the linearity of the nicking reaction as measured by the filter retention assay usually did not extend to greater than 70%, we routinely determined the activity from rate determinations. The values in Table I represent the average of at least two individual determinations on each cell line. The activity of the extracts was not changed by dialysis.

Fig. 1. Incision of Depurinated DNA by Fibroblast Extracts. Reaction conditions were described in "Materials and Methods." The open circles represent non-depurinated DNA with normal extract.

We had 40-50 nM of apurinic sites per assay, well above the Km value for the nuclease (6). The lack of linearity of the reaction could be due to several factors, including the presence of nucleases in the extracts. Using neutral isokinetic gradients, we inspected the reaction products at various extents, including stages beyond peak retention of DNA (Fig. 3). The analysis showed that the reaction proceeded to completion and overt digestion of the substrate to acid-solubility was present (loss of acid-insoluble
Fig. 2. Incision of depurinated DNA by fibroblast extracts. Reaction conditions were as described in "Materials and Methods".

TABLE I. Apurinic Endonuclease Activity of Cell Extracts

<table>
<thead>
<tr>
<th>Source</th>
<th>Cell Type</th>
<th>Endonuclease Activity (u/mg)</th>
<th>DNA Polymerase Activity (pmol TMP/mg)</th>
</tr>
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<tbody>
<tr>
<td>GM130</td>
<td>Control Lymphoblasts</td>
<td>250</td>
<td>2101</td>
</tr>
<tr>
<td>GM1056</td>
<td>Control Lymphoblasts</td>
<td>118</td>
<td>747</td>
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<td>GM892</td>
<td>Control Lymphoblasts</td>
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<td>GM1074</td>
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<td>133</td>
<td>644</td>
</tr>
<tr>
<td>GM1077</td>
<td>Control Lymphoblasts</td>
<td>193</td>
<td>N/D</td>
</tr>
<tr>
<td>GM1525</td>
<td>AT Lymphoblasts</td>
<td>109</td>
<td>760</td>
</tr>
<tr>
<td>GM716</td>
<td>AT Lymphoblasts</td>
<td>110</td>
<td>700</td>
</tr>
<tr>
<td>M.N.</td>
<td>Control Fibroblasts</td>
<td>229</td>
<td>200</td>
</tr>
<tr>
<td>S.G.</td>
<td>Control Fibroblasts</td>
<td>124</td>
<td>194</td>
</tr>
<tr>
<td>R.N.</td>
<td>Control Fibroblasts</td>
<td>201</td>
<td>228</td>
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<tr>
<td>D.P.</td>
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<td>249</td>
<td>161</td>
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<td>CRL1347</td>
<td>AT Fibroblasts</td>
<td>169</td>
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<td>CRL1343</td>
<td>AT Fibroblasts</td>
<td>190</td>
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<tr>
<td>GM648</td>
<td>AT Fibroblasts</td>
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<td>633</td>
</tr>
<tr>
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<td>173</td>
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<td>GM1492</td>
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<td>123</td>
</tr>
<tr>
<td>GM739</td>
<td>CS Fibroblasts</td>
<td>177</td>
<td>203</td>
</tr>
</tbody>
</table>

Cells were grown, harvested and assayed by the filter retention assay as described in "Materials and Methods." Endonuclease units were determined by the method of Kuhnlein et al. (6). The mean endonuclease activity for the five control lymphoblast lines was 160±55 u/mg. For the four control fibroblast lines the mean was 201±55 u/mg. All figures are the average of at least two determinations. Values indicated N/D were not done.
Fig. 3. Extent of Incision by Lymphoblast Extracts. Cell line GM130 was used under reaction conditions as described in "Materials and Methods." At the points indicated reactions were terminated for analysis by isokinetic gradient. Times were: A=0 min; B=2 min; C=20 min.

Material). Presumably this was due to exonucleolytic action at the nicks introduced into the apurinic substrate, or endonucleases specific for single-stranded regions since non-depurinated substrate was not digested by an equivalent amount of the extract.

For security in analyzing relative activities of extracts we feel it better to include comparison of normal and repair-deficient extracts in the same experiment than to rely upon determination of units. Results identical to those in Fig. 1 and 2 were obtained with other control preparations.

The activity of endonuclease activity per µg of protein appears approximately the same for lymphoblasts and fibroblasts. Lymphoblast extracts were susceptible to decreased activity with decreasing viability, a problem we encountered twice (once with control cells and once with AT cells). In such cases we observed very low levels of DNA polymerase activity. We did not encounter any fibroblast extracts displaying a lack of endonuclease activity. No extract tested displayed detectable endonuclease activity against nondepurinated DNA by the filter retention assay. Our observations of normal levels of DNA polymerase activity in all the cell lines tested are in agreement with the reports of others (26).

We confirmed our observations on the apurinic endonucleolytic activity in the two cell lines of greatest interest by using neutral isokinetic
Fig. 4. Isokinetic Gradient Analysis of Incision by Normal and AT Fibroblasts. Cell lines S.G. and CRL1347 were used. Reaction conditions were as in "Materials and Methods."

Fig. 5. Isokinetic Gradient Analysis of Incision by Normal and XP Fibroblasts. Cell lines S.G. and CRL1157 were used. Reaction conditions were as in "Materials and Methods."
gradient analysis. By this analysis RFI DNA is converted to a distinguishable species after nicking. For both AT and XP-D cells the nicking activity per unit of protein was comparable to normal cells (Fig. 4 and 5). This was true for limited and extensive reactions. These results confirmed our results with the filter retention assay. In general we found a good correlation between these two methods of analysis at limited extents (<50%) of nicking. Thus there appeared to be no defect in apurinic endonuclease activity in either of these cell lines by two criteria.

DISCUSSION

The results presented here indicate that there is no detectable defect in apurinic endonucleases in any of the cell lines tested. This conclusion is based on assays of multiple representatives of each cell type and by two methods of assays in the case of AT and XP-D cells. Failure to demonstrate a defect does not rule out the possibility that multiple apurinic endonucleases are present and that our conditions did not detect the absence of a single activity. Our observation is in agreement with recently reported normal levels of excision of γ-ray-altered bases in AT cells by Remsen and Cerutti (4). Other reports (2,3) indicate that AT cells have a normal ability to repair single strand breaks. We also observe normal levels of DNA polymerase activities in AT cells. Several possibilities remain for a repair defect in AT cells: it is possible that some step prior to incision is defective; there may exist multiple enzymes for the steps involved in repair (e.g., endonucleolytic and synthetic) and our analysis has failed to detect the abnormality of one such form; or there may be a defect in handling a type of lesion unlike the one we introduced. Of course there may exist multiple complementation groups in a disease such as AT, representing several defects in the γ-ray repair process. Thus it is possible that in our selection of cell lines we did not obtain one defective in the enzymes we assessed, but that such defects may exist within the clinical phenotype. However, six unrelated AT lines were tested.

Our results do not support those of Kuhnlein et al. (6) with regard to the XP-D cell lines. Since both studies were done with fibroblasts it seems likely that loss of viability, easily monitored in fibroblasts, is not a likely basis for the difference. It still is possible that a variation in growth conditions or harvesting might account for the different result. One possibility that also bears consideration is that there may be multiple apurinic endonucleases, as reported for placenta (12).
and that our conditions of assay do not illustrate the deficiency of one species.

In general we have found fibroblasts to be a more reliable source for nuclease assay than lymphoblasts. This appeared to be due to decreased viability in lymphocytes grown to high density. We presume this represents decay of enzyme. Alternatively lymphoblast extracts may contain competing activities that mask the endonuclease activity, such as an exonuclease activity that degrades nicked circles. The extracts from either fibroblasts or lymphoblasts are remarkable for not nicking non-depurinated DNA.

ACKNOWLEDGMENTS

We are indebted to Sharon L. Bryan and Marguerite Hampton for excellent technical assistance. We thank Dr. S. Linn for helpful discussion. This work was supported by grants from the U.S.P.H.S. ALB is an Investigator of the Howard Hughes Medical Institute.

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