An Enzyme Activity in Normal and Ataxia Telangiectasia Cell Lines which is involved in the Repair of γ -Irradiation-Induced DNA Damage

Malcolm J. EDWARDS, Alexander M. R. TAYLOR and Gillian DUCKWORTH

Department of Cancer Studies, The Medical School, University of Birmingham, Birmingham B15 2TJ, U.K.

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An enzyme that enhances the activity of DNA polymerase I (EC 2.7.7.7) for γ -irradiated calf thymus DNA was demonstrated in cellular extracts of normal human fibroblasts and lymphoid-cell lines. This enzyme was found to be deficient in all cellular extracts of fibroblasts and lymphoid-cell lines examined from patients with the autosomal recessive disease ataxia telangiectasia. The activity in cellular extracts from normal fibroblasts was removed when heated to 100°C for 2 min or when the assay was performed at 4°C. No significant deficiency in primer-activating enzyme activity was observed in cell-free extracts of lymphoid lines from patients with xeroderma pigmentosum, Huntington's chorea or neurofibromatosis, or from an ataxia telangiectasia heterozygote.

γ -Irradiation of DNA is known to produce complex damage in base and sugar moieties, as well as scissions in the phosphodiester linkage. Investigations into the repair of base damage caused by γ -irradiation of mammalian DNA is still at an early stage, but they indicate that such lesions may be removed from normal cells. Patients with the autosomal recessive disorder ataxia telangiectasia show a hypersensitivity to the effects of ionizing radiation, and the sensitivity is reflected at the cellular level (Taylor *et al.*, 1975). It has been demonstrated that extracts from skin fibroblasts of ataxia telangiectasia patients remove γ -radiation-induced 5,6-dihydro-5,6-dihydroxythymine-type damage normally from bacteriophage DNA and chromatin isolated from skin fibroblasts of normal subjects and ataxia telangiectasia patients under aerobic conditions (Cerutti & Remsen, 1977). A decreased ability of some ataxia telangiectasia strains to undergo repair replication after exposure to γ -radiation under anoxic conditions has been reported (Paterson *et al.*, 1976). Furthermore, Paterson *et al.* (1976) observed that normal human fibroblasts are capable of removing γ -endonuclease-sensitive sites from DNA after γ -irradiation, but in fibroblasts from some ataxia telangiectasia patients the repair process was present in a smaller amount.

It has been demonstrated that DNA repair in γ -irradiated cells of *Bacillus subtilis* requires DNA polymerase (EC 2.7.7.7) and DNA ligase (EC 6.5.1.1) for the rejoining of radiation-induced single-

strand breaks (Noguti & Kada, 1975). The DNA polymerase has an absolute requirement for a 3'-hydroxy terminal as an initiation site. Landbeck & Hagen (1973) showed that radiation-induced single-strand breaks contain inhibitory 3'-terminals which are ineffective as primer templates for DNA polymerase I, and treatment of γ -irradiated DNA with exonuclease III resulted in the release of several nucleotides and partial restoration of template activity. Noguti & Kada (1975) have isolated an enzyme activity from *B. subtilis* which enhances the activity of DNA polymerase I for γ -irradiated DNA. This enzyme activity is an endonuclease. They have also demonstrated that a second enzyme exists in *B. subtilis*. This enzyme is an exonuclease, which enhances the activity of DNA polymerase I (EC 2.7.7.7) for γ -irradiated DNA. From their results it was suggested that this exonuclease may function by removing γ -irradiation-induced damage from DNA and so provide effective sites for repair by DNA polymerase I. Inoue *et al.* (1977) have demonstrated that a single fibroblast line from ataxia telangiectasia patients was deficient in enhancing the activity of purified *Micrococcus luteus* DNA polymerase I for γ -irradiated colicin-E1 DNA. The precise defect was not identified, but was not due to an apurinic-site-specific endonuclease. In the present paper the procedure of Inoue *et al.* (1977) has been modified and used to investigate the ability of lymphoid-cell lines from normal subjects and from patients with ataxia telangiectasia, xeroderma pigmentosum, neurofibromatosis and Huntington's

chorea, and two fibroblast-cell lines from ataxia telangiectasia patients, to enhance the activity of DNA polymerase I for γ -irradiated DNA.

Materials and Methods

Materials

Reagents were A.R. grade (BDH Chemicals, Poole, Dorset, U.K.). [$8\text{-}^3\text{H}$]dATP (10–30 Ci/mmol), [$\text{methyl-}^3\text{H}$]thymidine 5'-triphosphate (40–60 Ci/mmol) and [$\text{thymine-2-}^{14}\text{C}$]DNA from *Escherichia coli* (15–50 $\mu\text{Ci/mg}$) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Calf thymus DNA, dATP, dCTP, dGTP, TTP, ATP and *Micrococcus luteus* DNA polymerase were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Transformed lymphoid-cell cultures from ataxia telangiectasia and other patients were obtained from the Human Genetic Mutant Cell Repository Institute for Medical Research, Camden, NJ, U.S.A. Normal transformed lymphoid-cell cultures were obtained from Dr. M. Steel, M.R.C. Clinical and Population Cytogenetics Unit, Edinburgh, U.K. Ham's F10 and RPMI tissue-culture media were obtained from Flow Laboratories, Irvine, Scotland, U.K.

Culture of human cells

Human transformed lymphoid-cell lines of ataxia telangiectasia (GM1525/AT2BI, GM1526/AT8BI, GM717), xeroderma pigmentosum (GM1646), ataxia telangiectasia heterozygote (GM736), neurofibromatosis (GM1641), and Huntington's chorea (GM2080) patients and two from normal subjects (LIZ, DEW) were grown in RPMI medium (Flow Laboratories) supplemented with 10% (v/v) foetal calf serum under an air/ CO_2 gas phase (19:1) at 37°C. Fibroblasts from two normal (Con100BI, Con101BI) and two ataxia telangiectasia (AT5BI, AT7BI) patients were grown as monolayers in Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with 10% foetal calf serum at 37°C under an air/ CO_2 gas phase (19:1).

Preparation and irradiation of DNA substrate

Calf thymus DNA (500 $\mu\text{g/ml}$) (Sigma) was dissolved in 20 mM-NaCl/10 mM-Tris/HCl buffer, pH 8.0, and irradiated under aerobic conditions with a ^{60}Co γ -ray source at a dose rate of 20 krad/min with a total dose of 50 krad. All the DNA preparations were dialysed against 20 vol. of 20 mM-NaCl/10 mM-Tris/HCl buffer, pH 8.0, for 24 h and stored at -20°C .

Preparation of DNA polymerase

DNA polymerase I (DNA nucleotidyltransferase, EC 2.7.7.7) from *M. luteus* (Sigma) was purified by the method of Zimmerman (1966). The

activity of purified DNA polymerase was measured by the incorporation of radioactive deoxyribonucleoside triphosphate into a deoxyribonuclease-I-activated calf thymus DNA template, as described by Hamilton (1974).

The reaction mixture (0.2 ml) contained: 20 μmol of Tris/HCl, pH 7.8, 0.8 μmol of MgCl_2 , 0.1 μmol of 2-mercaptoethanol, 10 nmol of each deoxyribonucleoside triphosphate, [$8\text{-}^3\text{H}$]dATP (0.12 μCi), activated calf thymus DNA (5 μg) and 10 μl of DNA polymerase extract. After incubation at 37°C for 30 min the reaction mixture was cooled in ice, 20 μl of carrier DNA (2.5 mg/ml) was added, the DNA product was precipitated with cold 5% (w/v) trichloroacetic acid/10 mM- $\text{Na}_4\text{P}_2\text{O}_7$ (0.5 ml) and kept at 4°C for 20 min. The acid-insoluble product was collected on GF/C filters (Whatman) and washed with 5% trichloroacetic acid/10 mM- $\text{Na}_4\text{P}_2\text{O}_7$ (5 ml $\times$ 10 ml). Filters were counted for radioactivity in scintillation fluid [containing 10 g of 2,5-diphenyloxazole, 0.25 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 100 g of naphthalene in 1 litre of dioxan].

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951). Bovine serum albumin (fraction V) was used as a standard.

Endonuclease assay

Endonuclease activity was measured in a reaction mixture essentially the same as that used for the determination of DNA polymerase activity, except that 5 μg of [$\text{thymine-2-}^{14}\text{C}$]DNA from *E. coli* was substituted for calf thymus DNA. After a 30 min incubation period an ice-cold solution (1 ml) containing 7 mM- Na_2HPO_4 , 0.4 mM- NaH_2PO_4 and 1 mM- Na_2EDTA , pH 7.06, was added. The solution was heated for 5 min at 100°C and cooled rapidly to denature the DNA. The capacity of the DNA to be retained on a nitrocellulose filter was then determined by the method of Geiduschek & Daniels (1965).

Preparation of cell-free extract

Human fibroblasts or transformed lymphocytes (6×10^7 cells/ml) were harvested and washed twice in 0.01 M-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl. The cells were resuspended in 50 mM-Tris/HCl buffer, pH 7.4, containing 10 mM- MgCl_2 and 5 mM-2-mercaptoethanol, and sonicated at 4A for 30 s at 4°C (instrument from Ultrasonics). Particulate material was removed by sedimentation at 10000 g for 30 min (r_{av} , 4.2 cm). The supernatant was dialysed at 4°C against 20 vol. of 20 mM-Tris/HCl buffer, pH 7.4, containing 10 mM- MgCl_2 and 5 mM-2-mercaptoethanol. The non-diffusible material was centrifuged at 10000 g for 20 min,

the precipitate was removed and the supernatant was stored at -20°C .

Assay of the primer-activating enzyme

The primer-activating-enzyme activities of cell-free extracts from ataxia telangiectasia and control subjects were measured by a modification of the Noguti & Kada (1975) method. Dilution of the enzyme extracts was done with 20 mM-Tris/HCl, pH 7.4, containing 10 mM-MgCl₂ and 5 mM-2-mercaptoethanol. The enzyme extract (20–100 μg of protein) was mixed with 0.5 vol. of γ -irradiated (50 krad) or unirradiated calf thymus DNA (500 $\mu\text{g}/\text{ml}$ in 20 mM-NaCl/HCl buffer, pH 7.4) and incubated at 37°C for 30 min. The reaction was stopped by the addition of ice-cold distilled water and the tubes were kept at 4°C .

The activity of DNA polymerase I with the preincubated γ -irradiated and non-irradiated DNA (20 μg) was measured in a reaction mixture (0.3 ml) containing 20 μmol of Tris/HCl, pH 7.4, 0.8 μmol of MgCl₂, 0.1 μmol of 2-mercaptoethanol, 100 μg of bovine serum albumin (fraction V), 40 nmol each of dCTP, dGTP, dATP and ATP, and 20 nmol of [*methyl*-³H]TTP (0.25 $\mu\text{Ci}/\text{nmol}$). The reaction was initiated by the addition of 0.5 unit (1 $\mu\text{mol}/\text{min}$) of DNA polymerase I and the samples were incubated for 5 min at 37°C . The reaction was terminated by the addition of 0.1 M-Na₂EDTA (0.1 ml), carrier DNA (200 μg), 1 M-HCl/0.01 M-Na₄P₂O₇ (0.1 ml) and 1 M-HClO₄ (0.5 ml). The acid-insoluble material was collected on GF/F filters (Whatman), washed with 1 M-HCl/0.01 M-Na₄P₂O₇ (5 ml $\times$ 10 ml) and the acid-insoluble material was counted for radioactivity on the filters in Intra-Gel (Packard Instrument Co.).

The ability of the cell-free extracts to increase the activity of DNA polymerase I on γ -irradiated DNA was calculated from the equation:

$$\text{Enhancement of DNA polymerase activity} = (a - b) - (c - d)$$

where a = radioactivity of enzyme-treated γ -irradiated DNA, b = radioactivity of non-treated γ -irradiated DNA, c = radioactivity of enzyme-treated non-irradiated DNA, and d = radioactivity of non-enzyme-treated non- γ -irradiated DNA. The quantity $(c - d)$ represents the increase of DNA polymerase I activity caused by nucleases which non-specifically introduce nicks with 3'-hydroxy terminals into DNA (Noguti & Kada, 1975).

Results

Primer-activating enzyme activity in cell-free extracts of cells from normal and ataxia telangiectasia subjects

Fibroblasts. Cell-free extracts of fibroblasts from two ataxia telangiectasia patients (AT5BI, AT7BI)

and two normal controls (Con100BI, Con101BI) were assayed for their ability to enhance the priming activity of DNA polymerase I for γ -irradiated DNA. Both γ -irradiated (50 krad) and unirradiated calf thymus DNA were incubated separately with increasing concentration of cell-free extract from fibroblasts of normal or ataxia telangiectasia subjects and the activity of DNA polymerase I was measured as described in the Materials and Methods section.

Fig. 1 shows that the activity of DNA polymerase I for γ -irradiated DNA increased linearly with increasing concentration of cell-free extract of fibroblasts from normal (Con100BI, Con101BI) and two ataxia telangiectasia (AT5BI, AT7BI) subjects.

Fig. 1. Increase in DNA polymerase I activity after preincubation of γ -irradiated DNA (50 krad) with increasing concentration of cell-free extracts from fibroblast-cell lines from normal (○, Con100BI; □, Con101BI) and two ataxia telangiectasia (●, AT5BI; ■, AT7BI) subjects

Cells were grown as monolayers at 37°C in Ham's F10 medium supplemented with 10% foetal calf serum. Cells were harvested and washed twice in Dulbecco's phosphate-buffered saline. Washed cells (5×10^6 cells/ml) were resuspended in assay buffer (1 ml), which contained 10 mM-MgCl₂ and 5 mM-2-mercaptoethanol in 0.1 M-Tris/HCl buffer, pH 7.4, and sonicated for 30 s at 4°C . The sonicated cellular extracts were centrifuged at 20000 g for 20 min and the supernatant was dialysed extensively against assay buffer. The non-diffusible material was used to assay the primer-activating-enzyme activity as described in the Materials and Methods section. The results were expressed as the incorporation of [*methyl*-³H]TTP into acid-insoluble material (pmol).

However, the cell-free extracts (AT5BI, AT7BI) from ataxia telangiectasia patients had lower extents of template activation when compared with normal fibroblasts.

Lymphoid-cell lines. Cell-free extracts from lymphoid-cell lines were obtained from two ataxia telangiectasia (GM1525/AT2BI, GM1526/AT8BI) patients, an ataxia telangiectasia heterozygote (GM736), and from two normal subjects as controls. Fig. 2 shows that the preincubation of γ -irradiated DNA (50krad) with cell-free extracts from the ataxia telangiectasia heterozygote (GM736) and the two normal patients greatly enhanced the activity of DNA polymerase I for γ -irradiated DNA. The preincubation of γ -irradiated DNA with increasing concentration of cell-free extract from normal cells produced a concen-

Fig. 2. Increase in DNA polymerase I activity after preincubation of γ -irradiated DNA (50krad) with increasing concentrations of cell-free extracts from lymphoid cell-lines from normal (O, LIZ: Δ, DEW), ataxia telangiectasia (●, GM1525; ■, GM1526) and ataxia telangiectasia heterozygote (□, GM736) subjects

Washed cells (6×10^7 cells/ml) were suspended in assay buffer, which contained 10 mM-MgCl₂ and 5 mM-2-mercaptoethanol in 0.1 M-Tris/HCl, pH 7.4, and sonicated for 30 s at 4°C. The sonicated cellular extracts were centrifuged at 20000g for 20 min and the supernatant was dialysed at 4°C against 20 vol. of assay buffer. The non-diffusible material from the cellular extracts was incubated at 37°C for 30 min in the presence of γ -irradiated or non-irradiated calf thymus DNA. The priming activity of DNA polymerase I from *M. luteus* for the extract-treated γ -irradiated DNA was measured as described in the Materials and Methods section. The results were expressed as the incorporation of [methyl-³H]TTP into acid-insoluble material in 5 min at 37°C (pmol).

tration-dependent linear increase in the activity of DNA polymerase I for γ -irradiated DNA. However, the cell-free extracts from ataxia telangiectasia patients (GM1525, GM1526) had lower activity when compared with normal lymphoid-cell lines.

Table 1 shows the activity of DNA polymerase I for γ -irradiated DNA which had been preincubated with cell-free extracts (100 μg) from lymphoid-cell lines from ataxia telangiectasia (GM717, GM1525/AT2BI, GM1526/AT8BI), xeroderma pigmentosum (GM1646), neurofibromatosis (GM1641) and Huntington's chorea (GM2080) patients and from two normal subjects as controls. The means and standard deviations obtained from experiments each performed in triplicate were analysed statistically by analysis of variance. The results in Table 1 show that the activity of DNA polymerase I for γ -irradiated DNA that had been preincubated with cell-free extracts from neurofibromatosis, xeroderma pigmentosum or Huntington's chorea patients and an ataxia telangiectasia heterozygote were not statistically different from those with normal cells. In contrast, the activity of DNA polymerase I for γ -irradiated DNA that had been preincubated with cell-free extract from three lymphoid-cell lines from ataxia telangiectasia patients (GM717, GM1525, GM1526) were significantly different ($P < 0.005$) from those with normal cells. Negligible enhancement of DNA polymerase activity was observed when the γ -irradiated DNA had been pre-incubated with cell-free extract that had been heated at 100°C for 2 min or when the preincubation was carried out at 4°C.

Discussion

A repair replication defect has been reported in some fibroblast-cell strains from ataxia telangiectasia patients after γ -irradiation under anoxic conditions (Paterson *et al.*, 1976). Under the same conditions they have also reported that fibroblast-cell strains from three ataxia telangiectasia heterozygotes show intermediate radiosensitivity and defective repair replication, to an extent intermediate between those of normal and ataxia telangiectasia homozygotes. However, these three ataxia telangiectasia heterozygotes exhibit normal sensitivity to γ -irradiation under aerobic conditions (Paterson *et al.*, 1979).

Inoue *et al.* (1977) have demonstrated the existence of a DNA-repair enzyme activity in cell-free extracts of normal human fibroblasts. This enzyme when incubated in the presence of γ -irradiated colicin-E1 DNA enhances the rate of deoxyribonucleoside triphosphate incorporation into DNA. Using a single homozygous ataxia telangiectasia cell strain, they demonstrated a deficiency in this activity. Our results support the findings of Inoue *et al.* (1977). The ataxia telangiectasia

Table 1. Increase in DNA polymerase I activity after a preincubation of γ -irradiated DNA with a fixed concentration of cell-free extracts of lymphoid lines from normal subjects (LIZ, DEW) and from neurofibromatosis (GM1641), ataxia telangiectasia (GM717, GM1525, GM1526), ataxia telangiectasia heterozygote (GM736), xeroderma pigmentosum (GM1646) and Huntington's chorea (GM2080) patients

Both γ -irradiated and non-irradiated calf thymus DNA (20 μ g) were incubated for 30 min at 37°C in the presence of sonicated cell-free extracts (100 μ g) of the above lines. The reaction was terminated with ice-cold distilled water (200 μ l). The priming activity of DNA polymerase I from *M. luteus* for the extract-treated DNA was measured in triplicate as described in the Materials and Methods section. Primer-activating-enzyme activity was expressed as the incorporation of [methyl-³H]TTP into acid-insoluble material in 5 min at 37°C (pmol/mg of protein). The numbers in parentheses represent the numbers of values, each performed in triplicate, used in calculating the mean $\pm$ S.E.M.

Cell type	Primer-activating activity	
	(pmol/mg of protein)	(%)
Normal (average of two lines)	170.5 $\pm$ 4.32 (4)	100
Neurofibromatosis	160.5 $\pm$ 3.88 (4)	94.2
Ataxia telangiectasia		
(a) (GM717)	79.5 $\pm$ 5.10 (2)	46.6
(b) (GM1526)	89.0 $\pm$ 2.04 (4)	52.2
(c) (GM1525)	35.0 $\pm$ 1.80 (4)	20.5
Ataxia telangiectasia heterozygote (GM736)	149.5 $\pm$ 2.88 (5)	87.7
Xeroderma pigmentosum (GM1646)	160.0 $\pm$ 4.28 (3)	93.8
Huntington's chorea (GM2080)	164 $\pm$ 4.72 (3)	96.2

fibroblasts (AT5BI, AT7BI) and lymphoid-cell lines (GM717, GM1525/AT2BI, GM1526/AT8BI) are deficient in this enzyme activity. We have been unable to demonstrate a similar defect in the ataxia telangiectasia heterozygote (GM736) lymphoid-cell line.

It is important to note that not all ataxia telangiectasia cell strains were reported to be defective in anoxic repair replication (Paterson *et al.*, 1977). However, all cell strains so far examined are radiosensitive, as measured by colony-survival assays, and the cell-survival curves after γ -irradiation are similar (Taylor *et al.*, 1975; Weichselbaum *et al.*, 1977; Cox *et al.*, 1978; Paterson *et al.*, 1979). Our results show that the ataxia telangiectasia fibroblast-cell lines (AT5BI, AT7BI) are deficient in an enzyme activity which removes γ -irradiation-induced damage in DNA under aerobic conditions. Paterson *et al.* (1977) have demonstrated that under anoxic conditions these two ataxia telangiectasia fibroblast-cell lines (AT5BI, AT7BI) were repair-replication proficient. Under anoxic conditions the ratio of γ -endonuclease-susceptible sites to strand breaks is greater, whereas under aerobic conditions the number of strand breaks increases 2–3-fold. Therefore it is possible that cell lines derived from different ataxia telangiectasia patients have a deficiency in more than one repair enzyme.

It is not clear what role these repair defects play in the lethal effects of γ -irradiation. There seems to be an analogous situation in xeroderma pigmentosum,

where several repair enzymes are defective in single strains (Strauss *et al.*, 1979). Since both ataxia telangiectasia and xeroderma pigmentosum are single gene disorders, it is possible that there is a more fundamental defect which exerts its effect over the regulation of repair-enzyme activities.

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