Reduced host cell reactivation of oxidatively damaged DNA in ageing human fibroblasts

ANDREW J. RAINBOW, NATALIE J. ZACAL and DERRIK M. LEACH

Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada

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Abstract. Many reports have linked oxidative damage to DNA and the associated avoidance and/or repair processes to carcinogenesis, ageing and neurodegeneration. Cancer incidence increases with age and there is evidence that oxidative stress plays a role in human ageing and neurodegeneration. Several reports have suggested that the accumulation of unrepaired DNA lesions plays a causal role in mammalian ageing. Since base excision repair (BER) is the main pathway for the repair of oxidative DNA lesions, the relationship of BER to human ageing and carcinogenesis is of considerable interest. The aim of the present study was to examine the relationship between donor age and increasing time of cells in tissue culture and the repair of oxidative DNA damage in primary human skin fibroblasts. Methylene blue (MB) acts as a photosensitizer and after excitation by visible light (VL) produces reactive oxygen species that result in oxidative damage to DNA. MB+VL produce predominantly 8-hydroxyguanine as well as other single base modifications in DNA that are repaired by BER. We used host cell reactivation (HCR) of a non-replicating recombinant human adenovirus, Ad5CMV*lacZ*, which expresses the β-galactosidase (β-gal) reporter gene, to measure BER of MB+VL-damaged DNA. HCR of β-gal activity for the MB+VL-treated reporter gene was examined in 10 fibroblast strains from normal donors of ages 2 to 82. The effect of cell passage number on HCR was also examined in human skin fibroblasts from 2 normal donors. We found a significant reduction in HCR with increasing cell passage number, indicating that BER decreases with increasing time of cells grown in tissue culture. We also found a significant correlation of donor age with HCR of the MB+VL-treated reporter gene for high passage number, but not for low passage number fibroblasts. The present study provides evidence that a decrease in BER of oxidatively damaged DNA may play a role in carcinogenesis, ageing and neurodegeneration.

Correspondence to: Dr Andrew J. Rainbow, Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada
E-mail: rainbow@mcmaster.ca

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Introduction

Many reports have linked oxidative damage to DNA and the associated avoidance and repair processes to carcinogenesis, ageing and neurodegeneration (1). Cancer incidence increases with age and there is substantial evidence that oxidative stress plays a role in human ageing and neurodegeneration. Since base excision repair (BER) is the main pathway for the repair of oxidative DNA lesions, the relationship of BER to human ageing and carcinogenesis is of considerable interest. Several reports have suggested that the accumulation of unrepaired DNA lesions plays a causal role in mammalian ageing. Cells from patients with Cockayne syndrome (CS), a rare inherited photosensitive progeroid syndrome, are defective in the repair of 8-oxoguanine and 7,8-dihydro-8-oxoadenine (8-OxoA) resulting from ROS, suggesting that disorders with defects in BER can result in phenotypes displaying premature ageing (2). In mammalian cells, the oxidative DNA lesions are removed by BER, which is initiated primarily by two glycosylases, OGG1 and NTH1. Chen *et al* (3) reported an age-dependent decrease in OGG1, and Szczesny *et al* (4) reported an age-dependent deficiency in the import of OGG1 into mitochondria. In additional, Cabelof *et al* (5) reported a lack of inducibility of DNA polymerase β and AP endonuclease following exposure to ageing mice as compared to young mice, suggesting an age-related loss in BER after exposure to oxidative stress.

Several, but not all, studies have shown that cells from individuals varying in age do have a different replicative lifespan *in vitro*. Ksiazek *et al* (6) reported that human peritoneal mesothelial cells (HPMCs) from aged donors (>75 years) have lower proliferative capacity and increased 8-OxoG content compared to cells from younger individuals (<25 years). However, even in early passage HPMCs, repair of 8-OxoG was delayed in cells from aged compared to young donors; a positive correlation was noted between donor age and 8-OxoG level, and an inverse relationship was noted between those 8-OxoG levels and subsequent cell lifespan.

Yuan *et al* (7) demonstrated that primary human skin fibroblasts were more susceptible to oxidative stress from hydrogen peroxide, the superoxide radical, or linoleic acid hydroperoxide at late passage compared to early passage,

presumably due to decreases in cellular reduced glutathione concentration and catalase activity. Surprisingly, a more recent study by Matsuo *et al* (8) reported that primary human skin fibroblasts derived from aged donors were more resistant to oxidative stress from hydrogen peroxide, linoleic acid hydroperoxide or UVB compared to fibroblasts from young donors. The activity of glutathione peroxidase was higher in fibroblasts from aged compared to young donors, suggesting that the fibroblasts from aged donors are more resistant to oxidative stress due to an increase in cellular glutathione peroxidase. Kaneko *et al* (9) showed that skin fibroblasts established from fetal tissue showed an increase in 8-oxo-2'-deoxyguanosine content and a similar decreased activity of 8-oxo-2'-deoxyguanosine endonucclease and DNA polymerases with increasing cell passage during *in vitro* cellular ageing. Increases in superoxide dismutase and glutathione peroxidase were observed prior to increased 8-OxoG, while catalase activity decreased with *in vitro* cellular ageing at late passage. These results suggest that defense mechanisms against oxidative stress remain sufficiently active in late passage fetal skin fibroblasts, but that repair systems against oxidative damage decrease at late passage. The 8-OxoG content in fibroblasts from fetal skin changed little up to 60 population doubling levels (PDL) and then increased significantly after 63 PDL (9). In contrast, the 8-OxoG content in fibroblasts from a 36-year-old adult donor changed little up to 38 PDL but increased beyond 44 PDL and in fibroblasts from a 72-year-old donor the 8-OxoG content changed little up to 32 PDL, but then increased after 36 PDL. These results suggest that repair systems against oxidative damage may start to decrease at an earlier cell passage number in fibroblasts from aged compared to young donors.

There are numerous factors that could contribute to ageing and lifespan, such as telomere shortening, hormone levels and multiple targets of reactive oxygen species, making it difficult to establish a direct role of BER in counteracting ageing. It was, therefore, considered important to examine the repair of oxidative DNA damage in skin fibroblasts from young and elder donors.

We previously reported a host cell reactivation (HCR) assay for examining BER of methylene blue plus visible light (MB+VL)-induced 8-OxoG lesions in a number of different cell strains including xeroderma pigmentosum fibroblasts from complementation group C and CS fibroblasts (10-13). The HCR assay utilizes a recombinant non-replicating adenovirus (Ad5CMV*lac*Z) expressing the β-galactosidase (β-gal) reporter gene under control of the cytomegalovirus immediate early (IE) promoter (14) to examine the ability of different cell types to remove damage and reactivate reporter gene expression. Methylene blue (MB), a type II photosensitizer, produces singlet oxygen ($^1O_2$) upon exposure to visible light (VL) in the presence of oxygen (15) which through interaction with DNA predominantly leads to the formation of 8-OxoG lesions with a small number of other single base oxidative lesions occurring (16,17).

In the present study, we examined HCR of a MB+VL-treated reporter gene in human skin fibroblasts from 10 donors of different ages and from fibroblasts from 2 donors as a function of increasing passage number of cells grown in tissue culture.

Materials and methods

Cell lines and virus. Skin fibroblasts from normal donors, GM9503, GM37G, GM38A, GM969, GM8399, GM8400, GM01706A, AG02261B, GM1863 and GM00288B were obtained from the National Institute of General Medical Sciences, Human Genetic Cell Repository, Coriell Institute for Medical Research (Camden, NJ, USA). Cell cultures were grown at 37˚C in a humidified incubator in 5% CO_2 and cultured in Eagle's α-minimal essential media (α-MEM) supplemented with 10% fetal bovine serum and antimycotic-antibiotic 100 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Gibco-BRL, Grand Island, NY, USA).

The recombinant adenovirus Ad5MCMV*lacZ* (AdCA35) (14) was obtained from Dr F.L. Graham, McMaster University. The virus was propagated, collected and titered as previously described (18).

Treatment of the virus with methylene blue plus visible light (MB+VL). Preparation of MB was as previously described (13). Treatment of the virus was performed as previously described (10). Briefly, 80 μl of AdCMV*LacZ* virus was added to 3.6 ml of phosphate-buffered saline (PBS) containing 20 μg/ml MB in 35-mm Petri dishes on ice. With continuous stirring, the virus suspensions were irradiated (or mock irradiated) with VL. VL irradiation of virus employed a General Electric 1000-watt halogen lamp (GE R1000) at a distance of 70 cm from the bulb. After each time point, 400 μl of irradiated virus was removed, diluted appropriately in unsupplemented α-MEM and used to infect the cell monolayers.

HCR of MB+VL-treated Ad5CMVLacZ. Primary human fibroblasts were seeded at a density of $1.5x10^4$ to $1.8x10^4$ cells/well in 96-well plates (Falcon, Franklin Lakes, NJ, USA). After seeding, cells were incubated for 18-24 h and subsequently infected with 40 μl of untreated or MB+VL-treated virus for 90 min at a multiplicity of infection (MOI) of 20-40 plaque forming units (pfu)/cell. Following infection, cells were overlaid with 160 μl of complete α-MEM and incubated for a further 12-40 h before harvesting. A single HCR experiment consisted of triplicate wells for each treatment of the virus, and triplicate wells of non-infected cells were used to obtain background levels of β-gal activity. β-gal activity was scored by measuring absorbance at 570 nm as previously described (19).

Results

HCR of the MB+VL-treated reporter gene in normal human skin fibroblasts from different passage numbers in tissue culture. Normal skin fibroblasts from 2 donors of ages 10 and 19 years were examined for HCR of the MB+VL-treated reporter gene in fibroblasts from different passage numbers in tissue culture. Representative survival curves for β-gal activity for MB+VL-treated virus for the different fibroblast strains are shown in Fig. 1. From the β-gal survival curves, the VL exposure required to reduce β-gal activity to 50% of that for non-VL-exposed virus (D_{50}) was used as a measure of HCR. It was noted that for both GM9503 and GM8399 cells, HCR of the MB+VL-treated reporter gene was greater in

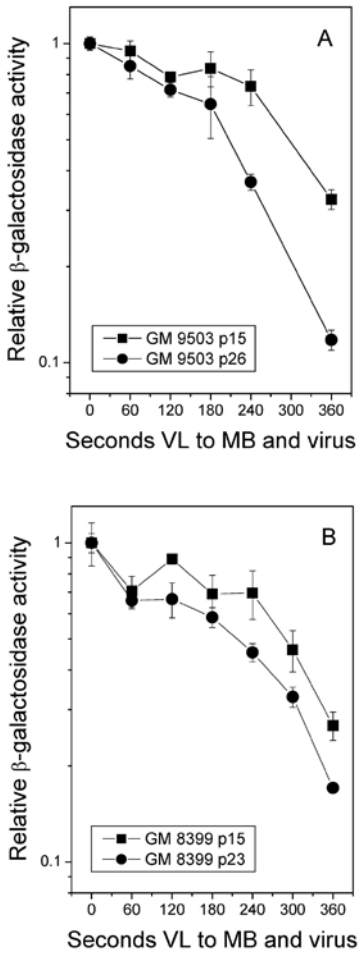

Figure 1. HCR of β-gal activity for MB+VL-treated Ad5CMV*lac*Z in normal skin fibroblasts from high and low passage number in tissue culture. Cells were scored for β-gal activity at 40 h after infection. Representative results of a single experiment for (A) GM9503 and (B) GM8399 normal fibroblasts. MB, methylene blue; VL, visible light; HCR, host cell reactivation.

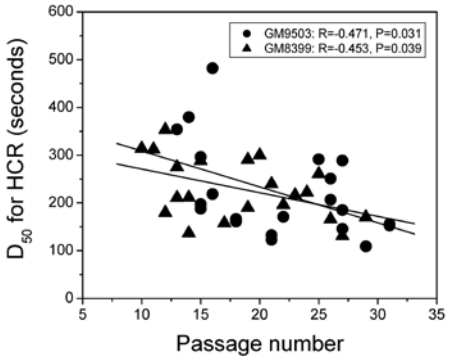

Figure 2. D_{50} for HCR of β-gal activity for MB+VL-treated Ad5CMV*lac*Z in normal skin fibroblasts from 2 different donors [GM9503 (●) and GM8399 (▲)] plotted as a function of cell passage number. MB, methylene blue; VL, visible light; HCR, host cell reactivation.

low passage compared to high passage number cells. Similar results were obtained in additional individual experiments where low passage number cells ranged from 13 to 21 for GM9503 and 10 to 19 for GM8399 and high passage numbers ranged from 22 to 31 for GM9503 and 19 to 29 for GM8399.

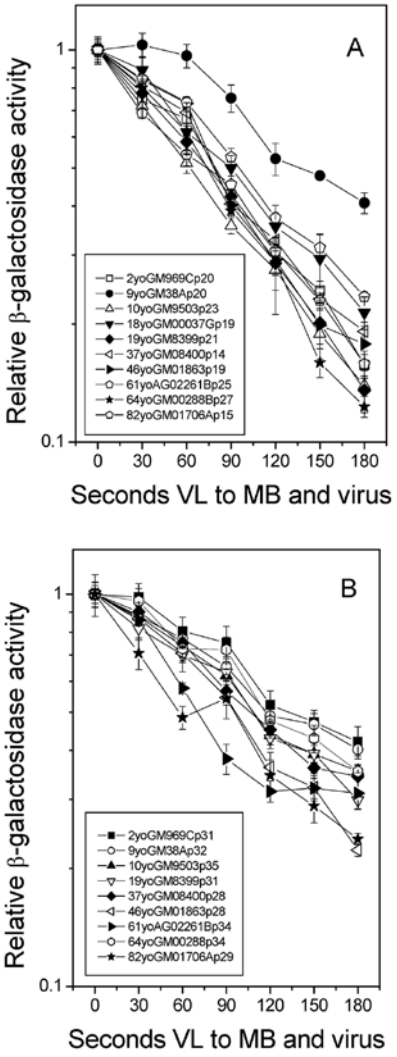

Figure 3. HCR of β-gal activity for MB+VL-treated Ad5CMV*lac*Z in several normal skin fibroblasts from donors of different ages. Cells were scored for β-gal activity at 40 h after infection. Representative results of a single experiment for (A) low (14-27) and (B) high (28-35) passage number. MB, methylene blue; VL, visible light; HCR, host cell reactivation.

Differences between low and high passage number for individual experiments ranged from 6 to 12 for GM9503 and 6 to 14 for GM8399. The D_{50} value for HCR of the MB+VL-treated reporter gene is plotted against passage number for GM9503 and GM8399 over a range of 10 to 32 cell passages in Fig. 2. A significant decrease in D_{50} value with increasing passage number was found for GM9503 (R=-0.471, P=0.031) and GM8399 (R=-0.453, P=0.039).

HCR of the MB+VL-treated reporter gene in normal human skin fibroblasts of different ages. In a second series of experiments, normal human fibroblasts from 10 donors of different ages ranging from 2 to 82 years at passage numbers 12 to 35 were examined for HCR of MB+VL-treated Ad5CMV*lac*Z. Each experiment was performed under a different condition of light output from the 1000-watt halogen lamp. Survival curves of β-gal activity for the MB+VL-treated virus in the various fibroblast strains are shown in Fig. 3 for representative experiments using low (A) and high (B) passage number.

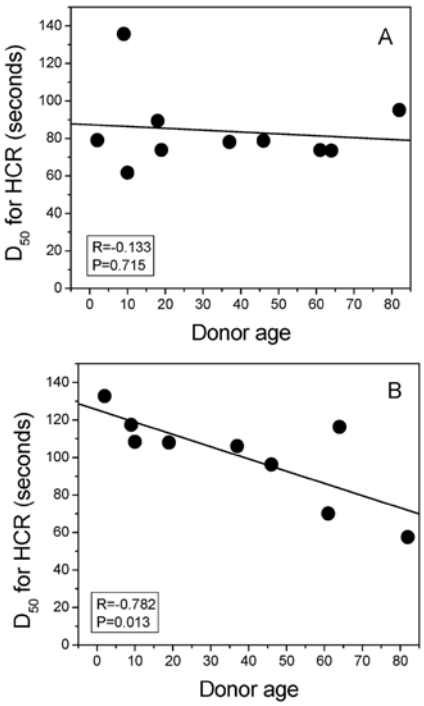

Figure 4. D_{50} for HCR of β-gal activity for MB+VL-treated Ad5CMV*lac*Z in several different normal skin fibroblasts plotted as a function of donor age. Representative results of a single experiment for (A) low (14-27) and (B) high (28-35) passage number. MB, methylene blue; VL, visible light; HCR, host cell reactivation.

For each experiment, the D_{50} value for HCR of the MB+VL-treated reporter gene was plotted against donor age in Fig. 4. For this sampling of normal skin fibroblasts from donors of different ages, a significant correlation of HCR was noted with donor age at high passage number (28-35) (R=-0.782, P=0.013) (Fig. 4B) but not at low passage number (14-27) (R=-0.133, P=0.715) (Fig. 4A).

Discussion

Reports on the repair of UV-induced DNA damage in skin fibroblasts from young compared to elder donors are conflicting. Using cells of a similar passage number of less than 13 PDL, Takahashi *et al* (20) reported a significant decline in HCR of a transfected UV-irradiated chloramphenicol acetyltransferase gene, but not in the actual removal of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts as determined using an ELISA assay, in skin fibroblasts from aged donors compared to fibroblasts from young donors. These authors also found reduced expression of DNA repair synthesis-related genes in skin fibroblasts from aged donors compared to young donors and suggest that the reduced HCR reflects a reduced post-UV repair capacity in cells from ageing donors that results from an impairment in a late step of NER due to the decrease in factors required for repair synthesis. In contrast, Merkle *et al* (21) reported no significant difference between normal skin from young and aged donors for host cell reactivation of a transfected UV-irradiated luciferase gene providing no indication that the higher incidence of skin cancer observed with increasing age is due to an age-related decrease in the ability to repair UV-induced DNA damage. In their experiments they compared fibroblasts from young and aged donors at cumulated PDL ranging from 7 to 15 in the various fibroblast strains. In addition, they found that fibroblasts from both young and aged donors showed a similar and significant decrease in HCR with increasing PDL over the range of 10 to 40 PDL.

In the present study, we found a significant reduction in HCR of the MB+VL-treated reporter gene with increasing cell passage number, indicating that BER decreases with increasing time of cells grown in tissue culture. We found also a significant reduction in HCR of the MB+VL-treated reporter gene with increasing donor age for high passage (passage 28-35), but not for low passage primary human fibroblasts (passage 14-27). Kaneko *et al* (9) reported that the 8-OxoG content of fibroblasts from a 72-year-old donor changed little up to 32 PDL and increased after 36 PDL, whereas the 8-OxoG content of fibroblasts from fetal skin changed little up to 60 PDL and then increased after 63 PDL. Although the study by Kaneko *et al* did not directly measure DNA repair, their report is consistent with a decrease in the repair of 8-OxoG at an earlier cell passage number in fibroblasts from aged compared to young donors as reported here.

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