Vol. 149, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1987 Pages 233-238

A DEFECT IN DNA TOPOISOMERASE II ACTIVITY IN ATAXIA-TELANGIECTASIA CELLS

Rahmah Mohamed[1], Surinder Pal Singh[2], Sharad Kumar[3], and Martin F. Lavin[2]

[1] Biochemistry Department, Faculty of Science, UKM, Banji 43600, Selangor, Malaysia

[2] Department of Biochemistry, University of Queensland, Brisbane, 4067 Australia

[3] CSIRO, Australian Animal Health Laboratory, Geelong, Victoria, 3220 Australia

Received September 14, 1987

SUMMARY: DNA topoisomerase type I and II activities were determined by serial dilution in nuclear extracts from control and ataxia-telangiectasia lymphoblastoid cells. Topoisomerase I activity, assayed by relaxation of supercoiled plasmid DNA, was found to be approximately the same in both cell types. In order to remove interference from topoisomerase I, the activity of topoisomerase II was measured by the unknotting of knotted P4 phage DNA in the presence of ATP. The activity of topoisomerase II was markedly reduced in two ataxia-telangiectasia cell lines, AT2ABR and AT8ABR, compared to controls. This reduction in activity was detected with increasing concentration of protein and in time course experiments at a single protein concentration. A third cell line, AT3ABR, did not have a detectably lower activity of topoisomerase II when assayed under these conditions. The difference in topoisomerase II activity in the ataxia-telangiectasia cell lines examined may reflect to some extent the heterogeneity observed in this syndrome.

DNA topoisomerases are enzymes that introduce transient single and double strand breaks into DNA and thus are capable of interconverting various DNA conformations (1-3). Eukaryotic topoisomerases catalyze a number of different reactions that include relaxation of both negatively and positively supercoiled DNA; intertwining of complementary single-stranded DNA circles; catenation and interstrand transfer (4, 5). The isolation of mutants in topoisomerase 1 (top I) and II (top 2) in yeast provides evidence for a role for these enzymes in chromosome segregation and maintenance of chromatin organization (6, 7). Top I mutants, with as low as 1% activity, are viable and exhibit no obvious growth defects since they are complemented by topoisomerase II (6). Top II mutants replicate DNA normally but are defective in the termination of DNA replication and the segregation of daughter chromosomes (7, 8). Increased levels of DNA topoisomerase II in regenerating liver (9), and in concanavalin-A-stimulated lymphocytes (10), paralleling a wave of DNA synthesis, provide further evidence for a role for this enzyme in DNA replication.

0006-291X/87 $1.50

Exposure of mammalian cells to ionizing radiation causes inhibition of initiation of DNA replication at lower radiation doses and in addition inhibition of the elongation step at higher doses(11-13). The extent of inhibition of DNA replication is considerably less in cells from patients with the human genetic disorder ataxia-telangiectasia (A-T) than that observed in normal human cells after exposure to ionizing radiation and several radiomimetic chemicals (14-18). It has been suggested that A-T cells are deficient in DNA processing which could account for the range of abnormalities described in these cells (19). In view of the likely role of topoisomerases in DNA replication, and their possible involvement in DNA repair and recombination, it was conceivable that a defect in one of these enzymes might explain the chromosomal and other anomalies observed in A-T cells. We report here that topoisomerase II activity is reduced in some A-T cell lines compared to controls.

MATERIALS AND METHODS

Cell Culture: EBV-transformed lymphoblastoid cells, derived from control and A-T homozygotes were used in this investigation. Cells were grown in suspension culture as described previously (14).

Preparation of cell extracts: A modification of the method described by Duguet et al. (9) was employed to prepare nuclear extracts from A-T and control cells. Cultures (4×10^7 cells) were centrifuged for 10 min, washed in 5 ml of extraction buffer containing 50 mM Tris-Cl pH 7.5, 25 mM KCl, 3 mM $MgCl_2$, 0.25 M sucrose, 1 mM PMSF, 2 mM DTT, 1 mM EDTA, and resuspended in 2 ml of the same buffer. The cells were homogenized in a Dounce homogenizer at 0°C until 98% of the cells were disrupted. The homogenate was adjusted to 400 mM KCl and centrifuged for 60 min. in a 50 Ti Beckman rotor. The supernatants were collected and adjusted to 70% saturation with $(NH_4)_2SO_4$ for 30 min. at 4°C. The precipitated proteins were collected by centrifugation in a Sorvall centrifuge for 15 min., redissolved in buffer (40 mM Tris-Cl pH 7.5, 2 mM DTT, 1 mM EDTA, 0.25 M sucrose) and dialyzed against the same buffer. Protein estimations were performed according to the method of Bradford (20).

DNA topoisomerase I assay: Enzyme activity was measured by relaxation of supercoiled plasmid, pR1-2 (kindly provided by U.Novak School of Veterinary Science, University of Melbourne). Activity was determined on serial dilutions of extracts as described previously (9). Analysis of the products of the reaction was achieved by electrophoresis on 0.75% agarose gels followed by staining with ethidium bromide.

DNA topoisomerase II assay: Strand-passing activity of DNA topoisomerase II was assayed by the P4 phage unknotting assay as described by Liu et al (21). P4 phage and the bacterial strains required for growth and titering were kindly provided by Leroy Liu, John Hopkins Medical School, Baltimore, U.S.A.

RESULTS

DNA topoisomerase I activity was measured in nuclear extracts, prepared from A-T and control lymphoblastoid cells, by relaxation of supercoiled plasmid in the absence of ATP. Fig.1 a,b compares activity in two sets of A-T and control cell lines. It is evident that the extent of relaxation of supercoiled DNA with increasing protein concentration, is approximately the same in all four extracts. Topoisomerase I levels were also comparable in other A-T and control cell lines (data not shown).

On the other hand determination of topoisomerase II activity revealed a significant difference between control and A-T extracts. Fig. 2a demonstrates that unknotting of P4 phage DNA, to produce circular DNA, is very much reduced in AT8ABR extracts compared to control (C12ABR) extracts. The production of circular DNA is greater than 10 fold higher with control than with A-T extracts over the protein concentration range 0.5-3 μg/incubation. This difference

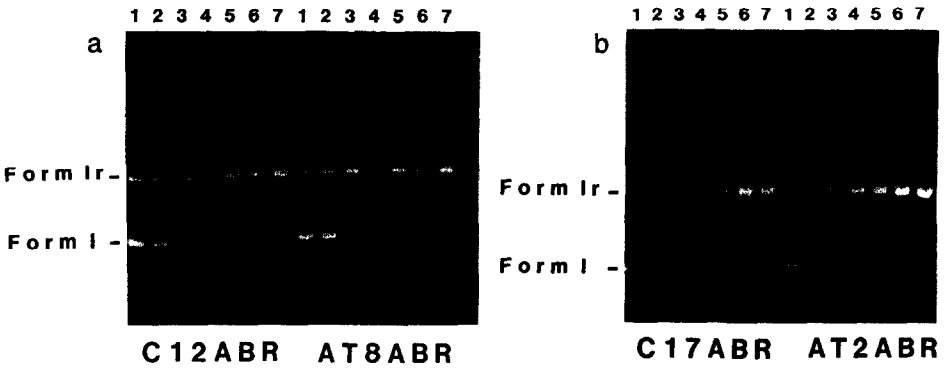

Figure 1. DNA topoisomerase I activity in (a) control (C12ABR) and A-T (AT8ABR) and (b) control (C17ABR) and A-T (AT2ABR) nuclear extracts. Lane 1, no protein; lane 2, 0.01 μg; lane 3, 0.025 μg; lane 4; 0.05 μg; lane 5, 0.1 μg; lane 6; 0.25 μg and lane 7, 0.5 μg. Topoisomerase I activity was determined by relaxation of supercoiled plasmid DNA. Form 1, supercoiled; Form 1r, relaxed.

in activity can also be visualized by decrease in knotted DNA (Fig. 2 a), and by appearance of catenated DNA in the wells (Fig. 2 b). It is also clear that the ratio of linear to circular DNA is considerably greater with increasing protein concentration in the case of AT8ABR. This observation is compatible with the accumulation of an intermediate in the reaction. A time course experiment at 2μg protein/incubation showed an increased amount of activity with time for control extracts and little or none for A-T (Fig. 2b). Comparison of a second set of control and A-T extracts also demonstrated considerably reduced activity of topoisomerase II in the A-T (AT2ABR) cells.. This was evident with both increasing protein concentration (Fig. 3a), and with increasing time at a single concentration of 2μg of protein/incubation (Fig. 3b). A third A-T cell line (AT3ABR) had similar levels of topoisomerase II activity to those in a control, C17ABR (Fig. 4a, b). Overall there was some variation in control activity but it is clear that 2 of the 3 A-T cell extracts showed very low topoisomerase II activity. This activity was found to be dependent on the presence of ATP.

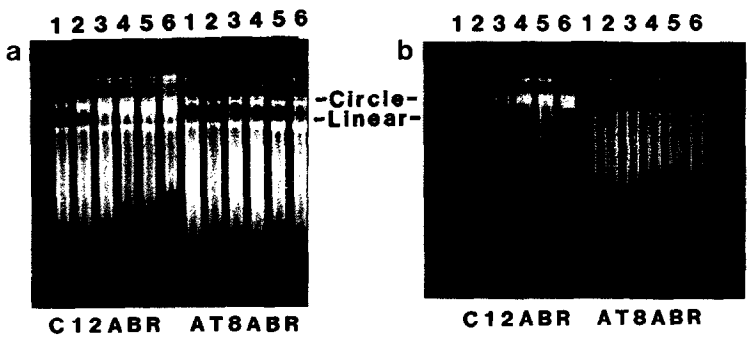

Figure 2. DNA topoisomerase II activity in control (C12ABR) and A-T (AT8ABR) nuclear extracts. (a) Increasing concentration of protein; lane 1, no protein; lane 2, 0.5 μg; lane 3, 1.0 μg; lane 4, 1.5 μg, lane 5, 2.0 μg and lane 6, 3.0 μg. (b) Time course of reaction using 2.0 μg of protein; lane 1, 0 time; lane 2, 10 min, lane 3, 20 min; lane 4, 30 min; lane 5, 40 min and lane 6, 60 min. Enzyme activity was determined by unknotting of P4 phage DNA.

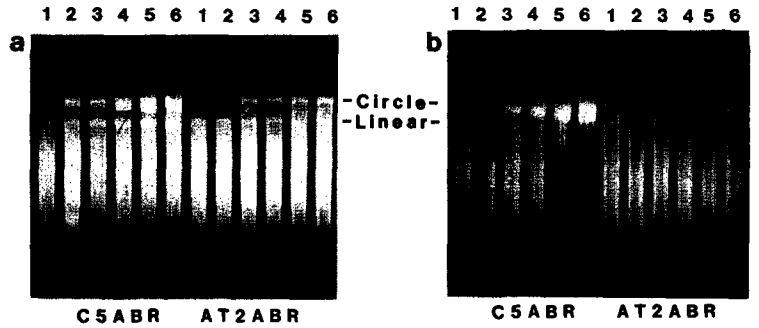

Figure 3. DNA topoisomerase II activity in control (C5ABR) and A-T (AT2ABR) nuclear extracts. (a) Increasing protein concentration; (b) Time course (2.0 μg protein). Order is the same as in legend to Figure 2 for protein concentration and time course experiments.

Since the extracts employed here were relatively crude it was important to rule out a significant contribution from endonuclease activity, although this would be minimized by the dilutions used in our assays (9). Nuclease activity was determined by production of nicked circular DNA using the pR1-2 plasmid. Relaxed circular DNA and the nicked circular form, which might arise from endonuclease activity, can be resolved in the presence of ethidium bromide (22). A small amount of nicked circular DNA was present in the absence of extract and this did not increase at protein concentrations up to 3μg of extract/incubation (date not shown). Furthermore no linear DNA was observed under conditions for topoisomerase I activity (Fig. 1). It is unlikely that some other activity is interfering with topoisomerase II in A-T extracts since mixing experiments between A-T and control extracts did not dramatically reduce topoisomerase II activity (results not shown). Finally, there was no evidence of loss of topoisomerase II activity into the cytoplasm in A-T cells. In both cell types activity was negligible in cytoplasmic extract compared to nuclear extract.

DISCUSSION

Ataxia-telangiectasia is a heterogeneous genetic disorder characterized by a variety of clinical, cellular and molecular abnormalities (23). A number of features that include

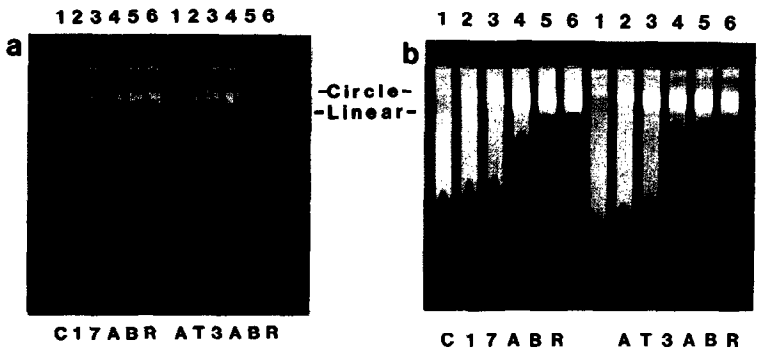

Figure 4. DNA topoisomerase II activity in control (C17ABR) and A-T (AT3ABR) nuclear extracts (a) Increasing protein concentration. (b) Time course (2.0 μg protein). Order is same as in legend to Figure 2.

chromosome instability, abnormal DNA rearrangements, defective DNA repair, radioresistant DNA synthesis and cell cycle anomalies can be explained by what has been termed a defect in DNA processing (24). To date it has not been possible to identify a common biochemical defect in A-T or indeed to understand how a defect in a single gene could explain the "DNA abnormalities" and the other characteristics of this syndrome. We have provided evidence in this report for reduced topoisomerase II activity in nuclear extracts from 2 out of 3 A-T cell lines. The change is very significant and represents a greater than 10-fold reduction in activity in both cases. Failure to observe a decrease in the other A-T cell line (AT3ABR), which is incidentally in a different complementation group to AT2ABR, could be due to a different site of mutation which might alter the enzyme in a different way. It should be pointed out that the three A-T lymphoblastoid cells used in this study have approximately the same doubling times as the controls and there is no evidence for a prolonged S phase (25). Consequently, if topoisomerase II plays an important role in DNA replication, it is unlikely that the defect in the enzyme is a drastic one *in situ*. It is more likely that mutations lead to different structural changes in the enzyme, and when it is released from the nuclear matrix different degrees of reduction in enzyme activity result,dependent upon the actual structural change. In its natural environment in the nuclear scaffold the protein would function almost the same as a normal control protein. However, when the cell is exposed to damaging agents such as ionizing radiation, that interfere with the supercoiled structure of chromatin, this may be sufficient to expose the defect in topoisomerase II leading to the abnormalities in DNA processing described in A-T.

Evidence in support of a defect in topoisomerase II in A-T cells has been reported previously. Henner and Blazka (26) have shown that 3 A-T fibroblast lines were 2-3 fold more sensitive to etoposide (VP-16-213) than control fibroblasts. Increased cell killing and elevated retention in G2 phase, after exposure to etoposide, have also been described in A-T fibroblasts, SV-40 transformed A-T cells, and in A-T lymphoblastoid cell lines (27). After exposure of A-T cells to ionizing radiation in G1 phase, a large fraction of the resulting double strand breaks in DNA (determined as prematurely condensed chromosome fragments) remained unrepaired (28). In addition increased cell killing by novobiocin (29), and misrepair of double strand DNA breaks in restriction enzyme cleaved recombinant plasmids, transfected into A-T cells (30), are also in keeping with a defect in topoisomerase II. The report of a defect in topoisomerase II described here for A-T cells, together with evidence of increased numbers of unrepaired double strand breaks, suggest that this enzyme may play an important role in the repair of double strand breaks induced in DNA by ionizing radiation and other radiomimetic chemicals.

The description of a defect in topoisomerase II provides a means of understanding the occurence of a variety of characteristics associated with A-T. A deficiency in this enzyme could clearly lead to anomalies in DNA replication, repair and recombination, all of which are observed to some extent in A-T cells. A-T thus is a useful model system linking a defect in a specific enzyme and predisposition to cancer.

ACKNOWLEDGEMENTS

We wish to acknowledge the support of the Australian Research Grants Scheme,the National Health and Medical Research Council and the Australian Institute of Nuclear Science and Engineering. We also would like to thank Priscilla le Poidevin and Suzanne Strother for technical assistance, and Pamela Knobel for preparing this manuscript.

REFERENCES

1. Wang, J.C. (1971) J. Mol. Biol. **55,** 523-533.
2. Gellert, M., Mizuuchi, K., O'Dea, M.H., and Nash, H.A. (1976) Proc. Natl. Acad. Sci. USA **73,** 3872-3876.
3. Wang, J.C. (1985) Ann. Rev. Biochem. **54,** 665-697.
4. Champoux, J. J., and Dulbecco, R. (1972) Proc. Natl. Acad. Sci. USA **69,** 143-146.
5. Hsieh, T.-S., and Brutlag, D. (1980) Cell **21,** 115-125.
6. Uemura, T., and Yanagida, M. (1984) EMBO J. **3,** 1737-1744.
7. Di Nardo, S., Voelkel, K., and Sternglanz, R. (1984) Proc. Natl. Acad. Sci. USA **81,** 2616-2620.
8. Holm, C., Goto, T., Wang, J.D., and Botstein, D. (1985) Cell *41*, 553-563.
9. Duguet, M., Lavenot, C., Harper, F., Mirambeau, G., and De Recondo, A-M. (1983) Nucleic Acids Res. **11,** 1059-1075.
10. Taudou, G., Mirambeau, G., Lavenot, C., der Garabedian, A., Vermeersch, J., and Duguet, M. (1984) FEBS **176,** 431-435.
11. Watanabe, I. (1974) Radiat. Res. **58,** 541-556.
12. Makino, F., and Okada, S. (1975) Radiat. Res. **62,** 37-51.
13. Painter, R.B., and Young, B.R. (1976) Biochim. Biophys. Acta **418,** 146-153.
14. Houldsworth, J., and Lavin, M.F. (1980) Nucleic Acids Res. **8,** 3709- 3720.
15. Edwards, M.J. and Taylor, A.M.R. (1980) Nature **287,** 745-747.
16. Painter, R.B. and Young, B.R. (1980) Proc. Natl. Acad. Sci. USA **77,** 7315-7317.
17. de Wit, J., Jaspers, N.G., and Bootsma, D. (1981) Mutation Res. **80,** 221-226.
18. Shiloh, Y., Tabor, E., and Becker, Y. (1985) In: Ataxia Telangiectasia, Genetics, Neuropathology and Immunology of a Degenerative Disease of Childhood (R.A. Gatti, D.R. Coman, eds) pp 111-121, Alan R. Liss, New York.
19. Hanawalt, P.C., and Painter, R. (1985) In: Ataxia Telangiectasia, Genetics, Neuropathology and Immunology of a Degenerative Disease of Childhood (R.A. Gatti, D.R. Coman, eds) pp 67-71, Alan R. Liss, New York.
20. Bradford, M.M. (1976) Anal. Biochem. **72,** 248-254.
21. Liu, L.F., Davis, J.L., and Calender, R.(1981) Nucleic Acids Res. **9,** 3979-3989.
22. Mohamed, R.(1985) Ph.D Thesis, University of Queensland.
23. Boder, (1985) In: Ataxia-Telangiectasia, Genetics, Neuropathology and Immunology of a Degenerative Disease of Childhood (R.A.Gatti,D.R. Coman, eds) pp 1-63, Alan R. Liss, New York.
24. Hanawalt, P.C., and Sarasin, A. (1986) Trends in Genetics **2,** 124-129.
25. Ford,M. (1983) Ph.D Thesis, University of Queensland.
26. Henner, W.D., and Blazka, M.E.(1986) JNCI **76,** 1007-1011.
27. Smith, P.J., Anderson, C.O., and Watson, J.V.(1986). Cancer Res. **46,** 5641-5645.
28. Cornforth, M.N., and Bedford, J.S. (1985) Science **227,** 1589-1591.
29. Debenham, P.G., Webb, M.B.T., Jones, N.J. and Cox, R. (1987) In: Molecular Biology of DNA repair (A. Collins, R.T. Johnson, J.M. Boyle, eds) pp 177-189, Cambridge: Company of Biologists.
30. Cox, R., Debenham, P.G., Masson, W.K. and Webb, M.B.T. (1986) Mol. Biol. Med. **3,** 229-244.