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Endogenous DNA damage clusters in human skin, 3-D model, and cultured skin cells

Paula V. Bennett^a, Noelle L. Cuomo^a, Sunirmal Paul^{a,1}, Stefan T. Tafrov^a, and Betsy M. Sutherland^{a,*}

^aBiology Department, Building 463, Brookhaven National Laboratory, Upton, NY 11973-5000, USA

Abstract

Clustered damages—two or more oxidized bases, abasic sites, or strand breaks on opposing DNA strands within a few helical turns—are formed in DNA by ionizing radiation. Clusters are difficult for cells to repair and thus pose significant challenges to genomic integrity. Although endogenous clusters were found in some permanent human cell lines, it was not known if clusters accumulated in human tissues or primary cells. Using high-sensitivity gel electrophoresis, electronic imaging, and number average length analysis, we determined endogenous cluster levels in DNA from human skin, a 3-D skin model, and primary cultured skin cells. DNA from dermis and epidermis of human skin contained extremely low levels of endogenous clusters (a few per gigabase). However, cultured skin fibroblasts and keratinocytes—whether in monolayer cultures or in 3-D model skin cultures—accumulated oxidized pyrimidine, oxidized purine, and abasic clusters. The levels of endogenous clusters were decreased by growing cells in the presence of selenium or by increasing cellular levels of Fpg protein, presumably by increasing processing of clustered damages. These results imply that the levels of endogenous clusters can be affected by the cells' external environment and their ability to deal with DNA damage.

Keywords

Endogenous DNA damage; DNA damage clusters; Human skin; 3-D skin model; Primary human cells; Free radicals

Endogenous DNA lesions, including oxidized bases and abasic sites, are formed by normal metabolism of cells living in an oxygen environment [1-3]. Unrepaired or misrepaired lesions have been linked to both aging and disease [4-6]. It has been firmly established that radiation induces oxidized lesions [7] and clustered damages—two or more oxidized bases, abasic sites, or strand breaks on opposing strands within a few helical turns [8]—and that cellular oxidative metabolism induces endogenous lesions as isolated sites [1,3,9]. However, it was only recently shown that some human cell lines accumulate endogenous bistranded damage clusters [10]. Endogenous lesions may be generated in cells by reactive oxygen species such as hydroxyl radicals or hydrogen peroxide.

Isolated oxidized bases and abasic sites in DNA can be removed effectively by a panoply of lesion-recognizing glycosylases and endonucleases [11]. However, clustered damages were hypothesized to be difficult to repair [12,13], and indeed both *in vitro* measurements of repair enzymes acting on synthetic oligonucleotides containing defined clusters [14-19] and studies of abasic cluster processing in repair-proficient human cells [20] indicate that many clusters are refractory to repair. Further, *in vitro* some clusters can be converted to double-strand breaks

*Corresponding author. Fax: +1 631 344 3407. E-mail address: bms@bnl.gov (B.M. Sutherland).

¹Present address: Department of Environmental Medicine, New York University School of Medicine, Tuxedo, NY 10987, USA.

(DSBs) [14-19], and irradiated mammalian cells with repair deficiencies can generate additional DSBs during postirradiation incubation [21,22], possibly at cluster sites.

Endogenous DSBs have been proposed as a major molecular origin of initial oncogenic events in human carcinogenesis [23]. Further, mice with deficiencies in both predominant paths of DSB processing—nonhomologous end-joining and homologous recombination—showed poor birth rates and short life spans, indicating the consequences of persistent, unrepaired or misrepaired DSBs, which are one kind of bistranded damage cluster. Non-DSB endogenous clustered damages could contribute to the burden of endogenous DSBs through cleavage on both strands at sites of lesions within a cluster. Additionally, clustered damages might pose direct challenges to genomic integrity by being refractory to repair processes, thus persisting as mutagenic or lethal lesions.

Because endogenous clustered damages have been demonstrated to accumulate in only two human hematopoietic cell lines, which had apparent repair deficiencies [10], it was not known if they are present in DNA from normal human tissue or primary cells. To answer this question, we measured the levels of endogenous clustered damages in DNA from skin tissue of two normal individuals, in a 3-D human epidermal skin model, and in four primary cultures of skin fibroblasts and keratinocytes. To test for the low levels of clusters found in unirradiated cells, we used approaches that we developed and validated previously [8,10,24-26]: DNA isolation methods that minimize artifactual lesion induction [27], high-sensitivity electrophoresis, quantitative electronic imaging, and number average length analysis [25].

The results show that the levels of clustered damages are extremely low in DNA from the epidermis of human neonatal foreskin and slightly higher in the dermis. The levels of clusters were substantially higher in cultured epidermal keratinocytes derived from the same epidermis, but those in cultured fibroblasts were only slightly higher than in the dermis. To distinguish whether three-dimensional tissues intrinsically contained lower levels of clusters than the corresponding monolayer cultures of their cells, we measured endogenous clustered damages in a 3-D epidermal model skin and in the corresponding cultured keratinocytes from the same cell stock. Both contained similar endogenous cluster levels, indicating that the three-dimensional systems do not intrinsically—by means of geometry, cell biology, or biochemistry—contain lower cluster levels. These results suggested that the difference might be the cellular milieu, e.g., the culture medium. Indeed, we found that the cluster levels could be reduced by addition of selenium to the culture medium and also by increasing the level of a pertinent repair enzyme, Fpg protein. These data show that normal human tissue and cells can accumulate endogenous clustered damages; furthermore, the cellular steady-state level of such damages can be modulated by both environmental and cellular factors, including the repair capacity of the cell.

Materials and methods

Human tissues and cells

Tissues and 3-D model skin cultures were obtained from commercial sources (see below). The tissues were harvested immediately after arrival at Brookhaven National Laboratory (BNL), and the 3-D model skin cultures were incubated in the medium supplied by the manufacturer. Cultured cells were obtained from cell banks or from cultures initiated in this laboratory. After primary cultures were established, cells were grown without antibiotics and were ascertained to be free of mycoplasma by periodic testing (Bionique, Saranac Lake, NY, USA). Cells were grown and cells and tissues were handled under yellow-filtered fluorescent bulbs to minimize light-induced cellular damage [28-30]. TK6 cells (CRL-8015 from the American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 (Gibco/BRL), 10% fetal bovine serum (HyClone, Logan, UT, USA) as recommended by the ATCC.

Normal human fibroblasts

All solutions and equipment were sterilized by appropriate means. Neonatal foreskins were obtained from Brookhaven Memorial Hospital (Patchogue, NY, USA) or the National Disease Research Interchange (Philadelphia, PA, USA) with BNL IRB approval as exempt human samples. Tissues were washed in phosphate-buffered saline (PBS; 171 mM NaCl, 3.4 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄), fat tissue was removed, and foreskins were minced into 1 × 1-mm pieces with cuticle scissors. Tissue was resuspended in MCDB 153 medium (Sigma, St. Louis, MO, USA) without serum, containing 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) and 1× Pen/Strep (penicillin–streptomycin, 100 units and 100 µg/ml, respectively), and placed at 4°C overnight. The tissue suspension was vortexed briefly, and the tissue was allowed to settle. The supernatant cell suspension was diluted into MCDB medium containing 50% iron-supplemented bovine serum (HyClone) to inactivate the trypsin. The remaining tissue was resuspended in 0.05% trypsin and repeatedly pipetted to dislodge cells from connective tissue. Cell suspensions were pooled and centrifuged (1200 rpm, 5 min) and the cells were resuspended at 10⁶ cells/ml in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; Invitrogen):MCDB 153 containing 10% fetal bovine serum (FBS; HyClone) and 1× Pen/Strep. The cells were seeded into Corning T-25 flasks (Corning, NY, USA) at 3 × 10⁶ cells/flask, gassed with 5% CO₂, placed into a 5% CO₂ incubator, and left undisturbed for 3 days. The medium was then replaced; after 2–4 days, the medium containing antibiotics was replaced by the same medium without antibiotics.

Human skin keratinocytes

All solutions and equipment were sterilized by appropriate means. Freshly collected neonatal foreskins were obtained from the National Disease Research Interchange. Upon delivery to BNL the tissue was washed five to six times by immersion in PBS to remove any traces of blood or other contaminants. Fat was removed using scissors, the skin was washed again and was then cut into 3- to 5-mm squares and transferred into Dispase II solution (Roche Applied Science; 0.25% in DMEM; Gibco, Grand Island, NY, USA). The tissue was digested overnight at 4°C. The following day the epithelium and dermis were separated using forceps, and the resulting fragments were washed several times with PBS to remove traces of Dispase II. The epithelia were transferred to 0.025% trypsin in PBS containing 0.5 mM EDTA and incubated at 37°C for 15–20 min with periodic shaking. The released keratinocytes were then collected by removing the supernatant and mixing it with keratinocyte medium (KC; 95% DMEM, 5% fetal bovine serum), followed by centrifugation at room temperature for 5 min, at 200g. The supernatant was removed and the cells were stored at room temperature, whereas the remaining pieces of epithelium were treated with trypsin as above. The released keratinocytes were pooled with the first sample of keratinocytes in KC medium.

3-D human skin model and companion keratinocyte monolayers

Cultures of normal human epithelial keratinocyte-based 3-D human skin model system (EpiDerm) in multiwell plates as well as monolayer cultures of the same cells were obtained from MatTek (Ashland, MA, USA). Cells were incubated at 37°C for 4 h after receipt, then dissociated by trypsin treatment, resuspended in a 1:1 mixture of DMEM and MCDB 153 containing 10% FBS, and then embedded in agarose plugs (see below).

Chinese hamster ovary cells

Isogenic Chinese hamster ovary (CHO) cell lines (CHO pSV2-FPG and CHO pSV2-neo) were kindly provided by Dr. Françoise Laval (INSERM, France). Transduced cells (CHO pSV2-FPG and CHO pSV2-neo) were generated by transfecting the parental CHO cells with pSV2-FPG or pSV2-neo plasmids [31]. Cells were cultured in DMEM supplemented with 10% dialyzed FBS (HyClone) without antibiotics. Geneticin (Gibco) was added (7.5 µg/ml) to CHO

psV2-FPG and CHO psV2-neo cell lines to maintain the cloned plasmids. The higher activity of Fpg protein was confirmed in the CHO pSV2-FPG line (Paul and Sutherland, manuscript in preparation) using oligonucleotides containing a single 8-oxo-dGuo as previously described [10].

DNA isolation

Cells in vials from tissue, 3-D model systems, or monolayer cultures were harvested by immersion into dry ice and were stored in liquid nitrogen until processed. To minimize artifactual induction of clustered damages, all solutions (where possible) used in DNA isolation were bubbled for 20 min with argon before use, and all tubes containing DNA plugs were filled with argon at each solution change [32]. The cells were thawed rapidly and EDTA was added to 83 mM. Cells were then embedded in agarose plugs, digested with proteinase K, then treated with TE containing 40 µg/ml phenylmethylsulfonyl fluoride, and finally soaked 2× (1 h each) in 10 mM Tris-HCl, 0.1 mM EDTA, pH 8, as previously described [27]. For damage analysis, DNA was digested with *AscI* according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA).

Complex DNA cluster determination

Homogeneous *Escherichia coli* Nfo protein was purified and characterized as previously described [10]. Homogeneous *E. coli* Nth protein and Fpg protein were kindly supplied by Dr. Jacques Laval (IGR, France). For digestion with a DNA glycosylase or endonuclease, companion plugs were transferred to 70 mM Hepes · KOH, 100 mM KCl, 0.1 mM EDTA, pH 7.6, and then to this buffer plus 1 mM DTT, 50 µg/ml bovine serum albumin. Plugs were digested with sufficient homogeneous, lesion class-specific enzyme to cleave at all specific cluster sites cleavable by that enzyme in 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, rinsed, and equilibrated into 10 mM Tris, 1 mM EDTA. Samples were electrophoresed (64.8 mM Tris borate buffer containing 0.36 mM EDTA) along with molecular length standard DNAs appropriate to the restriction digest; they included *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, and *Hansenula wingei* chromosomes; λ ladders; T7; *BglI* digest of T7; and λ-*HindIII* ladders. Neutral transverse alternating field electrophoresis [33] was used as previously described [32] for human DNA, whereas contour clamped homogeneous field electrophoresis was used for CHO DNA, using a pulsed-field gel regime to be described in detail elsewhere (Paul and Sutherland, manuscript in preparation). Gels were stained with ethidium bromide and destained, and a quantitative electronic image was obtained [34]. The DNA length standards were used to determine a dispersion curve, and the number average length (L_n) of each experimental DNA distribution was computed. The frequencies of DSBs and oxidized base or abasic clusters were then calculated from these L_n 's as previously described [8,26].

Results

Endogenous clusters can be quantified in unirradiated mammalian tissues or cells by isolation of high-molecular-length DNA in agarose plugs, treatment with lesion-specific enzymes, electrophoresis along with DNA length standards, quantitative electronic imaging, and number average length analysis. Fig. 1 shows a representative gel containing unirradiated DNA from 28SC human cells electrophoresed without treatment (lane 1) or after treatment with *E. coli* Nth protein (lane 2). The DNA size is reduced little, if at all, by treatment with the enzyme, indicating the presence of few, if any, endogenous oxypyrimidine clusters recognized by Nth protein. Such low level of Nth cleavage could result from either the presence of few endogenous cluster sites or an inactive enzyme. The latter possibility is excluded by the cleavage by the

same Nfo preparation of DNA from irradiated cells [cf. lanes 3 (+ radiation, no enzyme) and 4 (+ radiation, + Nth protein)].

We used this approach to ask if cells derived directly from human tissue contained endogenous clusters. Fig. 2 shows the results of cluster determinations in DNA from cells of the epidermis and dermis of neonatal foreskins from two individuals. In addition to oxypyrimidine clusters recognized by Nth protein, we determined the levels of oxypurine clusters recognized by *E. coli* Fpg protein and abasic clusters recognized by *E. coli* Nfo protein. The endogenous levels of these clusters were extremely low in cells from the dermis and epidermis of individual B and slightly higher in cells from individual A, especially in the dermis.

We then asked if cultured primary keratinocytes derived from epidermis A contained similarly low levels of endogenous clusters compared to the tissue from which they were derived. Fig. 3 shows the levels of the three cluster classes in neonatal foreskin epidermal keratinocytes and in primary neonatal fibroblasts from two different donors. Comparison of the cluster levels in the cultured epidermal keratinocytes with those of the epidermal tissue in Figs. 2A and 2B shows that DNA from the cultured cells had higher levels of all three endogenous cluster types than did that from skin tissue.

These data suggested two possibilities: that epidermal cells in a three-dimensional tissue have intrinsically lower endogenous cluster levels than those in monolayer culture or that the difference in cellular milieu between those present in tissue and those present in culture medium could affect the cluster levels. To distinguish between these possibilities, we obtained a 3-D epidermal skin model, "EpiDerm," containing only human epidermal keratinocytes as well as a culture of the same cells growing in monolayer culture. Fig. 4 shows that both the 3-D culture and the monolayer of the same cells in the same medium contained substantial levels of the three cluster types. These results suggest that the lower cluster levels in the human tissues were not a function of the three-dimensional property of the tissue but might reflect instead the surrounding cellular milieu, for example, the culture medium.

To test whether the medium could make a difference, we used a cell line previously shown to contain high levels of endogenous clusters [10] and grew the cells on either of two media. One set of TK6 cells, grown on RPMI 1640 (the medium recommended by the supplier, ATCC), contained high levels of both Fpg-oxypurine and Nth-oxypyrimidine clusters [10]. Fig. 5A shows the level of Fpg-recognized oxypurine clusters in cells grown in this medium and in a companion culture grown in the same medium supplemented with 17 $\mu\text{g/L}$ sodium selenite. These data show clearly that the composition of the culture medium can affect the level of endogenous clustered damages.

We also asked if the levels of cellular DNA repair enzymes could affect the cluster levels. We examined two companion CHO cell lines, one containing the SV2 vector plus the bacterial gene for the Fpg protein, as well as the same cell type containing only the empty vector. Fig. 5B shows that the Fpg-overproducing cells contained lower endogenous oxypurine cluster levels than did a companion culture containing the vector only. These results indicate that repair enzymes within the cell are active in maintaining low levels of endogenous clusters in genomic DNA and that increasing the levels of those enzymes is effective in lowering the cluster levels.

Discussion

Substantial evidence indicates that clustered damages are difficult for cellular enzymes to repair, and thus clusters pose a threat to genomic integrity. Although several cultured human cell lines were found to contain very low levels of endogenous oxidized base and abasic clusters, two were shown to accumulate substantial levels of oxidized base clusters [10]. It was

not clear, therefore, whether DNA in human tissue would accumulate measurable levels of endogenous clusters.

Human skin offers an ideal system for examination of endogenous clusters, as skin tissue, monolayer primary cultures of cells derived from skin, and three-dimensional model skin cultures are readily available. Although based on a limited number of specimens, the data in Fig. 2 show that the dermis and epidermis of skin from two individuals contain few if any endogenous clusters. However, cultured monolayer epidermal keratinocytes derived from one of these donors as well as monolayer fibroblast cultures from other individual accumulated detectable levels of all three classes of clustered damages (Figs. 3 and 4). Similar levels of clusters were observed in AGO1522 human fibroblasts (Sutherland and Bennett, manuscript in preparation).

Cells from skin can also be grown in three-dimensional models that have many similarities to skin tissue. We therefore asked if the low level of clusters seen in human skin tissue would also be observed in a 3-D skin model culture. To avoid possible interindividual variability, we used cells of the same origin and grew them both as monolayer cultures and in a 3-D model. Although the 3-D model culture might be expected to have levels of clusters similar to those in human skin tissue, the data in Fig. 4 show that the 3-D model culture contained significant cluster levels, similar to those in the companion monolayer keratinocytes as well as to cultured skin keratinocytes (initiated in this laboratory) derived from other individuals.

Systematic investigations of the levels of endogenous isolated base lesions have shown that the measured levels depend on the method of analysis [35]. The levels of 8-oxo-dGuo in human peripheral lymphocytes were found to be 4.24/Mb by high-performance liquid chromatography (HPLC) and estimated to be 0.34/Mb Fpg protein-sensitive sites by alkaline comet assays. Similarly, the level of 8-oxo-dGuo in HeLa cells was found to be 2.78/Mb by HPLC and only 0.5/Mb Fpg-sensitive sites by alkaline comet determinations.

Rodriguez et al. used gas chromatography/isotope-dilution mass spectrometry and liquid chromatography/isotope-dilution mass spectrometry to determine the levels of several DNA biomarkers in a tissue-engineered skin (TestSkin II; Organogenesis), along with those in several cultured human cell lines, human peripheral lymphocytes, and commercial calf thymus DNA [36]. These markers included both oxidized pyrimidines and oxidized purine lesions. DNAs from both the tissue-engineered skin and the cell lines and strains contained similar levels of endogenous oxidized bases, ~1 – 15 lesions per million bases. Calf thymus DNA contained higher levels of several oxidized bases. Comparing our results on cluster levels in engineered skin with those for lesion levels in similar cell constructs of Rodriguez et al., the levels of endogenous clustered damages we measure are approximately a thousandfold lower than those of the isolated oxidized lesions. However, the cluster data support a similar conclusion: that there is no intrinsic difference in oxidative damage—at the level of either lesions/Mb or clusters/Gbp—between 3-D tissue models and primary human cells or cell lines. The cluster data reveal, however, that DNA in human skin can contain lower levels of complex damage than DNA from cultured skin cells growing either as monolayers or in three-dimensional cultures.

The steady-state level of clustered damages probably reflects the net result of the cluster level induced by cellular metabolism or by exogenous factors in the cellular environment minus the level that the cells processed. As a first approximation, it can be assumed that cells use the same paths for processing radiation-induced and endogenous clusters. However, it must be noted that radiation-induced and endogenous clusters may differ in complexity (number of individual lesions per cluster), composition (identity of lesions comprising the cluster), and geometry (polarity and interlesion spacing). Thus cellular processing of clusters induced by

endogenous factors might differ from that of clustered damages induced by environmental agents such as radiation.

Human cells can deal with radiation-induced clustered damages by several paths. TK6 cells apparently convert radiation-induced clusters to DSBs [21]. However, these cells show higher radiation sensitivity than related cells and have been reported to be defective in double-strand break repair [37]. Thus DSB generation by these cells may be a consequence of defective repair. Radiation-resistant, repair-proficient 28SC cells exposed to X-rays apparently do not generate measurable levels of repair-related DSBs [20]. In these cells, bistranded abasic clusters persist until DNA replication and are then presumably converted to unistranded abasic lesions upon DNA synthesis. In addition, evidence from *in vitro* studies of complex clusters by extracts of radiation-resistant MRC5-V1 fibroblasts showed a hierarchical processing of the lesions comprising the cluster so that DSB induction was avoided [38]. Likewise, Malyarchuk and Harrison found that the majority of plasmids containing clustered damages that were transfected into HeLa cells did not suffer DSB induction, suggesting the presence of alternate paths of cluster processing [39].

The endogenous cluster data for skin-derived cultured cells—accumulation of all three cluster classes (Figs. 3 and 4)—differ strikingly from those in TK6 and WI-L2-NS cells, which contained measurable levels of oxidized base clusters but no detectable abasic clusters. However, these data are consistent with a unified hypothesis of cluster processing based on several lines of evidence: first, irradiated, repair-proficient human 28SC cells produce *de novo* abasic clusters [20], probably from glycosylase action without lyase action (consistent with the much higher glycosylase than lyase activity of several glycosylases [40-42]). TK6 and WI-L2-NS cells have lower glycosylase activities than 28SC cells [10] and do not effectively convert oxybase clusters to abasic clusters. Thus TK6 and WI-L2-NS cells accumulate endogenous oxybase clusters but no abasic clusters [10]. In normal, repair-proficient cells growing under low-stress conditions the levels of induced endogenous clusters are low; those that are induced can be processed successfully and thus these cells do not accumulate measurable levels of endogenous clusters of any kind [10]. In cells growing under conditions of higher oxidative stress, significant levels of oxidized base endogenous clusters are induced, and a portion of these clusters is processed to repair-intermediate abasic clusters, which are highly refractory to repair. Thus under conditions of stress, even normal human cells can accumulate significant levels of endogenous clusters of all classes.

Endogenous clusters apparently do accumulate and are likely to be repair refractory. In dividing cells, the levels of endogenous bistranded clusters should be “reset” to zero, because DNA synthesis would send lesions on one strand to one daughter DNA molecule and any lesions on the opposing strand to the companion daughter DNA molecule. Such DNA synthesis could be carried out by an error-prone polymerase (e.g., a Y-family polymerase [43] or POLQ polymerase [44]). Any resulting misincorporated bases could be removed by mismatch repair, and the remaining isolated lesion could then be repaired faithfully by BER (base excision repair). This cellular strategy would allow conversion of a repair-refractory bistranded clustered damage to unistranded lesions that can be readily repaired by normal BER pathways, circumventing both DSB formation and mutation induction.

If the hypothesis that the observed endogenous cluster levels result from the net of induced minus processed clusters is correct, then it should be possible to change the level of endogenous clusters by changing the cellular environment or the ability of cells to process them. The data in Fig. 5 indeed show that addition of Se to the medium or an increase in cellular repair enzyme levels can affect the net levels of endogenous clusters. Selenomethionine has anticarcinogenic properties in experimental animals [45] and reduces the levels of radiation-induced cellular oxidative stress in cultured cells [46], probably through its role as a component of critical repair

enzymes. It also increases the level of expression of the ATR gene [46], whose gene product ATR is an important component of the DNA damage response path [47]. In addition to these possibly indirect paths, increasing the cellular levels of the glycosylase Fpg should facilitate cluster processing. These data suggest that it should be possible to alter the level of endogenous clusters accumulated in cells, providing useful avenues for determining the mechanisms of endogenous cluster induction and cellular paths for dealing with them.

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Abbreviations

ATCC, American Type Culture Collection
BER, base excision repair
CHO, Chinese hamster ovary (cell)
DMEM, Dulbecco's modified Eagle's medium
DSB, double-strand break
FBS, fetal bovine serum
KC, keratinocyte (medium)
 L_n , number average molecular length
8-oxo-dGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine
Pen/Strep, penicillin/streptomycin
PBS, phosphate-buffered saline.

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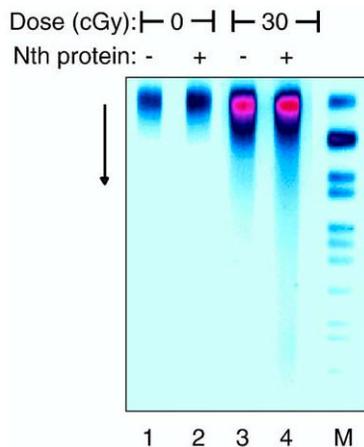


Fig. 1. Electronic image of electrophoretic gel containing DNA from human cells not exposed to radiation (lanes 1 and 2) or exposed to 30 cGy 100-kVp X-rays (lanes 3 and 4). Companion agar plugs were incubated with (lanes 2 and 4) or without (lanes 1 and 3) Nth protein for detection of endogenous or radiation-induced Nth-oxypyrimidine clusters. M, molecular length standards; *S. cerevisiae* chromosomes.

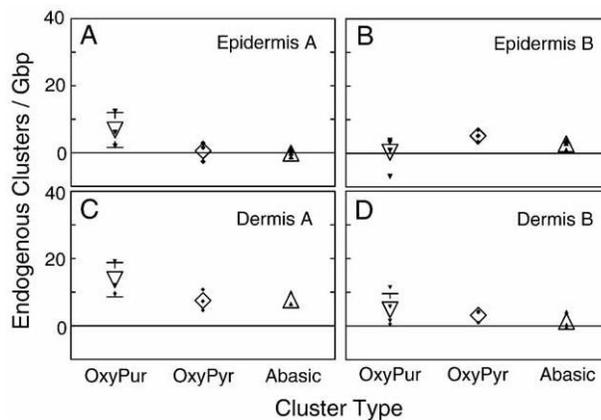


Fig. 2. Endogenous clusters in DNA from human neonatal foreskin tissue from two individuals. For donor A: (A) epidermis, (C) dermis. For donor B, (B) epidermis, (D) dermis. (\blacktriangledown , \triangledown) Fpg-oxypurine clusters, (\blacklozenge , \lozenge) Nth-oxypyrimidine clusters, and (\blacktriangle , \triangle) Nfo-abasic clusters. Solid symbols, individual measurements; open symbols, averages; error bars, SEM; where not shown, SEMs were smaller than the symbol showing the average.

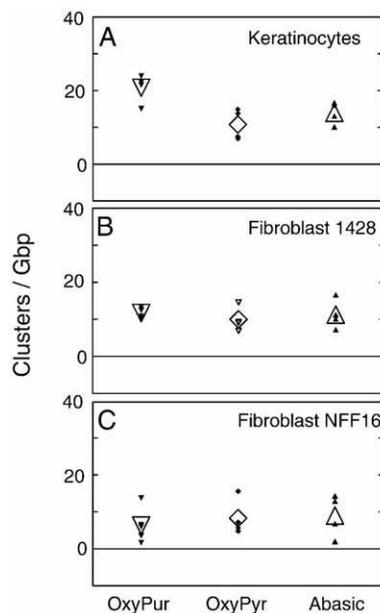


Fig. 3. Endogenous clustered damages in cultured primary (A) keratinocytes and (B) fibroblasts derived from donor A (see Fig. 2) and (C) in a primary skin fibroblast culture derived from a different individual. (\blacktriangledown , \triangledown) Fpg-oxypurine clusters, (\blacklozenge , \lozenge) Nth-oxypyrimidine clusters, and (\blacktriangle , \triangle) Nfoa-basic clusters. Solid symbols, individual measurements, open symbols, averages; error bars showing the SEMs were smaller than the symbols showing the average.

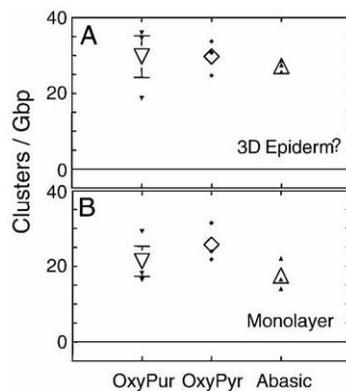


Fig. 4. Endogenous clusters in (A) a 3-D epidermal skin model (EpiDerm; MatTek) and in (B) companion monolayer cultures of the same cell strain. (\blacktriangledown , ∇) Fpg-oxypurine clusters, (\blacklozenge , \lozenge) Nth-oxypyrimidine clusters, and (\blacktriangle , \triangle) Nfo-abasic clusters. Solid symbols, individual measurements, open symbols, averages; error bars, SEM; where not shown, SEMs were smaller than the symbol showing the average.

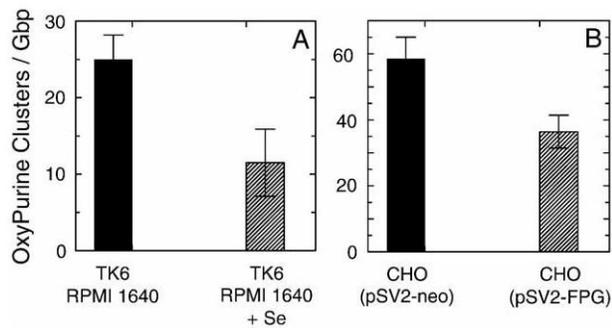


Fig. 5. Endogenous oxypurine clusters in (A) TK6 human cells grown in standard RPMI 1640 medium (solid bar) or in the same medium supplemented with 17 ng/L sodium selenite (diagonal striped bar) and in (B) CHO cells with only the vector construct (solid bar) or CHO cells containing the *E. coli* Fpg gene and expressing higher levels of Fpg protein (diagonal striped bar).