# The Role of DNA Repair in Aging of Cultured Fibroblasts from Xeroderma Pigmentosum and Normals[1] (35655)

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(Introduced by J. Fraser Mustard)
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Human diploid fibroblasts cultured *in vitro* provide an excellent model system for the study of aging (1). These cells have a finite lifetime so that after a period of active multiplication, their generation time increases, mitosis gradually ceases, followed finally by degeneration and death. Furthermore, the growth potential is inversely proportional to the age of the donor (1–3). However, physiological rather than chronological age seems to be important because fibroblasts from patients with diseases associated with premature aging have a shortened life-span when cultured *in vitro* (3, 4).

Xeroderma pigmentosum (XP) is a rare disease of autosomal recessive inheritance in which the skin is extremely sensitive to ultraviolet light (uv) and natural sunlight (5). Advanced cases have extreme atrophy and malignant tumors of the skin, both features which characteristically show an increased incidence during normal aging (5). A clinically variant form of XP, the deSanctis-Cacchione syndrome, also shows neurological disease (6). Autopsies on such individuals have revealed that in addition to skin and the central nervous system, internal organs, presumably shielded from sunlight, show abnormalities compatible with early senescence (7, 8). Cleaver has shown that fibroblasts cultured from the skin of such patients are unable to repair DNA damage following uv irradiation (9). Recently, he has localized the defect to an early step in the sequence of DNA repair (10), a mechanism normally present in all mammalian cells so far examined (10, 11).

Among the possibilities to be considered as the primary cause of declining growth of cultured fibroblasts is lack of DNA repair. Failure of this mechanism could impair DNA function and lead to senescence *in vitro*. It could also account for the atrophy and increased rate of carcinogenesis that occur not only in the skin of patients with XP, but also in many organs of normal individuals during normal aging (12).

To explore the possible role in aging of failure in DNA repair, studies have been carried out which compare the survival and DNA repair capacity following uv irradiation of skin fibroblasts from XP and normal individuals, at various stages of their *in vitro* lifetime.

*Materials and Methods. Fibroblast cultures.* Cell strains were established from apparently normal skin of the anterior forearm of a 20-year-old male and his 21-year-old sister with the deSanctis-Cacchione syndrome, and from normal subjects. Techniques of culture and plating have been described (2). Briefly, cultures were established and grown in Eagle's minimum essential medium supplemented with 15% fetal calf serum and nonessential amino acids in an atmosphere of 95% air:5% $CO_2$ at 37°. All passages were done with a 0.125% trypsin solution. Cultures were inspected each day and subdivided when confluent, at a 1:8 split ratio. In this way 1/8 of the cells liberated from the monolayer were inoculated into a petri dish of identical surface area, and three generations were counted when a confluent monolayer was attained (1, 2). A culture was "pro-

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nounced dead" when cells became swollen and granular and failed to grow to confluence despite frequent refeeding.

*uv irradiation.* The source was a 15-W germicidal lamp of predominantly 2537° Å wavelength with an incident dose rate of 4 erg/mm$^2$/sec. Cells ($2 \times 10^5$) were inoculated into 60 × 15-mm petri dishes containing a round, custom-fitted glass slide and allowed to grow to confluence. The growth medium was removed, cells adherent to the slide were rinsed with phosphate-buffered saline, then transferred with forceps to a 100 × 20-mm petri dish, where the slide was secured from below by a small pat of silicone grease. Cells were then irradiated through air. This method precludes a uv-shilding effect by the rim of the dish on cells growing at the periphery. Following irradiation, cells were liberated from the monolayer by trypsin, an aliquot was counted, the cells were diluted, and plated into three petri dishes for each uv dose. After incubation at 37° for 10–14 days, colonies were stained, scored, and the average number plotted.

*Autoradiography.* Cells ($4 \times 10^4$) were inoculated into 30 × 10-mm petri dishes containing a round 22-mm glass cover slip. After incubation for 16 hr, the dishes were drained and rinsed with phosphate-buffered saline, the cover slip was centered, and the cells were irradiated. To each dish was added regular growth medium containing $^3$H-thymidine, 10 μCi/ml, sp act 19.9 Ci/mmole (New England Nuclear). After 3 hr the medium was removed, the cells were rinsed twice with unlabeled thymidine $10^{-3}$ *M* in phosphate-buffered saline, and then again with phosphate-buffered saline, free of thymidine. Cells were fixed in acetic acid: ethanol (1:3) followed by 70% ethanol, then dried and mounted onto glass slides with Permount. Slides were dipped in NTB-2 emulsion (Kodak) and stored at 4° in light-proof boxes containing a desiccant. Since grain counts are linear with time of exposure (10), XP cells were exposed longer to allow more grains to appear. The preparations were then developed and stained with Wright–Giemsa. Grains overlying 50 lightly labeled nuclei were counted with good agreement by two observers, one who did not know the identity of the cells. Results were expressed as the mean grain count per nucleus per week of exposure.

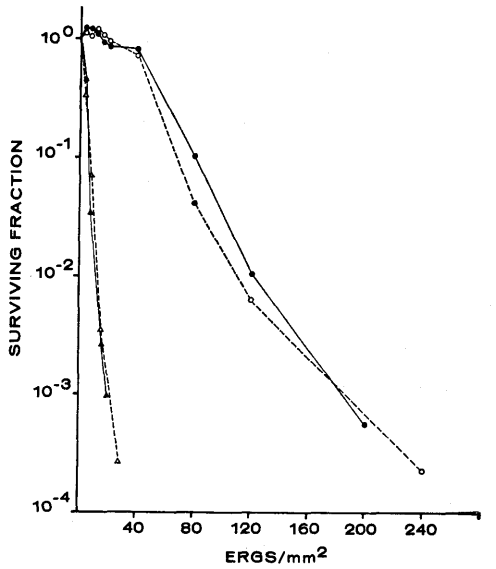


FIG. 1. Survival of early-passage cells from XP and normal controls following uv. The mean number of macroscopic colonies at each uv dose is compared to unirradiated controls which were assigned 100% survival. XP1 ▲—▲; XP2 △- -△; normal-1 ●—●; normal-2 O- -O.

*Results.* XP cells are very sensitive to uv compared to cells from age-matched normal controls (Fig. 1 and Table I). Table I also shows that the uv sensitivity of fibroblasts derived from elderly donors is indistinguishable from cells at similar stages of passage from normal donors who are fetal, newborn, and young adult. Repeated passage of fetal and newborn cells results in decreasing plating efficiency (Table I), a criterion of early senescence *in vitro* (2, 13). However, no change in sensitivity to uv is evident until late stages of culture. Cells from both patients with XP had life-spans *in vitro* which did not differ significantly from age-matched controls. On combining the data from all cell strains, an inverse correlation was found between the age of the donor and the life-span *in vitro* ($r = -.799$, $p < .01$), as has been reported previously (1–3).

While colony-forming ability following uv provides an index of DNA repair in individu-

TABLE I. Origin, Life-span, and Sensitivity to uv of Human Skin Fibroblasts Cultured *in Vitro*.

| Strain | Age of donors (years) | Life-span *in vitro*[a] (generations) | Age of culture when irradiated (generations) | Plating efficiency[b] of unirradiated controls (%) | Parameters of cell survival after uv[c] | |
|---|---|---|---|---|---|---|
| | | | | | $\tilde{D}o$ (erg/mm$^2$) | $\tilde{n}$ |
| XP-1 | 20 | 63 | 22 | 14.6 | 2 | 1 |
| XP-2 | 21 | 60 | 22 | 13.0 | 2 | 1 |
| Normal-1 | 21 | 63 | 18 | 10.8 | 23 | 4 |
| Normal-2 | 22 | 84 | 21 | 15.4 | 24 | 4 |
| Normal-3 | 75 | 48 | 24 | 18.0 | 22 | 3 |
| Normal-4 | 83 | 52 | 25 | 12.0 | 24 | 4 |
| Normal-5 | 1 week | 87 | 20 | 17.2 | 24 | 4 |
| | | | 44 | 9.8 | 25 | 4 |
| | | | 76 | 1.2 | 20 | 3 |
| Normal-6 | First trimester fetus | 99 | 20 | 12.7 | 23 | 4 |
| | | | 56 | 8.4 | 24 | 4 |
| | | | 84 | 0.6 | 18 | 3 |

[a] Ten generations were arbitrarily assigned to the phase of establishment of cell strains from explants. Subsequently, passage was performed at a 1:8 split ratio until cessation of mitosis (see Methods).

[b] Plating efficiency equals 100 times the mean number of colonies divided by the number of cells inoculated.

[c] Survival curves in radiobiology are routinely fitted to a multitarget model (18). The radiosensitivity of cells is described by a slope $\tilde{D}o$ and an extrapolation number $\tilde{n}$. $\tilde{D}o$ is the dose required to reduce survival by the factor 1/e ($\sim$37%) on the linear portion of the curve. $\tilde{n}$ is the point at which the ordinate is intersected by extrapolating the linear portion back to zero dose.

al cells, the DNA repair mechanism may be examined more directly by another method. Normally, DNA synthesis in mammalian cells is strictly scheduled, occurring only during the S phase of the cell cycle. Following exposure to uv, repair of damaged DNA occurs during all phases and is thus called unscheduled DNA synthesis (9–11). After exposing a population of asynchronously dividing cells to uv, S phase cells still incorporate large amounts of $^3$H-thymidine while those in other phases, performing unscheduled synthesis, incorporate isotope only into small patches of DNA. Autoradiographically, two types of nuclei, heavily labeled and lightly labeled, respectively, can clearly be discerned. DNA repair may be quantitated by averaging the number of grains over lightly labeled nuclei. Figure 2 shows that normal strains at early and intermediate stages of culture have virtually the same numbers of grains per nucleus at all uv doses, so that the same straight line fits both sets of points. Normal cells at late passage have somewhat fewer grains while XP cells clearly have the fewest grains at all uv doses.

*Discussion.* The results of this study are of interest in three areas. First, fibroblasts from XP are extremely sensitive to uv, confirming an earlier report on skin cells from the nonneurological form of XP (14). Thus, the defect in DNA repair is expressed phenotypically as markedly reduced survival of cultured fibroblasts following uv.

Secondly, the data show that DNA repair and survival of cells following uv irradiation may decrease at late stages of passage *in vitro*. However, no change can be detected in either parameter at earlier stages, when cultures have already begun their senescent decline. Moreover, cultured cells from elderly donors have uv sensitivities virtually identical to those of young donors at similar stages of passage. Most important, it appears that a serious defect in DNA repair does not materially curtail the life-spans of XP strains, even without taking special precautions to shield them from ambient light during routine manipulations. These findings, therefore, fail to implicate the DNA repair mechanism in

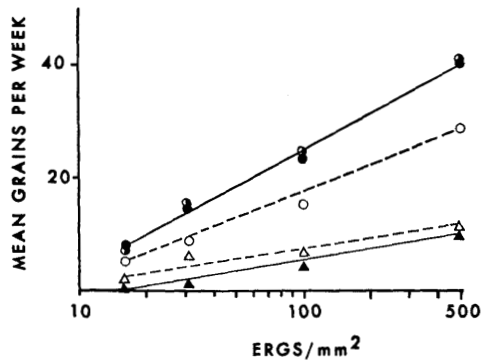


FIG. 2. Unscheduled DNA synthesis following uv in XP and normal cells at various stages of passage. Results are expressed as the mean number of grains per nucleus per week of exposure to photographic emulsion. Standard errors were ±15% as reported by others (9–11). XP1 ▲—▲; XP2 △- -△ (both at 22 generations). Normal: 20 generations ●—●; 56 generations ◑—◑; 87 generations ○- - -○. Significance of the difference between the slopes of linear regression equations for normal cells at 20 and 56 generations versus XP1 and XP2: $p<.01$; for normal cells at 20 and 56 generations versus normal cells at 87 generations: $p<.05$.

the aging process. In this context, Cleaver has reported finding normal DNA repair in fibroblasts cultured from individuals with progeria and Rothmund-Thomson syndrome (10), two disorders associated with premature aging.

Finally, the results show that a defect in DNA repair is not associated with spontaneous transformation to permanent cell lines. This is the case both in XP cells, where the defect is severe throughout the life-span *in vitro*, and in normal cells where a mild defect becomes apparent at late stages only. On the other hand, it has been reported that transformation by oncogenic viruses is more easily effected with cultured fibroblasts which are either at later stages of passage (15), or derived from older donors (16). These are important considerations since permanent lines have many similarities to malignant tumors (1, 15, 16). However, the recent report that XP cells do not show an abnormally high viral transformation frequency (17), plus the observation that abundant DNA repair is found in malignant cells (10, 11), militate against a simple connection between deficient DNA repair and carcinogenesis.

In conclusion, on the basis of techniques used in this study, it is suggested that failure in DNA repair is not causal to senescence *in vitro*. Whether the process of aging *in vivo* and the associated increased incidence of carcinogenesis are related to the accumulation of irreparable faults in informational macromolecules and/or a program in the genome, remains to be elucidated.

*Summary.* Deficient DNA repair in fibroblasts from xeroderma pigmentosum is expressed as markedly reduced survival after low doses of uv. However, the life-span *in vitro* of XP cells is not reduced below normal age-matched controls. Senescence of normal cells *in vitro*, measured by decreased plating efficiency, appears before any measurable decline in either the capacity for DNA repair or survival following uv. In no cases was spontaneous transformation to permanent cell lines observed. It is concluded that failure in DNA repair is probably not causal to aging *in vitro* or *in vivo*.

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1. Hayflick L., Exp. Cell Res. **37**, 614 (1965).

2. Goldstein, S., Littlefield, J. W., and Soeldner, J. S., Proc. Nat. Acad. Sci. U.S.A. **64**, 155 (1969).

3. Martin, G. M., Sprague, C. A., and Epstein, C. J., Lab. Invest. **23**, 86 (1970).

4. Goldstein, S., Lancet **1**, 424 (1969).

5. Lever, W. F., "Histopathology of the Skin," 794 pp. Lippincott, Philadelphia (1967).

6. Reed, W. B., May, S. B., and Nickel, W. R., Arch. Dermatol. **91**, 224 (1965).

7. Yano, K., Folia Psychiat. Neurol. Jap. **4**, 143 (1950).

8. Reed, W. B., Landing, B., Sugarman, G., Cleaver, J. E., and Melnyk, J., J. Amer. Med. Ass. **207**, 2073 (1969).

9. Cleaver, J. E., Nature (London) **218**, 652 (1968).

10. Cleaver, J. E., J. Invest. Dermatol. **54**, 181 (1970).

11. Rasmussen, R. E., and Painter, R. B., J. Cell Biol. **29**, 11 (1966).

12. Doll, R., Gerontol. Clin. **4**, 211 (1962).

13. Merz, G. S., and Ross, J. E., J. Cell Physiol. **74**, 219 (1969).

14. Cleaver, J. E., Int. J. Radiat. Biol. **18**, 557 (1970).

15. Jensen, F., Koprowski, H., and Pontén, J. A., Proc. Nat. Acad. Sci. U.S.A. **50**, 348 (1963).

16. Todaro, G. J., and Aaronson, S. A., Proc. Nat. Acad. Sci. U.S.A. **61**, 1272 (1968).

17. Aaronson, S. A., and Lytle, C. D., Nature (London) **228**, 361 (1970).

18. Elkind, M. M., and Whitmore, G. F., "The Radiology of Cultured Mammalian Cells," 615 pp. Gordon & Breach, New York (1967).

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