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VARIATION OF DNA REPAIR CAPACITY IN PROGERIA CELLS UNRELATED TO GROWTH CONDITIONS

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SUMMARY - Fibroblasts from two progeria patients were tested for their capacity to repair γ-irradiated DNA. An immunofluorescence host cell reactivation (HCR) assay was employed to compare normal, progeria, and Xeroderma Pigmentosum strains. HCR decreased markedly over a three day period in confluent cultures, indicating that growth conditions can substantially effect the DNA repair observed in HCR of cultured fibroblasts. Using a variety of growth conditions, one progeria strain showed decreased HCR, while a second progeria strain showed normal HCR. These results suggest both that heterogeneity for DNA repair capacity exists among progeria fibroblast strains and that decreased DNA repair capacity is unlikely to be the basic genetic defect in progeria.

INTRODUCTION

Several genetic diseases appear to show marked in vitro sensitivity to X-irradiation by a variety of assays. These include Ataxia Telangectasia (1), Xeroderma Pigmentosum (XP,2), Hereditary Retinoblastoma (3), partial trisomy 13 (3), Gardner's syndrome (4), and possibly progeria. The basis for these defects are unknown, though in the case of Xeroderma Pigmentosum (XP) a deficiency of an enzymatic step involved in the repair of UV irradiated DNA seems likely (2).

The Hutchinson-Gilford Progeria Syndrome (26410) is a rare human genetic disease with striking features resembling accelerated aging (5). Progeria patients are usually of average intelligence and appear clinically normal at birth. However, in early childhood, they develop severe growth retardation and a senescent phenotype including: balding, aged-appearing skin, generalized atherosclerosis, and strokes. They die prematurely as a result of myocardial infarctions with an average age of 13. The primary defect in progeria is unknown.

Since progeria patients have a remarkable clinical presentation which resembles premature aging, it has been of considerable interest to determine whether

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cultured cells from these patients exhibit a defect which could be related to the etiology of their disease and of aging. Epstein et al (6) first suggested that single strand rejoining of γ-irradiated DNA might be defective in progeroid cells. They subsequently showed decreased rates of single-strand rejoining of irradiated cellular DNA in 4 progeric strains (7,8). Brown et al (9,10) showed that co-cultivation of 2 progeria strains with normals or with each other reversed the single strand DNA rejoining defect, and suggested that complementation groups for DNA repair induced by γ-irradiation exist in progeria. Using a somewhat different assay of single strand rejoining, differing results were obtained by Regan and Setlow, who observed no differences between 1 progeric strain, and 2 atypical progeric strains, as compared to normal strains (11). Weichselbaum et al (3) assayed the X-ray sensitivity of human diploid fibroblasts *in vitro* by measuring their ability to form colonies following irradiation. They found 2 progeric strains with increased radiosensitivity and 3 progeric strains with relatively normal sensitivity. The above studies left open the possibility that the radiation damage of cellular components other than DNA may have been responsible for the increased radiosensitivity observed. However, Rainbow reported that 2 progeric fibroblast strains showed a DNA repair defect using a sensitive host cell reactivation (HCR) assay of γ-irradiated adenovirus (12,13), thus adding other evidence to suggest a defect in DNA repair exists for progeria.

In the past, studies of DNA repair dealing with specific genetic diseases have not emphasized the potentially important role of the state of growth of the cell. Altered *in vitro* growth kinetics may influence the rate of DNA repair. For example, there is evidence that cellular sensitivity to X-ray damage is greatest in S phase and least in G_0 (14,15). This suggests that differences in X-ray sensitivity observed between cell types may partly reflect the relative fractions of cell types in the various stages of the cell cycle. To address this question, and to help resolve the controversy as to whether progeria cells show defective DNA repair, we compared the HCR capacity of normal, progeric, and XP fibroblasts as a function of the growth conditions and time in stationary culture.

MATERIALS AND METHODS

Diploid human fibroblasts were cultured in plastic 75 cm^2 flasks (Falcon) in a 5 % CO_2 atmosphere at 37°C with 90-100% humidity. The growth medium consisted of Fagle's minimal essential medium (MEM) with 10% fetal bovine serum (FBS). Normal strains IMR-90 (fetal lung), IMR-91s (skin), and XP strain GM-544 (complementation group A) were obtained from the Institute for Medical Research, Camden, NJ. Two patients with classical Hutchinson-Gilford Progeria were examined by one of us (WTB). Fibroblast strains 441 (KC) and 234 (SK) obtained from skin biopsies of these 2 patients were kindly supplied by Drs. R. Erbe and J. Epstein. Cells were passaged weekly at a split ratio of 1:4. Experiments were conducted with cultures between passages 10 to 20 during which time no marked differences in the rates of proliferation were observed among the different cell strains.

Adenovirus type 2 (Ad2), obtained from Drs. Harold Ginsberg and C. H. Y. Young, was grown in KB cells. Stock viral preparations, containing 1012 particles (1010 plaque-forming-units/ml), were suspended in phosphate-buffered-saline (PBS). Aliquots were γ-irradiated on dry ice at -76°C with either 0, 0.5, 1.5, and 3 million rads at a dose rate of 4 Mrads/hr using a commercial cobalt source (Isomedix, Parssipany, NJ). The dose was calibrated by a Harwell Rad Dosimeter.

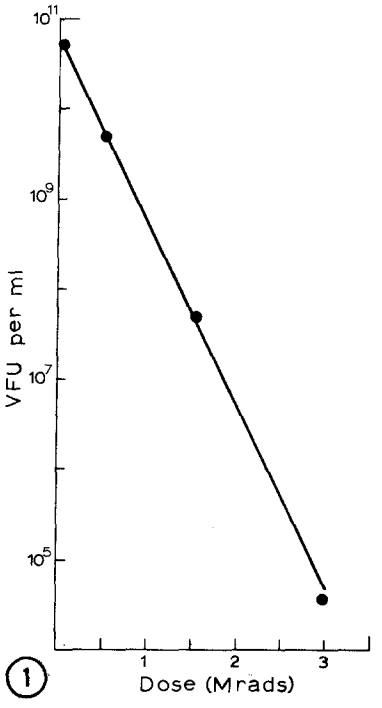
The efficiency of DNA repair of irradiated Ad2 was determined using previously described methods (16) based on the immunofluorescence detection of V antigen (Ad2 capsid proteins) present only in virus-producing cells. Cell suspensions at a concentration of 1.5-3.0 x 10^5 cells/ml were distributed with a Hamilton repeating syringe using 10 ul aliquots into 60 well (Falcon 3034) microtiter plates. At various times after seeding, media was shaken from the wells and the cultures were inoculated with serial dilutions of Ad2 in MEM with 1 % FBS. Nine serial 3-fold dilutions were used with 6 replicate wells per dilution. The 10th row served as an uninfected control. Indirect immunoflourescent staining was performed 24-48 hrs postinfection following washing of the monolayers with PBS and fixation with absolute ethanol as previously described (17). Cells were stained by dispensing 5 ul of rabbit anti-Ad2 serum diluted 1:100 with PBS to each well, incubating for 45 min at 37°C, washing 3-5 times with PBS, and incubating for 30 min with 5 ul of fluoroscein isothiocyante conjugated goat anti-rabbit IgG immunoglobulin (Antibodies Incorporated) diluted 1:20 with PBS. The plates were washed with PBS and viewed at a magnification of 252 with a Leitz Diavert microscope equipped for epifluorescence excitation at 495 nm and suppression at 525 nm.

The number of V antigen-forming-units (VFU) per virus inoculum was calculated from the average number of V antigen positive cells in 6 replicate wells at 3 to 5 dilutions. The range of variation for the 6 replicate wells was usually within +/- 10 % of the mean. Each plate was scored independently by 2 investigators. The average VFU per ml of irradiated and unirradiated virus stock was calculated for each cell strain. HCR is expressed as the surviving fraction of VFU, determined as the ratio of the titers of VFU of irradiated to unirradiated Ad2 determined in each cell strain.

A dose of 1.5 Mrads was chosen for comparisons of progeric with XP and normal cell strains. To determine the effect upon DNA repair of length of time in stationary culture, HCR was studied in progeric, normal, and XP strains at 6, 24, and 72 hours after cell passage. The cells were plated at a confluent density of 3 x $10^5/cm^2$ and by 6 hours, over 90 % of the cells had attached to the substrate and appeared flattened. By 24 hours the cells had oriented themselves in packed parallel patterns typical of confluent fibroblast cultures. In addition, HCR was assayed in the presence of either 1 or 10 % FBS in stationary or growing cultures as described in results.

RESULTS

The titer of the irradiated virus decreased in a linear fashion as dose of irradiation increased, as shown in figure 1. A decrease of 6 orders of magnitude was found for a dose of 3 Mrads.



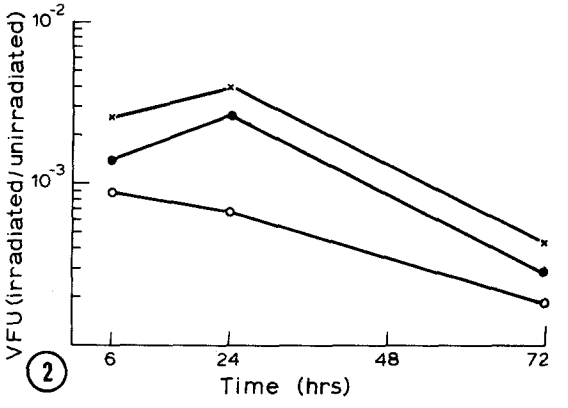


Figure 1. The titer of V antigen forming units (VFU) per ml of irradiated adenovirus decreased in a linear fashion with increasing dose of γ-irradiation. Titers were obtained using infected monolayer HeLa cell cultures.

Figure 2. Time course of HCR following subcultivation at confluent density. VFU is expressed as a ratio of the number of cells positive following infection with virus irradiated with 1.5 Krads to the number of cells positive following infection with unirradiated virus. X = normal (91s), ● = progeria (234), and O = XP (541).

DNA repair capacity was examined in two progeric strains, two normal strains, and one XP strain. HCR was assayed at 6, 24, and 72 hrs after plating. All three cell types showed a marked decrease in the level of HCR between 24 and 72 hours as shown in figure 2. The XP cells showed the lowest level of HCR, while the progeric strain 234 was intermediate to the high level of the normals.

Since prolonged culture past confluence caused a decline in the overall HCR, we tested the effect of cell density, cell proliferation, and serum factors upon DNA repair. Cells were plated at a subconfluent density of $1.5 \times 10^5/cm^2$, and allowed to grow in 10 % serum for 24 hrs. Cultures were then incubated in 1 % serum for 3 days to arrest growth. These cells remained subconfluent during the 3 days. One of a replicate set of cultures was refed with 10 % serum 24 hrs prior to infection to stimulate DNA synthesis and cell cycle progression. At the end of

this 24 hr period these cells were nearly confluent. The other replicate culture was refed with 1 % serum to continue growth arrest which maintained a subconfluent cell density. As a further control, a normal and a progeric culture were infected 6 hrs after plating at a subconfluent density and kept in 10 % serum to maintain active proliferation. All cultures were then assayed for HCR 48 hrs following infection. Under these conditions, neither the concentration of serum factors nor cell density, nor the stimulation of cell proliferation by serum or subcultivation affected HCR. As shown in figure 3, the progeric strain 234 showed consistently reduced HCR compared to the two normal strains, while the second progeric strain 441 showed normal levels of HCR. The XP line showed the most reduced HCR under all conditions as compared to the normals.

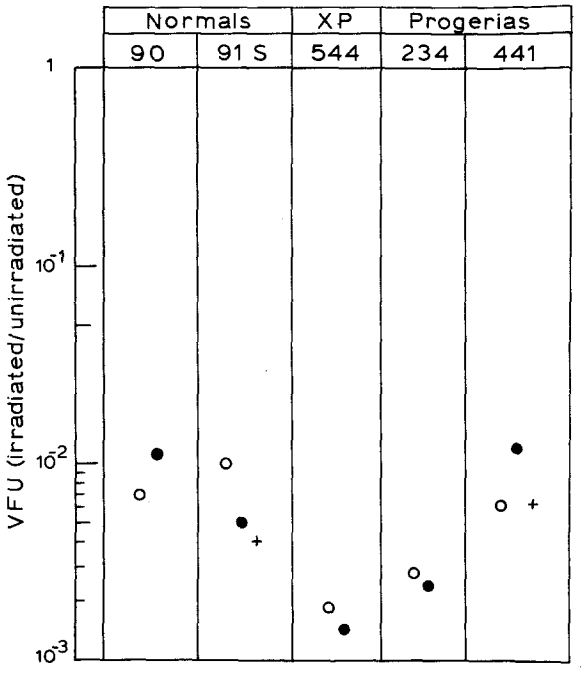


Figure 3. Host cell reactivation of irradiated adenovirus in Progeria, Xeroderma Pigmentosum, and Normal fibroblasts as a function of growth state. Following plating for 24 hours in 10% FCS, the cells were grown for 72 hours in 1% serum. Duplicate cultures were either refed 1% serum (○) or 10 % serum (●) for 24 hours prior to infection with either irradiated (1.5 Mrads) or uniradiated virus. As a control, two strains were infected at 6 hours following subcultivation and refed with 10 % serum (+).

DISCUSSION

Our results using the HCR assay, showed decreased repair of γ-irradiated adenovirus DNA in one progeric strain (234) while normal repair was found in a second progeric strain (441). The normal level of HCR for progeric strain 441 confirms the normal level of X-ray sensitivity demonstrated for a different fibroblast isolate (442) from the same progeric patient by Weichselbaum et al (3). Although their results were obtained by assaying colony-forming-ability following irradiation of cells which leaves open the question of possible cellular damage, the HCR assay we employed relies upon the expression of a viral gene product requiring repair of the irradiated viral DNA. This has the advantage that the cells to be tested are not themselves irradiated. The lethal effect of X-ray damage is thus likely to be on DNA and not, for example on RNA, or on the cellular mitotic apparatus.

Using a similar HCR assay system Rainbow (3) has reported a decrease in HCR capaciy in two progeric strains. Compared to Rainbow's findings we found a normal level of HCR for one progeric strain (441) and a decreased level for the second strain (234). Our findings of decreased HCR for the XP strain (541) do however confirm his results for XP (2). The D_0, that dose of radiation which would produce a reduction to 1/e of remaining virus, can be calculated (2) for the HCR assay by extrapolating between the titer of irradiated virus and unirradiated virus to the point where 37% (an average of one hit per virus) of the titer remains. For the strains in figure 3, the averages are normals - 31.7 and 30.5, progerics - 32.5 and 25.2, and XP - 23.3 ($\times 10^4$ Rads). Compared to Rainbow's findings (3), our normal and XP strains showed moderately lower D_0's which could indicate some difference in the performance of the assay or the extent of DNA damage induced under these circumstances.

The results indicated that the presence of serum factors did not greatly influence the HCR assay under the circumstances employed. Also, little difference in HCR was seen between stationary and proliferationg cells of each strain at either subconfluent or confluent cell density. Under these conditions in which

cells are not irradiated, there was no suggestion of variation in DNA repair efficiency during the cell cycle. However, longer times in stationary culture (G_0) reduced DNA repair capacity in normals and progerics alike. This suggests that prolonged culture in G_0 leads to a decreased level of some factor or factors involoved in the repair of γ-irradiated DNA which is reflected by decreased HCR.

The results suggest there may be genetic heterogeneity among strains of progeric fibroblasts as assayed by HCR and in progeria, DNA repair capacity is not a consistent marker for the disease. Failure to find consistent differences between normals and all progerics was independent of culture conditions. We suggest the variable defect of DNA repair as assayed by HCR seen in progeria may reflect a more primary metabolic defect only indirectly related to DNA repair.

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