A HUMAN ENZYME THAT LIBERATES URACIL FROM DNA

Mutsuo Sekiguchi[1], Hiroshi Hayakawa[1], Fumiko Makino[2], Kiyoji Tanaka[3] and Yoshio Okada[3]

[1] Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812, Japan; [2] National Cancer Center Research Institute, Tokyo 104; [3] Research Institute for Microbial Diseases, Osaka University, Osaka 565.

Received July 29,1976

SUMMARY: An enzyme activity which acts specifically on uracil-containing DNA was found in human placenta and cultured fibroblasts. The enzyme liberates uracil from DNA in the presence of EDTA at pH 7.5. Almost equal levels of the activity were found in normal and xeroderma pigmentosum cell lines (complementation group A).

Mechanism of repair of DNA damaged by ultraviolet light has been studied extensively in human cells. It has been demonstrated that fibroblasts from normal skin carry out ultraviolet-induced unscheduled DNA synthesis (repair replication) whereas cells from XP patients do not (1). XP cells have been classified into 5 complementation groups (2), all of which are likely to be defective in the first incision step of excision repair (3).

Recently, Day (4) reported that nitrous acid-treated adenovirus 2 shows more plaque-forming ability in normal cells than when XP cells, belonging to the complementation group A, were used as hosts. This implies that normal human cells are able to repair damages of DNA induced by nitrous acid as well. It has been shown that nitrous acid deaminates cytosine and other bases of nucleic acid and this accounts, at least in part, for the high mutagenic activity of the compound (5, 6). These results prompted us to search an enzyme activity which acts on DNA containing abnormal bases and removes them from the DNA. In this paper we report that such an enzyme is indeed present in human cells.

Abbreviation : XP, xeroderma pigmentosum.

MATERIALS AND METHODS

DNA: PBS1 DNA, which contains uracil in place of thymine (7), was used as substrate for the enzyme assay. PBS1 phage was propagated in Bacillus subtilis Marburg in a synthetic medium containing [^{3}H]deoxyuridine or [^{3}H]deoxyadenosine and purified by CsCl equilibrium centrifugation. DNA was extracted by phenol, treated with RNase T_1 and pancreatic RNase and then subjected to the second cycle of phenol extraction and filtration through Sephadex G-100 column. Analysis by alkaline digestion showed that the DNA preparations contain no RNA. [^{3}H]Thymine-labeled Escherichia coli DNA was prepared as described previously (8).

Extracts: A part of human placenta (5 g) was cut into small pieces and homogenized in a Potter homogenizer with 20 ml of cold 20 mM Tris·HCl (pH 7.5)-2 mM EDTA. The homogenate was filtered through gauze and the filtrate was spun at 10,000 x g for 10 min. The precipitate was suspended in 10 ml of the buffer and treated for 2 min in an ultrasonic disintegrator while the supernatant fluid (Fraction I) was retained. The sonicated materials were centrifuged at 10,000 x g for 10 min and the supernatant fluid was taken (Fraction II).

Human fibroblasts derived from skin of normal and XP subjects (H. Takebe and K. Tanaka, in preparation) were grown as monolayers in Eagle's minimum essential medium supplemented with 10 % fetal calf serum (3). Cells were collected by centrifugation, washed with balanced salt solution and resuspended in 10 mM Tris·HCl (pH 7.5)-10 % ethylene glycol-10 mM 2-mercaptoethanol. The suspension was treated for 1 min in an ultrasonic disintegrator and then centrifuged at 10,000 x g for 10 min. The supernatant solution was taken as extract.

Enzyme assay: Reaction mixture contains 0.32 μg of DNA, 50 μmoles of Tris·maleate (pH 7.5), 25 μmoles of EDTA and an extract in 0.5 ml. Unless otherwise noted, the reaction was performed at 37°C for 10 min in the above reaction mixture. At the end of incubation, 0.3 ml of 1 mg/ml bovine serum albumin and 0.2 ml of 25 % trichloroacetic acid were added. The mixture was kept in ice for 10 min and then centrifuged at 1000 x g for 10 min. The radioactivity of the acid-soluble fraction was determined in a liquid scintillation counter. One unit of the enzyme was defined as the activity which releases 0.1 nmole of uracil from DNA in 10 min at 37°C.

Chromatographic analyses: The reaction mixture was acidified with 0.5 N perchloric acid. The acid-soluble fraction was neutralized with KOH and the resulting precipitate was removed. The neutralized solution was applied to a small column (0.6 x 5 cm) of Dowex-1 (x 8, 200 - 400 mesh) and the column was washed with 25 ml of 0.2 N formic acid. The filtrate and the washing were combined (uracil fraction). The column was eluted with 15 ml of 4 N formic-0.2 M ammonium formate and the eluate was collected (dUMP fraction).

For paper chromatography, the sample was acidified and adsorbed to a small column (0.6 x 1 cm) of charcoal. After washing with distilled water, the column was eluted with 30 ml of 2 % NH_4OH-20 % ethanol at 45°C. The eluate was concentrated and applied to a Whatman 3 MM paper. Together with the sample, non-radioactive uracil, uridine, deoxyuridine, UMP and dUMP were applied. The paper was developed in ammoniacal water (pH 10) by the descending technique (9) and the radioactivity in each spot was determined.

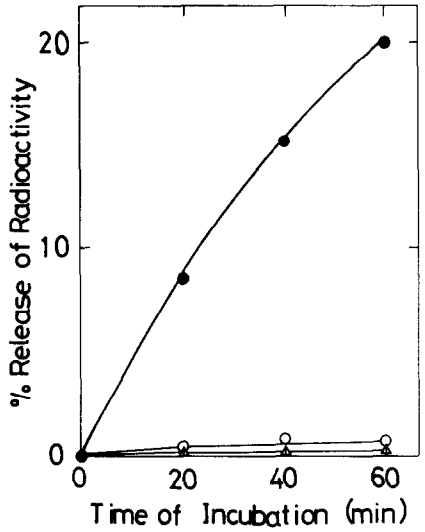

Fig. 1. Release of radioactive materials from DNA by an extract of human placenta. Three types of DNA samples were incubated at 37°C in the following reaction mixture; 0.32 μg of DNA, 50 μmoles of Tris·maleate (pH 7.5), 25 μmoles of EDTA and 145 μg protein of an extract of human placenta (Fraction II) in 0.5 ml. At the times indicated, the mixture was acidified and the radioactivity in the acid-soluble fraction was determined. ●—●, [^{3}H]deoxyuridine-labeled PBS1 DNA (3200 c.p.m./μg); o—o, [^{3}H]deoxyadenosine-labeled PBS1 DNA (6030 c.p.m./μg); Δ—Δ, [^{3}H]thymine-labeled *E. coli* DNA (10120 c.p.m./μg).

RESULTS

Enzyme activity specific for uracil-containing DNA:

Fig. 1 shows the time-course of the reaction of an extract of human placenta (Fraction II) with three types of DNAs. The radioactivity was released rapidly from [^{3}H]deoxyuridine-labeled PBS1 DNA whereas no appreciable radioactivity was released from [^{3}H]thymine-labeled *E. coli* DNA. It has been shown that DNA of PBS1 phage contains uracil in place of thymine (7). Thus, this assay measures specifically an enzyme activity that acts on uracil-containing DNA. Specific activities of the enzyme in Fraction I and II were 0.03 and 0.31 units/mg protein, respectively. It appears that the enzyme is mainely present in cell nuclei.

Of much interest is the observation that there is no or only little release of the radioactivity from [^{3}H]deoxyadenosine-labeled PBS1 DNA under the same conditions. Since the same

Table 1. Release of uracil from PBS1 DNA by an extract of human placenta

Reaction	Radioactivity (c.p.m.)	
	Uracil	dUMP
0-time	17	36
60 min	878	57
60 min (+dUMP)	810	44

1.28 μg of [^{3}H]deoxyuridine-labeled PBS1 DNA (4000 c.p.m.) was incubated at 37°C with 0.58 mg protein of an extract of human placenta (Fraction II) in 2 ml of 0.1 M Tris·maleate (pH 7.5)-0.05 M EDTA. To one tube dUMP (150 μg/ml) was added. The acid-soluble fractions were analyzed by Dowex-1 column chromatography.

amounts of DNA and of the enzyme were used for these reactions, it seems that the enzyme liberates uracil without concomitant release of other bases.

Release of uracil from DNA: The products of the reaction with [^{3}H]deoxyuridine-labeled PBS1 DNA were analyzed by Dowex-1 column chromatography. As shown in Table 1, almost all the radioactivity was found in the filtrate, indicating that the products are free bases or nucleosides but not nucleotides. The radioactive material found in the filtrate was identified as uracil by paper chromatography in ammoniacal water.

To exclude the possibility that nucleotide was first released from DNA and then converted to uracil, the reaction was performed in the presence of excess non-radioactive dUMP. In this case, too, all the radioactivity released was found in uracil (Table 1). Thus, uracil is liberated directly from DNA.

Activity in XP cells: The enzyme activities to release uracil in normal and XP cell lines were compared. Strain UNK, derived from normal skin, and strain XP100S, which was

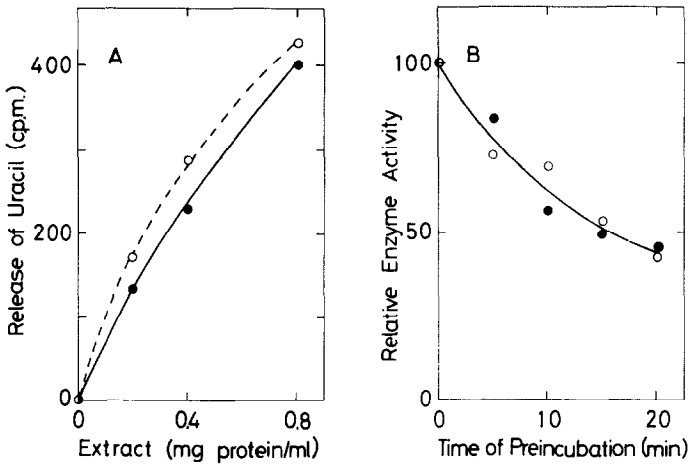

Fig. 2. Uracil-releasing enzyme in normal and XP cell lines. (A) Levels of enzyme activity. Reaction was performed at 37°C for 60 min under the standard assay conditions using [^{3}H]deoxyuridine-labeled PBS1 DNA (2380 c.p.m./μg). (B) Heat stability. The enzymes were pre-incubated in 10 mM Tris·HCl (pH 7.5)-10 % ethylene glycol-10 mM 2-mercaptoethal at 45°C for various times. ●——●, an extract of normal cell (UNK), o---o, an extract of XP cells (XP10OS).

from a XP patient and has been classified in the complementation group A, were used in the experiment. Fibroblasts were grown in Eagle's minimum essential medium supplemented with 10 % fetal calf serum, and extracts were prepared.

Fig. 2A shows that the activity is present to a similar degree in normal and XP cell lines. The heat inactivation curves of the normal and XP enzymes are indistinguishable from each other (Fig. 2B). It was shown, moreover, that the two enzymes possess the same pH dependency; they are most active between pH 6.0 and 8.0 and about 50 % of the maximum activity was obtained at either pH 5.5 or 9.5. So far as experiment goes, there is no quantitative and qualitative difference between the enzymes in normal and XP (A group) cell lines.

DISCUSSION

Lindahl (10) first found that an extract of E. coli contains an enzyme activity that releases deaminated cytosine residues from alkali-treated DNA. The enzyme, purified 30 fold from the extract, cleaves N-glycoside linkage without interrupting phosphodiester bond of the DNA. By monitering the activity

with PBS1 DNA, we were able to purify the enzyme 250 fold over the crude extract (H. Hayakawa and M. Sekiguchi, unpublished result). A similar enzyme activity was also found in B. subtilis (11).

The human enzyme found in the present studies resembles the E. coli enzyme in many respects. It acts specifically on DNA containing uracil and liberates uracil but not adenine. The enzyme reaction proceeds at neutral pH in the absence of Mg^{2+}. It is likely that the enzyme cleaves N-glycosidic bond between uracil and deoxyribose, thus releasing uracil from the DNA without concomitant breakage of phosphodiester bond. To show this more definitely, it is necessary to purify the enzyme and to characterize the reaction products. Studies are in progress in the laboratory at Kyushu University.

The widespread occurence of N-glycosidase suggests that the enzyme plays some important role in cellular metabolism. Uracil is not a normal constituent of DNA, but it may be formed by the deamination of cytosine in DNA or may erroneously be incorporated into DNA. It has been shown that chemicals, such as nitrous acid and sodium bisulfite, deaminate cytosine residues of nucleic acid in mild physiological conditions (5, 12). Such changes cause the GC to AT transition, which is sometimes fatal to organisms. A major role of N-glycosidase is likely to be repair of such DNA.

It has been shown that human cells contains an endonuclease specific for apurinic sites (13). This enzyme induces strand breaks near the positions where purine bases were removed by heat or alkylating agents. It is reasonable to assume that the enzyme also acts on apyrimidinic sites. Thus, once uracil is removed by N-glycosidase, a strand break is induced near the lesion by the endonuclease. The subsequent steps of repair may follow the pathway for excision repair of ultraviolet-damaged DNA.

Recently, it was shown that XP cells (group A and D) possess an altered endonuclease activity upon depurinated DNA (14). The results presented in this paper indicate that N-glycosidase in XP cells (group A) is normal in both quantitative and qualitative respects. The decreased ability of repair of nitrous acid-damaged DNA in the XP cells (4) may be attributed to their defect in the endonuclease activity.

ACKNOWLEDGEMENTS: We thank Dr. H. Tanooka (National Cancer Center Research Institute) for discussion, and Drs. N. Kuwano and T. Shibata (Kyushu University Medical School) for supplying some of the materials used. This work was supported in part by research grants from the Ministry of Education, Science, and Culture of Japan and the Japan Securities Scholarship Foundation.

REFERENCES

1. Cleaver, J. E. (1968) Nature 218, 652 - 656.
2. Kraemer, K. H., Weerd-Kastelein, E. A. de, Robbins, J. H., Keijzer, W., Barrett, S. F., Petinga, R. A., and Bootsma, D. (1975) Mutation Res. 33, 327 - 340.
3. Tanaka, K., Sekiguchi, M., and Okada, Y. (1975) Proc. Nat. Acad. Sci. USA. 72, 4071 - 4075.
4. Day, III, R. S. (1975) Mutation Res. 27, 407 - 409.
5. Shuster, H., and Schramm, G. (1958) Z. Naturforschg. 13b, 697 - 704.
6. Tessman, I., Poddar, R. K., and Kumar, S. (1964) J. Mol. Biol. 9, 352 - 363.
7. Takahashi, I., and Marmur, J. (1963) Nature 197, 794 - 795.
8. Sekiguchi, M., Yasuda, S., Okubo, S., Nakayama, H., Shimada, K., and Takagi, Y. (1970) J. Mol. Biol. 47, 231 - 242.
9. Wyatt, G. R. (1955) The Nucleic Acid (ed. by E. Chargaff and J. N. Davidson) vol. 1, pp 243 - 265, Academic Press, New York.
10. Lindahl, T. (1974) Proc. Nat. Acad. Sci. USA. 71, 3649 - 3653.
11. Friedberg, E. C., Ganesan, A. K., and Minton, K. (1975) J. Virol. 16, 315 - 321.
12. Shapiro, R., Braverman, B., Louis, J. B., and Servis, R. E. (1973) J. Biol. Chem. 248, 4060 - 4064.
13. Teebor, G. W., and Duker, N. J. (1975) Nature 258, 544 - 547.
14. Kuhnlein, U., Penhoet, E. E., and Linn, S. (1976) Proc. Nat. Acad. Sci. USA. 73, 1169 - 1173.