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SCIENCE DIRECT

Free Radical Biology & Medicine 39 (2005) 832 – 839

www.elsevier.com/locate/freeradbiomed

Original Contribution

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

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Received 22 February 2005; revised 23 April 2005; accepted 9 May 2005

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.
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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 (DSBs) [14–19], and irradiated mammalian cells with repair deficiencies can generate additional DSBs during

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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0891-5849/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.freeradbiomed.2005.05.008

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 Na_2HPO_4, 1.8 mM KH_2PO_4), 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 10^6 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 × 10^6 cells/flask, gassed with 5% CO_2, placed into a 5% CO_2 incubator, and left undisturbed for 3 days. The medium