Experimental Cell Research **256,** 308–314 (2000)
doi:10.1006/excr.2000.4826, available online at http://www.idealibrary.com on IDEAL®

Gene-Specific DNA Repair of Pyrimidine Dimers Does Not Decline during Cellular Aging *in Vitro*

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A large number of studies have demonstrated that various kinds of DNA damage accumulate during aging and one of the causes for this could be a decrease in DNA repair capacity. However, the level of total genomic repair has not been strongly correlated with aging. DNA repair of certain kinds of damage is known to be closely connected to the transcription process; thus, we chose to investigate the level of gene-specific repair of UV-induced damage using *in vitro* aging of human diploid skin fibroblasts and trabecular osteoblasts as model systems for aging. We find that the total genomic repair is not significantly affected during cellular aging of cultures of both human skin fibroblasts and trabecular osteoblasts. Gene-specific repair was analyzed during cellular aging in the dihydrofolate reductase housekeeping gene, the p53 tumor suppressor gene, and the inactive region X_{754}. There was no clear difference in the capacity of young and old cells to repair UV-induced pyrimidine dimers in any of the analyzed genes. Thus, *in vitro* senescent cells can sustain the ability to repair externally induced damage.

Key Words: **DNA repair; human; aging; p53; DHFR.**

INTRODUCTION

Cells are continuously challenged by a large number of internally and externally induced chemical changes in DNA that, if left unrepaired, result in mutations. Reduced maintenance of genomic stability may contribute to the aging process through changes in gene expression, and several studies have shown that DNA damage and mutations accumulate during aging [1, 2]. It has been hypothesized that the age-related increase in DNA damage and mutations is due to a decrease in DNA repair and during the past 20 years several investigators have studied the relationship between DNA repair and aging. A large proportion of these studies have involved repair measurements only at the level of total genomic DNA, using the amount of unscheduled DNA synthesis (UDS) and other similar techniques after UV-irradiation as a measure of repair capacity. However, results have been contradictory. DNA repair capacity has been reported either to decrease [3], to not change [4], or even to increase [5] as a function of age within the same model system, *in vitro* aging of fibroblasts.

UV-C irradiation of cells generates mainly cyclobutane pyrimidine dimers (CPDs) in genomic DNA, and in mammalian cells this DNA lesion is known to be removed by the nucleotide excision repair (NER) pathway. In general, NER removes a broad spectrum of lesions and collectively these are characterized as bulky adducts. The NER mechanism is rather complex, involving at least six different proteins and protein complexes in the DNA damage recognition and excision steps [6]. XPA, RPA, and XPC are involved in damage recognition and stabilization of the preincision complex, the transcription factor TFIIH, with its associated helicases XPD and XPB, serves to open up the DNA helix around the damage, and XPG and XPF are nucleases that incise 5′ and 3′, repectively, to the lesion [6]. It has been shown that CPDs are repaired faster from transcriptionally active DNA than from the overall genome [7]. This efficient repair is due to differences in chromatin structure and, more importantly, it is due to the rapid removal of CPDs from the DNA strand transcribed by RNA polymerase II through a transcription-coupled repair (TCR) mechanism [8]. DNA repair involving transcription coupling is termed preferential repair and transcriptionally independent DNA repair is termed global genomic repair. The importance of NER and TCR is immediately apparent when looking at human diseases. Xeroderma pigmentosum (XP) and Cockayne syndrome (CS) are rare autosomal recessive diseases, which are characterized at the molecular level by defects in nucleotide excision repair. XP is diagnosed by the occurrence of extreme photosensitivity, early onset of skin cancers, and neurological abnormalities [9]. Seven different complementation groups, designated XPA to XPG, have been identified and all the genes have been shown

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0014-4827/00 $35.00

to participate in NER [10]. CS patients show dwarfism, sunlight sensitivity, neurological abnormalities, and premature aging [9]. Two genes, CSA and CSB, have been cloned and are found to be involved in both transcription and repair, and cells from CS patients are deficient in TCR [8].

Very little is currently known about the correlation between aging and repair capacity in specific DNA regions. In an *in vivo* study recently reported by Guo and coworkers [11], hepatocytes from old rats showed a reduced rate of repair in the transcriptionally active and preferentially repaired albumin gene compared with hepatocytes from young rats. Furthermore, they showed an age-related reduced extent of repair in the total genome and in two transcriptionally inactive genes, *H-ras* and embryonic myosin heavy chain (MHC^{emb}), which were not repaired preferentially [11]. However, no age-related studies on gene-specific NER in human cells have been reported.

In the present study we have analyzed the correlation between *in vitro* age and nucleotide excision repair in UV-C-irradiated human diploid fibroblasts and trabecular osteoblasts. The removal of pyrimidine dimers has been assessed in the total genome, in two actively transcribed genes, the dihydrofolate reductase (DHFR) gene, which is a housekeeping gene, and the p53 tumor suppressor gene, and finally in the inactive X_{754} region. We did not see any significant differences between young and senescent cells of either cell type in any of the analyzed genes; thus, senescent human cells seem to retain the capacity to repair pyrimidine dimers *in vitro*.

MATERIALS AND METHODS

Cell Cultures

Human diploid skin fibroblast culture, designated ASS-2, established from breast biopsies from a 20-year-old healthy female donor [12], and normal human trabecular osteoblast culture, designated M57, established from trabecular bone biopsies from a 57-year-old healthy male following a procedure described by Kassem *et al.* [13], were used in this study. Cells were grown in complete medium consisting of Dulbecco's Minimal Essential Medium (DMEM; Bio Whittaker, Belgium) supplemented with 10% fetal calf serum (FCS; *In vitro,* Denmark), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Bio Whittaker). Cells were grown at 37°C in humidified 5% CO_2 and 95% air. G_1-arrested, synchronized cells were obtained by serum-starvation of cells for 48 h in DMEM containing 2% FCS for the ASS-2 cells and 0.2% FCS for the M57 cells, and synchronization was tested by the incorporation of 5′-bromo-2′-deoxy-uridine following a protocol supplied by Boehringer Mannheim with Labeling and Detection Kit II.

Serial Passaging

Almost confluent cultures of ASS-2 and M57 were trypsinized and split at a 1:8, 1:4, or 1:2 ratio, depending on the cell density, and the medium was changed twice a week. Serially passaged cultures were considered to have become senescent and to have completed their replicative lifespan when no increase in cell number had occurred within 1 month. At each subculturing, the number of cells was counted using a Coulter Counter (Coulter Electronics, UK), and the number of population doublings (PD) was calculated as $\log(N/N_0)/\log 2$, where N is the output cell density at the time of splitting and N_0 is the input cell density. Cumulative population doubling level (CPDL) attained after serial passaging was referred to as 100% lifespan completed. In this study cells were considered to be young if they had completed <40% lifespan and cells were considered to be old if they had completed >90% lifespan.

Cells at different PD levels were checked for senescence-associated β-galactosidase activity at neutral pH, which is considered a marker of cellular aging [14]. In brief, cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS) for 5 min, washed in PBS, and stained overnight at 37°C in 1 mg/ml X-gal, 40 mM citric acid/sodium phosphate buffer (pH 6), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride and 2 mM magnesium chloride. For analysis of morphological changes, cells were fixed in −20°C methanol for 30 min and thereafter stained with Giemsa stain for 1 h. After washing in tap water, cells were observed under a microscope.

Telomere Restriction Fragment Length Analysis

Extraction and restriction enzyme digestion of DNA. DNA was extracted from young and senescent cultures of human diploid skin fibroblasts and normal human trabecular osteoblasts using a high-salt procedure. Cells were washed and lysed in lysis buffer (1% SDS, 0.5 M Tris, pH 8.0, 20 mM EDTA, pH 8.0, 10 mM NaCl, 0.5 mg/ml Proteinase K) and the lysates were incubated at 37°C for >16 h. Then, $\frac{1}{4}$ vol saturated NaCl was added and protein precipitate was removed by centrifugation at 7*g* for 30 min. Next, $2\frac{1}{2}$ vol 96% ethanol was added to the supernatant and DNA was collected and dissolved in TE (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0). Samples were RNase-treated (0.1 mg/ml) for 3 h at 37°C. DNA was precipitated using $\frac{1}{5}$ vol 11 M ammonium acetate and $2\frac{1}{2}$ vol 96% ethanol, redissolved in TE, and stored at 4°C. DNA was quantified by measuring absorbance at 260 nm and digested with *Hin*fI and *Rsa*I (3 units/μg DNA, New England Biolabs) for 8–20 h at 37°C.

In gel hybridization and restriction fragment length analysis. Electrophoresis of 1 μg DNA was performed in 0.5% agarose gels in $\frac{1}{2}\times$ TBE at 30 V for 18 h. Detection of the telomere-containing restriction fragments was performed as described by Mather [15]. Gels were dried under vacuum at 55°C for 45 min, denatured in 0.5 M NaOH, 1.5 M NaCl for 10 min and neutralized in 0.5 M Tris, pH 7.5, 1.5 M NaCl for 10 min. Gels were then immersed in hybridization solution (5× Denhardts solution, 5× SSC, 0.1 M Na_2HPO_4, 0.01 M $Na_4P_2O_7$, 33 μg/ml salmon sperm DNA, 0.1 μM ATP). The telomeric probe $(CCCTAA)_4$ was end-labeled using a T4 polynucleotide kinase (New England Biolab) and [γ-^{32}P]dATP (Amersham) [16], 250,000 cpm/ml hybridization solution was added, and hybridization was performed for 12–16 h at 37°C. Gels were washed 3× for 5 min in 0.24× SSC at room temperature. Mean telomere restriction fragment (TRF) length was determined after exposure of the gel to a Molecular Dynamics phosphor-imaging screen and analyzed using Imagequant software (Molecular Dynamics Inc.). Differences between TRF length in young and old cells were analyzed using Student's *t* test.

DNA Repair Assay

The procedure for measuring repair of UV-induced pyrimidine dimers described previously [7] was followed with a few exceptions.

Introduction of UV damage. Cells were seeded onto 150-mm plates and maintained until 70% confluent; then, the cells were synchronized by serum deprivation 2 days prior to UV irradiation. Cells were washed 2× in Hank's buffer (ice-cold for cells to be lysed immediately and 37°C for incubation) and UV-irradiated at 10 J/m^2 at 254 nm. Cells were incubated with low-serum medium during

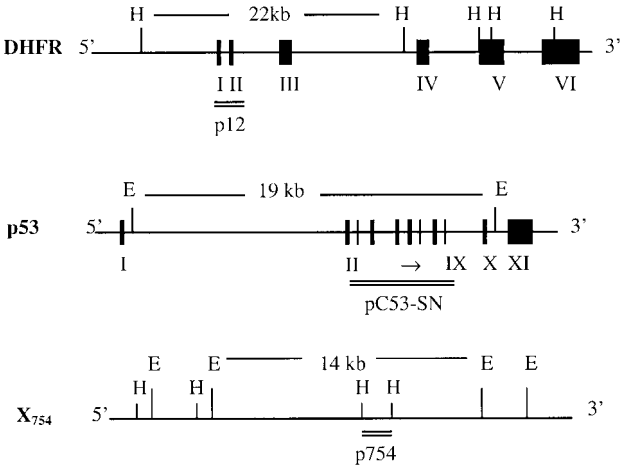

FIG. 1. Genomic maps of the DHFR and p53 genes and the X_{754} region. The letter H indicates *Hin*dIII restriction sites in the DHFR gene and the X_{754} region, and the letter E indicates *Eco*RI restriction sites in the p53 gene and the X_{754} region. The black boxes represent exons. The positions of the DNA probes used in this study are shown as black lines below the gene.

repair, thus preventing cell division to avoid the need of CsCl-gradient centrifugation of the DNA to separate parental and replicated DNA.

Extraction and restriction enzyme digestion of DNA. DNA was extracted at different time points after UV irradiation, as described for TRF analysis. DNA samples were restricted with *Hin*dIII or *Eco*RI (5 U/μg DNA, 6 h, 37°C, New England Biolab) and were used directly, as no DNA replication had occurred.

Southern blot analysis. Ten micrograms of each sample was either treated with T4 endonuclease V, resulting in recognition and incision at pyrimidine dimers, or mock-treated with buffer at 37°C for 15 min. Southern blotting was performed as described previously [16]. In brief, the DNA was electrophoresed in 0.6% alkaline agarose gels, the gels were washed for 1 h in neutral gel wash (0.5 M Tris, pH 7.5, 1.5 M NaCl), and ethidiumbromide (EtBr) was added after 30 min. Then, the gels were washed for 30 min in H_2O, after which the gel was photographed for analysis of total genomic repair. DNA was nicked for 20 min in 0.25 M HCl and neutralized for 20 min in 0.5 M NaOH, 1.5 M NaCl. The DNA was blotted onto Hybond N+ (Amersham) nylon membrane. Prehybridization was performed in 0.5 M Na_2HPO_4, pH 7.2, 7% SDS and 1.25 mM EDTA, pH 8.0 at 68°C for >15 min, after which the random ^{32}P-labeled probe was added and hybridization was performed for >20 h. After hybridization, the blot was washed briefly at room temperature and for 45 min at 68°C in 40 mM Na_2HPO_4, pH 7.2, 1% SDS, 2 mM EDTA, pH 8.0, then for 45 min at 68°C in 40 mM Na_2HPO_4, pH 7.2, 0.1% SDS, 2 mM EDTA, pH 8.0, and finally for 10 min at 68°C in 100 mM Na_2HPO_4, pH 7.2, 2 mM EDTA, pH 8.2. The membranes were exposed to Molecular Dynamics phosphor-imaging screen (Molecular Dynamics Inc.) and analyzed as described below.

Quantification of CPDs. After EtBr staining of alkaline agarose gels as described above, the fluorescence of the gel was recorded and the frequency of CPDs in the genome overall was determined as described by Freeman *et al.* [17]. The frequency of CPDs in specific gene fragments was found by quantification using Imagequant software (Molecular Dynamics Inc.) of radioactivity in DNA bands from membranes exposed to phosphor-imaging screen. The numbers of T4 endo V sensitive sites (i.e., CPDs) in DNA fragments containing the DHFR, p53, and X_{754} genes were calculated as described previously [7]. DNA repair was measured as the disappearance of CPDs over time.

Probes

The map of the three genes in which gene specific DNA repair was assessed is presented in Fig. 1. The 1.8-kb *Eco*RI fragment of the DHFR gene obtained from Guiseppe Attardi, California Institute of Technology, detects a 21.5-kb fragment in *Hin*dIII-digested genomic DNA. Bert Vogelstein, Johns Hopkins University, kindly supplied a 1.8-kb p53 cDNA probe which detects a 19-kb fragment in *Eco*RI-restricted DNA. Leon Mullenders, University of Leiden, kindly provided a 2.0-kb *Hin*dIII fragment of the X_{754} region which detects a 14-kb fragment in *Eco*RI-restricted DNA. The 754 locus on the X chromosome is transcriptionally inactive in primary human fibroblasts [18].

Statistical Analysis

Results from multiple experiments are reported as mean ± standard deviation. Differences between young and old cells were assessed by one-way analysis of variance followed by Student's t test, and $P < 0.05$ was considered to be significant.

RESULTS

Serially passaged normal human diploid skin fibroblasts ASS-2 and trabecular osteoblasts M57 exhibited the typical phenomenon of cellular aging and a limited proliferative capacity in culture. Representative longevity curves (Fig. 2) show that the ASS-2 cell strain underwent 35 CPDL in a period of 206 days and was comparable to that reported previously [12]. The osteoblast strain attained 47 CPDL within a period of 507 days. To monitor the senescence status of the fibroblast and osteoblast cultures, cells were stained for senescence-associated β-galactosidase (SA-β-gal) activity and with Giemsa stain to characterize the morphological changes that these cells undergo during their lifespan. The cell cultures exhibited increased cell size, increased amounts of debris, and the expected age-related increase in SA-β-gal activity, such that less than 1% of the cells stained positive in young cultures (<30% lifespan completed), whereas more than 90% of the cells stained positive in old cultures (>95% lifespan

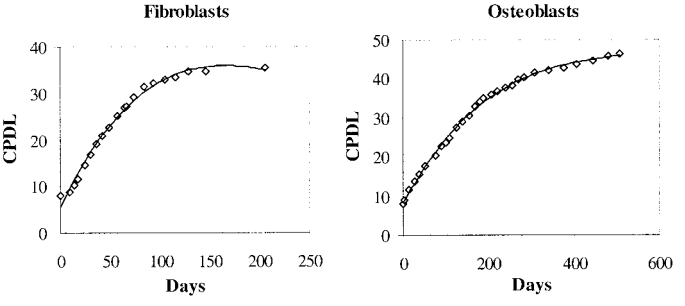

FIG. 2. Representative longevity curves showing cumulative population doubling level (CPDL) of the human skin fibroblast cell strain and the human trabecular osteoblast cell strain employed in this study.

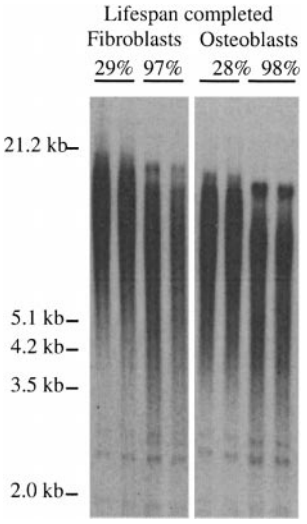

FIG. 3. Telomere restriction fragment length of young and old human diploid skin fibroblasts and trabecular osteoblasts serially passaged *in vitro.* Genomic DNA was digested, electrophoresed, and hybridized with a telomeric probe. The size in kb and position of the DNA marker are indicated.

completed, data not shown). In addition, we characterized the degree of telomere shortening in the two cell strains used in this study. The mean telomere restriction fragment length decreased from 9.9 kb in young fibroblasts to 7.9 kb in old fibroblasts (Fig. 3). This constitutes a significant decrease in the TRF length of about 2 kb ($P < 0.05$), which corresponds to a loss of about 80 bp per cell division. Similarly, a significant decrease of about 1 kb was observed for the osteoblast strain, which was comparable to a recent report from our laboratory [19].

After demonstrating that both cell strains underwent *in vitro* aging, we examined the repair characteristics of young and senescent cells. The removal of UV-induced cyclobutane pyrimidine dimers from the genome overall was determined by calculating the average molecular weight of DNA run in ethidium bromide-stained agarose gels and comparing lanes that were and were not treated with T4 endonuclease V, which recognizes and incises at CPDs. The initial dimer frequency after exposure to 10 J/m^2 UV-C was about 0.5 dimer/20 kb for young and old cells from both cell strains. As shown in Fig. 4, no significant changes were seen when comparing the repair capacity of young and senescent human diploid fibroblast cultures. Both young and old cells removed 65% of the dimers in the total genome within 24 h. In the osteoblast strain also, we did not see any dramatic decrease in total genomic repair; however, this cell strain seemed to repair the genomic DNA at a slower rate than that of the fibroblasts.

We then measured the removal of CPDs from two actively transcribed genes, DHFR and p53, and from the inactive 754 region of the X chromosome during cellular senescence. We chose the housekeeping DHFR gene because gene-specific repair in this gene has been characterized rather extensively and the p53 gene because the protein plays an essential role in the maintenance of genomic stability and is involved in induction of cell cycle arrest, promoting repair and apoptosis. Finally, we analyzed the repair in an inactive region, which is known not to be preferentially repaired. Figure 5A shows representative autoradiograms of repair experiments performed after irradiating young and senescent fibroblasts with 10 J/m^2 UV-C. In Table 1, initial dimer frequencies after 10 J/m^2 are shown and no differences between young and old cells were observed. As can be seen from Fig. 5, neither the actively transcribed genes nor the inactive region was repaired at a different rate or to a different extent in young and old fibroblasts. These results were confirmed in the osteoblast strain (Fig. 5).

DISCUSSION

In this study we have used serial passaging of two different normal human cell types, diploid skin fibroblasts and trabecular osteoblasts, as model systems of aging. We have shown that both cell strains exhibit the typical Hayflick phenomenon and undergo expected changes during *in vitro* aging, including changes in cell morphology, limitation of proliferative capacity, increase in senescence-associated β-galactosidase activity, and significant reduction of telomere length. The observed reduction of 2 kb in the fibroblast strain is similar to that reported previously for human fibroblasts [20].

There is evidence both for and against the causal involvement of nucleotide excision repair in cellular senescence and aging. Using unscheduled DNA synthesis after UV irradiation as a measure of nucleotide excision repair, some researchers have shown a reduction with *in vitro* age [3, 21], others have not been able

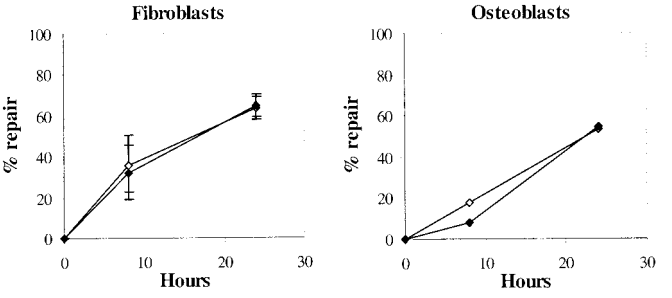

FIG. 4. Repair in the total genome in diploid fibroblasts and trabecular osteoblasts. ◇, young cells; ◆, senescent cells. Points represent the mean ± standard deviation of at least three different biological experiments for the fibroblast strain and the mean of two biological experiments for the osteoblast strain.

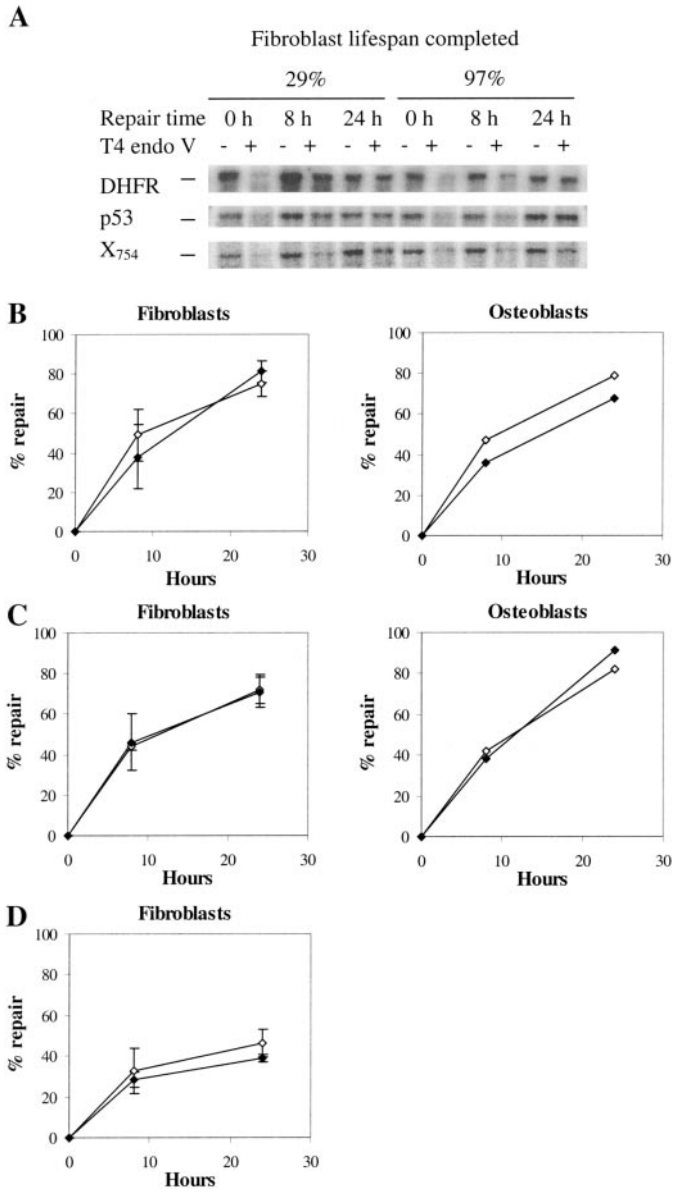

FIG. 5. (A) Representative autoradiograms showing CPD removal in the DHFR, p53, and X_{754} genes by young and old human diploid fibroblasts 0, 8, and 24 h after irradiation with 10J/m^2; (−) without or (+) with T4 endonuclease V treatment. Repair of pyrimidine dimers in (B) the DHFR gene, (C) the p53 gene, and (D) the X_{754} region in diploid fibroblasts and trabecular osteoblasts. ◇, young cells; ◆, senescent cells. Points represent the mean ± SD of at least three different biological experiments for the fibroblast strain and the mean of two biological experiments for the osteoblast strain.

to detect a difference [4, 22], and repair has, in addition, been reported to increase [5]. Furthermore, Kunisada *et al.* [23] did not find any difference in the capacity of young and senescent fibroblasts to repair a plasmid vector containing a transcribed gene. It can be argued that if a defect in NER is in fact involved in determining cellular lifespan *in vitro,* then NER-deficient cells should have a reduced lifespan. This is, however, not the case; the lifespan of fibroblasts obtained from xeroderma pigmentosum patients, which are deficient in NER, have a comparatively normal *in vitro* lifespan [24]. This was also the case for cells obtained from Cockayne syndrome patients [24], and these cells are deficient in transcription-coupled NER. Our results in the present study do not lend support to the hypothesis that decreased NER during serial passaging gives rise to genomic instability, resulting in the senescence phenotype. We did not see any age-related changes in NER, either global genomic or gene specific. The analysis of repair in both the DHFR gene and the p53 gene revealed no differences between young, actively dividing cells and old, irreversibly arrested cells. Also, the analysis of DNA repair in a transcriptionally inactive region did not show age-related changes.

However, *in vivo,* the correlation between age and NER is considerably stronger. Comparing the UDS capacity of different species shows a correlation between species lifespan and UDS capacity (for a compilation see [25]). It has been shown that old rats exhibit a decreased ability to repair UV-induced damage compared to young rats [26, 27]. This decrease could furthermore be reversed by caloric restriction, which is the only known treatment that can prolong the lifespan of mammals [26]. In a more recent report [11], reduced gene-specific repair was shown in hepatocytes from old rats, and calorically restricted animals of the same age had a repair capacity comparable to young rats. Investigations of human *in vivo* aging also show an age-related decline in DNA repair capacity. Hartwig and Korner [28] reported an age-related decline in incision at UV-induced lesions, and the ability of peripheral blood T lymphocytes and cultured skin fibroblasts from donors of different ages to repair an UV-irradiated plasmid decreased about 0.6% per year [29, 30]. Thus, it seems likely that there is a difference in the mechanisms underlying *in vivo* and *in vitro* aging and that NER could play a role in aging *in vivo.*

Therefore, it is necessary to look for other possible mechanisms leading to cellular senescence, and it could be argued that oxidative damage and repair of oxidative damage might be a potential primary cause

TABLE 1

Initial Cyclobutane Pyrimidine Dimer (CPD) Frequency per DNA Fragment after 10 J/m^2 (mean ± SD)

	Initial CPDs in fibroblasts	
	Young	Old
DHFR	1.1 ± 0.3	1.0 ± 0.3
p53	1.1 ± 0.1	1.3 ± 0.2
X_{754}	0.9 ± 0.2	0.9 ± 0.1

of cellular senescence. Cells grown in culture are continuously subjected to a relatively high amount of environmental oxidative stress due to the high partial O_2 pressure in the atmosphere. Reducing the amount of stress either by growing under low-oxygen conditions or by adding antioxidants to the growth medium can prolong the lifespan of fibroblasts in culture [31, 32], and increasing the oxidative stress by H_2O_2 treatment or growing at high oxygen partial pressure actually decreases the *in vitro* lifespan of fibroblasts [31, 33]. It was recently shown that accelerated senescence induced by oncogenic Ras occurs through an alteration in the intracellular level of reactive oxygen species (ROS), thereby supporting the theory of ROS being causally involved in cellular senescence. Telomeres are known to shorten due to the problem of DNA replication at the ends of chromosomes, and prevention of this shortening by induction of telomerase results in an extension of lifespan [34]. Furthermore, it has been shown that oxidative stress rather specifically causes accelerated shortening of the telomeres, thus implying that oxidative conditions, telomere length, and thereby cellular lifespan are linked [32]. Finally, the occurrence of the oxidative lesion 8-oxodeoxyguanosine has been shown to increase during cellular aging and, concurrently, the repair of this lesion was shown to be reduced in senescent cells [31, 35].

In summary, we did not see any change in either global genomic or gene-specific DNA repair of UV-induced damage with *in vitro* age. Thus, this study does not support the idea that nucleotide excision repair is involved in *in vitro* aging. However, it does not rule out the possibility that nucleotide excision repair is involved in *in vivo* aging.

We thank the Danish Research Council for support.

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Received November 26, 1999
Revised version received January 13, 2000