Deficient DNA Repair in Human Progeroid Cells

(Hutchinson-Gilford progeria syndrome/x-irradiation/DNA strand breaks/senescence)

J. EPSTEIN, JERRY R. WILLIAMS, AND JOHN B. LITTLE

Laboratory of Radiobiology, Department of Physiology, Harvard University School of Public Health, Boston, Massachusetts 02115

Communicated by Paul C. Zamecnik, January 24, 1973

ABSTRACT Skin fibroblasts biopsied from a patient with clinical symptoms of the Hutchinson-Gilford progeria syndrome failed to show evidence of normal DNA strand rejoining *in vitro* after exposure to cobalt-60 gamma irradiation. Control human diploid fibroblasts of fetal origin in early passage, skin fibroblasts of adult origin, and an established line of human liver cells (LICH) all showed essentially complete rejoining of radiation-induced strand breaks within 30 min after irradiation, as evidenced by DNA sedimentation profiles in alkaline sucrose gradients. The results suggest that an enzyme involved in DNA repair may be absent or greatly reduced in efficiency in cells from this patient with the progeria syndrome. Such a repair-deficient cell strain may prove useful in the study of the molecular events associated with x-ray-type damage and its repair in mammalian cells.

It would be useful to isolate or identify mutants in mammalian cells that are defective for mechanisms of DNA repair. Such mutants have been widely studied in bacteria, and one such mutation has recently been identified in mammalian cells (1). The mutation, a deficient capability for repair of ultraviolet light (UV)-induced DNA damage, has been found in human fibroblasts explanted from patients with the autosomal recessive trait xeroderma pigmentosum. This finding has not only established the biochemical basis for the clinical syndrome in xeroderma patients, but has led to a firmer understanding of the mechanisms involved in DNA repair for the specific damage induced by UV light. The molecular mechanisms involved in the repair of the physically distinct types of DNA damage caused by x-rays and UV light, however, appear to differ (2). Furthermore, it is now well documented that xeroderma cells exhibit defective repair only for UV damage, and are fully capable of restoring x-ray induced DNA damage (3, 4). These observations have allowed the characterization of the DNA damage caused by various carcinogenic agents as being either UV-like or x-ray-like.

Attempts to isolate mutant mammalian cells deficient for repair of x-ray-type damage have been unsuccessful, although the value of such cells to the study of repair events at the molecular level and their correlation with cellular changes is obvious. We have been attempting to isolate both induced and naturally occurring mutants deficient in their ability to rejoin x-ray-induced single-strand breaks. The rapid repair of such strand breaks has been a universal finding in all mammalian cells studied (5-8), and repair is not influenced by a wide variety of environmental factors, such as exposure to many metabolic inhibitors (7, 8).

In this preliminary report we describe a primary human fibroblast strain derived from a progeroid patient that appears incapable of the normal rejoining of DNA strand breaks

induced by cobalt-60 gamma rays. We cannot determine at present whether this repair deficiency is manifested as a result of a primary genetic lesion in the progeroid cells, or rather as a secondary consequence of the progeria phenomenon. However, the response of these cells is uniquely deficient compared with that of other primary fibroblast strains derived from normal patients or of a human aneuploid line.

METHODS AND MATERIALS

Cell Cultures. Skin fibroblasts biopsied from a patient exhibiting clinical symptoms of the Hutchinson-Gilford progeria syndrome of precocious aging were received during their fourth generation after primary explant.* These cells, designated KH, were grown in monolayer in 8-oz glass prescription bottles in a 37°, humidified, 5% CO₂ atmosphere. Progeroid cells have been observed to undergo a limited number of generations *in vitro* and are reported to be somewhat difficult to maintain (9-11). We found that the growth of KH cells was enhanced by maintenance in conditioned medium freshly decanted from normal diploid human fibroblasts. Consequently, a normal strain of diploid human skin fibroblasts was maintained in logarithmic growth in Eagle's minimal essential medium supplemented with nonessential amino acids, 900 mg/ml of glucose, 6.6 mg/ml of sodium pyruvate, 0.6 mg/ml of ferrous nitrate, 50 U/ml of potassium penicillin, 50 µg/ml of streptomycin sulfate, 2.5-5.0 µ/ml of amphotericin-B, and 15% heat-inactivated calf serum. This medium was changed daily, and the conditioned medium was filtered through a 0.22-µm membrane filter and used to feed KH cultures. The conditioned medium in KH cultures was renewed every 24-48 hr. The experiments described herein were performed during the 8th, 9th, and 10th generations after primary explant. The cells could not be carried beyond the 11th generation.

Ex-1, a normal diploid skin fibroblast, was derived from a biopsy of an adult man. Ex-4, a normal diploid skin fibroblast, was obtained from a 9-week fetus at hysterotomy. These cell strains were grown in 100-mm diameter plastic petri dishes and fed Eagle's minimal essential medium supplemented as described above, except that 15% fetal-calf serum was used in place of the heat-inactivated calf serum. LICH cells, a human aneuploid cell line originally derived from a liver explant (12), were maintained as reported elsewhere (13). All cell lines used in these experiments were tested for and

* These cells were kindly supplied by Dr. Robert Hay. The clinical history and other pertinent information was received from Drs. William Reichel and J. W. Retann.

Fig. 1. DNA sedimentation profiles for KH progeroid cells and Ex-1 adult human fibroblasts for 0 irradiation (A); 10 krad (B); 10 krad followed by 30 min repair incubation at 37° (C); and 10 krad followed by a 90-min repair incubation at 37° (D).

found free of mycoplasma. Before irradiation and centrifugation, the cultures were incubated for 24–50 hr with either [*methyl*-³H]thymidine (6.7 Ci/mmol, 0.25 μ Ci/ml) or [*2*-¹⁴C]-thymidine (53.7 Ci/mol, 0.50 μ Ci/ml) in their respective complete media.

Irradiation. Cells were detached from the monolayer by incubation with 0.05% trypsin in phosphate-buffered saline (pH 7.4), resuspended in complete medium, and adjusted to a final density of 2×10^5 cells per ml. The suspended cells were irradiated at 4° in a cobalt-60 gamma irradiator; they were given 10⁴ rads in an elapsed time of about 30 sec (dose-rate 315 rads/sec).

Sucrose Gradients. Immediately after irradiation, 5000 cells in a volume of 25 μ l were placed in a high alkali-salt lysing solution layered on top of a linear 5–20% alkaline sucrose gradient. This top layer was composed of 0.5 M NaCl–0.45 M NaOH–0.05 M diNa-EDTA. The gradients were composed of 0.90 M NaCl–0.10 M NaOH–0.05 M diNa-EDTA in a total volume of 5 ml. Lysis was allowed to continue for 4.5 hr at room temperature (25°) in the dark. Centrifugation after 4.5 hr at room temperature allows observation of a homodisperse DNA subunit (5, 6, 14, 15). This subunit has been estimated to be of a size of 165 S (15) and 180 S (14).

Centrifugation. After the 4.5-hr lysis period, the gradients were spun at 5000 rpm ($1700 \times g$) for 15 min. Then they were centrifuged for 60 min at 36,000 rpm and 15° in an SW 50.1 swinging bucket rotor in a Beckman L2-65B ultracentrifuge. Thirty 10-drop fractions were collected onto glass fiber filters by displacement from the bottom of the tube with 40%

sucrose. Scintillation counting was performed by dropping the dried filters directly into a toluene-base solution containing 4.0 g/liter PPO and 50 mg/liter POPOP. Results are expressed as the percent of the total label recovered that was present in each fraction. Mean fraction numbers were obtained by calculation of the mean of all fractions of a gradient that exceeded 50% of the radioactivity contained in the maximum fraction.

RESULTS

Fig. 1 presents the results of an experiment in which DNA sedimentation patterns were studied in cells from a progeria patient (KH) and from a normal individual (Ex-1) that were returned to the incubator for various periods of time after gamma irradiation. The cells were incubated with labeled thymidine for 48 hr before irradiation, as described above, and were lysed for 4.5 hr on top of the gradient before centrifugation. The sedimentation profiles for DNA from unirradiated KH and Ex-1 cells (Fig. 1A) are similar to those obtained with Ex-4 and LICH cells in this laboratory, as well as those reported by other workers using various strains of mammalian cells.

The size of the DNA subunit observed by this technique (165–180 S) would indicate that each cell contains about 10⁴ of these molecules (5, 14). A given radiation dose must break a substantial number of these subunits before a measurable shift of the DNA sedimentation pattern will occur. Fig. 1B shows the sedimentation patterns obtained when cells were irradiated with 10 krad, then immediately lysed and centrifuged. Panels C and D show the effect of allowing repair to occur after irradiation by incubation of the cells at 37° in complete medium for 30 or 90 min before they were placed in the lysing solution. KH cells failed to rejoin radiation-induced strand breaks during this repair interval. Ex-1 cells, however, had repaired most, if not all, breaks after 30 min of repair incubation, as is evidenced by the return of the DNA sedimentation pattern to an unirradiated configuration. (Panel A).

The results from several experiments, such as that in Fig. 1, are summarized in Fig. 2. The mean fraction number of the DNA sedimentation peak of each gradient is plotted as a function of the time interval of repair incubation after irradiation with 10 krad. The results demonstrate clearly the unique response of the KH cells as compared with that of the other three cell strains. With a 270-min repair interval, there was a small shift of the sedimentation peak of KH cells toward larger molecular size. This result suggests that the repair capability may not be completely lacking in the KH cells, but greatly reduced in efficiency.

Further experiments were designed to determine if KH cells were unusually sensitive to low dose-rate irradiation, such as might be expected if they were deficient in repair capability. In these experiments (Fig. 3) sedimentation profiles were obtained from KH cells exposed for a fixed time interval to continuous beta irradiation from different amounts of incorporated radioactive thymidine. The DNA sedimentation patterns of KH cells containing an average of 0.63 dpm per cell of [¹⁴C]thymidine (Fig. 3A) was similar to that of normal cell lines containing tritium-labeled thymidine at much higher levels (Fig. 3B and C). When KH cells contained 1.1 and 5.9 dpm per cell; however, a significant shift in the sedimentation pattern towards a lower molecular weight

FIG. 2. Variation of the mean fraction number of the DNA sedimentation peak against minutes of repair incubation at 37° after 10 krad of gamma irradiation for KH progeroid cells (O), Ex-1 adult human fibroblasts (□), Ex-4 fetal human fibroblasts (Δ), or LICH established human aneuploid liver cells (◇).

occurred. This change is consistent with the accumulation of single-strand breaks in these cells.

By measuring the amount of incorporated isotope in the cells, and by using the data of Cleaver *et al.* (16) and Lett and Sun (17), we can estimate the total dose, dose rate, and rate of induction of DNA strand breaks under each condition. These data are tabulated in Table 1 and may be used to estimate the rate of strand breakage that exceeds the rejoining capacity of KH cells. Although there are no direct data for the rate of strand breakage induced by tritium decay

in cells incubated at 37°, Cleaver *et al.* (16) report a rate of 2.1 breaks per tritium decay and 1 break per 115 eV of x-irradiation in frozen Chinese hamster cells. Lett and Sun (17) calculated that x-rays produced about 1 break per 60 eV of energy absorbed in cells irradiated at 4°. If we assume from these data that tritium decays in our cells produced a maximum of 4.2 breaks per decay, we can relate tritium decay

TABLE 1. Effect of incorporated tritium on the production of DNA strand breaks

	Cell line			
	KH	KH	Ex-1	LICH
Incorporation per labeled cell (dpm)	1.12	5.9	1.15	9.37
Maximum rate of break production per cell (breaks/min)	<4.7	<23.8	<4.8	<39.2
Maximum dose-rate (rads/min)	<0.54	<2.8	<0.56	<4.5
Maximum possible total dose (rads)	<1,600	<8,000	<1,700	<13,000
Effect on DNA profile	+	++	0	0
Estimated external x-irradiation for same effect on DNA profile (rads)	750-1500	3000-6000	0	0

FIG. 3. DNA sedimentation profiles for: (A) KH progeroid cells containing 0.63 dpm per cell [¹⁴C]thymidine (Δ); 1.12 dpm per cell [³H]thymidine (×); and 5.9 dpm per cell [³H]thymidine (O). (B) Ex-1 normal human fibroblasts containing 1.15 dpm per cell [³H]thymidine. (C) LICH human aneuploid liver cells containing 9.37 dpm per cell [³H]thymidine.

rate to break production rate, and the total number of decays to the total number of breaks produced.

The total cellular radiation dose and dose-rate from tritium decay (Table 1) were obtained from the determination of Cleaver *et al.* (16) of 0.48 rads per decay, and on the assumption that the radioactivity at the time of lysis was present during the entire period of incubation in [³H]thymidine. Both of these are maximum estimates; the data in Table 1 are, therefore, also maximum estimates, and actual rates may be considerably less.

DISCUSSION

KH cells have a greatly diminished capacity for rejoining DNA strand breaks whether induced by large acute doses of ionizing irradiation (Figs. 1 and 2) or by low dose-rate beta irradiation from incorporated radioactivity (Fig. 3). To our knowledge, no mammalian cells have been previously described that fail to rejoin completely x-ray-induced single-strand breaks within 30–90 min after irradiation. Indeed, it has proven very difficult to depress the normal rejoining process by treatments such as incubation with various metabolic inhibitors (7, 8).

The fact that a small increase in mean molecular weight in KH cells was consistently seen after 270 min of repair incubation (Fig. 2) suggests that these cells do possess a limited rejoining capacity. This finding is supported by the observation that KH cells labeled with [¹⁴C]thymidine of low activity show control DNA profiles similar to those of normal cells (Fig. 3). Under these conditions, the role of break production in KH cells apparently does not exceed their rejoining capacity. The data in Table 1 indicate, however, that if breaks are produced by tritium disintegrations at a rate of 4.7 breaks per min per cell, the rejoining capacity of KH cells is exceeded and breaks accumulate. Normal cells, on the other hand, can rejoin at a rate that can be estimated from Fig. 2 by observing that all breaks produced by 10 krad, about 1 to 2×10^4 per cell, are rejoined within 30 min. This would indicate a rejoining rate in normal cells in excess of 300 breaks per min per cell.

As can be seen in Fig. 2, KH cells lysed immediately after irradiation with 10 krad (0 time) showed a consistently greater reduction in DNA molecular weight than did normal cells. For cells labeled with [³H]thymidine, part of this difference may be due to accumulated strand breaks produced by the incorporated tritium label (Fig. 3). Otherwise, this difference may result from a rapid repair system, present in normal cells but deficient in KH cells, which is able to rejoin some breaks at 4° in the short time from the beginning of irradiation until the cells are placed in the lysing solution. Such a fast component of repair has been demonstrated in bacteria (18), and its existence in mammalian cells has been discussed by Lett and Sun (17).

The KH cells used in these experiments incorporated thymidine at a rate comparable to normal fibroblasts (Ex-1) cultured under similar conditions. They were thus viable, metabolically active cells. Furthermore, the DNA of KH cells was synthesized into subunits of a size comparable to normal fibroblasts and to established cell lines. Although different investigators do not agree on the exact size of this subunit, it is certainly larger than 10^8 daltons. From the work of Huberman and Riggs (19) among others, it appears that DNA is synthesized into small fragments that must be joined to

produce molecules of 10^8 daltons. This fact would indicate that a competent ligase must exist in these repair-deficient cells. If this be the case, either: (1) the ligase involved in normal DNA synthesis and the ligase required for rejoining radiation-induced strand breaks must differ; or (2) other enzymatic steps necessary to produce the correct ligase substrate or to allow ligase-substrate contact must be deficient or greatly reduced in KH cells.

The alkaline-sucrose gradient method used here to assay DNA molecular weight is essentially an end-stage assay. That is, while rejoining of strand breaks could very well require a multi-step, enzyme-mediated process, this assay will only reveal fully rejoined strands. If a ligase is the final enzyme required, rejoining will be detected only if that step, the resynthesis of a phosphodiester bond by esterification of a 5'-OH phosphate to a 3'-OH group in a well-aligned helix, is carried to completion (20). However, there is evidence to suggest that single-strand breaks may require removal and reinsertion of nucleotide subunits before the ligase can physically rejoin the DNA phosphodiester backbone (21). Such a multi-step mechanism requiring excision and resynthesis before strand rejoining would be analogous to that reported for repair of UV-induced damage (3).

In this context, it is interesting to note the report that the amount of unscheduled DNA synthesis found after UV exposure in cells cultured from a progeric patient was similar to that found in cells from normal individuals (22). Two possibilities may explain this result in light of our findings. First, the two types of repair may involve different enzymatic processes. Second, a cell could show unscheduled DNA synthesis due to insertion of labeled nucleotides, but be unable to undergo the final step in repair—the rejoining of the strand breaks by a ligase.

The progeria condition exhibits a wide range of clinical and biochemical abnormalities (23, 24). In culture, progeria cells exhibit a markedly reduced life span (number of generations) (9–11), but it is not at all clear if this phenomenon is the true result of the progeria process or merely a secondary manifestation of it. Presumably, the decreased *in vitro* vitality could result from any number of causes secondary or peripheral to the true progeria phenomenon. Consequently, the observed lack of repair reported here cannot yet be causally related to the progeria syndrome observed clinically, nor do we know whether this trait is common to all progeria patients. Even though experiments with other progeria cells, preferably from patients with different degrees of clinical severity, must be performed, the obvious hypothesis that decreased capacity for the repair of DNA is in some way associated with precocious aging is fascinating and could open a fruitful line of research.

We thank Dr. Stanley Chism for valuable discussions during the early stages of this work; Dr. Robert Hay for supplying us with the KH cells, and Drs. William Reichel and J. W. Retann for supplying the clinical history and other information on this patient; Dr. Dorothy Villee for advice and for providing biopsy specimens; and Trudy Lanman for technical assistance in obtaining the specimens. This work was supported by Grants CA-11751 and ES-00002, and by Training Grant ES-00044 (to J. E.), from the National Institutes of Health.

1. Cleaver, J. E. (1968) "Defective repair replication of DNA in xeroderma pigmentosum," *Nature* **218**, 652–656.
2. Regan, J. D. (1972) "Two forms of DNA repair in human cells following damage by chemical carcinogens," *Sym-*

- posium American Association for Cancer Research, 4-6 May, 1972, Boston, Mass.
3. Cleaver, J. E. (1969) "Xeroderma pigmentosum: A human disease in which an initial stage of DNA repair is defective," *Proc. Nat. Acad. Sci. USA* **63**, 428-435.
 4. Kleijer, W. J., Lohman, P. H. M., Ulder, M. P. & Bootsma, D. (1970) "Repair of x-ray damage in DNA of cultivated cells from patients having xeroderma pigmentosum," *Mutat. Res.* **9**, 517-523.
 5. Lett, J. T., Caldwell, I., Dean, C. J. & Alexander, P. (1967) "Rejoining of x-ray induced breaks in the DNA of leukaemia cells," *Nature* **214**, 790-792.
 6. Elkind, M. M. & Kamper, C. (1970) "Two forms of repair of DNA in mammalian cells following irradiation," *Biophys. J.* **10**, 237-244.
 7. Tsuboi, A. & Terasima, T. (1970) "Rejoining of single breaks of DNA induced by x-rays in mammalian cells: Effects of metabolic inhibitors," *Mol. Gen. Genet.* **108**, 117-128.
 8. Ormerod, M. G. & Stevens, U. (1971) "The rejoining of x-ray induced strand breaks in the DNA of a murine lymphoma cell," *Biochim. Biophys. Acta* **232**, 72-82.
 9. Goldstein, S. (1969) "Life-span of cultured cells in progeria," *Lancet* **i**, 424.
 10. Martin, G. M., Sprague, C. A. & Epstein, C. J. (1970) "Replicative life-span of cultivated human cells," *J. Lab. Invest.* **23**, 86-92.
 11. Danes, B. S. (1971) "Progeria: A cell culture study on aging," *J. Clin. Invest.* **50**, 2000-2003.
 12. Chang, R. S. (1954) "Continuous subcultivation of epithelial-like cells from normal human tissues," *Proc. Soc. Exp. Biol. Med.* **87**, 440-443.
 13. Little, J. B. (1969) "Differential response of rapidly and slowly proliferating human cells to x-irradiation," *Radiology* **93**, 307-313.
 14. McBurney, N. W., Graham, F. L. & Whitmore, G. F. (1972) "Sedimentation analysis of DNA from irradiated and unirradiated L-cells," *Biophys. J.* **12**, 369-383.
 15. Lett, J. T., Klucis, E. S. & Sun, C. (1970) "On the size of the DNA in the mammalian chromosome structural subunit," *Biophys. J.* **10**, 277-292.
 16. Cleaver, J. E., Thomas, G. H. & Burki, H. J. (1972) "Biological damage from intranuclear tritium: DNA strand breaks and their repair," *Science* **177**, 996-998.
 17. Lett, J. T. & Sun, C. (1970) "The production of strand breaks in mammalian DNA by x-rays: At different stages in the cell cycle," *Radiat. Res.* **44**, 771-787.
 18. Town, C. D., Smith, K. C. & Kaplan, H. S. (1972) "Influence of ultrafast repair processes (independent of DNA polymerase I) on the yield of DNA single-strand breaks in *Escherichia coli* K-12 irradiated in the presence or absence of oxygen," *Radiat. Res.* **52**, 99-114.
 19. Huberman, J. A. & Riggs, A. D. (1968) "On the mechanism of DNA replication in mammalian chromosomes," *J. Mol. Biol.* **32**, 327-341.
 20. Richardson, C. (1969) "Enzymes in DNA metabolism," *Annu. Rev. Biochem.* **38**, 795-840.
 21. Kapp, D. S. & Smith, K. C. (1969) "Lack of *in vitro* repair of x-ray induced chain breaks in DNA by the polynucleotide-joining enzyme," *Int. J. Radiat. Biol.* **14**, 567-571.
 22. Cleaver, J. E. (1970) "DNA damage and repair in light-sensitive human skin disease," *J. Invest. Dermatol.* **54**, 181-195.
 23. Villee, D. B., Nichols, G. & Talbot, N. B. (1969) "Metabolic studies in two boys with classical progeria," *Pediatrics* **43**, 207-217.
 24. DeBusk, F. L. (1972) "The Hutchinson-Gilford progeria syndrome," *Pediatrics* **80**, part 2, 697-724.