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Original Contribution

Endogenous DNA damage clusters in human hematopoietic stem and progenitor cells

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ABSTRACT

Clustered DNA damages—multiple oxidized bases, abasic sites, or strand breaks within a few helical turns—are potentially mutagenic and lethal alterations induced by ionizing radiation. Endogenous clusters are found at low frequencies in unirradiated normal human cells and tissues. Radiation-sensitive hematopoietic cells with low glycosylase levels (TK6 and WI-L2-NS) accumulate oxidized base clusters but not abasic clusters, indicating that cellular repair genotype affects endogenous cluster levels. We asked whether other factors, i.e., in the cellular microenvironment, affect endogenous cluster levels and composition in hematopoietic cells. TK6 and WI-L2-NS cells were grown in standard medium (RPMI 1640) alone or supplemented with folate and/or selenium; oxidized base cluster levels were highest in RPMI 1640 and reduced in selenium-supplemented medium. Abasic clusters were low under all conditions. In primary hematopoietic stem and progenitor cells from four non-tobacco-using donors, cluster levels were low. However, in cells from tobacco users, we observed high oxidized base clusters and also abasic clusters, previously observed only in irradiated cells. Protein levels and activity of the abasic endonuclease Ape1 were similar in the tobacco users and nonusers. These data suggest that in highly damaging environments, even normal DNA repair capacity can be overwhelmed, leaving highly repair-resistant clustered damages.

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DNA damage clusters—two or more closely opposed oxidized bases, strand breaks, or abasic sites—are repair resistant, persistent, and potentially highly lethal and mutagenic damages [1,2]. Ionizing radiation induces bistranded clustered damages – double-strand breaks (DSBs) and oxidized pyrimidine (OxyPyr), oxidized purine (OxyPur), and abasic clusters – in a characteristic ratio or spectrum of approximately equal levels of DSBs, OxyPyr clusters, and OxyPur clusters and slightly fewer abasic clusters. Clusters are induced by a single radiation "hit," i.e., the ionizing photon or particle and its radical clouds [1,3].

Clusters are also found in unirradiated cells. Cellular metabolism in an aerobic atmosphere generates radicals [4,5], including reactive oxygen species (ROS), known to induce oxidized bases and abasic sites in unirradiated human cells and tissues [6]. Such species can also produce endogenous clusters in cultured cells [7–9]. Although most normal human hematopoietic cell lines contain low levels of clustered damages, two radiation-sensitive lines (TK6 and WI-L2-NS) accumulate OxyPur clusters and OxyPyr clusters (but extremely low levels of abasic clusters) [8], suggesting an important role for repair genotype in determining endogenous cluster levels in cultured cells. Whether other factors play a role in steady-state cluster levels was not known. To determine the effect of cell environment on cluster levels, we grew these two lines on standard and supplemented media and found a substantial lowering of endogenous cluster levels by selenium.

Primary human skin tissue contains low endogenous cluster levels [9]. This does not seem to be the result of its three-dimensional architecture, because cultured skin cells in either two-dimensional (monolayer) or three-dimensional cultures contained higher cluster levels than those in human skin. To determine whether primary human hematopoietic cells also have low endogenous cluster levels, we determined cluster levels in DNAs isolated from unirradiated, primary human bone marrow stem and progenitor cells from eight healthy donors. Samples from four non-tobacco-users contained extremely low levels of all cluster classes, similar to most cultured human hematopoietic cells. DNAs from four tobacco-user donors contained high levels of all clusters—including abasic clusters, previously found in cells of hematopoietic origin only after ionizing radiation exposure [3]. Protein levels and enzyme activity toward abasic clusters of the abasic endonuclease human (h) Ape1 indicated no systematic reduction in repair in tobacco-users' cells. These results suggest that levels of DNA-damaging agents in these tobacco-using

Abbreviations: AP, abasic (apurinic or apyrimidinic); αT, α-anomeric 2′-thymidine; BSA, bovine serum albumin; DHU, 5,6-dihydrouridine; DSB, double-strand break; HRP, horseradish peroxidase; OxyBase, oxidized base (cluster); OxyPur, oxidized purine; OxyPyr, oxidized pyrimidine; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; THF, tetrahydrofuranyl; UDG, uracil DNA glycosylase.

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donors are sufficient to overwhelm cellular repair capacity, allowing the accumulation of potentially mutagenic clustered damages, even in tissue not directly exposed to tobacco constituents. The data from both cultured hematopoietic lines and primary stem and progenitor cells show that cell environment plays a major role in determining the level of endogenous clustered damages.

Materials and methods

Human cells

Human cells [28SC monocytes; American Type Culture Collection (ATCC) CRL 9855] were grown on Iscove's modified Dulbecco's medium (IMDM; Gibco BRL, Grand Island, NY, USA), 10% fetal bovine serum (Hyclone, Logan, UT, USA). Other human cells (TK6, ATCC CRL-8015; WI-L2-NS, ATCC CRL-8155) were grown in RPMI 1640, 10% fetal bovine serum (as recommended by the ATCC). In some experiments, RPMI 1640 was supplemented with folic acid (4 mg/L), sodium selenite (0.017 mg/L), or both. All cells were grown without antibiotics and were ascertained mycoplasma-free by periodic testing (Bionique, Saranac Lake, NY, USA). Primary human bone marrow stem and progenitor cells were from Cambrex BioScience (Walkersville, MD, USA). Cells were grown and handled under yellow lights to minimize light-induced cellular damage [10]. All cells were obtained and handled under Brookhaven National Laboratory IRB-approved conditions.

DNA isolation

Cells in medium (1×10^6 cells/0.25 ml in small vials, chilled on ice) were harvested by immersing the vial into dry ice and then stored at −80°C. Each vial of frozen cells was thawed rapidly and EDTA added to a final concentration of 83 mM; the cell suspension was mixed with an equal volume of 2% agarose (InCert; Cambrex) in TE (10 mM Tris–Cl, pH 7.5, 1 mM EDTA) and formed into plugs. DNA samples were isolated with minimum exposure to air to prevent formation of additional lesions [3,11] and with minimum mechanical shear, which can reduce the size of DNA isolated in agarose plugs. All solutions used during DNA isolation (except those containing detergents or enzymes) were Ar-bubbled for 20 min immediately before use. The solidified plugs were placed into 4-ml plastic tubes containing lysis solution [L buffer (20 mM NaCl, 0.1 M EDTA, 10 mM Tris–Cl, pH 8.3) bubbled with Ar for 20 min before the addition of proteinase K (Roche Molecular Biochemicals, Indianapolis, IN, USA) and *n*-lauroylsarcosine to 1 mg/ml and 1.0%, respectively]. At each change of solution, the tubes were gassed with Ar before capping. Samples were incubated at 37°C for 96 h in Ar in a Billups–Rothenberg chamber (Billups–Rothenberg, Del Mar, CA, USA), with daily changes of lysis solution. Digested plugs were rinsed with TE to remove detergent, treated with 10 vol of TE containing 40 μg/ml phenylmethylsulfonyl fluoride (PMSF), and then soaked 2× (1 h each) in 10 mM Tris–HCl, 150 mM NaCl, 0.1 mM EDTA, pH 8. For damage analysis, DNA from the cell lines was digested with NotI and that from stem/progenitor cells with AscI according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA).

Enzymes

Two preparations of *Escherichia coli* Nfo protein (endonuclease IV) were purified as previously described (Nfo BMS [12]; Nfo MS [13]). Homogeneous *E. coli* Nth protein and Fpg protein were prepared as previously described [14,15] or in the Sutherland laboratory [14]. Protein homogeneity was verified by SDS–PAGE electrophoresis. Activities of the various proteins were confirmed under appropriate reaction conditions using well-defined DNA base modifications within synthetic oligonucleotides and/or by cleavage of supercoiled substrates containing fewer than one lesion (for that enzyme) per molecule. Their absence of nonspecific scission (essential for determination of clusters at very low frequencies) was tested using undamaged DNA (see, e.g., Bennett et al. [8]). The *E. coli* Nfo and human Ape1 AP endonucleases were devoid of detectable AP-lyase and DNA glycosylase activities on DNA substrates containing 8-oxo-7,8-dihydro-2′-deoxyguanosine, uracil, ethenobases, or other modified bases.

Complex DNA cluster determination

For DNA glycosylase treatment, duplicate plugs were transferred to 70 mM Hepes·KOH, 100 mM KCl, 1 mM EDTA, pH 7.6, and then to the same buffer containing 1 mM DTT, 10 mg/ml bovine serum albumin (BSA). Plugs were treated with sufficient homogeneous, lesion-class-specific enzyme (for 1 plug containing 500 ng human genomic DNA, 1.2 μg Nth protein, 40 ng endonuclease IV, or 40 ng Fpg protein) to cleave at all substrate sites in positive control DNA from cells irradiated with 50 cGy of 50-kVp X-rays. Plugs were then treated with proteinase K (1 mg/ml), 1.0% Sarkosyl in L buffer. Plugs were rinsed and equilibrated into neutral electrophoresis buffer (10 mM Tris, 87 mM acetic acid, 0.5 mM EDTA free acid, pH 8). Samples and molecular length standard DNAs (*Saccharomyces cerevisiae* chromosomes, λ ladders) were electrophoresed using neutral transverse alternating field electrophoresis [7,16]. Gels were stained with ethidium bromide and destained, and a quantitative electronic image was obtained using a charge-coupled device (CCD)-based imaging system [17]. A DNA dispersion curve was determined using the DNA length standards, and the number average length of each DNA distribution was computed. The DSB, oxidized base, or abasic cluster frequencies were then calculated [12,18], and then for comparison with radiation-induced damage yields, the values were normalized to the DSB level produced by 50 cGy of 100-kVp X-rays.

Clusters in cell lines were determined in at least duplicate experiments, with the following numbers of replicate gels per cell line, given in order for oxypurine clusters, abasic clusters, and Fpg clusters: WIL-2 NS, all 3–5 replicates; WTK1, all 3 replicates; 28SC, 8, 10, and 9 replicates, respectively; and TK6, all 2–6 replicates. Only single primary bone marrow samples were available for each donor; determinations were carried out from each sample in at least 4 replicate measurements given in order for oxypurine clusters, abasic clusters, and Fpg clusters: 1390 C, all 5 replicates; 1148F, 5, 5, and 4 replicates, respectively; 1179D, 7, 7, and 6 replicates; 1240A, 7, 7, and 6 replicates; 1238A, all 5 replicates; 1119A, all 5 replicates; 1119A, incubated overnight, all 5 replicates; 2070B, 5, 6, and 6 replicates; and 2289C, 6, 4, and 6 replicates, respectively.

Oligonucleotides containing single lesions

Oligonucleotides (17-mers) containing a single α-anomeric 2′-thymidine (αT) nucleotide were prepared as described [19,20]. Other oligodeoxyribonucleotides were purchased from Eurogentec (Seraing, Belgium), including those containing tetrahydrofuranyl (THF) and 5,6-dihydrouridine (DHU) nucleosides and complementary oligonucleotides containing dA, dG, dC, or T opposite the adduct. The oligonucleotide sequences were 5′-AGCATTCGXGACTGGGT containing αT or THF and d(TGACTGCATAXGCATGTAGACGATGTGCAT) containing DHU, where X is the position of the modified nucleotide. These sequences have been previously used to study the repair of α-anomeric deoxynucleotides and dihydropyrimidines in DNA [20–22]. Oligonucleotides were end-labeled as previously described [21]. The labeled oligonucleotides were annealed to their appropriate complementary oligonucleotides in 50 mM NaCl, 10 mM Hepes/KOH (pH 7.5) at 65°C for 3 min as previously described [23]. The resulting duplex oligonucleotides are referred to as X·C (G, A, T), where X is αT, THF, or DHU.

The standard assay mixture for DNA-damage-specific nucleotide incision activity (20 μl final volume) contained 5 nM 5′-[^{32}P]THF·T or

αT·A or 20 nM 3′-[^{32}P]ddAMP-end-labeled DHU·G oligonucleotide duplex in 20 mM Hepes/KOH (pH 7.6), 50 mM KCl, 1 mM DTT, 100 μg BSA/ml, and 0.1 nM Nfo protein (or 1 nM DHU·G) as previously described [13]. Reactions were carried out for 5 min (10 min for THF·T; THF·T was selected because it was better repaired by Nfo compared to the other base pairs, THF·A, G, C) at 37°C. Reaction products were analyzed by electrophoresis in denaturing 20% (w/v) polyacrylamide gels [7 M urea, 0.5× TBE (100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.3)], visualized by a PhosphorImager Storm 840 (Molecular Dynamics, Sunnyvale, CA, USA), and quantified using ImageQuant software.

Fluorophore-labeled oligonucleotide duplexes containing an abasic cluster

Oligonucleotides A1 (51 bp) and the components of B (see below) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The double-stranded oligonucleotide A1·B1 was constructed from A1, a full-length 51-mer containing a uracil residue, and three complementary oligonucleotides, Ba and Bc (two constant terminal oligonucleotides) and Bb, the central cassette containing a uracil residue (see Table 1). Strand B (51 bp) was assembled from Bb (21 bp), ligated to the 3′ Ba [labeled with the fluorophore 6-FAM (6-carboxyfluorescein)] and the 5′ Bc terminal oligonucleotides (15 bp each) as follows: equimolar quantities of oligonucleotides A, Ba, Bb, and Bc were denatured (5 min at 80°C) and then annealed with slow cooling to 20°C (at 0.01°C/s). They were held for 15 min at 20°C and then cooled to 4°C at 0.01°C/s. The components of B were ligated using T4 DNA ligase according to the manufacturer's (New England Biolabs, Ipswich, MA, USA) recommended procedures. Ligated constructs were checked for completeness of ligation, and only preparations with 90% or more ligation were used in cleavage experiments. Finally, DNA duplexes were equilibrated at 5 pmol/μl in 10 mM Tris, pH 7.5.

For conversion of uracils into abasic sites, 5 pmol duplex oligonucleotide in 5 μl buffer (50 mM Hepes, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 50 μg/ml BSA) was incubated with 1 unit uracil–DNA glycosylase (UDG; New England Biolabs) for 30 min at 37°C and placed on ice. The completeness of conversion of uracil residues to abasic sites was verified by cleavage with 0.5 M NaOH or by adding 100 pg hApe1 (a kind gift from Dr. David Wilson, National Institute on Aging) in Ape1 buffer (100 mM Hepes, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 50 μg/ml BSA) to a total volume of 10 μl and incubating for 15 min at 37°C. Reactions were stopped by adding 10 μl deionized formamide and 2.5 μl loading buffer (30% glycerol, bromophenol blue in 1× TBE) and heating to 80°C for 5 min. Then 2 μl stop mix (0.1 M SDS, 30% glycerol, containing bromophenol blue in TBE buffer) was added, and the samples were incubated for 10 min at 65°C. Samples were electrophoresed on 15% denaturing polyacrylamide gels.

Cleavage of abasic clusters by human cell extracts

Protein extracts were prepared as previously described [2]. In brief, 250 μl of cell suspension (2.5×10^6 cells/ml) was washed in PBS, resuspended in 250 μl extraction buffer (100 mM Tris, pH 7.5, 100 mM KCl, 0.2 mM EDTA, 1% glycerol, 1 mM PMSF), vortexed, sonicated on ice 4×20 s, incubated on ice 30 min, and vortexed again. Cell debris was removed by centrifugation at 12,000 g at 4°C. The supernatant was transferred into a fresh reaction tube and small aliquots were frozen on dry ice. Protein concentrations were measured using a Bio-Rad (Hercules, CA, USA) protein assay.

Abasic cluster cleavage activities of extracts were measured using DNA duplex A1·B1 as a substrate containing an abasic cluster (Table 1). Cell extract (100 ng protein) in Ape1 buffer was added as above and the samples were incubated at 37°C. Reactions were stopped and separated on 15% native polyacrylamide gels. All experiments were carried out three times.

Table 1
Sequence and construction of a fluorescein-labeled double-stranded oligonucleotide containing an abasic cluster (X, produced from uracil residues at the indicated position by UDG)

```
A1  5′  6-FAM-ATCAGACTCTACTGCCACAGAGGAXATGTATGTATGGAGAGGCCGGACGAT
B1  3′        TAGTCTGAGATGACGGTGTCTCCTATACAXACATACCTCTCCGGCCTGCTA
                                ▲                 ▲
```

The arrows show the delineations of the three smaller oligonucleotides from which strand B1 was assembled: invariant 3′ Ba and 5′ Bc terminal segments (15 bp each) and a central 21-bp cassette, Bb.

Visualizing the DNA in polyacrylamide gels

Gels were illuminated with an epi-illuminator emitting UV radiation centered at 340 nm. Images were obtained by a Princeton Instruments VersArray CCD camera (Model 7380-0001; Roper Scientific, Trenton, NJ, USA) equipped with a filter selecting 6-FAM emission (cut-on 510 nm, cutoff 530 nm, peak transmission >65%; Omega Optical, Brattleboro, VT, USA). Quantitative electronic images were obtained [17,24,25] and stored, and the FAM fluorescence associated with each DNA species was calculated using an area analysis program.

Detection of Ape1 protein

Thirty micrograms of cell extract was loaded per lane and separated on 10% polyacrylamide gels containing SDS, and proteins were then transferred to nitrocellulose membranes (Bio-Rad) via tank blotting in Towbin buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) containing 20% ethanol. Completed protein transfer and equal sample loading were controlled for by staining for 2 min in 0.1% Ponceau S in 5% acetic acid and destaining in water. Blots were blocked in PBS with 0.1% Tween 20 and 5% Blocking Grade blocker (Bio-Rad) and then washed with PBS containing 0.01% Tween 20. Ape1 was detected by mouse anti-Ref-1 (C4) [sc-17774 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA (1:2500)] and further labeled with anti-mouse IgG–HRP (1:5000) from Bio-Rad. ECL detection reagents from GE HealthCare (Amersham; Piscataway, NJ, USA) were used to visualize the protein bands, and results were recorded on X-Omat V film (Kodak, Rochester, NY, USA).

Results

Cellular genotype can affect the levels of endogenous clusters [8], but the roles of other factors are not known. Because cellular environment influences the level of endogenous isolated bases and abasic sites, we tested whether cell growth conditions can also affect endogenous cluster levels. Fig. 1A illustrates the low levels of endogenous OxyPyr, OxyPur, and abasic clusters in 28SC human monocytes, a repair-proficient human hematopoietic cell line. In contrast, Figs. 1B and 1C show that two radiation-sensitive human cell lines—WI-L2-NS and TK6—accumulate substantial levels of OxyPyr and OxyPur clusters. TK6 cells may be deficient in DSB repair [26] (but also see [27]). Further, they contain lower levels of hOGG1 activity than 28SC cells [8], possibly contributing to their accumulation of endogenous clusters.

To determine whether the cell microenvironment affects endogenous cluster levels, we grew these cells in five media—those suggested by the supplier for these cells, plus that medium supplemented with folate and/or selenium—and then determined cluster levels. TK6 and WI-L2-NS cells were grown in RPMI 1640 (solid bars in Figs. 1B and 1C); companion 28SCs were also grown in RPMI 1640 (solid bars in Fig. 1A). These cells were then grown on IMDM

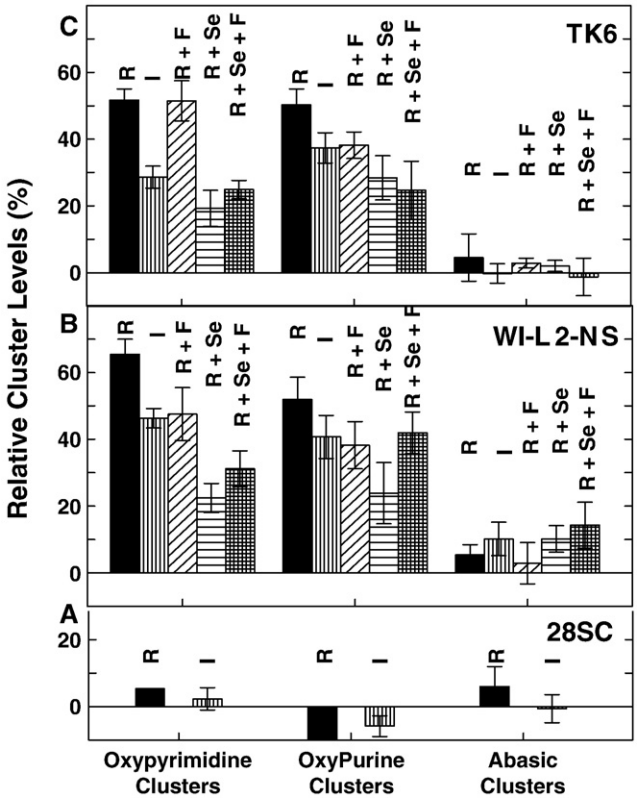


Fig. 1. Endogenous clustered damages in human hematopoietic cell lines. (A) 28SC human monocytes grown in RPMI 1640 (R) or IMDM (I). (B) WI-L2-NS cells grown in RPMI 1640, IMDM (bars as in A), RPMI 1640 plus folate (R+ F; 4 mg/L), RPMI 1640 plus selenium (R+ Se, 0.017 mg/L), or RPMI 1640 plus folate (4 mg/L) and selenium (0.017 mg/L) (R+ Se+ F). (C) TK6 cells grown in the same media as in B. Data for cells in RPMI 1640 (solid bars only) are from [8].

(ATCC-recommended for 28SCs), and the endogenous cluster levels were determined (vertically striped bars in Fig. 1). WI-L2-NS and TK6 cells grown in Iscove's contained lower levels of OxyPyr clusters than companion cultures grown in RPMI 1640 [TK6, Student's t (8 df)= 3.05, $p< 0.05$; WI-L2-NS, Student's t (6 df)= 4.09, $p< 0.01$], suggesting that endogenous OxyPyr cluster levels are affected by the cellular microenvironment. Neither cell line showed significant differences in OxyPur cluster levels when grown on RPMI 1640 vs Iscove's [TK6, Student's t (8 df)= 1.14, not significant at the 5% level; WI-L2-NS, Student's t (6 df)= 2.19, not significant at the 5% level].

RPMI 1640 has lower levels of many nutrients than IMDM. The absence of sodium selenite in RPMI 1640 and the presence of folic acid at 0.001 g/L (25% the IMDM level) could affect oxidized base levels [28,29]. Growth of the cells in RPMI 1640 containing folate at the level in IMDM reduced the levels of some OxyBase clusters slightly (diagonally striped bars in Fig. 1). However, sodium selenite at 17 mg/L (the IMDM level) reduced the OxyBase cluster levels in TK6 and WI-L2-NS cells approximately threefold (horizontally striped bars in Fig. 1). [For TK6, RPMI 1640 vs RPMI+ Se: OxyPyr, Student's t (7 df)= 5.83, $p< 0.01$; OxyPur, Student's t (7 df)= 2.46, $p< 0.05$. For WI-L2-NS, RPMI 1640 vs RPMI+ Se: OxyPy, Student's t (6 df)= 5.06, $p< 0.01$; OxyPur, Student's t (6 df)= 3.14, $p< 0.05$.] Addition of sodium selenite and folate to the above levels did not reduce further the OxyBase cluster levels (cross-hatched bars). Abasic cluster levels were low in TK6 and WI-L2-NS cells under all conditions (Fig. 1).

We next asked if the tissue origin of primary human cells could affect endogenous cluster levels. Previous studies showed that endogenous cluster levels in human skin tissue were low – consistently lower than in cultured fibroblasts or keratinocytes from the same donor [9]. To determine if DNA of other human tissues also contained low cluster frequencies, we determined the steady-state levels of damage clusters in the bone marrow stem and progenitor cells from eight healthy human volunteers. Table 2 shows the characteristics (as provided by the vendor) of these donors. All samples were negative for HIV, hepatitis B, and hepatitis C. Measurement of the cluster levels in the bone marrow cells showed two strikingly different groups—one with low cluster levels and the other with high cluster levels (Table 2). Fig. 2 shows clearly that cells from all non-tobacco users contain low levels of all clustered damage types, but the stem and progenitor cells from all tobacco users tested contain high total levels of clusters. We observed that the four tobacco users are associated with the four highest cluster levels and the four nonusers are associated with the four lowest cluster levels for all three (OxyPyr, OxyPur, and abasic) cluster types. Assuming that cluster level and tobacco use are independent and follow a hypergeometric distribution, the probability that this experimental outcome would occur by chance is equal to 0.0143. Thus we have evidence at the 5% level (p= 0.0143) that cluster level and tobacco use are not independent for our eight subjects.

Fig. 2 also shows the cluster spectra – the relative levels of OxyPyr, OxyPur, and abasic clusters – among the eight donors: Fig. 2A shows the low cluster levels in non-tobacco users, and Fig. 2B, the high cluster levels among tobacco users. A nonparametric rank sum test was used to test the significance of the differences between the four tobacco users and the four nonusers for each of the cluster types. For all three cluster types, OxyPyr, OxyPur, and abasic, the differences were significant with p= 0.05. Moreover, the cluster spectrum in smokers is quite different from those in TK6 and WI-L2-NS cells, which accumulate high levels of OxyPur and OxyPyr clusters (Figs. 1B and 1C), but low levels of abasic clusters. However, the cluster spectrum for the tobacco users (Fig. 2B) contains high total cluster levels, plus substantial levels of abasic clusters. This clustered damage spectrum was more like that produced by ionizing radiation, ~1 DSB:1 OxyPur cluster:0.9 OxyPyr cluster:0.75 abasic cluster [3]. Notably, in DNA from tobacco users, abasic clusters were produced at levels comparable to those of other clusters.

Cluster identification depends critically on the properties of the diagnostic enzymes to cleave at their cognate substrate sites. Ishenko et al. showed that the substrate specificities of Nfo protein preparations depend on the purification procedure [13]. Because some preparations of Nfo protein can incise at oxidized pyrimidines and abasic sites [30], clusters identified by these preparations would include OxyPyr as well as abasic clusters. To determine unambiguously the identity of clusters recognized by homogeneous Nfo protein isolated in the Sutherland laboratory (Nfo BMS), we asked whether this preparation also cleaved at oxidized pyrimidines in defined sequence double-stranded oligonucleotides containing an abasic site analog or one of two altered pyrimidines (dihydrouracil and α-thymine, an anoxic radiation product of thymine irradiation). We characterized the ability of Nfo BMS with respect to a preparation (Nfo MS) known to be able to incise 5′ next to αT.

Table 2
Properties of bone marrow stem cell donors[a]

Code	Age (years)	Gender	Origin	Harvest date	Alcohol use	Tobacco use	Endogenous cluster level
1148F	25	M	AA	3/11/03	No	No	Low
1238A	44	F	C	7/11/02	Yes	Yes	High
1240A	18	F	C	11/11/02	Yes	No	Low
1390C	24	M	AA	11/11/03	NR	No	Low
1779D	22	F	NR	12/12/05	NR	No	Low
2070B	24	M	AA	12/12/05	NR	Yes	High
2289C	29	M	AA	13/12/05	NR	Yes	High
1119A	26	F	His	29/04/02	NR	Yes	High

[a] Specimens and donor information from Bio-Whittaker (Cambrex). All samples were negative for HIV, hepatitis B, and hepatitis C. AA, African American; C, Caucasian, His, Hispanic, NR, not reported.

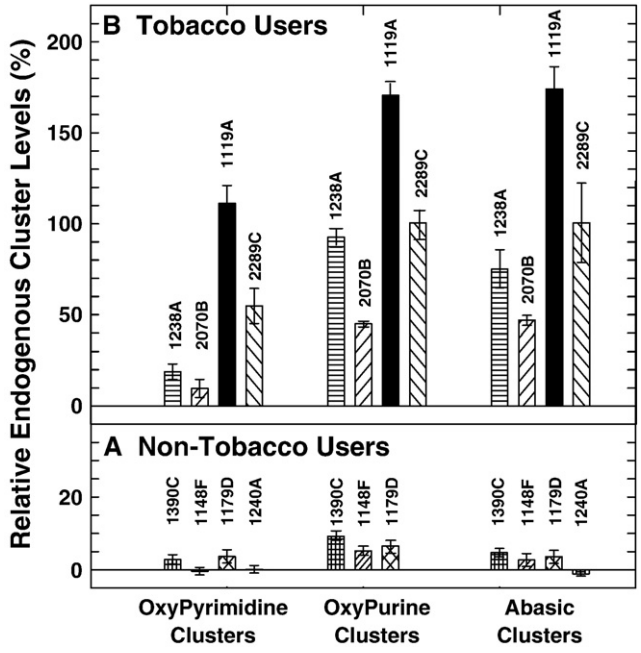
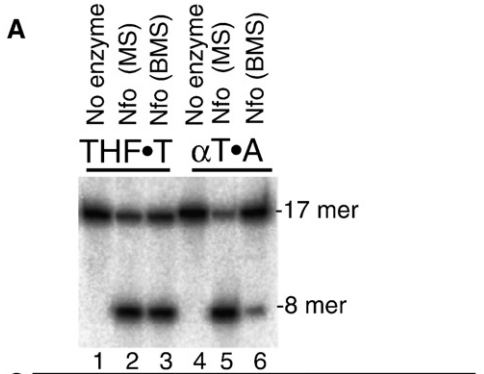


Fig. 2. Clustered damages in hematopoietic stem and progenitor cells from bone marrow of healthy humans. (A) OxyPyr, OxyPur, and abasic cluster levels in cells from four non-tobacco-user donors, 1390C, 1148F, 1179D, and 1240A. (OxyPur clusters were not measured for donor 1240A owing to small sample size). (B) Levels of the three cluster classes in four donors who identified themselves as tobacco users: 1238A, 2070B, 1119A, and 2289C. Error bars, SEM.

Fig. 3 shows the results of such experiments. Lanes 1–3 of Fig. 3A show that both preparations effectively cleave at tetrahydrofuran, a synthetic abasic site, opposite to T. Although Nfo MS cleaved the substrate containing αT·A, Nfo BMS had minimal activity toward this substrate (lanes 4–6). Nfo MS cleaved the DHU·G oligonucleotide duplex better (4.9%) than Nfo BMS (1.5%) (Fig. 3B). Fig. 3C summarizes the quantitative results: Nfo BMS, used to identify abasic clusters in this work, cleaves primarily at abasic sites, with little activity toward oxidized pyrimidines. Thus the "abasic clusters" detected in the tobacco users were true abasic clusters, not OxyPyr clusters.



C

Enzyme	Substrate	Per Cent Incision
Nfo protein (MS)	THF	71.2
Nfo protein (BMS)	"	57.3
Nfo protein (MS)	α T	83.4
Nfo protein (BMS)	"	4.4
Nfo protein (MS)	DHU	4.9
Nfo protein (BMS)	"	1.5

Fig. 3. Specificity of Nfo protein used in recognition of abasic clusters in DNA from human cells. (A) Electronic image of a polyacrylamide gel showing incision of oligonucleotides containing THF·T or α-T·A by a preparation of Nfo protein (Nfo MS) previously characterized to incise at both lesion sites, or by the Nfo protein preparation used to quantify abasic clusters in this work (Nfo BMS). (B) Electronic image of a gel testing incision at DHU·G sites by the same Nfo protein preparations as in (A). (C) Quantitative results obtained from gels such as those in (A) and (B).

The increased cluster levels in tobacco users could come from lower content of repair enzyme protein or inactivation of their enzymatic function. Alternatively, repair enzyme levels in the tobacco users' cells could be normal, but their DNA could contain cluster levels too high for the normal repair enzyme capacity to process effectively. To distinguish these possibilities, we measured cluster levels in tobacco-user cells immediately upon arrival (Fig. 4A) and then after 24 h incubation in fresh medium (Fig. 4B). There was a substantial decrease in all cluster classes. Thus, in the absence of damaging agents, the decreased burden of DNA damage allows repair enzymes

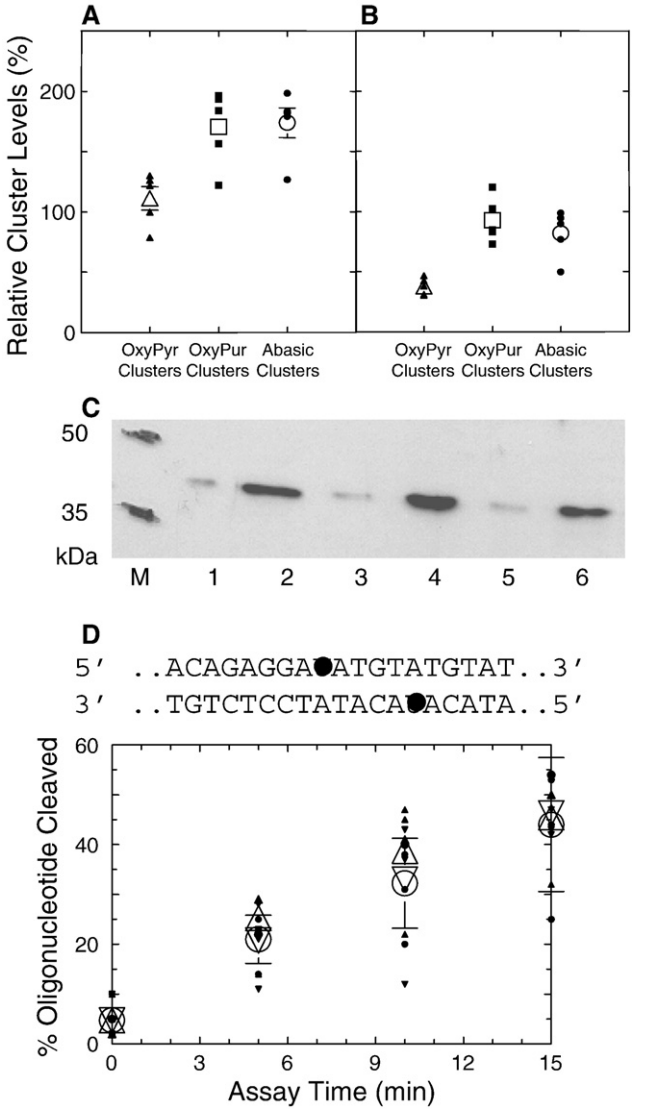


Fig. 4. Clustered damage levels, Ape1 protein levels, and activities in human hematopoietic stem and progenitor cells of healthy non-tobacco-user and tobacco-user donors. (A) Clustered damage levels in tobacco-user donor 9362 immediately after receipt from the vendor and (B) in 9362 cells after incubation for 24 h at 37°C in fresh medium. (C) Ape1 protein detection with anti-Ape antibody in 15 or 30 μg of cell extract from non-tobacco-user donor 1779 (lanes 1 and 2) and from tobacco-using donors 2070 (lanes 3 and 4) and 2289 (lanes 5 and 6). M, protein size standards, sizes shown in kDa. (D) Ape1 activity in 100 ng of extracts of one non-tobacco user (1779, triangles) and in two tobacco users (2070, inverted triangles, and 2289, circles). Small filled symbols, individual measurements; large open symbols, averages; error bars, SEM.

to reduce the cluster level substantially – even in cells from a tobacco user.

In the tobacco users' bone marrow cells there are significant levels of abasic clusters, which are not present in other unirradiated primary human cells. This high abasic cluster level could result from lower levels of Ape1 protein in tobacco users' cells. Even if the Ape1 protein levels were similar in tobacco users and nonusers, Ape1 enzymatic activity in tobacco users could be decreased, perhaps owing to some inactivation mechanism. Alternatively, even if Ape1 protein and enzymatic activity levels are similar in users and nonusers, the DNA damage levels in tobacco users' cells could simply overwhelm the ability of normal Ape1 levels to effectively process the abasic clusters.

We first asked if the Ape1 protein concentrations were similar in tobacco users and nonusers. Western blots with anti-Ape1 antibody suggested similar levels of Ape1 in cells from tobacco users and nonusers (Fig. 4C). Because this did not exclude the possibility of decreased Ape1 enzymatic activity in tobacco users' cells, we determined Ape1 activity in cell extracts from non-tobacco-user and tobacco-user donors. To relate this enzyme activity to the endogenous cluster levels, we used as substrate an oligonucleotide containing an abasic cluster readily cleaved by purified hApe1. Fig. 4D (top) illustrates the central cluster-containing portion of the A1·B1 double-stranded 51-mer and the resulting cleavage data in the graph (bottom). Ape1 activity levels in the two tobacco users and a non-tobacco user are virtually identical. These data show that the increased level of abasic clusters in tobacco users does not stem from decreased cellular Ape1 cluster-cleaving activity.

Discussion

Cellular metabolism in an aerobic environment generates ROS that can damage DNA and other cellular components. In TK6 and WI-L2-NS cells, which are radiation-sensitive and contain lower glycosylase levels, even standard growth conditions result in accumulation of OxyBase clusters [8]. Growth in either a multiply-supplemented medium or RPMI 1640 supplemented with selenium decreases the steady-state OxyBase cluster level. Thus the steady-state endogenous cluster level reflects cellular environment, e.g., the growth medium. Strikingly, endogenous cluster levels in repair-proficient cells are only slightly altered by more protective medium, indicating that their repair capacity can deal with higher damage levels induced in less protective media. The mechanism by which selenium supplementation results in a decrease in endogenous cluster levels is not clear. Selenium is a known cancer-preventive agent in experimental animals (for a review, see [31]), and dietary selenium supplementation has been shown to decrease chromosome breaks in humans who are BRCA1 carriers. Further, Kennedy et al. found that selenium decreased cell killing and transformation induced by ionizing radiation [32]. Selenium can interact with zinc finger proteins, including several involved in various aspects of DNA repair [33,34]. Whether these interactions can affect steady-state levels of enzymes critical in repair of endogenous clustered damages is yet to be determined.

Tissue metabolism also produces ROS. Thus DNA in tobacco users' tissues is subjected to metabolism-engendered ROS plus a plethora of damaging agents from tobacco. In addition to its role in producing lung cancer, cigarette smoke is also associated with induction of specific hematological malignancies [35–38]. Cigarette smoke contains thousands of compounds [39], including the oxidants NO and NO_2 as well as a variety of radicals that can react with biomolecules [40]. Tobacco also contains ^{210}Po, a naturally occurring, α-particle-emitting radionuclide. It is easy to associate chemicals and radioactivity in tobacco with cellular damage in tissues exposed directly to cigarette smoke or to oral use of tobacco or in cultured cells exposed to activated or nonactivated tobacco or smoke constituents [41,42]. Moreover, strong evidence shows that aberrant signaling paths induced by cigarette smoke exposure are similar to those produced by H_2O_2, a DNA-damaging agent [43].

However, chemicals and radioisotopes from tobacco are detectable in tissues that are not exposed directly to tobacco or to smoke [44–46]. The increased concentration of DNA-damaging agents in smokers would be expected to produce higher levels of altered sites. Although increased levels of some oxidized DNA bases (e.g., 8-oxoguanine) have been reported in cells of tobacco users [47,48], other investigations produced null or inverse results [49]. Because both oxidized bases [50] and oxidized base clusters [8] can be induced during DNA isolation, early investigations may have been compromised by such artifacts. Our current DNA isolation methods were designed to minimize artifactual induction of OxyBase clusters [8].

Human cells have a battery of repair pathways for dealing with DNA damages: nucleotide excision repair (especially for bulky adducts), base excision and nucleotide incision repair (for processing oxidized bases and abasic sites) [30]. Despite the coordination and substrate overlap of these paths, some damages remain unrepaired for substantial periods, persisting until DNA replication and thus posing lethal and mutagenic consequences. Clustered damages are strong candidates for these repair-resistant damages. In vitro studies of specific clusters in defined oligonucleotides show that many cluster configurations are resistant to repair-enzyme action [51–54]. Radiation-sensitive cells exposed to high radiation doses generate de novo DSBs, possibly at cluster sites [55,56]. At lower doses, repair-competent cells rapidly rejoin radiation-induced DSBs, whereas repair of abasic clusters is substantially delayed [2], perhaps even until replication.

Tissues from normal humans—both neonatal and adult—contain extremely low steady-state levels of clusters [9]. Because endogenous cluster levels are the net result of damage induction vs cellular repair, these data show that the sum of the coordinated repair processes in these individuals removes the vast majority of DNA damages induced by endogenous factors and exogenous agents. In all these cases, the levels of three major classes of clustered, non-DSB damages—OxyPur, OxyPyr, and abasic clusters—are low, almost at the limit of detection.

Cluster levels in hematopoietic stem and progenitor cells from tobacco users differ strikingly from this paradigm, with both high total cluster levels and the presence of abasic clusters. Two hypotheses could explain these data: tobacco users' cells might have decreased levels of essential repair enzymes and thus be unable to remove critical clusters at normal rates. Alternately, damage induction in tobacco users could be high enough to overwhelm normal DNA repair. Thus stem and progenitor cells in tobacco users would be unable to reduce the steady-state cluster levels to those found in normal donors who do not smoke.

Two lines of evidence make the possibility of decreased repair capacity unlikely. First, after 24 h incubation in fresh medium, cluster levels in stem and progenitor cells from a tobacco user donor were substantially reduced (Fig. 4A). Second, the levels of Ape1 protein in extracts of cells from tobacco users were similar to non-tobacco-user levels (Fig. 4C). Additionally, Ape1 cleavage activity on authentic abasic clusters in cells from tobacco users was similar to that from nonusers (Fig. 4D). The presence in tobacco of ^{210}Po, a highly radioactive α-particle emitter, suggests an origin for the abasic clusters, which we have previously observed only in directly irradiated cells. Although present at very low levels, they might be sufficient to induce damage clusters, which are rare lesions present only at the per-gigabase-pair level.

Thus, despite the presence of an active repair system, if damage induction is high (as in a tobacco user) substantial levels of OxyBase and abasic clusters can accumulate. These clusters are of high mutagenic and oncogenic potential and may contribute to significant biological damage—possibly including hematological malignancies—in these stem and progenitor cells.

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References

[1] Sutherland, B. M.; Bennett, P. V.; Sidorkina, O.; Laval, J. DNA damage clusters induced by ionizing radiation in isolated DNA and in human cells. *Proc. Natl. Acad. Sci. USA* **97:**103–108; 2000.
[2] Georgakilas, A. G.; Bennett, P. V.; Wilson, D. M., 3rd; Sutherland, B. M. Processing of bistranded abasic DNA clusters in gamma-irradiated human hematopoietic cells. *Nucleic Acids Res.* **32:**5609–5620; 2004.
[3] Sutherland, B. M.; Bennett, P. V.; Sutherland, J. C.; Laval, J. Clustered DNA damages induced by x-rays in human cells. *Radiat. Res.* **157:**611–616; 2002.
[4] Beckman, K. B.; Ames, B. N. The free radical theory of aging matures. *Physiol. Rev.* **78:**547–581; 1998.
[5] Halliwell, B. Oxidative stress and cancer: have we moved forward? Biochem. *J.* **401:**1–11; 2007.
[6] Kamminga, L.; de Haan, G. Cellular memory and hematopoietic stem cell aging. *Stem Cells* **24:**1143–1149; 2006.
[7] Sutherland, B. M.; Bennett, P. V.; Cintron, N. S.; Guida, P.; Laval, J. Low levels of endogenous oxidative damage cluster levels in unirradiated viral and human DNAs. *Free Radic. Biol. Med.* **35:**495–503; 2003.
[8] Bennett, P. V.; Cintron, N. S.; Gros, L.; Laval, J.; Sutherland, B. M. Are endogenous clustered DNA damages induced in human cells? Free Radic. *Biol. Med.* **37:**488–499; 2004.
[9] Bennett, P. V.; Cuomo, N. L.; Paul, S.; Tafrov, S. T.; Sutherland, B. M. Endogenous DNA damage clusters in human skin, 3-D model, and cultured skin cells. *Free Radic. Biol. Med.* **39:**832–839; 2005.
[10] Wang, R. J. Lethal effect of "daylight" fluorescent light on human cells in tissue-culture medium. *Photochem. Photobiol.* **21:**373–375; 1975.
[11] Sutherland, B.; Bennett, P.; Cintron-Torres, N.; Hada, M.; Trunk, J.; Monteleone, D.; Sutherland, J.; Laval, J.; Stanislaus, M.; Gewirtz, A. Clustered DNA damages induced in human hematopoietic cells by low doses of ionizing radiation. *J. Radiat. Res. (Tokyo)* **43** (Suppl.)**:**S149–S152; 2002.
[12] Sutherland, B. M.; Bennett, P. V.; Georgakilas, A. G.; Sutherland, J. C. Evaluation of number average length analysis in quantifying double strand breaks in genomic DNAs. *Biochemistry* **42:**3375–3384; 2003.
[13] Ishchenko, A.; Deprez, E.; Maksimenko, A.; Brochon, J.; Tauc, P.; Saparbaev, M. Uncoupling of the base excision and nucleotide incision repair pathways reveals their respective biological roles. *Proc. Natl. Acad. Sci. USA* **103:**2564–2569; 2006.
[14] Boiteux, S.; Gajewski, E.; Laval, J.; Dizdaroglu, M. Substrate specificity of the *Escherichia coli* Fpg protein (formamidopyrimidine-DNA glycosylase): excision of purine lesions in DNA produced by ionizing radiation or photosensitization. *Biochemistry* **31:**106–110; 1992.
[15] Dizdaroglu, M.; Laval, J.; Boiteux, S. Substrate specificity of the *E. coli* endonuclease III: excision of thymine and cytosine-derived lesions in DNA produced by radiation-generated free radicals. *Biochemistry* **32:**12105–12111; 1993.
[16] Gardiner, K.; Laas, W.; Patterson, D. Fractionation of large mammalian DNA restriction fragments using vertical pulsed-field gradient gel electrophoresis. *Somatic Cell Mol. Genet.* **12:**185–195; 1986.
[17] Sutherland, J. C.; Lin, B.; Monteleone, D. C.; Mugavero, J.; Sutherland, B. M.; Trunk, J. Electronic imaging system for direct and rapid quantitation of fluorescence from electrophoretic gels: application to ethidium bromide-stained DNA. *Anal. Biochem.* **163:**446–457; 1987.
[18] Sutherland, B. M.; Bennett, P. V.; Sidorkina, O.; Laval, J. Clustered damages and total lesions induced in DNA by ionizing radiation: oxidized bases and strand breaks. *Biochemistry* **39:**8026–8031; 2000.
[19] Ide, H.; Okagami, M.; Murayama, H.; Kimura, Y.; Makino, K. Synthesis and characterization of oligonucleotides containing the alpha-anomer of deoxyadenosine to study its influence on DNA replication. *Biochem. Mol. Biol. Int.* **31:**485–491; 1993.
[20] Ide, H.; Tedzuka, K.; Shimzu, H.; Kimura, Y.; Purmal, A. A.; Wallace, S. S.; Kow, Y. W. Alpha-deoxyadenosine, a major anoxic radiolysis product of adenine in DNA, is a substrate for Escherichia coli endonuclease IV. *Biochemistry* **33:**7842–7847; 1994.
[21] Ishchenko, A.; Sanz, G.; Privezentzev, C.; Maksimenko, A.; Saparbaev, M. Characterisation of new substrate specificities of Escherichia coli and Saccharomyces cerevisiae AP endonucleases. *Nucleic Acids Res.* **31:**6344–6353; 2003.
[22] Ishchenko, A. A.; Ide, H.; Ramotar, D.; Nevinsky, G.; Saparbaev, M. Alpha-anomeric deoxynucleotides, anoxic products of ionizing radiation, are substrates for the endonuclease IV-type AP endonucleases. *Biochemistry* **43:**15210–15216; 2004.
[23] Saparbaev, M.; Laval, J. Excision of hypoxanthine from DNA containing dIMP residues by the Escherichia coli, yeast, rat, and human alkylpurine DNA glycosylases. *Proc. Natl. Acad. Sci. USA* **91:**5873–5877; 1994.
[24] Sutherland, J. C.; Sutherland, B. M.; Emrick, A.; Monteleone, D. C.; Ribeiro, E. A.; Trunk, J.; Son, M.; Serwer, P.; Poddar, S. K.; Maniloff, J. Quantitative electronic imaging of gel fluorescence with charged coupled device cameras: applications in molecular biology. *BioTechniques* **10:**492–497; 1991.
[25] Sutherland, J. C.; Monteleone, D. C.; Trunk, J. G.; Bennett, P. V.; Sutherland, B. M. Quantifying DNA damage by gel electrophoresis, electronic imaging and number-average length analysis. *Electrophoresis* **22:**843–854; 2001.
[26] Evans, H. H.; Ricanati, M.; Horng, M. F.; Jian, Q.; Mencl, J.; Olive, P. DNA double-strand break rejoining deficiency in TK6 and other human B-lymphoblast cell lines. *Radiat. Res.* **134:**307–315; 1993.
[27] Schwartz, J. L.; Jordan, R.; Sedita, B. A.; Swenningson, M. J.; Banath, J. P.; Olive, P. L. Different sensitivity to cell killing and chromosome mutation induction by gamma rays in two human lymphoblastoid cell lines derived from a single donor: possible role of apoptosis. *Mutagenesis* **10:**227–233; 1995.
[28] Blount, B. C.; Mack, M. M.; Wehr, C. M.; MacGregor, J. T.; Hiatt, R. A.; Wang, G.; Wickramasinghe, S. N.; Everson, R. B.; Ames, B. N. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc. Natl. Acad. Sci. USA* **94:**3290–3295; 1997.
[29] Wan, X.; Ware, J.; Zhou, Z.; Donahue, J.; Guan, J.; Kennedy, A. Protection against radiation-induced oxidative stress in cultured human epithelial cells by treatment with antioxidant agents. *Int. J. Radiat. Oncol. Biol. Phys.* **64:**1474–1481; 2006.
[30] Ischenko, A. A.; Saparbaev, M. K. Alternative nucleotide incision repair pathway for oxidative DNA damage. *Nature* **415:**183–187; 2002.
[31] Combs, G. F., Jr.; Gray, W. P. Chemopreventive agents: selenium. *Pharmacol. Ther.* **79:**179–192; 1998.
[32] Kennedy, A. R.; Ware, J. H.; Guan, J.; Donahue, J. J.; Biaglow, J. E.; Zhou, Z.; Stewart, J.; Vazquez, M.; Wan, X. S. Selenomethionine protects against adverse biological effects induced by space radiation. *Free Radic. Biol. Med.* **36:**259–266; 2004.
[33] Fischer, J. L.; Lancia, J. K.; Mathur, A.; Smith, M. L. Selenium protection from DNA damage involves a Ref1/p53/Brca1 protein complex. *Anticancer Res.* **26:**899–904; 2006.
[34] Blessing, H.; Kraus, S.; Heindl, P.; Bal, W.; Hartwig, A. Interaction of selenium compounds with zinc finger proteins involved in DNA repair. *Eur. J. Biochem.* **271:**3190–3199; 2004.
[35] Doll, R. Cancers weakly related to smoking. *Br. Med. Bull.* **52:**35–49; 1996.
[36] Bjork, J.; Albin, M.; Mauritzson, N.; Stromberg, U.; Johansson, B.; Hagmar, L. Smoking and acute myeloid leukemia: associations with morphology and karyotypic patterns and evaluation of dose–response relations. *Leuk. Res.* **25:**865–872; 2001.
[37] Stagnaro, E.; Ramazzotti, V.; Crosignani, P.; Fontana, A.; Masala, G.; Miligi, L.; Nanni, O.; Neri, M.; Rodella, S.; Costantini, A. S.; Tumino, R.; Vigano, C.; Vindigni, C.; Vineis, P. Smoking and hematolymphopoietic malignancies. *Cancer Causes Control* **12:**325–334; 2001.
[38] Kasim, K.; Levallois, P.; Abdous, B.; Auger, P.; Johnson, K. C. Lifestyle factors and the risk of adult leukemia in Canada. *Cancer Causes Control* **16:**489–500; 2005.
[39] Wynder, E. L.; Hoffmann, D. Tobacco and Tobacco Smoke. Academic Press, New York; 1967.
[40] Church, D. F.; Pryor, W. A. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ. Health Perspect.* **64:**111–126; 1985.
[41] Albino, A. P.; Huang, X.; Jorgensen, E. D.; Gietl, D.; Traganos, F.; Darzynkiewicz, Z. Induction of DNA double-strand breaks in A549 and normal human pulmonary epithelial cells by cigarette smoke is mediated by free radicals. *Int. J. Oncol.* **28:**1491–1505; 2006.
[42] Kato, T.; Nagasawa, H.; Warner, C.; Okayasu, R.; Bedford, J. S. Cytotoxicity of cigarette smoke condensate is not due to DNA double strand breaks: comparative studies using radiosensitive mutant and wild-type CHO cells. *Int. J. Radiat. Biol.* **83:**583–591; 2007.
[43] Khan, E. M.; Lanir, R.; Danielson, A. R.; Goldkorn, T. Epidermal growth factor receptor exposed to cigarette smoke is aberrantly activated and undergoes perinuclear trafficking. *FASEB J.* **22:**910–917; 2008.
[44] Radford, E. P. J.; Hunt, V. R. Polonium-210: a volatile radioelement in cigarettes. *Science* **143:**247–249; 1964.
[45] Little, J. B.; Radford, E. P. J. Polonium-210 in bronchial epithelium of cigarette smokers. *Science* **155:**606–607; 1967.
[46] Holzman, R. B.; Ilcewicz, F. H. Lead-210 and polonium-210 in tissues of cigarette smokers. *Science* **153:**1259–1260; 1966.
[47] Fraga, C. G.; Motchnik, P. A.; Wyrobek, A. J.; Rempel, D. M.; Ames, B. N. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat. Res./Fundam. Mol. Mech. Mutagen.* **351:**199–203; 1996.
[48] Wako, Y.; Satoh, M.; Suzuki, K. 8-Hydroxydeoxyguanosine levels in white blood cell DNA and ex vivo oxidation resistance of plasma in smokers. *Tohoku J. Exp. Med.* **194:**99–106; 2001.
[49] Besaratinia, A.; Van Schooten, F. J.; Schilderman, P. A.; De Kok, T. M.; Haenen, G. R.; Van Herwijnen, M. H.; Van Agen, E.; Pachen, D.; Kleinjans, J. C. A multi-

biomarker approach to study the effects of smoking on oxidative DNA damage and repair and antioxidative defense mechanisms. *Carcinogenesis* **22:**395–401; 2001.

[50] Helbock, H. J.; Beckman, K. B.; Ames, B. N. 8-Hydroxydeoxyguanosine and 8-hydroxyguanine as biomarkers of oxidative DNA damage. *Methods Enzymol.* **300:**156–166; 1999.

[51] Chaudhry, M. A.; Weinfeld, M. The action of *Escherichia coli* endonuclease III on multiply damaged sites in DNA. *J. Mol. Biol.* **249:**914–922; 1995.

[52] Harrison, L.; Hatahet, Z.; Wallace, S. *In vitro* repair of synthetic ionizing radiation-induced multiply damaged DNA sites. *J. Mol. Biol.* **290:**667–684; 1999.

[53] Cunniffe, S. M. T.; O'Neill, P. Cell extracts reveal complexity of DNA damage. 11th International Congress of Radiation Research, Dublin, Vol. 1,p. 103; 1999.

[54] David-Cordonnier, M. H.; Laval, J.; O'Neill, P. Clustered DNA damage: influence on damage excision by XRS5 nuclear extracts and *Escherichia coli* Nth and Fpg proteins. *J. Biol. Chem.* **275:**11865–11873; 2000.

[55] Ahnstrom, G.; Bryant, P. E. DNA double-strand breaks generated by the repair of X-ray damage in Chinese hamster cells. *Int. J. Radiat. Biol.* **41:**671–676; 1982.

[56] Yang, N.; Galick, H.; Wallace, S. S. Attempted base excision repair of ionizing radiation damage in human lymphoblastoid cells produces lethal and mutagenic double strand breaks. *DNA Repair (Amsterdam)* **3:**1323–1334; 2004.